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BIOCATALYSIS FOR THE SUSTAINABLE PRODUCTION OF BIO-BASED INGREDIENTS FROM RENEWABLE RESOURCES

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Abstract

This research aims at obtaining high-added value chemicals from renewable resources by using either a chemical or an enzymatic approach, as well as a combination of both. On one hand, glycerophosphoinositol (GPI) and glycerophosphocholine (GPC) which have anti-inflammatory and cognitive enhancer properties, respectively, were obtained from sunflower lecithin, a by-product of oil extraction and refining, by (phospho)lipase-catalyzed hydrolysis of pre-treated lecithin. On the other hand, sugar fatty acid esters (SFAE), which are non-ionic emulsifiers, were synthesized by using lactose as well as its hydrolysis products (glucose and galactose) as raw materials. Lactose is the most abundant component of cheese whey, the main waste stream of dairy industry, that can be upgraded, indeed, as a pool of sugars and as a carbon source for microbial production of fatty acids/glycerides which constitute the "hydrophobic tail" of SFAE. A fine-tuning of the enzymatic/chemoenzymatic esterification/transesterification of the sugars with fatty acids/esters, respectively, was needed to overcome the opposite solubility profiles of the sugar "polar head" and the fatty acid "tail". Derivatization of the sugar, investigation of non-conventional media (DES), and organocatalysis were explored to this aim.

Riassunto

L'obiettivo di questa ricerca è l'ottenimento di prodotti ad alto valore aggiunto da fonte rinnovabile mediante un approccio chimico, enzimatico, o chemoenzimatico. Nella prima parte della Tesi, il glicerofosfoinositolo (GPI) e la glicerofosfocolina (GPC), aventi rispettivamente proprietà antinfiammatorie e di *cognitive enhancer*, sono stati ottenuti dalla lecitina di girasole, un sottoprodotto dell'estrazione e della raffinazione dell'olio, mediante idrolisi enzimatica con lipasi e/o fosfolipasi. Nella seconda parte della Tesi, è stata intrapresa la sintesi di *sugar fatty acid esters* (SFAE), emulsionanti non ionici, utilizzando il lattosio e i suoi prodotti di idrolisi (glucosio e galattosio) come materia prima. Il lattosio è il componente più abbondante del siero di latte, il principale sottoprodotto dell'industria lattiero-casearia, che può quindi essere valorizzato come biomassa, nonchè come fonte di carbonio per la produzione, mediante fermentazione, di acidi grassi/gliceridi utilizzati per la sintesi di SFAE. Per lo studio delle reazioni di esterificazione/transesterificazione enzimatica/chemoenzimatica degli zuccheri con acidi grassi/esteri, rispettivamente, sono state applicate diverse strategie: derivatizzazione dello zucchero, uso di solventi non convenzionali (DES) e organocatalisi.

Abbreviations

2M2B	2-Methyl-2-butanol
ACN	Acetonitrile
AO	Amine oxidase(s)
APG	Alkyl polyglycosides
API	Active Pharmaceutical Ingredients
ATA117	*ω-T*ransaminase from *Aspergillus terreus*
AXE	Acetyl xylan esterase from *Bacillus pumilus*
BOD	Biological Oxygen Demand
BV	Bed volume
BV333ATA	*ω-T*ransaminase from *Streptomyces sp.*
CalB	Lipase from *Candida antarctica*
CAR	Carboxylic acid reductase(s)
CEN	European Commission of Standardization
ChCl	Choline chloride
CHU	Polyphosphate kinase from *Cytophaga hutchinsonii*
CLEA	Cross-Linked Enzyme Aggregates
CLEC	Cross-Linked Enzyme Crystals
COD	Chemical Oxygen Demand
cPLA2	Cytosolic Phospholipase A2
*Cv*ATA	*ω-T*ransaminase from *Chromobacterium violaceum*
CW	Cheese whey
CWP	Cheese whey permeate
DA	Decanoic acid
DBU	1,8-Diazabicyclo [5.4.0] undec-7-ene
DES	Deep Eutectic Solvents
DHA	Docosapexaenoic acid
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethylsulfoxide
ELSD	Evaporative light scattering detector
ENSL	Ethanol non-soluble lecithin
EPA	Eicosapentaenoic acid
ESL	Ethanol soluble lecithin
FAME	Fatty acid methyl ester(s)
FDA	Food and Drug Administration
FFA	Free fatty acid(s)
fGOS	Fructosyl-galacto-oligosaccharides
GluA1	Glutamic acid residue 1
GluA2	Glutamic acid residue 2
GOS	Galacto-oligosaccharides

GPAE	Glycerophosphoric acid ester(s)
GPC	Glycerophosphocholine
GPI	Glycerophosphoinositol
GRAS	Generally Recognized As Safe
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
HLB	Hydrophilic-Lipophilic Balance
IcP	Inositol 1,2 cyclic phosphate
IL	Ionic liquids
iPLA2	Calcium independent phospholipase A2
IRED	Iminoreductase(s)
LA	Lauric acid
Lac	Lactose
Lid	Lidocaine
MBA	(a)-Methyl benzylamine
MD	Molecular dynamics
MEG	*N*-Methyl glucamine(s)
MEGA	*N*-Acyl-*N*-methyl glucamide(s)
MEK	Methyl ethyl ketone
MS	Mass spectroscopy
NMR	Nuclear Magnetic Resonance
NuH	Nucleophile
PA	Palmitic acid
PDA	Phosphatidic acid
PC	Phosphatidylcholine
PCL	Lipase from *Pseudomonas cepacia*
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PHA	Polyhydroxyalkanoate(s)
PI	Phosphatidylinositol
PI-PLC	Phosphoinositide specific phospholipase C
PL	Phospholipid(s)
PLA1	Phospholipase A1
PLA2	Phospholipase A2
PLB	Phospholipase B
PLC	Phospholipase C
PLD	Phospholipase D
PLP	Pyridoxal-5'-phosphate
PMP	Pyridoxamine
PS	Phosphatidylserine
PSL	Lipase from *Pseudomonas stutzeri*

*p*TSA	*p-T*oluen sulfonic acid
Py	Pyridine
RID	Refractive index detector
Sac	Saccharose
SCO	Single Cell Oil
SFAE	Sugar Fatty Acid Esters
sPLA2	Secreted phospholipase A2
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TLL	Lipase from *Thermomyces lanuginosus*
TOS	Transgalactosylated oligosaccharides
U	Urea
VD	Vinyl decanoate
VE	Vinyl esters
*Vf*ATA	ω-Transaminase from *Vibrio fluvialis*
VL	Vinyl laurate
VP	Vinyl palmitate
β-Gal	β-Galactosidase(s)
ω-ATA	ω-Transaminase(s)

Chapter 1: Introduction

1.1 Green Chemistry and Circular Economy

Green Chemistry is the design of chemical products and processes that reduce or eliminate the use or generation of hazardous substances[1] besides promoting a more efficient use of the raw materials to reduce waste.[2] The 12 principles of Green Chemistry (**Table 1**) were formulated by Anastas and Warner.[3]

Table 1: The "12 Principles of Green Chemistry[3]"

N°	Principle
1.	It is better to **prevent waste** than to treat or clean up waste after it is formed
2.	Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final products (**atom economy**)
3.	Wherever practicable, synthetic methodologies should be designed to use and generate substances that possess little or **no toxicity** to human health and the environment
4.	Chemical products should be designed to **preserve efficacy and function** while reducing toxicity
5.	The use of **auxiliary substances** (*e.g*. solvents, separation agents, etc.) should be made unnecessary wherever possible and innocuous when used
6.	Energy requirements should be recognized for their environmental and economic impacts and should be minimized. Synthetic methods should be conducted at **ambient temperature and pressure**
7.	A raw material of feedstock should be **renewable** rather than depleting wherever technically and economically practicable
8.	Unnecessary **derivatization** (blocking groups, protection/deprotection, temporary modification of physical/chemical processes) should be avoided wherever possible
9.	**Catalytic reagents** (as selective as possible) are superior to stoichiometric reagents
10.	Chemical products should be designed so that at the end of their function they do not persist in the environment and break down into **innocuous degradation** products
11.	**Analytical methodologies** need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances
12.	Substances and the form of a substance used in a chemical process should be chosen so as to **minimize the potential for chemical accidents**, including releases, explosions, and fires

On the other hand, sustainability is defined as "*meeting the needs of the present without compromising the ability of future generations to meet their own needs*."[4] Sustainability is thus a holistic approach that considers ecological, social and economic dimensions, recognizing that all must be considered together to find lasting prosperity. Sustainability can be reached not only by the development of safer and more environmentally friendly processes, but also through a more efficient use of natural sources, and this includes the valorization/upgrading of waste and by-products. That is why Green Chemistry can be strictly correlated to Circular Economy, a model defined as "*restorative and regenerative by design, that aims to keep products, components and materials at their highest utility and value at all times"*,[5] which is gradually taking the place of the older linear route of production based on the principle "*take-make-use-dispose*". This new paradigm answers also the goals of the European Green Deal, the new strategy and policy of the EU for a resource-efficient and climate-neutral continent by 2050.[6] Since, basically, every process generates waste and the products become waste themselves after that they have served for their purpose, one of the foundations of the Circular Economy is the recycle of waste and its use as starting material for the preparation of new products. This scenario has recently boosted the research and development of new processes for upgrading waste and by-products from the agri-food industry into high-value chemicals and/or energy, with the aim of reducing the environmental impact of chemical processes.[7] This model answers the goals of the 2030 United Nations agenda for sustainable development (**Figure 1**), in particular the 12th goal: "*sustainable management and efficient use of natural resources, reducing waste generation through prevention, reduction, recycling and reuse..."*

Figure 1: 2030 Sustainable Development Goals (https://www.undp.org/sustainable-development-goals)

It is not surprising that the 12 principles of Green Chemistry have evolved into the 12 principles of Circular Chemistry (**Table 2**)[8] that provide a sort of guidelines for the design of a chemical process according to circularity, by extending the concept of sustainability through the entire lifetime of a product by both developing reactions to reuse and recycle chemicals, and the rational and efficient use of resources along the whole product value chain.

Table 2: The "12 Principles of Circular Chemistry"

N°	Principle
1.	**Collect and use waste**: waste is a valuable resource that should be transformed into marketable products
2.	**Maximize atom circulation**: circular processes should aim to maximize the utility of all atoms in existing molecules
3.	**Optimize resource efficiency**: resource conservation should be targeted, promoting reuse and preserving finite feedstocks
4.	**Strive for energy persistence**: energy efficiency should be maximized
5.	**Enhance process efficiency**: innovations should continuously improve in- and post-process reuse and recycling, preferably on-site
6.	**No out-of-plant toxicity**: chemical processes should not release any toxic compounds into the environment
7.	**Target optimal design**: design should be based on the highest end-of-life options, accounting for separation, purification and degradation
8.	**Assess sustainability**: environmental assessments (typified by the LCA, Life Cycle Assessment) should become prevalent to identify inefficiencies in chemical processes
9.	**Apply ladder of circularity**: the end-off life options for a product should strive for the highest possibilities on the ladder of circularity
10.	**Sell service, not product**: producers should employ service-based business models such as chemical leasing, promoting efficiency over production rate
11.	**Reject lock-in**: business and regulatory environment should be flexible to allow the implementation of innovations
12.	**Unify industry and provide coherent policy framework**: the industry and policy should be unified to create an optimal environment to enable circularity in chemical processes

The 12 principles of Green Chemistry have somehow "re-shaped" chemistry in the recent years by providing the guidelines for re-designing and optimizing chemical processes, thus leading to less environmentally demanding chemistry practices. The development of Sustainable Chemistry in a context of Circular Economy requires re-thinking chemical processes in terms of circularity as well and, indeed, of products which, ideally, can be continuously used and reused, and for which energy is the only input. Circular Chemistry, as it has been recently defined, can thus play a pivotal role in the management of waste, scarcity of resources, and climate change, through the development of both new chemical products and processes based on the use of waste as raw materials.[8]

1.2 Biocatalysis in a nutshell

Biocatalysis is the use of isolated enzymes and/or whole cells to catalyze chemical reactions. Biocatalysis has many attractive features in the context of Green Chemistry and Sustainable Development.[9] In other words, the "green credentials" of biocatalysis can be summarized as follows:

1. The catalyst, an enzyme, is derived from renewable resources and is biocompatible (sometimes even edible), biodegradable, and essentially non-hazardous; thus, it fulfils the criteria of sustainability.

2. Biocatalysis avoids the use of scarce and precious metals such as palladium, platinum, and rhodium, and contamination of end products, thereof. Removing traces of noble metals to an acceptable level from the final products is crucial.

3. Reactions are usually performed under mild conditions (physiological pH and ambient temperature and pressure) and, often, in environmentally friendly solvent (water).

4. Reactions of multifunctional molecules proceed with high activities and chemo-, regio-, and stereoselectivities and generally without the need for functional group activation, protection, and deprotection steps required in traditional organic syntheses. This affords processes that are more step economic and more efficient in energy and raw material consumption, that generate less waste and are both environmentally and economically more attractive than conventional routes.

5. As a direct result of the higher selectivity and milder reaction conditions, biocatalytic processes often afford products in higher purity than “conventional” chemical or chemo-catalytic processes.

6. Enzymatic processes (but not fermentations) can be performed in standard multipurpose batch reactors and, hence, do not require any extra investments as for high-pressure equipments.

7. It is relatively easy to integrate multiple enzymatic reactions into eco-efficient catalytic cascade processes since biocatalytic reactions are carried out roughly under the same conditions of temperature and pressure.[10]

Enzymes are physiologically able to perform reactions that are very challenging through conventional chemistry; this feature makes biocatalysts very useful in organic synthesis. For example, enzymes are able to perform chemo-, regio- and stereoselective reactions in one step and, generally speaking, to transform a single functional group within a complex molecule under mild conditions, thus avoiding multi-step processes that involve protection and deprotection reactions.[11]

All these features make biocatalysis very attractive in many industrial sectors ranging from food and beverage industry to pharmaceuticals and fine chemicals. The selectivity of enzymes and the mild reaction conditions typically used in biotransformations frequently result in “greener” synthetic schemes. Measuring the “greenness” of a product and/or process is not that simple, though.

The E-factor (Environmental factor[12,13]) is widely regarded as a quick assessment of the environmental compatibility of a process and often reflects the complexity of the synthesis. The E-factor indicates the actual amount of waste produced in the process, defined as anything but the desired product (E-factor= kg waste/kg product). Unlike the atom economy, the E-factor takes into account the chemical yield and includes reagents, solvents and materials used in the process, except water (while any inorganic salts and/or organic molecules in it are considered) and energy. Therefore, the higher is the E-factor, the greater is the environmental impact of the process, being zero the ideal E-factor. It is hardly surprising that the pharmaceutical industry is recognized as the chemical sector characterized by the greatest environmental impact (**Table 3**)[12] as well

as the sector in which the integration of biocatalysis with conventional synthetic routes can be more advantageous.

Table 3: E factor in chemical industry (modified from Sheldon et al.[12])

Industry	Production tonnage	E-factor (kg waste/kg product)
Oil refining	10^6-10^8	<0.1
Bulk chemicals	10^4-10^6	<1-5
Fine chemicals	10^2-10^4	5-50
Pharmaceutical	10-10^3	25-100

Once that the "right" biocatalyst has been identified for a specific biotransformation, typical shortcomings are its stability under operative conditions and its recovery after use. Enzyme immobilization is frequently used to have easily handling and stabilized, heterogeneous biocatalysts.[14]

The first definition of immobilized enzyme was given on the occasion of the First Enzyme Engineering Conference in Henniker (NH, USA, 1971) almost 50 years ago: an immobilized enzyme is *"physically confined or localized in a certain defined region of space with retention to their catalytic activities, and which can be used repeatedly and continuously".* This statement resumes the main advantages of immobilization as mentioned above: improved stability of the biocatalyst, recyclability and reusability. Moreover, it suggests that, upon immobilization, product downstream can be straightforward as the catalyst can be removed from the reaction mixture by a simple filtration, thus minimizing any interference with product isolation.[15–18]

Immobilization techniques can be based on physical (these methods involve the entrapment or encapsulation of the enzymes in a matrix), or chemical interactions between the enzyme and the carrier; cross-linking belongs to the chemical methods.

Chemical interactions can occur through van de Waals, ionic or covalent bonds: ionic and covalent interactions are stronger and generally result in a higher stability of the biocatalyst; protein leaching phenomena are also minimized in these cases.

Cross-linking of protein aggregates (CLEA, Cross-Linked Enzyme Aggregates) or crystallized proteins (CLEC, Cross-Linked Enzyme Crystals) is a carrier-free immobilization.[17] Cross-linking is frequently used also as post-immobilization technique

to further stabilize the biocatalyst through the use of polyfunctional reagents such as poly-aldehydes which react both with the enzyme and the carrier.[19]

Covalent immobilization is generally preferred when the biocatalyst is intended for the synthesis of API or API intermediates because of the low risk of protein leaching. This type of immobilization requires the presence, on the carrier, of chemical groups which can react with reactive groups belonging to superficial amino acid side-chains of the enzyme, typically ε-amino groups of lysine or carboxylic groups of aspartic and glutamic acids. A wide array of commercially available carriers that are already functionalized is commercially available; however, several polymers, such as polysaccharides, can be used as well since they are easy and versatile to functionalize.[20]

As a matter of fact, a "universal" immobilization procedure does not exist; therefore, the best immobilization technique should be experimentally determined depending on enzyme features, the envisaged application, and immobilization conditions (temperature, pH, buffer) dictated by the binding chemistry between the carrier and the protein. The purity, the source, and structural characteristics of the enzyme, when available, can address, in principle, the choice of the carrier and the technique.[18] The immobilization technique should satisfy both the catalytic (productivity, stability, selectivity, and time/space yield) and non-catalytic (recovery of the catalyst, process control, downstream) requirements of the reaction.[19]

Immobilization can modify the "microenvironment" around the enzyme: for example, the carrier can create a steric hindrance which can hamper a fruitful interaction between substrates and the catalytic site.[21] On the other hand, with information about enzyme structure and surface amino acids distribution, it is possible either designing biocatalysts in which the catalytic site is more prone to the binding with the substrate due to a more favorable orientation, or designing mutations far from the catalytic site to drive the immobilization toward a specific area of the protein.[22,23]

Post immobilization techniques are often necessary to deactivate functional groups of the carrier which have not reacted with the enzyme. These quenching reactions avoid unwanted interactions between the carrier and the molecules, reagents and/or products, involved in the bioconversion. Moreover, the quenching step minimizes possible enzyme distortions. For example, quenching of epoxy groups after the immobilization is carried out by using highly concentrated solutions of amino acids or

amino alcohols. The nature of the quenching agents can modify the hydro-/lipophilic characteristics of the carrier, thus affecting the properties of the biocatalyst.[18]

In this Thesis, the enzymes (all commercially available) were used both as "free" (non-immobilized) proteins (**Chapter 2.2**), and as immobilized biocatalysts (**Chapter 3**). Specifically, the β-galactosidase from *Aspergillus oryzae* described in **Chapter 3.2** was immobilized "in house".

Biocatalysis was applied herein to the valorization of different biomasses in view of combining the numerous advantages of the enzymes with the principles of Circular Economy. Some examples of this combined approach are discussed in the next paragraph.

1.2.1 Biocatalysis for biomass valorization

As mentioned before, the green credentials of biocatalysis make it a powerful tool for feedstock biotransformation and bio-based chemistry. For this reason, bioeconomy and circularity are becoming a new driver to biocatalysis development, besides its recognized benefits impacting pharmaceutical and fine chemicals industries.[24]

Recently, the bio-based industry has promoted the use of agro and food wastestream as a source of biomasses and high-value chemicals, taking into account that approximately 40% of all food is wasted and it generates by-products that are very rich in carbohydrates, lipids and proteins.[25] Carbohydrates are the most abundant organic compounds worldwide. They represent 95% of the biomass volume generated annually.[26] The oldest example of biomass transformation is the recovery of carbohydrates through the hydrolysis of starch by amylases, which dates back to 1970 and that currently represents the largest enzymatic process implemented at industrial scale.[24]

The high biological oxygen demand (BOD) of food waste prevents its disposal in wastewater.[25] On the other hand, all these substances can be transformed through biocatalysis to high value chemicals, thus reducing the cost of the waste handling and going back into the market, according to the Circular Economy model. In this frame, biocatalytic reactions include oxidation, hydrolysis, acylation, and phosphorylation of carbohydrates, but also hydrogenation, epoxidation and (trans)esterification of lipids,

and hydrolysis of proteins to peptides.[25] Some selected examples are summarized in **Table 4**.

Table 4: Examples of biocatalysis applied to biomass transformations (modified from Pellis et al. **2018**[24])

Starting material	Biocatalyst	Biotransformation	Product
Rapeseed oil	Lipases	Epoxidation	Epoxyacids
Palm olein	Lipases	Amidation	Fatty amides
Vegetable waste oils	Lipases	Alcoholysis	Biodiesel
Palm oil	Lipases	Interesterification	Cocoa butter substitute
Fish oil	Lipases	Selective concentration of EPA and DHA	Omega-3 concentrates
Soy lecithin	Phospholipases A and lipases	Hydrolysis	Glycerophosphocholine (GPC)
Cellulose (corn cob)	Cellulases	Hydrolysis	Glucose
Cellulose (sugar beet pulp)	Cellulases	Hydrolysis	Cellobiose
Sugar cane biomass	Cellulases and xylanases	Hydrolysis	Ethanol
Starch	Amylases	Hydrolysis	Maltose, glucose
Cheese whey (lactose)	β-galactosidase	Hydrolysis	Glucose and galactose
Cheese whey (lactose)	β-galactosidase	Transglycosylation	Galactooligosaccharides

The large amount of carbohydrates, oils and fats contained in the agri-food wastes can be a great opportunity (and also, a big challenge) for research groups and companies interested in the production of specialty chemicals such as natural surfactants. For example, sugar fatty acid esters (SFAE) can be obtained by the enzymatic condensation of sugars with fatty acids. This scenario paved the way to the development of integrated processes for the preparation of surfactants completely based on natural substrates. Siebenhaller and co-workers[27] developed a process based on lignocellulosic substrates. The beechwood hydrolysate was used as a source of glucose and xylose (the "polar heads" of the surfactant) and was incorporated in a deep eutectic solvent (DES) with choline chloride (molar ratio 1:1). The same biomass was also used as a carbon source for the cultivation of the oleaginous yeast *Cryptococcus curvatus* which accumulated

fatty acids ("hydrophobic tails"), that were then converted into the corresponding methyl esters (FAME). The transesterification between the FAME and the sugar DES was performed by a commercial immobilized lipase from *Candida antarctica* (CalB). Thus, the hydrolysate acted also as part of the reaction medium in a nice "all in one" process. A similar process was reported also by Hoyos and co-workers.[28] In this case, peach palm fruit shells were used as source of both the substrates. Fatty acids were extracted with ethanol and the de-oiled biomass was submitted to three sequential enzymatic reactions to recover simple sugars. The highest conversions were observed by using a system of choline chloride: glycerol 1:2 DES/DMSO/*t*-butanol 1/1/3.

The BioSurf project (https://www.biosurfproject.it/), which this Thesis is a part of, follows the same integrated approach by using cheese whey permeate (CWP) as a source of both the components of the surfactants. Cheese whey permeate was used, indeed, both as the source of the "polar heads" (through the enzymatic modification of lactose) and as the feedstock for the fermentation process to produce microbial lipids using *Cutaneotrichosporon oleaginosus.*[29] Also in this case, one of the substrates (fatty acid) was used as the reaction medium for the esterification reaction ("solvent free" system) (**Chapters 3.1**-**3.2**).

In the next paragraphs, the two biomasses used in this Thesis (sunflower lecithin and cheese whey permeate) will be introduced. Their composition, the possible modifications and the high-value chemicals that can be obtained from these biomasses will be described.

1.2.2 Reaction media in biocatalysis: the quest for the ideal solvent

"Ideal" solvents in biocatalysis have to fulfil a large number of requirements, such as high substrate solubility as well as high enzyme activity and stability. Moreover, as a general rule, solvents should not negatively affect the reaction equilibrium and the downstream process, besides being safe for people and the environment. Water media are the most frequently used solvents in biocatalysis. However, water is less than an "ideal" solvent for some applications. One major drawback of water as a solvent in biotransformations is its high polarity that is in contrast with the hydrophobic nature of many organic reactants; this turns into a low solubility of the substrates in the aqueous reaction media.[30] Moreover, some reactions such as (trans)esterifications and

amidations cannot be performed in water owing to equilibrium limitations and/or product hydrolysis. Therefore, there is a long-standing interest in non-conventional reaction media for biocatalytic applications. Biocatalysis in organic solvents[31] has dramatically overtaken the old "dogma" of enzymes working exclusively in water. The use of organic media assists the easier recovery of the product, eliminates the risk of microbial contamination, and allows to achieve higher concentrations of poorly water-soluble substrates and/or products. However, the use of organic solvents (even as co-solvents) can result in biocatalyst denaturation and/or inhibition. Enzyme stabilization in the presence of organic solvents can be achieved by immobilization, as mentioned above (**Chapter 1.2**). Moreover, within a reaction system, solvents are recognized to account for the highest environmental impact. The production and use of conventional organic solvents is also plagued by the effort and energy required for their disposal. Therefore, "alternative" solvents better defined as "non-conventional media" are actively being sought. Besides solvents derived from biomass,[32] ionic liquids (IL) and deep eutectic solvents (DES) are currently a "hot topic" in the quest for the ideal solvent. Ionic liquids (IL) are "non-conventional" media widely used in biocatalysis (at least at a lab scale) owing to their salt- and water-like character. IL are liquid at, or close to, ambient temperature and are composed entirely of ions. Based on their non-volatility, they have been widely proposed as potentially attractive alternatives to volatile organic solvents, and much attention has been devoted to their use as reaction media for catalytic processes.[33] However, their technical applicability and "greenness" are often limited, mainly due to high costs, poor biodegradability, poor biocompatibility, and low sustainability.

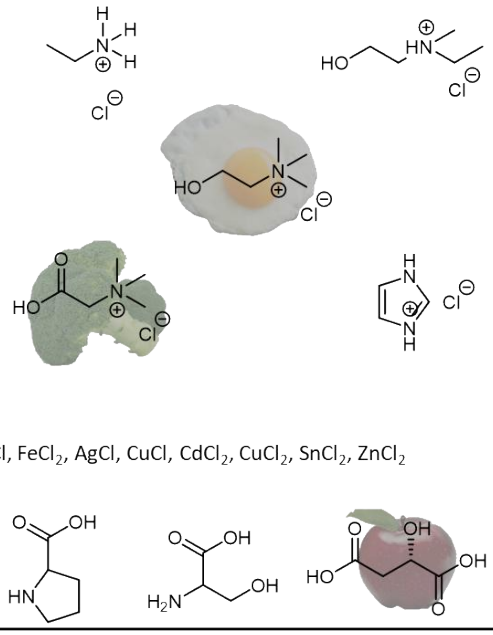
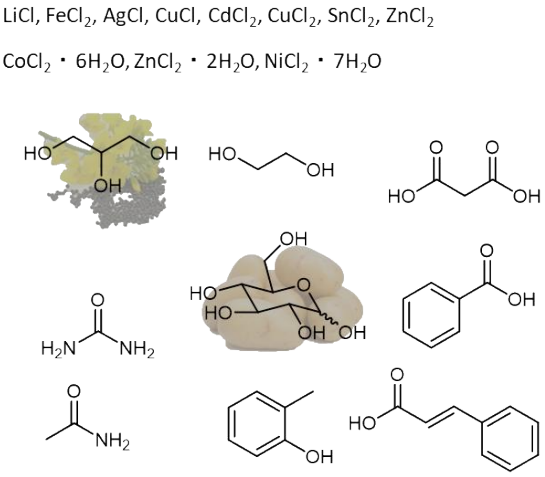
Deep eutectic solvents (DES) are mixtures of two or more molecules which act as hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA), respectively. HBA and HBD are mixed in appropriate molar ratios and heated under stirring, thus originating, through hydrogen bond formation, a eutectic mixture with a melting point much lower than its components, such as a liquid. It is worth mentioning that the synthesis of DES is very easy and produces no waste. Moreover, the atom economy associated to the DES formation is 100%, because all initial components are included in the final mixture.[34] There is a rising attention towards DES because they can be designed as non-toxic, non-volatile, non-flammable, or even biodegradable liquids. Therefore, DES are considered

as a green solvent class. Another reason for their increasing use is the easy preparation and the wide array of possible raw materials (including molecules derived from renewable sources) that can be used to develop a suitable DES for a certain application (**Table 5**).

Biocatalysis is one of the areas in which DES express better their potential. DES may circumvent the drawbacks of the organic solvents by combining the solubility of substrates with a greater compatibility with enzymes, both when used as additive and neat solvent.

Interestingly, while DES had been used exclusively as additives or as alternative solvents to routinely used solvents in early investigations, more recently some research groups have investigated reactions where the substrates are part of the DES.[35] This "2-in-1" approach was shown to be promising for certain applications in terms of conversion, yield, and atom efficiency.[30]

Table 5: Some molecules and salts used as DES components

Component 1	Component 2
	
LiCl, $FeCl_2$, AgCl, CuCl, $CdCl_2$, $CuCl_2$, $SnCl_2$, $ZnCl_2$	LiCl, $FeCl_2$, AgCl, CuCl, $CdCl_2$, $CuCl_2$, $SnCl_2$, $ZnCl_2$ $CoCl_2 \cdot 6H_2O$, $ZnCl_2 \cdot 2H_2O$, $NiCl_2 \cdot 7H_2O$
	

Despite their advantages, the use of DES comes also with some drawbacks. DES have a low volatility which is not a good point for a solvent in the view of pulling out the product(s) from the mixture.[34] Moreover, DES are a mixture of two or more molecules. Thus, the isolation of products from DES can be very tedious, especially if the product is hydrophilic. Even if DES are considered the greenest non-aqueous solvents existing

nowadays, due to the large number of molecules that can be used in DES, the statement that they are non-toxic and fully biodegradable cannot be generalized.

Considering all the possible molecules and their combinations at different molar ratios, the number of DES is almost endless. On one hand, the high number of potentially available DES to the organic chemist might be considered as a resource. On the other hand, the plethora of DES is associated with the lack of a deep knowledge on physico-chemical properties of DES and on how they really work. Thus, there is a great need for predictive models to allow a more rational approach to this quite new technology.

Deep eutectic solvents were studied in this Thesis for the enzymatic preparation of lactose fatty acid esters. In this part of the work, the assistance of computational methods was crucial for understanding the complexity of these systems (**Chapter 3.3**).

1.3 Lecithin

Lecithin is the commercial name for a natural mixture of phospholipids (PL). Phospholipids derive from natural sources, e.g., soybean, rapeseed, and sunflower seed, to name a few. Lecithin mainly contains phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylglycerol (PG), and phosphatidic acid (PA), with the corresponding lyso forms (with only one chain on the glycerol moiety) in addition to tri-, di- and monoacylglycerols and some vitamins.[36]

The structures of the most common phospholipids are reported in **Figure 2**; phospholipid and fatty acid composition of soy and sunflower lecithin is reported in **Tables 6-7**.

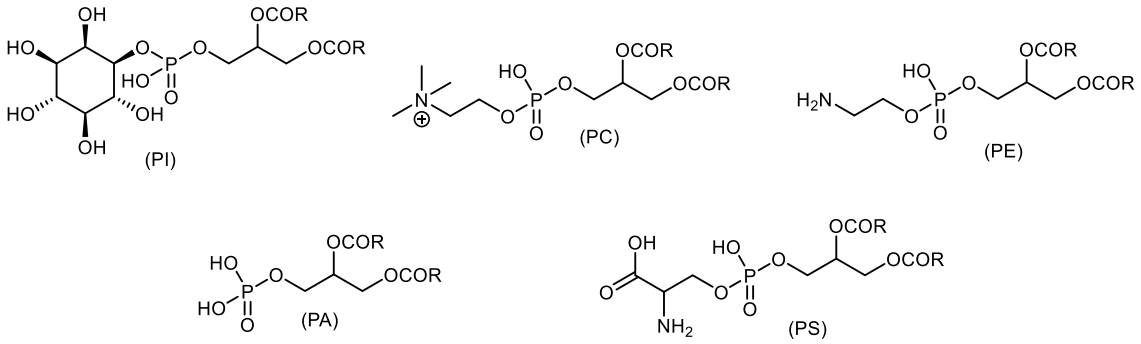


Figure 2: Main phospholipids contained in lecithin. R = alkyl or alkenyl chain

Soybean lecithin represents 90% of the available lecithin products dominating over the vegetable lecithin market. In Europe, the market for sunflower and rapeseed lecithin, which are locally produced, is increasing.[37] However, data about rapeseed or sunflower lecithin are still lacking since most of the studies have been carried out using soy lecithin.

Table 6: Phospholipid composition (%) of soy and sunflower lecithin[37]

Phospholipid	Soy lecithin (%)	Sunflower lecithin (%)
Phosphatidylcholine (PC)	15	16
Phosphatidylethanolamine (PE)	11	8
Phosphatidylinositol (PI)	10	14
Phosphatidic acid (PA)	4	3
Other phospholipids	7	6
Total PL %	47	47

Table 7: Fatty acid composition (%) of soy and sunflower lecithin[37]

Fatty acid	Soy lecithin (%)	Sunflower lecithin (%)
C16:0	16	11
C18:0	4	4
C18:1	17	18
C18:2	55	63
C18:3	7	0
Others	1	4

The first step for the production of lecithins from soybean is cold pressing of seeds or hexane extraction in order to obtain the crude oil. Water is added to crude oil to hydrate the phosphatides and the water–oil mixture is then heated at 70 °C for 30–60 min. Afterwards, the oil-insoluble lecithin fraction (a wet gum known as lecithin hydrate) is separated by centrifugation. Lecithin is then dried to a content of <1% moisture to ensure a long-time shelf life and fluidity. To prevent post-darkening, a final rapid cooling process below 50 °C in a heat exchanger needs to be done. The final product will consist of roughly 46% phospholipids, 5% sugars, 11% glycolipids, 37% oil, and 1% water.

Lecithin can be stored at 20–30 °C for years without significant changes in product quality and functional properties.[38]

Due to the presence of hydrophilic and lipophilic segments in their molecular structure, that account for the amphiphilic nature of these molecules, lecithin is largely used as emulsifier for a vast range of food, feed, pharmaceutical and technical applications. Emulsifiers concentrate at the interface between oil and water and subsequently reduce the interfacial tension. Physically fractionated and enzymatically hydrolyzed lecithin (E 322) is an authorized food additive in the EU, covering all standards for use in the food industry. In the US, lecithins are Generally Recognized As Safe (GRAS) by the FDA.[39] Lecithins are employed as pharmaceutical excipients; they have a very wide range of applications in drug delivery systems due to their amphiphilic nature and biodegradability.[40] Lecithin, consisting of various phospholipids including polyunsaturated fatty acids, has skin softening properties and it is therefore used in cosmetic formulations such as skin creams and lotions, foundations and cleansing creams, sunscreens, soaps, bath oils, shampoos, and hair conditioners. Lecithin can also reduce the undesirable oily feeling in cosmetics containing oils and supports moisturizing effects. Phospholipids penetrate the skin and facilitate the penetration of other essential cosmetic ingredients.[39]

Phospholipids are non-crystalline materials difficult to purify; they form stable emulsions and aggregates with a wide variety of compounds of different classes and their separation from unreacted material is not always an easy task. In order to obtain pure PL or enriched fractions of specific PL, lecithin can be submitted to chemical or enzymatic modifications, as well as to a combination of both.

“Oil-free” lecithin can be obtained by de-oiling with acetone. Polar lipids such as PL are insoluble in acetone in contrast to neutral lipids (triglycerides).

Lecithin can be treated with organic solvents to perform the selective extraction of some of its components, thus obtaining fractions enriched in specific PL. This step is useful to reduce the high complexity of lecithin before using it for other reactions. Moreover, enriched lecithin can have different properties depending on its composition.[41] The most interesting example of selective extraction, which was used also in this Thesis (**Chapter 2.3**) is the separation of PC and PI. These two main components of lecithin have a different solubility in short-chain alcohols such as MeOH, EtOH or EtOH/H_2O

mixtures. Specifically, after treatment of lecithin with EtOH, the so-called "soluble fraction" is a "PC-enriched fraction" which contains 73% PC, 24% PE, and 3% PI, based on the total PL content, whereas the "insoluble fraction" can be considered as a "PI-enriched fraction" since it contains 39% PI, still 26% PC, and 35% PE. The outcome of the EtOH treatment is strongly affected by the protocol used (i.e. volume of solvent, time, etc.), therefore the enrichment procedure needs to be finely tuned and standardized in order to have a reproducible yield of the "PC-enriched fraction". The PC-fraction as such is used in food emulsions and instant powders, as a frying agent in margarines and health supplement.[42]

Besides extraction in organic solvents, lecithin composition can be modified by exploiting the difference in the "polar heads" of phospholipids and, indeed, their different chemical reactivity. Free amino groups, such as that of ethanolamine in PE (**Figure 2**), can be acetylated with acetic anhydride, thus making PE soluble (as *N*-acetyl-PE) in acetone and separable from the remaining phospholipid mixture; PE can be thus removed from the "PC-enriched fraction" after alcohol fractionation.

The enzymatic treatments of lecithin are strictly correlated to the modification of its main component, the phospholipids, through different types of phospholipases.

1.4 Phospholipids

Phospholipids (PL) are lipids containing phosphorus, a polar portion and a non-polar portion in their structures; they can be classified into glycerophospholipids and sphingomyelins. They are generally classified on the basis of the polar head (PI, PC, PE, PA, PS, see **Figure 2**). According to their sources, PL can be further classified into natural and synthetic. Natural PL are derived from renewable sources and are produced by more ecologically friendly processes; they are available at a larger scale and are cheaper than synthetic PL. Phospholipids are widely distributed in animals and plants.

Phospholipids are structural and functional components of all cell membranes and play a crucial role in the communication between extra- and intracellular space. Owing to their excellent biocompatibility and amphiphilicity, they are employed as pharmaceutical excipients and they have a very wide range of applications in drug delivery systems and drug targeting. Due to their physiological role, PL possess a very low toxicity profile and can be used for any route of administration: liposomes, oil-in-

water emulsions, mixed micelles for intravenous use and slow release, vaccine vehicles and drug suspensions for intramuscular and subcutaneous administration.[40]

The main sources of PL include vegetable oils (e.g. soybean, sunflower, cotton seed, corn and rapeseed) and animal tissues (e.g. egg yolk and bovine brain). Synthetic PL contain chemically specific, defined polar head groups and fatty acids and are usually obtained by a chemical approach. Depending on the synthetic route, PL may contain intermediates or by-products and unnatural enantiomers may be formed. Synthetic PL are only available in relatively small amounts at a very high cost.[43]

Natural Pl can be isolated from lecithin by column chromatography in high purity. Different adsorbents can be used such as aluminum oxide, silica gel or amberlite, by using different solvent systems. Although chromatography is highly performing, the main issues associated to this technique are costs and time, especially when it is applied to large amounts of lecithin. The large volume of solvents used is another concern.

Considering their applications and costs, PL are for sure a class of high-value chemicals obtainable from lecithin and, generally speaking, from renewable sources. Also in this case, biocatalysis can be a powerful tool for the modification of these molecules.

1.5 Enzymatic modifications of phospholipids

Modification of PL relies on the use of phospholipases and lipases, that hydrolyze the hydrophobic ester bonds of PL and triacylglycerols, respectively. These enzymes catalyze a plethora of other reactions: lipases catalyze also esterification, transesterification, and interesterification reactions, whereas phospholipases show also acyltransferase, transacylase, and transphosphatidylation activities. Hence, lipases and phospholipases are versatile biocatalysts that are widely used for many industrial applications.

Phospholipases (EC 3.1.) form a large class of lipolytic enzymes that are well distributed in most living organisms and play a crucial role in cellular regulation; in fact, they hydrolyze membrane PL, thus generating smaller molecules which are considered as second messengers or membrane signalling agents. Phospholipases hydrolyze PL in different positions according to their specificities, as shown in **Figure 3**.

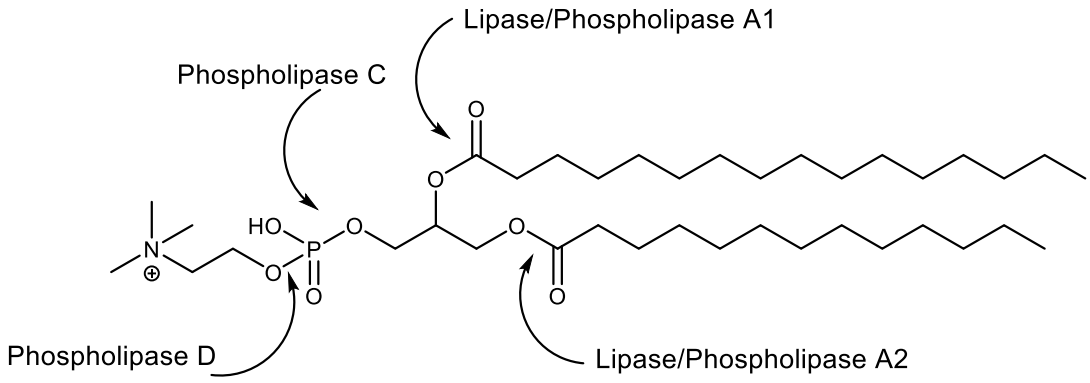


Figure 3: Enzymes involved in phospholipid modifications

PLA1 (EC 3.1.1.32) catalyzes the hydrolysis of fatty acids (FA) exclusively at the *sn*-1 position of PL. The products of a PLA1-catalyzed reaction are free fatty acid (FFA) and a lysophospholipid. PLA1 activities have been detected by measuring hydrolysis of PC to lysophosphatidylcholine in many cells and tissues from various organisms. PLA1 are ubiquitous enzymes in animals, plants, yeast and fungi, protozoa and bacteria. They can be used for hydrolytic reactions in emulsion systems in a free or immobilized form.[44]

PLA2 (EC 3.1.1.4) are the most widely studied phospholipases. They are routinely classified into three categories: secreted PLA2 (sPLA2), cytosolic PLA2 (cPLA2), and Ca^{2+}-independent PLA2 (iPLA2). Hydrolysis of a PL by PLA2 yields a lysophospholipid and a fatty acid.[45]

PLB have been purified from plants, microorganisms, bee venom, and mammalian tissues. They are non-specific enzymes that hydrolyze ester bonds, exploiting a lytic (destructive) activity against biological membranes.[46]

PLC (EC 3.1.4.11) belong to a large superfamily of enzymes called phosphoinositide-specific phospholipases C (PI-PLC). PLC hydrolyze the phosphodiester bond between the phospholipid glycerol residue and its polar phosphate group. PLC isoforms are involved in multiple physiological processes, including growth, cell differentiation, and gene expression. They have been found in a broad spectrum of organisms including bacteria, simple eukaryotes, plants and animals.[46]

PLD (EC 3.1.4.4) catalyze the hydrolysis of the phosphodiester bond of PL to generate phosphatidic acid (PA) and an alcohol moiety. In addition to the hydrolytic activity, PLD also catalyze the conversion of the polar head groups of PL in a reaction called transphosphatidylation. Using this reaction, it is possible to synthesize PL of low natural abundance from highly abundant ones such as PC or lecithin and the corresponding

hydroxyl compounds. This class of phospholipases was first identified in plants; subsequently, a number of PLD isoforms were detected and characterized in mammals, bacteria and yeasts.[44]

Lipases catalyze a broad spectrum of reactions that use triglycerides as substrates; however, owing to their catalytic versatility, they can also be applied to modify the *sn*-1 position of PL. Indeed, many lipases share amino-acid sequences identical to PLA1 and show phospholipase activity.

Phospholipases are used industrially to modify PL. These modifications mainly include the hydrolysis of PL, the synthesis of specific PL, and the conversion from one phospholipid class to another. The hydrolysis of FFA from the glycerol backbone of PL is carried out by using PLA1, PLA2, as well as lipases; in this latter case, the reaction is carried out using specific 1,3-lipases, which are also specific for the *sn*-1 position of PL. To give an example which is related to the second part of this project, phospholipases are used in the production of dairy products. During cheese production, phospholipases are added to milk and allowed to act before the addition of coagulant. The treatment reduces fat losses in whey and cooking water, thus increasing cheese yield, improving fat and moisture retention in the cheese curd without changing the cheese properties.[47]

For the conversion of one PL class to another, PLD-catalyzed transphosphatidylation is used. This is of particular interest for the industrial production of unnatural or low-abundance PL with nutraceutical or pharmacological properties.[48]

1.6 Glycerophosphoric acid esters

Glycerophosphoric acid esters (GPAE) represent a further group of high-value chemicals derived from lecithin; GPAE are PL derivatives in which both the fatty acid chains are removed. The deacylated derivatives of PC, PE, PI and PS find application in many industrial sectors. L-α-Glycerophosphocholine (GPC) is useful as a cognitive enhancer, whereas glycerophospho-*myo*-inositol (GPI) acts as a natural anti-inflammatory agent.[49] GPAE can be prepared by chemical or enzymatic routes. GPI and GPC (**Figure 4**) are the target molecules of the first part of this Thesis. Their biological activity and applications were reviewed along with those of the other GPAE in **Chapter 2.1**.

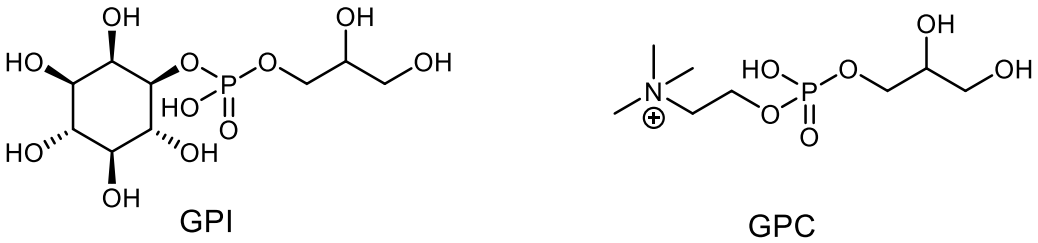


Figure 4: Glycerophosphoinositol (GPI) and glycerophosphocholine (GPC)

1.7 Cheese whey

As already mentioned, carbohydrates are the most abundant organic compounds found in agri-food by-products. Specifically, cheese whey (CW) is the main by-product of the dairy industry. It is the residual liquid after the coagulation of casein, the main milk protein. The coagulation is generally performed by chymosin or by acids (both organic and inorganic). Cheese whey is known since 3000 years when milk coagulated during the storage into veals' stomach.[50,51]

In 2020, about 148 million of tons of milk were produced in Europe and 50% of milk was used for cheese production. The derived CW was estimated to be 55 million of tons, corresponding to 80-90% total volume of the milk used.[52]

Cheese whey is an aqueous medium containing 50% of the hydrosoluble components of milk. Lactose is the most abundant of these components (75%) followed by proteins, minerals and other substances (**Figure 5** and **Table 8**).

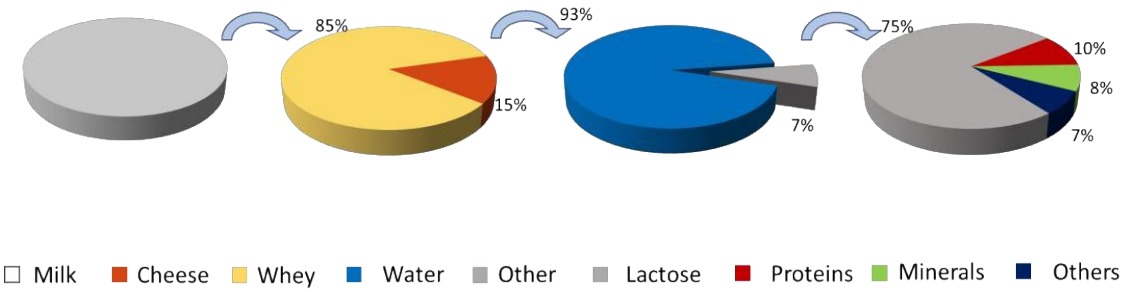


Figure 5: Composition of cheese whey and cheese whey permeate (modified from Tsermoula et al.[53])

Table 8: Composition of milk and cheese whey (modified from Tsermoula et al.[53])

Components	Amount (% p/v)	
	Milk	Cheese whey
Casein	2.8	<0.1
Proteins	0.7	0.7
Lipids	3.7	0.1
Ashes	0.7	0.5
Lactose	4.9	4.9
Total solid	12.8	6.3

Cheese whey is the main by-product of dairy industry. Owing to the high disposal costs and the environmental burden associated to CW, processes for its recycle/reuse are actively being sought. Although CW does not contain toxic substances, it has high BOD

(Biochemical Oxygen Demand, 30-50 g/L) and COD (Chemical Oxygen Demand, 60-80 g/L) values which do not allow to dispose it in the wastewater.

The high costs of CW disposal, the availability of new technologies, the rapidly growing food and nutraceutical market, the rising awareness about the threats of climate change and the decrease of natural sources, have raised the attention towards CW upgrading, which is not considered a dairy waste anymore, but a relevant co-product of cheese manufacturing.[54] Due to the different nature of the valuable molecules contained in CW, its fractionation is a key step for fully exploiting it. Ultrafiltration and diafiltration allow the separation and the recovery of the whey proteins, thus leaving cheese whey permeate (CWP), which contains lactose, mineral salts and traces of vitamins.[54,55]

As mentioned above, CW contains up to 20% proteins and it can be thus used as a source of bioactive peptides suitable as food additives. The CW protein hydrolysates are generally produced by enzymatic hydrolysis because this process is easier to control than chemical hydrolysis or fermentation. The market of bioactive peptides is estimated to reach 48 billion USD in 2025. Moreover, the CW proteins contain essential amino acids, leucine, isoleucine, valine (regulators of the metabolism of proteins, lipids and glucose), cysteine and methionine (precursors of glutathione).[55]

Beside the recovery of proteins, CW and CWP can be converted into useful products by microbial fermentation. Many of these products have a high industrial interest and methods to produce them from this biomass have been extensively studied and reviewed over time. Carboxylic acids (lactic, propionic, acetic and succinic acids), bioalcohols (ethanol and 1-butanol), and biogases are some of the bio-products that can be obtained in this frame. Biopolymers such as dextran, xantan, and polyhydroxyalkanoates (PHA), received lesser attention, although their production could provide sustainability and economical boost for several food-related applications.[56] Polyhydroxyalkanoates, in particular, were reported as the only “bio-plastics” with a whole “green” life-cycle: renewable resources act as feedstock of the production (bio-based), living cells are responsible for both the synthesis of their monomeric building blocks and their subsequent polymerization (bio-synthesized), the products do not show adverse effects on the biosphere (biocompatibility) and, lastly, they endure degradation by the action of living organisms (biodegradability), such as

gram-positive and gram-negative bacteria. Nevertheless, production of PHA on industrial scale is still challenging for the economical sustainability.[56]

The valorization of CW and CWP through fermentation also includes the production of single-cell oil (SCO) accumulated by oleaginous yeasts. Donzella and co-workers[29] developed and optimized a two-step fermentation process that employs the oleaginous yeast *Cutaneotrichosporon oleaginosus* for the production of oil from liquid CWP. In the second step of the fermentation, another food waste (syrup from candied fruit processing) was also used. Microbial lipids were accumulated at a final concentration of 38 g/L. These lipids can be then used as hydrophobic tails for the enzymatic synthesis of sugar-based surfactants, one of the goal of this Thesis.

Last but not least, CWP is a source of lactose and all the high-value chemicals derivable from it (especially prebiotics) which are discussed in the next paragraphs.

1.8 Lactose

Lactose is the main component of milk. It is a natural disaccharide composed of D-galactose and D-glucose units connected by a β (1-4) glycosidic bond. Lactose is a reducing sugar and gives mutarotation in water thus generating the two anomers (α and β). The mutarotation is minimal at pH 5 and low temperature, while it occurs rapidly at high temperature (75 °C) and/or alkaline pH.[57] Lactose has a peculiar solubility profile: at low temperatures (15 °C) the β anomer is much more soluble (50 g/L) than the α anomer (7 g/L). At room temperature, the α anomer is favored by mutarotation equilibrium, thus resulting in a low solubility. Lactose solubility in water is only 10% of that of sucrose; lactose is poorly soluble also in alcohols.[58]

In the pharmaceutical industry, lactose is routinely used as bulking agent in many formulations. In fact, it can be obtained in different crystalline forms which display different chemical, physical, and technological properties.[59]

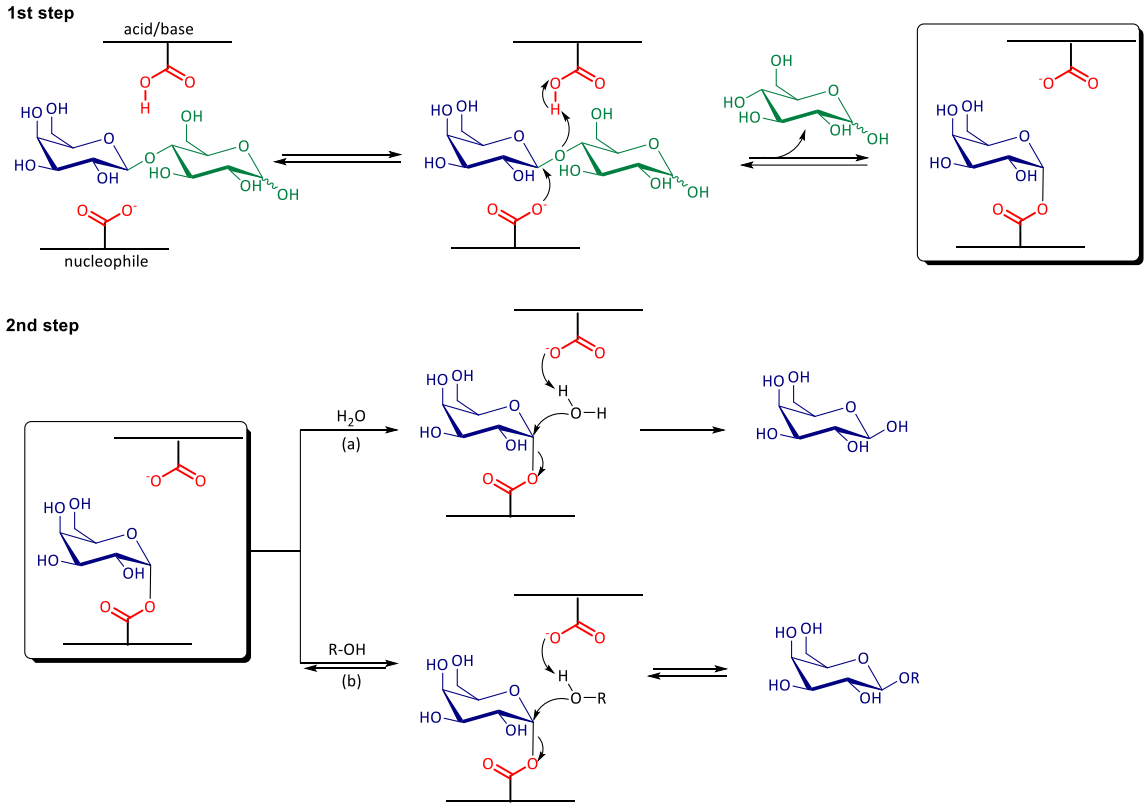
Lactose is a low-value commodity derived from CW, but has a high potential as raw material.[54]

1.9 Enzymatic modifications of lactose

Talking about biocatalysis and lactose (or CW), it is impossible not to mention β-galactosidases (β-Gal). β-D-Galactoside-galactohydrolase (E.C. 3.2.1.23) catalyzes the

hydrolysis of a glycosidic bond between a terminal non-reducing β-D-galactoside unit and an aglycone moiety. β-Galactosidases belong to the glycosidases family, whose mechanism is reported in **Scheme 1**.

The enzyme active site contains two glutamic acid residues (GluA1 and GluA2), which can act as an acid or a base, respectively. In the first step, Glu1 acts as an acid by protonation of the anomeric oxygen, making the (oligo)saccharide moiety [RO] a good leaving group, while the glycosyl residue is bound to the enzyme via GluA2 as oxonium ion. Then, the leaving group ROH is displaced by the incoming nucleophile NuH (usually water) via diffusion. In the second step, the nucleophile is deprotonated by Glu1 and the glycosyl-enzyme intermediate attacks from the same face from which the leaving group R-OH was expelled. Since both steps constitute an SN_2-reaction, double inversion results in net retention of configuration.[11] It is important to underline that inverting glycosidases also exist, but all the known β-Gal share the retaining mechanism.[60]



Scheme 1: Catalytic mechanism of β-Gal for the hydrolysis and transglycosylation of lactose (modified from Faber[11])

Most applications of this enzyme are related to the hydrolysis of lactose; for this reason, β-Gal has been commonly referred to as lactase. However, its biological role is not limited to such a reaction and, in fact, not all the β-galactosidases are able to hydrolyze

lactose. The enzymatic hydrolysis of lactose into its monosaccharide units (glucose and galactose) catalyzed by β-Gal made it one of the most important industrial enzymes, that has been used for many decades in the dairy industry to produce low-lactose or lactose-free milk and products, lactase tablets for lactose-intolerant people, production of whey syrups, and for dairy waste management.[60] The enzymatic hydrolysis is preferred to the acid hydrolysis because the latter requires the absence of protein and a tedious work-up to remove colored by-products.[61]

As depicted in **Scheme 1**, if water is replaced by an alcohol, the transglycosylation reaction can occur, thus broadening the applications of β-Gal (in the case of these specific enzymes, the term "*transgalactosylation*" is often used). These applications involve a strategy that favors transglycosylation over hydrolysis. This includes the use of very high substrate concentrations, the use of organic (co)-solvents, the use of ionic liquids or any other low water activity media. However, these reactions are still challenging because the use of non-aqueous media may impair the enzyme activity and stability, and severely reduces lactose solubility. Moreover, since transglycosylation is a kinetically controlled reaction, the production of transglycosylated compounds reaches a maximum. After that, the rate of hydrolysis and transglycosylation become equal and the former prevails, thus hydrolyzing the desired transglycosylated product (decreasing conversions and yields). For these reasons, the reaction must be quickly arrested and this is an easier operation when an immobilized enzyme is used.[60]

It is important to underline that glycosidases can be used also for the direct glycosylation (known also as "reverse hydrolysis"). However, this condensation reaction releases water as the by-product which can easily start hydrolysis. High concentrations of both the monosaccharide and the nucleophilic component (carbohydrate or alcohol) must be used. Therefore, yields in these reactions are generally low. Moreover, this reaction is hampered by the low solubility of sugars in apolar solvents. Some strategies to improve the reaction yield is the use of biphasic systems, very low temperature (0 °C), or the selective adsorption of the product but, in general, this approach is not ideal.[11] However, the reverse hydrolysis has been recently reported for the synthesis of alkyl glucosides with good results, thanks to the use of unconventional, low-water activity media such as ionic liquids and deep eutectic solvents.[62]

Going back to the transglycosylation, this reaction was applied to the synthesis of lactose-derived prebiotics (compounds able to resist to acid and enzymatic degradation and reach gut microbiota, where they act on bacteria associated with well-being, like *Lactobacilli*)[54] such as galacto-oligosaccharides (GOS) and lactulose (4-*O*-β-D-galactopyranosyl-D-fructose). The former are produced almost exclusively by biocatalysis since the chemical route results in a mixture of oligosaccharides with α and β linkages and anhydro sugars, not suitable for the prebiotic activity.[60]

As for lactulose, its synthesis by transglycosylation of lactose is much more complex than the GOS synthesis because it involves two sugars (lactose and fructose). Thus, along with lactulose synthesis, transgalactosylated oligosaccharides (TOS), namely GOS and fructosyl-galacto-oligosaccharides (fGOS), are formed and the reaction becomes challenging to control. For these reasons, this approach is suitable for the preparation of prebiotic mixtures, but not for the synthesis of pure lactulose for pharmaceutical use (lactulose is used for the treatment of hepatic encephalopathy and constipation). For this latter use, lactulose is still produced by chemical isomerization of lactose.[54]

If the sugar is replaced by an alkyl alcohol, the transglycosylation results in the formation of alkyl-β-galactosides. These compounds are biodegradable, non-toxic and hypoallergenic non-ionic surfactants that can be a sound alternative for the substitution of *p*-nonylphenol and their ethoxylated derivatives.[60] In fact, they are structurally similar to alkyl polyglycosides, an important class of commercial carbohydrate-based surfactants (see **Chapter 1.10**). Their preparation by enzymatic transglycosylation is also a more sustainable alternative to the chemical production (Fischer glycosidation), which uses harsh conditions and has a low selectivity (formation of α and β isomers and mixtures of pyranosidic and furanosidic forms).

The chain of alkyl galactosides should contain at least eight carbon atoms to exert surfactant properties, but the β-Gal productivity is quite poor with long alcohols.[60] This represents a shortcoming in the application of these enzymes in the synthesis of these sugar-based surfactants. On the other hand, a number of shorter chain (1-6 carbon atoms) alkyl β-galactosides was reported with good yields (**Table 9**). This step can be considered a smart modification of the sugar to increase its solubility in organic solvents, thus making it suitable for an acylation step to obtain a sugar fatty acid ester.[63,64] As it is

reported in this Thesis, this sugar modification does not negatively affect the surfactant properties of the final product (**Chapters 3.1-3.2**)

Table 9: Synthesis of alkyl β-galactosides catalyzed by β-galactosidases from different sources. Modified from Vera et al.[60]

β-Gal source	Donor (mM)	Acceptor	T (°C)	pH	Y (mol/mol)
A. oryzae	Lactose (13.3)	1-butanol	25	4.5	0.87
	Lactose (29)	1-hexanol	45		0.79
	Lactose (16.6)		35		0.06
	Butyl-β-galactoside (16.6)		35		0.49
	Propyl-β-galactoside (16.6)				0.47
	o-Nitrophenyl-β-D-galactopyranoside (16.6)				0.59
	Lactulose (16.6)				0.12
B. pseudofirmus	Lactose (150)	1-hexanol	40	9.5	0.5
K. lactis	Lactose (500)	methanol	40	6.8	0.71
	Lactose (1000)	ethanol			0.36
Pseudoalteromonas sp. 22b	Lactose (487)	1-butanol	30	8	0.18
		1-hexanol			0.11
S. solfataricus	Lactose (93)	1-hexanol	75	7	0.51
A. oryzae	Lactose (290)	ethanol	40	4.5	0.27
		1-propanol			0.25

Lactose itself can also be used as the polar head of sugar fatty acid ester surfactants. Even if its solubility issues make it less suitable than other disaccharides like sucrose,[55] some examples of lactose esterification by lipases have been reported.[65–67]

For completeness, there are other high-value chemicals derivable from lactose (**Figure 6**):

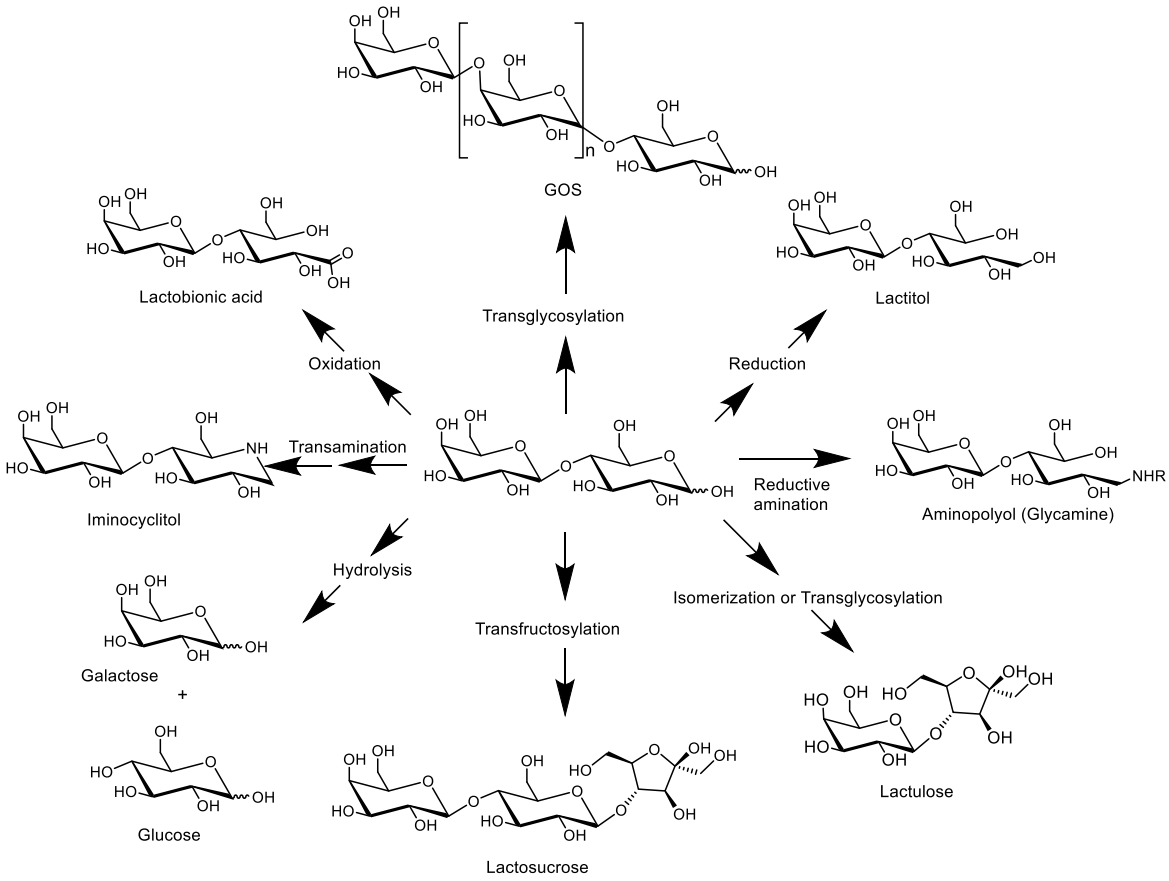


Figure 6: High-value molecules derived from lactose

- Lactosucrose(*O*-β-D-galactopyranosyl-(1,4)-*O*-α-glucopyranosyl(1,2)-β-D-fructofuranoside): a synthetic trisaccharide mostly produced by transfructosylation of lactose in a reaction catalyzed by β-fructofuranosidase and by enzymatic transgalactosylation of sucrose with *B. circulans* β-Gal. It has prebiotic properties.[54]
- Lactobionic acid: obtained by the oxidation of lactose. It functions as an antioxidant, chelating, humectant, and low-calorie sweetener. It can be prepared by chemical reaction, fermentation, or by biocatalysis by coupling cellobiose dehydrogenase and laccase.[68]
- Lactitol: it is the lactose-derived sugar alcohol (4-*O*-β-D-galactopyranosyl-D-glucitol). It is not involved in the Maillard reaction. Lactitol provides several health benefits, such as reduction of obesity, diabetes, cardiovascular diseases, hepatic encephalopathy, and constipation. It is produced exclusively by chemical approaches.[68]

- Tagatose: it is a considerable sweetener playing an important role in preventing tooth decay, controlling obesity and blood sucrose level. Tagatose can be produced by a chemo-enzymatic isomerization process in which lactose is hydrolyzed by lactase followed by the transformation of galactose into tagatose in the presence of a calcium catalyst.[68] The second isomerization step can be performed also by the enzyme L-arabinose isomerase.[54]

1.10 Carbohydrate-based surfactants

Surfactants are amphiphilic substances that show an affinity for hydrophilic and lipophilic phases thanks to their structure, made of a hydrophilic moiety (which can be ionic, zwitterionic or non-ionic) and a hydrophobic moiety (usually a hydrocarbon chain). These compounds, owing to their unique structure, can decrease the surface tension between liquid and gas or interfacial tension between two immiscible liquids or between liquid and solid, thus changing the wettability of the solid and increasing the solubility of hydrophilic solids. Surfactants also associate forming micelles and other organized structures that allow to increase solubilization effects.[26,69] For these reasons, surfactants are used as emulsifiers, dispersants, wetting agents, and foaming agents in countless industrial and consumer products. Surfactants are the most common compounds used in personal care industry. This market sector includes cosmetics and personal hygiene products (oral care, deodorants, hair and skin care).[70] Moreover, surfactants are also used in engineering and industrial manufacturing sectors such as: pulp and paper processing, fiber and textile manufacturing, metal and mineral processing, food processing, pigments, paints and coatings industry, crude oil production, lubricant industry. In the pharmaceutical industry, surfactants are important constituents of several transdermal topical formulations, medicinal compositions, and drug and gene delivery systems.[71]

Surfactants are widely used chemical products that can be classified as bulk and specialty chemicals accounting for very high production volumes worldwide. Surfactants are routinely manufactured from petrochemical feedstock (e.g. ethylene oxide for the ethoxylation or the polymerization of alkenes) or by using a combination of renewable and petrochemical feedstock.[72] Developing new sustainable approaches to lower the carbon footprint and to reduce the dependence on petrochemicals is a priority, in

agreement with the Agenda 2030 for sustainable development that underlined, for the years to come, the need for developing technologies based on sustainable chemistry and engineering. A lot of manufacturing companies are now investing on biosurfactants or surfactants from renewable sources and their market is expected to overtake that of synthetic surfactants in the near future.[72]

As described in **Chapter 1.2.1**, carbohydrates are ideal feedstock for chemicals since they are the most abundant organic compounds worldwide. Carbohydrates represent 95% of the biomass volume generated annually.[26]

With these premises, it is not surprising that bio-based surfactants with carbohydrate sourced head groups, known also as glycolipids, are one of the most interesting and studied chemicals derivable from carbohydrates and one of the most important classes of surfactants. Their structure consists of a carbohydrate connected with a lipophilic moiety through a covalent bond. Considering the different reactivity of hydroxyl groups of carbohydrates, the nature and the number of the bonds which connect the hydrophilic moiety to the hydrophobic one, and the nature of the hydrophobic moiety itself, the combinations are almost endless.[73] The classification of carbohydrate-based surfactants is not univoque. Glycolipids can be classified according to their amphiphilic structure, which depends on the polar headgroup, the apolar tail, but also to the linkage between these main entities (**Table 10**). Some examples of carbohydrate derived surfactants are reported in **Figure 7**.

Table 10: Classification of glycolipids (modified from Razafindralambo et al.[73])

<table>
<tr><th>Glycolipid</th><th>Hydrophilic head</th><th>Hydrophobic tail(s)</th><th>Linkers</th></tr>
<tr><td>Monocatenary</td><td rowspan="5">One headgroup</td><td>One tail</td><td rowspan="7">Ester, ether, thioester, amide, amine</td></tr>
<tr><td>Bicatenary</td><td>Two tails</td></tr>
<tr><td>Multicatenary</td><td>Multi tails</td></tr>
<tr><td>Glycoglycerolipids</td><td>Acyl glycerol</td></tr>
<tr><td>Glycosphingolipids</td><td>Sphingoides</td></tr>
<tr><td>Bolaforms</td><td rowspan="2">Two headgroups</td><td>One spacer</td></tr>
<tr><td>Geminis</td><td>One spacer/two tails</td></tr>
<tr><td>Alkylpolyglycosides</td><td>Multi headgroups</td><td>One or multi tails</td><td></td></tr>
</table>

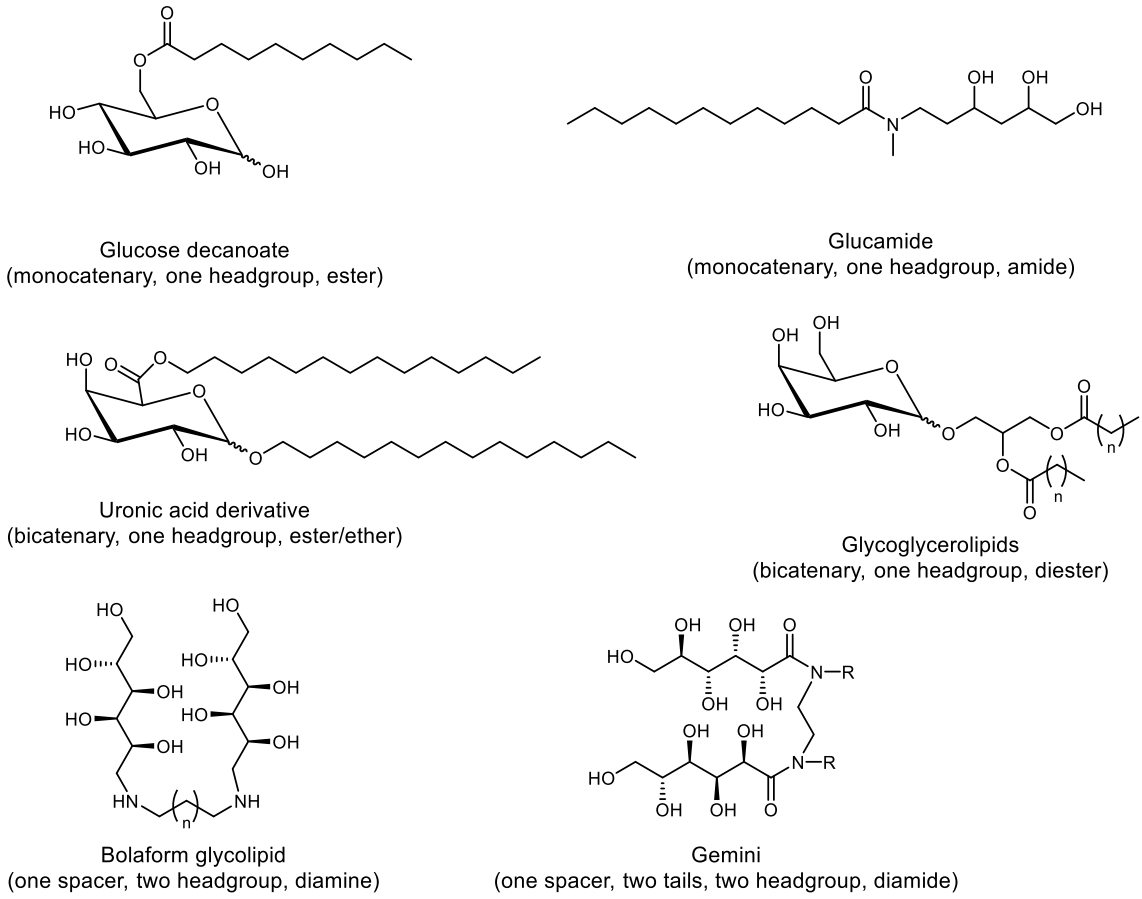


Figure 7: Chemical structures of some of the glycolipids reported in **Table 10**

About glycolipids, it is common to find the terms "*biosurfactants*", "*biobased surfactants"*, "*natural surfactants*", and "*surfactants derived from natural sources"* often used as synonyms. More precisely, biobased surfactants are derived in whole, or by significant part of biological products, renewable agricultural materials (including plant, animal, and marine materials), or forestry materials. The European Commission of Standardization (CEN) derived a classification of biobased surfactants based on their biobased content (i.e., percentage of carbon atoms derived from renewable resources): wholly biobased (>95%), majority biobased (50%–94%), minority biobased (5%–49%), and nonbiobased (<5%).[74] Natural surfactants are molecules that derive directly from a natural source such as gangliosides and cerebrosides from animals and butanolide glycosides from plants. Biosurfactants refer specifically to the natural surfactants from microbial sources[74] like sophorolipids, rhamnolipids, and mannosylerythritol lipids.[69] A glycolipid produced by one or more reactions starting from natural building blocks

should be technically defined as a surfactant derived from natural sources. However, it is possible to label a molecule as “natural” if biocatalysis is used for its preparation. Moreover, in comparison with the microbial glycolipids, the surfactants obtained by the use of single enzymes are simpler in structure and can be designed to have the desired physicochemical features.[75]

The commercially available carbohydrate-derived surfactants are sucrose esters, alkyl polyglycosides and sorbitan esters (**Table 11** and **Figure 8**).

Table 11: Market of carbohydrate-based surfactants (www.marketsandmarkets.com/Market-Reports/natural-surfactant-market-25221394.html)

Sugar-based surfactants	Fields of application	Market impact (million €)
Sucrose esters	Food, personal care, pharmaceuticals	70 by 2020
Alkyl polyglycosides (APG)	Personal care, detergents, agrochemicals	1250 by 2024
Sorbitan esters	Pharmaceuticals, personal care, food, agrochemicals, coatings	670 by 2023

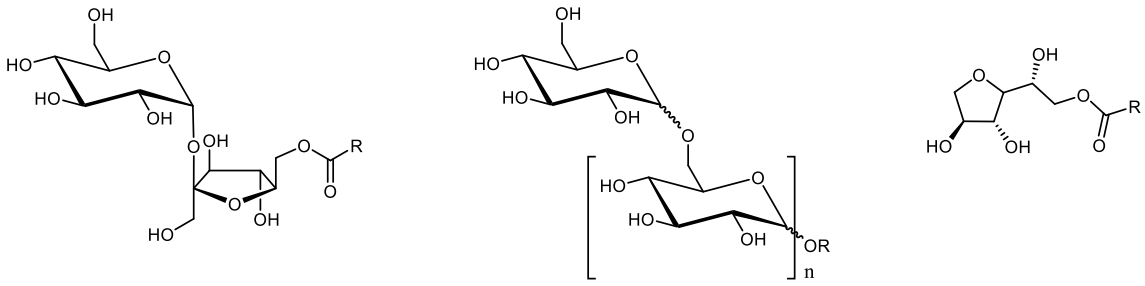


Figure 8: Chemical structures of commercial carbohydrate-based surfactants. From left: sucrose esters, alkyl polyglycosides (APG) and sorbitan esters

Sorbitan esters are typically synthesized via direct esterification of sorbitol with fatty acids and an alkaline catalyst to give a mixture of mono-, di- or trisorbitan esters that are suitable as water-in-oil emulsifiers. Sorbitan stearate and sorbitan isosterate are recorded as the most used surfactants in personal care.[70]

Alkyl polyglycosides are often preferred as surfactants due to their good biodegradability, dermatological and ocular safety and origin from renewable feedstock.

In fact, their glucose-based polar head group is derived from corn, whereas the fatty alcohol group is mainly derived from palm kernel oil.[75]

Sucrose esters will be discussed in **Chapter 1.10.1** since they are sugar fatty acid esters, the sub-class of carbohydrate-based surfactants that was investigated in this Thesis.

1.10.1 Sugar fatty acid esters

Sugar fatty acid esters (SFAE) are non-ionic surfactants, which contain one or more saccharide rings, for example, sucrose, linked to one or more hydrophobic fatty acid chains.[76] The most common fatty acids found in sugar esters are lauric (C12:0), myristic (C14:0), palmitic (C16:0), stearic (18:0), oleic (C18:1), behenic (C22:0), and erucic acid (C22:1), while the most common polar heads are glucose, fructose and sucrose; lactose esters have received less attention to date, despite recent studies have shown their high potential.[77] Sucrose esters are already commercially available for a variety of uses.[76]

SFAE are nontoxic and biodegradable amphiphilic molecules, which have a range of applications as emulsifiers in food, cosmetics, and pharmaceutical products.[74,78] In addition, SFAE have been reported to have antimicrobial, antitumor and plant growth inhibition[79] properties. For these reasons, SFAE demonstrated to be interesting for the pharmaceutical industry[80] as biologically active molecules or as excipients for drug delivery.

Moreover, being tasteless, odorless, nontoxic, and biodegradable, these features make them excellent biocompatible food emulsifiers.[81] Their use in food industry is not limited to emulsions. In fact, sucrose laurate can be used for delivering flavors in food and beverages. Another sucrose ester with a high degree of esterification is contained in Olestra, a noncaloric substitute for diary fat which is not adsorbed from the small intestine and has a fat-like taste. On the other hand, amongst the few examples of lactose esters, lactose laurate showed to inhibit the growth of strain cocktail of *L. monocytogenes* in yogurt and cheese, thus highlighting promising applications in preventing food contamination. SFAE are also used in skin-care products since they are not irritating.[66,76]

Depending on the sugar head and the length of the acyl chain(s), SFAE can be tailored for a specific application to fine tune hydrophilic-lipophilic balance (HLB) and, indeed, their physicochemical properties. Sugar esters with low HLB values (HLB 5-6) are good

water-in-oil emulsifiers, whereas medium HLB value (7-9) correlates with good wetting properties, and a high HLB value (10-16) makes the SFAE suitable emulsifiers for oil-in-water emulsions. For instance, sugar esters with high HLB values yield low viscosity emulsions suitable for thin skin lotions; for example, sucrose esters of coconut oil-derived fatty acids are widely used as emulsifiers in skin moisturizers.[76]

As mentioned before, carbohydrate-based surfactants are attractive because of their higher biodegradability than that of older synthetic surfactants and, upon biodegradation, they would be able to release quantitative carbon, which has been previously used by plants to build renewable feedstocks.[71] In particular, SFAE undergo easily to biodegradation because numerous varieties of esterases are already present in the environment, thus ensuring biodegradation of any substrate-containing ester linkage.[71] However, this outcome cannot be generalized because of the great number of possible molecules (different sugars, fatty chains, degree of esterification) and should be evaluated case by case.

These valuable chemicals can be prepared by chemical and/or enzymatic approaches, as explained in the next paragraphs.

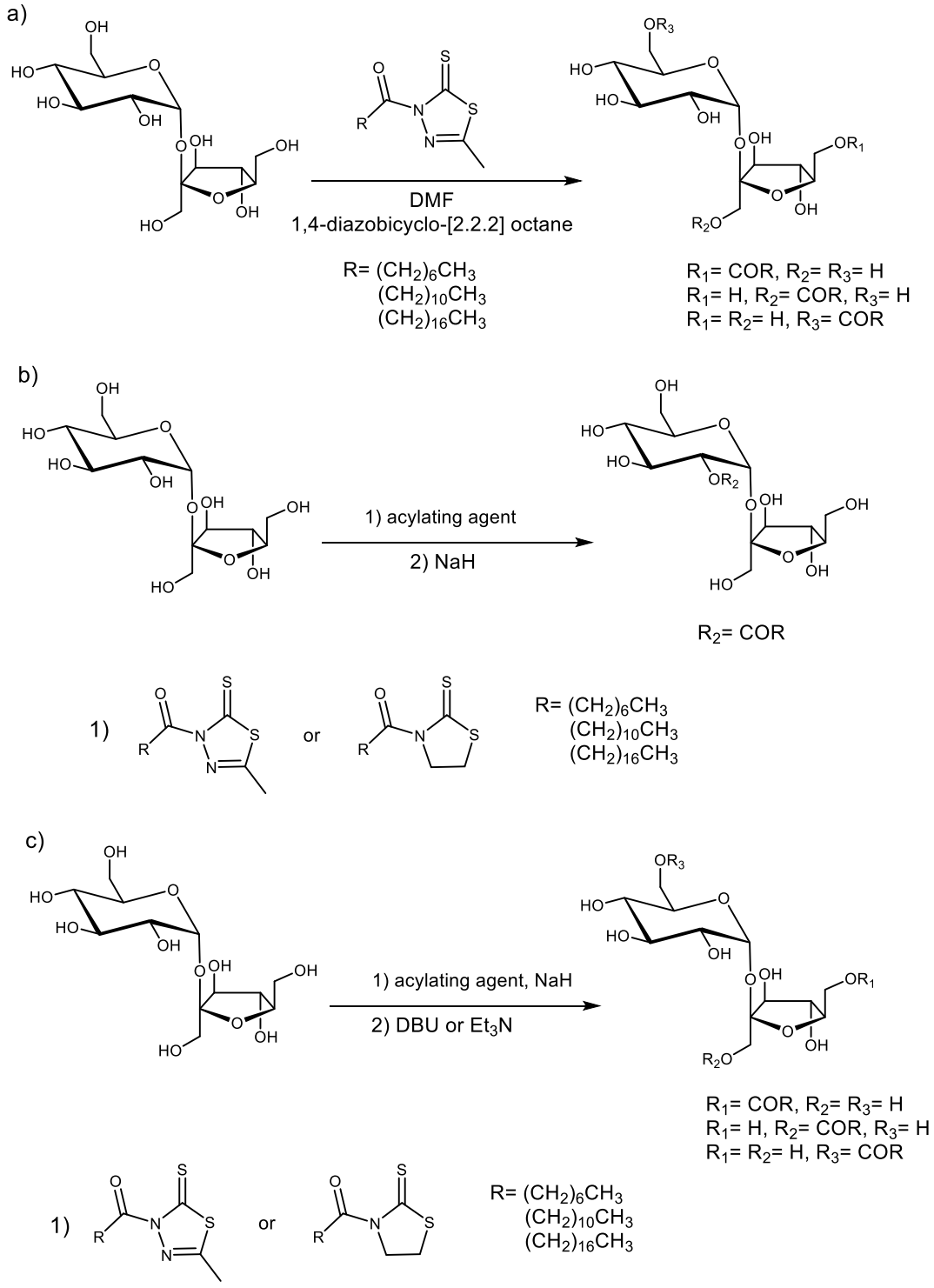
1.11 Synthesis of sugar fatty acid esters

1.11.1 Chemical synthesis

Generally speaking, the chemical synthesis of SFAE by esterification/transesterification requires high temperatures and the use of reduced pressure to remove the reaction by-products (water or alcohols). As a result, the high energy consumption as well as the formation of undesired products derived from discoloration, dehydration and polymerization of the sugars constitute a severe shortcoming of this approach. Moreover, the chemical synthesis of SFAE is plagued by low regioselectivity that has an impact especially on sucrose esters, due to the presence of three primary alcohols with a similar reactivity.[76,82]

However, several examples of regioselective chemical acylation of sugars have been reported. To name a few, the regioselective 6'-*O*-acylation of sucrose was achieved using 3-acyl-5-methyl-1,3,4-thiadiazole-2(3H)thiones as acyl donor in DMF and 1,4-diazobicyclo-[2.2.2] octane at low temperatures with modest (35-43%) yield (**Scheme**

2a). The same research group obtained also 2-*O*-acyl sucrose derivatives by using a different acyl donor (3-acyl-thiazoledine-2-thione) in presence of NaH (**Scheme 2b**).[83] Another research group reported the intramolecular isomerization of 2-*O*-acyl sucrose (prepared with similar acylating agents) using 1,8-diazabicyclo [5.4.0] undec-7-ene (DBU) or aqueous Et_3N, thus obtaining 6'-*O*-acyl sucrose (**Scheme 2c**).



Scheme 2: Chemical synthesis of sucrose esters (modified from Pérez et al.[76])

Another strategy consists of the acylation of the sugars with highly activated fatty acid derivatives like anhydrides and acyl chlorides, as reported to prepare different lactose fatty acid esters using *N*-methyl-2-pyrrolidinone and pyridine at 70 °C as the reaction medium.[77] The same type of reaction was reported also at lower temperatures, but with the previous modification of the sugars as dibutylstannylene acetals.[76,77]

The most recent and industrially used chemical method for the preparation of commercial sucrose esters consists of the transesterification of the unprotected sugar with fatty acid methyl esters (FAME) at 90-95 °C in DMF and in presence of K_2CO_3. After neutralization of the catalyst and evaporation, the product is obtained as a mixture containing 70% monoester and 30% higher (di and tri) esters. This process was recently analyzed according to the Green Chemistry principles.[70] From this assessment, atom economy (2nd principle) was 94% (if the esters are used in mixture) and the conditions used answered the 4th principle (safe chemicals), the 7th principle (renewable feedstock), the 10th principle (biodegradability), the 9th principle (catalysis) with respect to the established production of sucrose esters (and of alkylpolyglycosides as well, analyzed in the same review). On the other hand, this process did not answer the 3rd, 5th and 6th principles at all because of the toxicity of DMF and CH_3OH and the use of high temperatures. Thus, there is still room for improvement. In this frame, the authors underlined that the use of biocatalysis and a solventless system can play a key role in the improvement of the "green credentials" of SFAE and the synthetic processes to obtain them.[70]

1.11.2 Enzymatic synthesis

Enzymatic esterification is a more friendly and food compatible approach for the preparation of SFAE. The use of enzymes combines milder reaction conditions to higher regio- and stereoselectivity which allow to obtain a specific sugar ester without the need of protecting groups (see **Chapter 1.2**).

Lipases (EC 3.1.1.3) or triacylglycerol acyl hydrolases belong to the serine hydrolase family; they are also called carboxylic acid esterases and are abundantly present in nature.[84] As suggested by their second name, the natural role of lipases is the hydrolysis of the ester bond between carboxylic acids and glycerol in triglycerides. However, in organic and/or low water media, they can catalyze also direct esterification, alcoholysis,

interesterification and transfer of acyl groups from esters to other nucleophiles like amines or thiols involving long- to short-chain carboxylic acids. Lipases are probably the most used class of enzymes since the early days of biocatalysis and the knowledge of their structure, catalytic mechanisms, as well as their applications are continuously growing.

The acylation site and the degree of esterification are correlated to the structures of the substrates (sugar and acyl donors), the enzyme used, and the reaction medium.[76]

Several commercial lipases were reported for sugar acylation under very different conditions (e.g. solvents, temperature and time).

The enzymatic acylation of unprotected sugars in organic solvents was pioneered by Klibanov and co-workers. In 1986, they reported the transesterification of glucose and other monosaccharides with trichloroethyl esters of different carboxylic acids catalyzed by lipase from porcine pancreas in pyridine with good yields and regioselectivity (the acylation occurred on the primary hydroxyl group).[85] The same group further investigated the regioselectivity of lipases from different sources (porcine pancreas, bacterial, yeast, and fungal) for the secondary hydroxyl groups of C-6 blocked sugars in THF or CH_2Cl_2, thus observing the acylation of C-2 and/or C-3 depending on the enzyme.[86]

The same methodology was extended to the preparation of potential biosurfactants by reacting sugar alcohols (which resulted more reactive than the corresponding sugars) with different vegetable and animal oils (e.g. corn, apricot, cotton seed and others) in dry pyridine, thus further highlighting the feasibility of lipases for the acylation of carbohydrates.[87]

Despite the advantages of the enzymatic approach, the synthesis of SFAE is still challenging because of the opposite solubility of sugars and fatty acids. For the synthetic reaction, solvents should not contain water to avoid the hydrolysis of the ester formed. Water miscible solvents can dissolve high amounts of sugars without the need of solubilization reagents (e.g. organoboronic acids), but they generally have a negative effect on the hydration status of the enzyme, thus affecting the esterification. Such solvents include DMF, DMSO, THF, and pyridine which are quite good solvents for the acyl donors, but they are all not “preferred” solvents for safety and environmental issues, besides being less than ideal in some cases for the product downstream.[88] On

the other hand, apolar solvents such as aliphatic and aromatic hydrocarbons are generally well-tolerated by lipases, but sugars are practically insoluble, thus resulting in very low conversions after long reaction time.[65,76,89] Amongst the solvents suitable for enzymatic acylation of sugars, tertiary alcohols (e.g. *tert*-butanol) were also reported because they combine a good stability of the enzymes with a partial solubilization of the sugars, including disaccharides.[90] Moreover, they are less toxic and less challenging than DMF, DMSO and pyridine.

It is important to underline that proteases are another enzyme class suitable for the synthesis of SFAE, as reported in other papers.[82] In fact, serine proteases share with lipases a common active center head nucleophile that is mechanistically essential for a transesterification reaction.[91] Subtilisin (protease from *Bacillus subtilis*) was found to be stable in DMF and it was used for the acylation of sugars, including disaccharides (sucrose, cellobiose, lactose, and maltose) and oligosaccharides. 2,2,2 Trichloroethyl butyrate was used as acyl donor using the same approach reported by Therisod & Klibanov.[85] Moreover, thanks to the broad substrate specificity of subtilisin, the same process was extended also to molecules containing sugar and bulky aglycons like riboflavin and nucleotides.[91]

The same process was applied to the acylation of di- and oligosaccharides containing a fructose moiety, including lactulose, a lactose derived prebiotic (see **Chapter 1.9**).[92,93]

Beside the acylation catalyzed by subtilisin, authors reported also a hydrolytic step catalyzed by a glycosidase to cleave the glycosidic bond, thus obtaining 1-*O*-fructose esters.[93] Interestingly, a very similar combination of enzymes was used (for a different purpose) also in this Thesis (see **Chapter 3.2**).

Despite the versatility of subtilisin, the recent literature on the biocatalysis for glycolipids synthesis is more focused on lipases, which remain the most studied enzyme class.

A recent review[79] gave a systematic and clear picture of the recent advancement about the enzymatic synthesis of SFAE catalyzed by CalB, as depicted in **Figure 9a-b**.

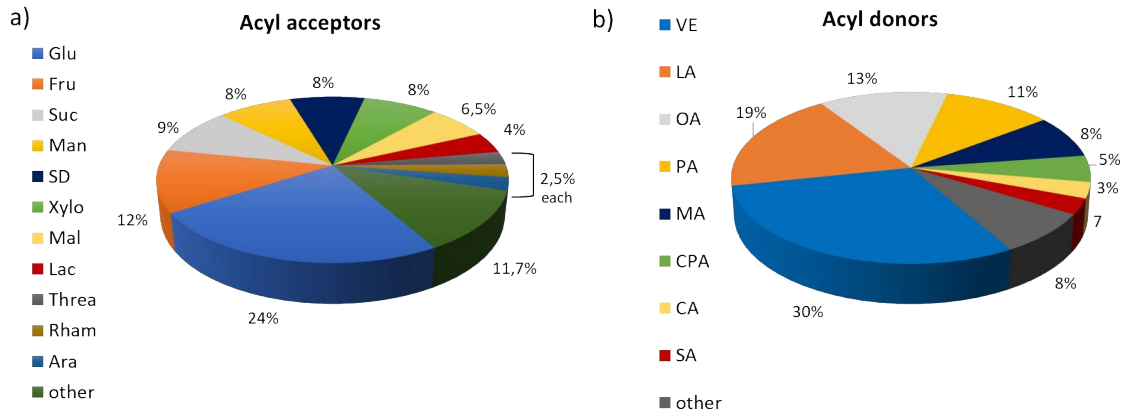


Figure 9a-b: Occurrence (%) of acyl acceptors and acyl donors in the CalB-catalyzed synthesis of SFAE (modified from Gonçalves et al.[79]). Glu= glucose; Fru= fructose; Suc= sucrose; Man= mannose; SD= sugar derivatives; Xylo= xylose; Mal= maltose; Lac= lactose; Threa= threalose; Rham= rhamnose; Ara= arabinose; VE= vinyl esters; LA= lauric acid; OA= oleic acid; PA= palmitic acid; MA= myristic acid; CPA= caprylic acid; CA= capric acid; SA=stearic acid

According to **Figure 9a-b**, glucose is the most studied acyl acceptor (24%) followed by fructose (12%) and sucrose (9%). The use of raw sugar mixtures (e.g. honey and agave syrup) as acyl acceptor is also mentioned (even if in less than 3% of the set).

This report showed that the use of vinyl esters as activated acyl donors is still largely used (30%), despite the toxicity of acetaldehyde. Amongst the free fatty acids, the most common acyl donors are lauric acid (19%), oleic acid (13%) and palmitic acid (11%).

The occurrence of the solvents used in the synthesis of SFAE was also evaluated, showing that *tert*-butanol is the most reported solvent (18%) closely followed by 2M2B (12%). The former is suitable for the synthesis of sugar esters since it is sufficiently hydrophobic (logP of 0.80) to prevent lipase inactivation, but sufficiently hydrophilic to dissolve the carbohydrates.[94]

Interestingly, ionic liquids (IL) have a high occurrence (12%) as well, thus demonstrating that they are a suitable option. However, the use of these solvents is very challenging, especially due to their high cost, long reaction time, low reaction yield, enzyme denaturation, difficult recoveries, and reuse of both the IL and the enzyme.

Thanks to the network analysis, the authors evaluated also the co-occurrence of the items (substrates, solvents, and methodologies) in the set of publications. It was found that 40% of the publications reported the use of more than one solvent and/or a combination of different solvents to obtain the highest solubilization of the components. Moreover, the use of immobilized biocatalysts, both commercial (e.g.

Novozym 435) and produced "in house" is the most used technology for these reactions (95% of the selected publications).[79]

It is important to underline that this study was limited to the lipase B from *Candida anctartica.* Taking into account that this enzyme is one of the most studied and used lipases, the trend can be considered highly representative.

The study was very useful also to highlight the gaps that still need to be filled in this research area. For example, only a few analyzed publications (4%) reported lactose as reagent. Galactose, the monosaccharide derived from lactose hydrolysis other than glucose, is not even mentioned. As for the solvents, even if *tert*-butanol is generally considered a "preferred" solvent,[88] a poor attention was given to food-grade or eco-friendly solvents such as methyl ethyl ketone (MEK, only 2%). The use of solvent-free systems or DES had also a low occurrence (3% and 5%, respectively). Eventually, it was observed that a few of the selected publications investigated the comparison of different acyl donors and/or acceptors and their effect on the reaction outcome.[79]

However, there are some papers not included in the above cited study which partially fills those gaps. As for the occurrence of lactose as acyl acceptor, different lactose fatty acid esters were prepared both by direct esterification with free fatty acids[65] and transesterification with vinyl esters.[67] In these papers, the effect of the different acyl donors was also investigated. Similarly, Walsh and co-workers[89] evaluated the influence of different lipases and solvents for the synthesis of lactose monolaurate and sucrose laurate, highlighting some interesting differences. For example, the acyl acceptor (sucrose or lactose) did not influence the reaction yield in 2M2B when the lipase from *Thermomyces lanuginosus* (TLL) and CalB were used as biocatalysts. On the contrary, the change of the acyl acceptor resulted in very different yields for the lipase from *Mucor miehei* in all the tested solvents.

Another recent paper[95] reported the synthesis of glucose laurate by direct esterification in the new "green" solvent 2-methyltetrahydrofuran-3-one which is compared with 2M2B as reference solvent and with 2-methyltetrahydrofuran, known for its interesting properties in terms of safety and biodegradability. The furanone resulted in the highest conversion and its ready biodegradability was evaluated.

The synthesis of SFAE was extended not only to new green "conventional" solvents but also to the so-called non-conventional media. Besides the already mentioned IL, DES are

worth mentioning. These solvents, as reported in **Chapter 1.2.2**, are obtained by mixing two or more molecules under stirring and heating. The formation of a hydrogen bond network deeply decreases the melting point of the mixture, thus generating a clear liquid. The toxicity of DES is very low and their biodegradability is extraordinarily high because it is possible to use natural molecules (including sugars and fatty acids) to prepare them. They are also characterized by high thermal stability and are easy to prepare. Moreover, it is possible to tune the properties of DES by changing the molar ratio of HBA and HBD or by the addition of a few amount of water.[34]

The use of DES for the synthesis of glucose decanoate, a SFAE having foaming properties, was deeply investigated by Hollenbach and co-workers.[96,97] The synthesis consisted of the transesterification of glucose with vinyl decanoate catalyzed by immobilized CalB. The process was studied both in hydrophilic (ChCl:U 1:2+5% v/v water)[96] and hydrophobic DES (menthol: decanoic acid 1:1).[97] In both cases, the formation of the product was detected by HPLC-ELSD. However, the hydrophobic DES resulted in a higher conversion, and it was very well tolerated by the enzyme (at least 5 reaction cycles). Moreover, in this system, the acyl donor (decanoic acid) acted both as solvent and substrate at the same time, thus avoiding the use of the vinyl ester. The same "2-in-1" system was also investigated by using glucose as the component of a hydrophilic DES (ChCl:glucose:water 5:2:5), but it gave worse results than ChCl:U.[96] These studies highlighted that DES are suitable reaction media for the synthesis of SFAE, but they need to be further studied before being able to replace the conventional organic solvents.

Another strategy to circumvent the solubility issue of sugars and fatty acyl donors is the sugar modification or the addition of adjuvants. Organoboronic acids (e.g. phenylboronic acid) can solubilize sugars by forming carbohydrate-boronate complexes through reversible condensation reactions. These complexes are soluble in nonpolar solvents, thus making the esterification of sugars possible in organic solvents.[76,98] However, organoboronic acids are toxic.

The sugars can be also converted into the corresponding acetals by reaction with dimethoxypropane in the presence of *p*-toluene sulfonic acid (*p*TSA). However, this is a protective group which needs to be removed, thus resulting in a multi-step process. This strategy was recently used to prepare lactose fatty acid esters by enzymatic

esterification of lactose tetra acetal with fatty acids catalyzed by TLL in toluene. The acetal was then removed by tetrafluoroboric acid diethyl ether complex.[99,100]

A promising alternative is to introduce in the sugar a permanent modification which does not affect the surfactant properties of the final product, by converting the sugar into the corresponding alkyl glycoside. This modification can be performed by Fischer glycosidation, or by an enzymatic approach using glycosidases, that are enzymes "designed by Nature" for sugar modification as mentioned in **Chapter 1.9**. In this latter case, the sugar ester can be prepared by a two-step enzymatic process. The less polar sugar derivative (glycoside) can be then used as the polar head for the synthesis of SFAE. This was the approach followed in this Thesis starting from literature data[64] and it is described in **Chapters 3.1-3.2**.

1.12 Fatty acid glucamides

A further modification of the sugar which does not affect the properties of the final surfactant is the reductive amination. When this reaction is performed on glucose by using methyl amine, *N*-methyl glucamine is obtained. *N*-Methyl glucamine is a non-toxic and cheap compound approved as drug excipient.[101] Later on, this intermediate can be reacted with an acyl donor to form *N*-acyl-*N*-methyl-glucamide, a tertiary amide endowed with non-ionic surfactant properties even if it is not fully biobased. These compounds, known also with the acronym MEGA (MEthylGlucAmides), have properties similar to those of alkyl glucosides, making them suitable in laundry detergents and haircare products.[74] In 2017, the swiss company Clariant commercialized *N*-cocoyl-*N*-methyl glucamide (**Figure 10**) as a 100% renewable surfactant (GlucoPure®).

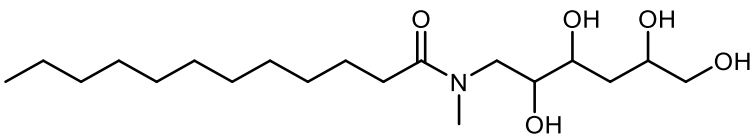


Figure 10: *N*-Cocoyl-*N*-methyl glucamide (GlucoPure®), the surfactant commercialized by Clariant

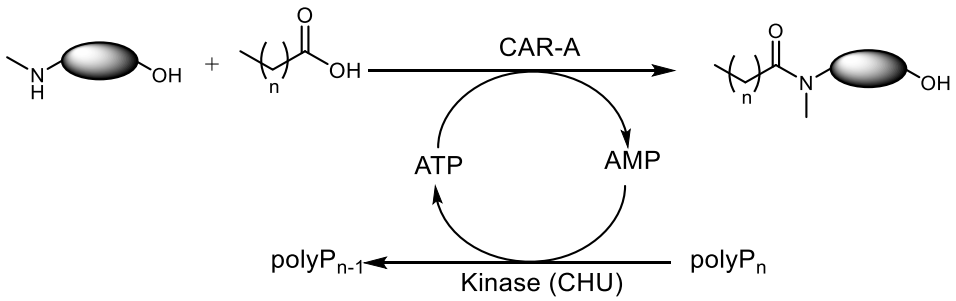
The chemical synthesis of fatty acid glucamides is generally performed using FAME and sodium methoxide as catalyst in completely dry conditions. This scenario, once again, calls for the need of more sustainable processes for the production of carbohydrate-based surfactants.[101]

Lipases demonstrated to be suitable also for the synthesis of fatty acid glucamides. The acylation of *N*-methylglucamine with oleic acid was performed in 2M2B at 90 °C affording almost quantitative conversion after 50 h.[102] The authors observed also the formation of two by-products: 6-*O*-oleyl-*N*-methylglucamine (esterification of primary alcohol of the glycamine) and *N*,*O*-dioleyl-*N*-methylglucamide (diacylated product). However, their formation was minimized by tuning the reaction conditions. The optimized conditions were then applied to the synthesis of a library of MEGA.

More recently, the same reaction was performed in a solvent-free system. Also in this case, the formation of the diacylated product was observed. For this reason, at the acylation endpoint, the reaction mixture was incubated in aqueous NaOH (0.05 M) at room temperature to fully convert the diacylated product into the target glycamide. This process gave a 99% final yield of the amide and a product essentially free of the substrate, *N*-methylglucamine (MEG). This is crucial since MEG can potentially be converted to carcinogenic nitrosamines.[103] The environmental assessment of the process was also reported.

The reductive amination of lactose and its hydrolysis product was performed in this Thesis: a small library of potential polar heads for the preparation of new glycamide surfactants was prepared. This part of the project is discussed in **Chapter 3.5**.

A recent work by Prof. Turner's group highlighted the potential of a new class of enzymes for the synthesis of MEGA: carboxylic acid reductases (CAR), which are ATP- and NADPH-dependent enzymes able to selectively reduce carboxylic acids to aldehydes.[101] Carboxylic acid reductases catalyze the formation of amide bonds when the NADPH is substituted for an excess of amine nucleophile (**Scheme 3**). Moreover, these enzymes are able to perform the amidation in water containing media.



Scheme 3: Synthesis of MEGA surfactants catalyzed by carboxylic acid reductase (CAR-A). CHU: polyphosphate kinase from *Cytophaga hutchinsonii*. Modified from Lubberink et al.[101]

The potential of these enzymes was exemplified by the synthesis of 16 amides including several commercially relevant molecules. However, this class of enzymes was not investigated in this Thesis.

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Chapter 2: From lecithin to phospholipid-derived ingredients

Glycerophosphoric acid esters (GPAE) are derivatives of glycerophospholipids in which both the fatty acid chains in *sn*-1 and *sn*-2 positions are removed. Glycerophosphoric acid esters derived from the main natural phospholipids were reviewed in **Chapter 2.1**. After an overview about their nomenclature, the natural sources they derive from, and their biological activities, the attention was focused on the chemical and enzymatic approaches to their preparation.

Chapter 2.2 describes the preparation of both glycerophosphocholine (GPC), a precursor of acetylcholine used as a cognitive enhancer, and glycerophosphoinositol (GPI), which has been demonstrated to act as an anti-inflammatory agent, being part of a negative feedback loop that inhibits the *de novo* synthesis of pro-inflammatory and pro-thrombotic compounds. These molecules were obtained by enzymatic hydrolysis of two different fractions of sunflower lecithin. These fractions were obtained by a pre-treatment of lecithin (*N*-acetylation, de-oiling, fractionation). This three-step sequential procedure was necessary for the enrichment in PI and PC (the precursors of GPI and GPC, respectively) and allowed to reduce, indeed, the complexity of the starting material, to achieve a higher selectivity in the following enzyme-catalyzed transformations, and to assist the product downstream. The target molecules were purified in moderate yield by silica chromatography or by a combination of ion exchange and silica chromatography and were characterized by ^{1}H and ^{31}P NMR spectroscopy. An in-depth analytical characterization was performed for GPI (^{13}C NMR, COSY, HSQC, HMBC, HPLC-ELSD, ESI-MS).

The chemical synthesis and the characterization of an intermediate for the preparation of inositol phosphates, including GPI, is described in **Chapter 2.3**.

Chapter 2.1 Chemical and enzymatic approaches to esters of *sn*-glycero-3-phosphoric acid

Riccardo Semproli, Marina S. Robescu, Mattia Cambò, Klodiana Mema, Teodora Bavaro, Marco Rabuffetti, Daniela Ubiali,* Giovanna Speranza*

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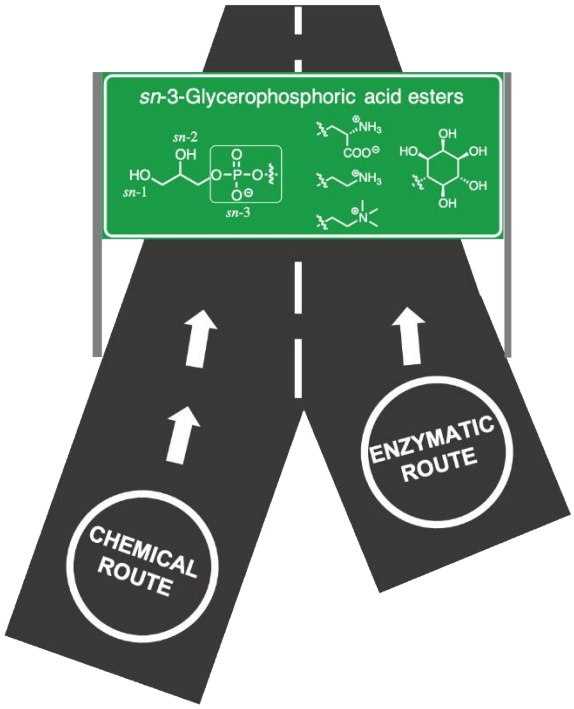

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Chemical and Enzymatic Approaches to Esters of *sn*-Glycero-3-Phosphoric Acid

Riccardo Semproli,[a] Marina S. Robescu,[a] Mattia Cambò,[b] Klodiana Mema,[a, c] Teodora Bavaro,[a] Marco Rabuffetti,[b] Daniela Ubiali,*[a] and Giovanna Speranza*[b]

Dedicated to Professor Franco Cozzi on the occasion of his 70th birthday.

Esters of *sn*-glycero-3-phosphoric acid (GPAE) are derivatives of glycerophospholipids in which both the fatty acid chains in *sn*-1 and *sn*-2 positions are removed. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) are the most abundant glycerophospholipids in Nature. Their deacylated derivatives find application in many industrial sectors; for example, *sn*-glycero-3-phosphocholine (GPC) is useful as a cognitive enhancer and as a food ingredient, whereas *sn*-glycero-3-phosphoinositol (GPI) acts as a natural anti-inflammatory agent. This minireview covers the state-of-the-art of chemical and biocatalytic methods for the obtainment of these lipid-derived components.

1. Introduction

Glycerophospholipids (GPL, Figure 1A) are the major components of cell membranes with important structural and functional properties.[1] They are a class of lipids composed of a glycerol backbone which is esterified in *sn*-1 and *sn*-2 positions with fatty acids and *sn*-3 position with a phosphate group. Although the length and degree of unsaturation of the fatty acids vary, a saturated fatty acid is typically present in *sn*-1 position, whereas an unsaturated or a poly-unsaturated fatty acid is found in *sn*-2 position.[2] The simplest GPL is phosphatidic acid (PA, Figure 1A); in other GPL the phosphate moiety of PA is further esterified with choline, *myo*-inositol, ethanolamine, or serine, thus giving rise to the most biologically important classes of GPL, *i.e.* 3-*sn*-phosphatidylcholine (phosphatidylcholine, PC), 3-*sn*-phosphatidyl-1-*myo*-inositol (phosphatidylinositol, PI), 3-*sn*-phosphatidylethanolamine (phosphatidylethanolamine, PE), and 3-*sn*-phosphatidylserine (phosphatidylserine, PS).[3]

When the two fatty acid chains of natural GPL are removed, the esters of *sn*-glycero-3-phosphoric acids are obtained (GPAE) (Figure 1B). In the last years, these latter deacylated derivatives have attracted increasing interest due to their biological and pharmacological properties. *sn*-Glycero-3-phosphoric acid (GPA, Figure 1B) is the precursor of all esters described herein. In this minireview, we aim at presenting the state-of-the-art of chemical and biocatalytic methods for the obtainment of these important lipid-derived compounds.

1.1. Nomenclature

There has been confusion in the literature about the chemical names of GPL and GPAE. The need for a novel departure in the nomenclature of lipids and, in particular, in distinguishing stereoisomers, resulted in the publication of recommended guidelines by IUPAC-IUB Commission on Biochemical Nomenclature (CBN) in 1967,[4] then revised later on.[5,6]

A stereospecific numbering (*sn*) system was, indeed, recommended by the IUPAC-IUB Commission to designate the stereochemistry of glycerol derivatives. The descriptors *sn*-1, *sn*-2, and *sn*-3 are used to identify the esterification position of carbon 1, 2, and 3 on the glycerol molecule, as depicted in Figure 2: if the secondary hydroxyl group is shown to the left of C-2 in a Fischer projection, the carbon atom above this then becomes C-1 while that below becomes C-3, and the prefix *sn* is placed before the stem name of the compound. *sn*-3-Glycerophosphoric acid (previously known as either L-α-glycerophosphoric acid or as D-glycerol-1-phosphate) is the precursor of all esters described herein.[5,6]

The system of "*sn*" numbering introduced by CBN in 1967 has been well-accepted in the field of glycerol derivatives and is still retained nowadays.[6]

3-*sn*-Phosphatidic acid (PA) can be considered a derivative of glycerophosphoric acid in which both remaining hydroxyl groups of glycerol are esterified with fatty acids. For the most common 3-*sn*-phosphatidic acids and their derivatives (see Figure 1), the locants are often omitted. This rule will be applied for the designation of the 3-*sn*-glycerophosphoric acid esters

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(GPAE) which are deacylated derivatives of GPL as follows: *sn*-glycero-3-phosphocholine (glycerophosphocholine, GPC), *sn*-glycero-3-phospho-1-*myo*-inositol (glycerophosphoinositol, GPI), *sn*-glycero-3-phosphoethanolamine (glycerophosphoethanolamine, GPE) and *sn*-glycero-3-phosphoserine (glycerophosphoserine, GPS). Glycerophospholipids in which one of the two acyl groups have been removed from either the *sn*-1 or *sn*-2 position are named lysophospholipids.[7] The old prefix 'lyso' originated from the fact that these compounds are hemolytic. It has been redefined to indicate limited hydrolysis of the phosphatidyl derivative (*i.e.*, 'deacyl').[5]

It is worth mentioning that the inositols are the nine isomeric forms of cyclohexanehexol and constitute a subgroup of a broader class of compounds known as cyclitols, *i.e.* cycloalkanes in which three or more ring atoms are each substituted with one hydroxyl group.[6] *myo*-Inositol, or *cis*-1,2,3,5-*trans*-4,6-cyclohexanehexol, is the most widespread isomeric form of inositol that can be found in nature and in food.[8] In this review, later mentions of 'inositol' refer only to *myo*-inositol.

Further useful specifications about nomenclature include the term 'plasmalogen', which refers to GPL in which the glycerol moiety bears a 1-alkenyl ether group, and 'lecithin', which is a mixture of GPL, but is commonly used to indicate its main component, *i.e.* 3-*sn*-phosphatidylcholine (PC), whose systematic name is 1,2-diacyl-*sn*-glycero-3-phosphocholine.[5]

1.2. Source

Esters of *sn*-glycero-3-phosphoric acid can be produced from GPL of plant, animal, and aquatic origin (*e.g.* soy, sunflower and rapeseed oils, egg yolk, cheese whey, fish, and Antarctic krill). Vegetable lecithins are the main commercial source of GPL.[9,10]

The commercial term lecithin is referred to a complex mixture of phospholipids obtained as by-products during the vegetable oil refining process (degumming). The degumming process removes the substances containing phosphorus and glycolipids from unrefined vegetable oil, thus enhancing oil stability against sedimentation as well as facilitating further refining processes.[11] Many degumming processes are reported in the literature. Briefly, the vegetable oil is heated and stirred in presence of water which causes the hydration of polar lipids, making them insoluble and allowing their separation from oil. The obtained gum is then dried and cooled to obtain lecithin. Temperature, water amount, and mixing times as well as drying and cooling conditions may vary depending on the vegetable source.[12] As mentioned before, lecithin is also the trivial name for PC, but common usage refers to the array of GPL present in all crude vegetable oils. The most abundant GPL in lecithin are usually PC, PE, PI, and PA (Figure 1). However, slight differences can be observed in phospholipid composition depending on the natural sources and the conditions applied in the extraction and quantification process (Table 1). In most plant species, PC and PE are predominant; however, in the case of sunflower, the main GPL are PC and PI. For this reason, this source is preferred for the obtainment of the latter GPL.[13]

Due to their surface-active properties, lecithins are among the most widely used emulsifiers in a vast range of food, feed, pharmaceutical, cosmetic and technical applications.[14]

Starting from GPL, GPAE can be obtained through both chemical and enzymatic approaches. Chemical approaches generally consist of the hydrolysis of lecithin in alcohols using organic or inorganic bases. In this case, a mixture of all possible GPAE is obtained since the hydrolysis is not selective and the complexity of the mixture depends on the used lecithin and/or pre-treatment of starting material before hydrolysis. Solvent extraction and silica or ion-exchange chromatography are then applied to the mixture to purify the desired GPAE (see paragraphs 2–5).

Alternatively, GPL can be hydrolyzed by using enzymes as biocatalysts. Typically, biotransformations are carried out by incubating lecithin or pre-treated lecithin with the selected enzyme(s) (as soluble or immobilized proteins) in a fully aqueous medium or in non-conventional media such as, for instance, biphasic systems. Isolation and purification procedures are the same as for chemical hydrolysis.

Lipases and phospholipases are hydrolytic enzymes suitable for this purpose. Lipases are the most used hydrolases in the industry (*e.g.* for the formulation of laundry detergents, treatment of wastewater, and hydrolysis of triglycerides) and their applications in esterification/transesterification reactions have also been widely exploited.[15] Phospholipases can also be used to modify GPL. There are four classes of phospholipases that catalyze the cleavage of a specific ester bond, as reported in Figure 3.

Phospholipases A_1 (PLA_1) and A_2 (PLA_2) are the most suitable for the obtainment of GPAE since they hydrolyze carboxyl esters

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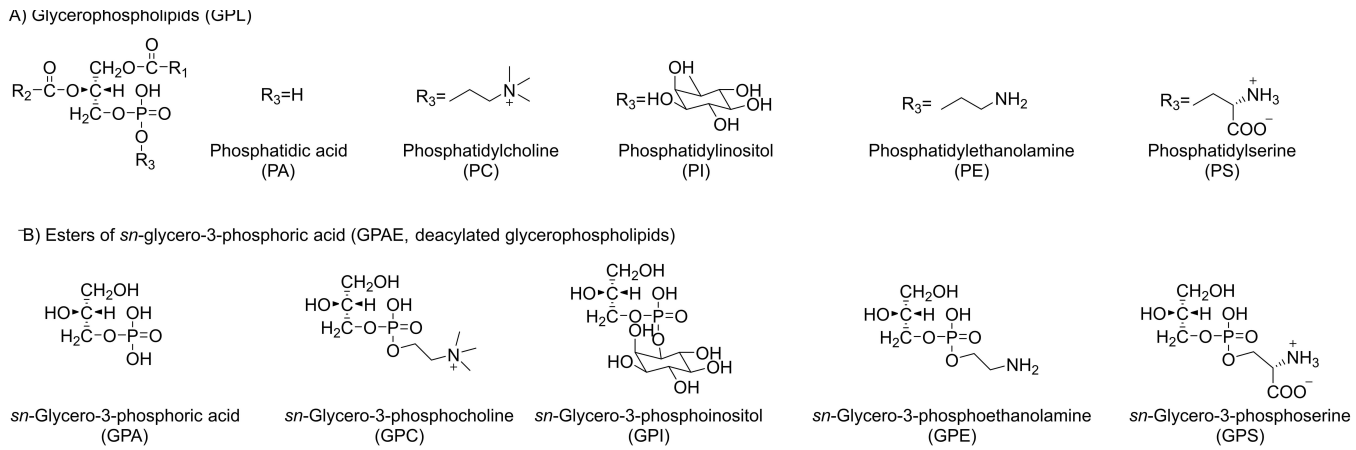


Figure 1. A) Structure of the most common glycerophospholipids (GPL); B) Structure of the most common esters of *sn*-glycero-3-phosphoric acid (GPA). R_1; R_2 = alkyl or alkenyl groups.

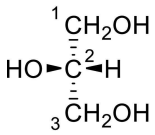


Figure 2. Glycerol "*sn*" numbering.

Table 1. Average composition of GPL in the main plant sources (adapted from [13]).

Lecithin	Composition [%]		
	PC	PE	PI
Soybean	25.5	17.6	15.6
Sunflower	28.2	12.0	20.2
Rapeseed	38.5	23.1	16.7

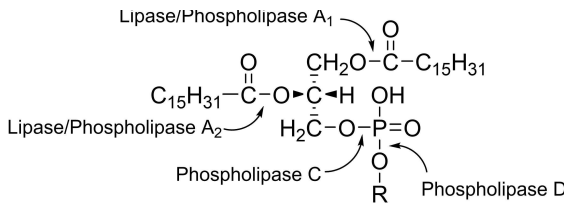


Figure 3. Enzymatic toolbox for GPL modification. R=H, choline, inositol, ethanolamine, or serine. Ester of 1,2-dipalmitoyl *sn*-glycero-3-phosphoric acid is depicted.

at the *sn*-1 and *sn*-2 position releasing free fatty acids similarly to lipases. Phospholipases C and D (PLC and PLD, respectively) have phosphodiesterase activity instead. PLC cleaves the glycerophosphate bond, thus producing a natural diacylglycerol and a phosphoric ester; PLD cleaves the terminal phosphodiester bond releasing phosphatidic acid and the polar head. As for lipases, the phospholipase-catalyzed reaction can be reversed to synthesize specific GPL and their derivatives.[2,16] Enzymatic catalysis is expected to simplify the purification procedure. Due to the intrinsic selectivity and specificity of enzymes, fewer by-products are generally formed.[2]

1.3. Biological activity

In the last years, many biological and pharmacological properties of GPAE have been reported.

Glycerophosphoinositols, which include non-phosphorylated glycerophosphoinositol (GPI) and its phosphorylated derivatives glycerophosphoinositol 4-phosphate and glycerophosphoinositol 4,5-bisphosphate, are ubiquitous, water-soluble metabolites. They constitute a class of biologically active lipid-derived mediators whose intracellular levels are modulated during physiological and pathological cellular processes. The functional roles and mechanisms of action of these different glycerophosphoinositols have been investigated in different cellular contexts and it has been observed that they can exert different functions based on the specific target/receptor to which they bind. As a result, roles for these mediators in cell proliferation, immune and inflammatory responses have been recently defined and reviewed by Corda et al.[17–20] After an inflammatory stimulus such as exposure of monocytes and macrophages to bacterial lipopolysaccharide (LPS), GPI is generated from membrane PI carrying arachidonic acid in position *sn*-2 of the glycerol backbone through two sequential deacylation reactions both catalyzed by PLA_2IVα, which has intrinsic PLA_2 and lysolipase activities.[20] It has been demonstrated that GPI can act as an anti-inflammatory agent, being part of a negative feedback loop that inhibits the *de novo* synthesis of pro-inflammatory and pro-thrombotic compounds. GPI can inhibit the signaling of Toll-like receptor 4 (TLR4) induced by LPS leading to a decrease in the nuclear translocation and binding of the transcription factor NF-kB to promoters, thus reducing the transcription of inflammatory genes,[20] and in parallel inhibiting both the expression[21] and activity of PLA_2.[22] Based on these *in vitro* results, the potential efficacy of GPI as an anti-inflammatory agent was also

demonstrated in an *in vivo* model of endotoxin shock in mice[20] and its use in sepsis syndrome has been recently patented.[23] The anti-inflammatory properties of GPI have been also demonstrated *in vitro* at blood-brain barrier (BBB) level and compared to corticosteroid dexamethasone, showing comparable effects in preventing culture-induced BBB decline after several days, as well in promoting recovery of function in compromised cells.[24]

A plethora of clinical and scientific studies have documented the central role of GPC in human health. GPC is a component of breast milk and an important source of choline required by infants for organ growth and membrane biosynthesis. Most important from an applicative viewpoint, GPC has the status of 'Generally Recognized as Safe' (GRAS) as an ingredient in beverages and foods when consumed at a level not to exceed 196.2 mg/person/day.[25,26] The use of GPC as a food additive and in health products makes its application increasingly important in numerous industries.[27] GPC was identified as the "meat factor" which is reported to enhance non-heme iron absorption, and this finding provides new opportunities for iron fortification of foods.[28]

In addition, GPC is present in brain tissue as a product of phospholipid metabolism[29–31] and has a well-established use as a therapeutic agent in the field of cognitive disorders.[32] GPC is the direct precursor of acetylcholine (ACh) in the brain and has shown significant advantages for increasing learning, memory abilities among stroke patients, and has been demonstrated to be effective in the treatment of Alzheimer's disease (AD) and dementia.[33–35] A primary trait of AD is the degeneration of basal forebrain cholinergic neurons, which causes a remarkable deficit of avenues of cortical cholinergic neurotransmission, such as ACh synthesis, release, and uptake, and choline acetyltransferase and acetylcholinesterase (AChE) activities.[36] Reduction of AChE activity is related with the degree of dementia and neuropathological hallmarks of AD, confirming a close association between cholinergic biochemical abnormalities and the disease.[37] Under conditions of reduced cholinergic synthesis and increased neuronal demand, neurons increase their ability to incorporate exogenous choline;[38] this suggests that the systemic administration of a choline precursor, such GPC, should antagonize biochemical disorders of the cholinergic system, thereby improving cognitive function. In fact, GPC is metabolized into phosphorylcholine that is able to reach cholinergic synaptic endings where it enhances ACh synthesis and release. Moreover, GPC positively affects the expression of choline uptake transporter (CHT) and vesicular Ach transporter (VAChT), thus improving synaptic efficiency.[39] Recent clinical studies indicated that the association between the AChE inhibitor donepezil (1-benzyl-4-[(5,6-dimethoxy-1-indanon-2-yl) methyl]piperidine) and GPC is accompanied by an improvement in several cognitive tests which is superior to that induced by donepezil alone.[40] It was suggested that this association may represent a therapeutic option to prolong the beneficial effects of cholinergic therapies in AD's patients with concomitant ischemic cerebrovascular disorders.[40] Among many other reported functions, being a putative ACh precursor, GPC can enhance growth hormone secretion and fat oxidation in young adults.[41] Finally, GPC is also involved in the balance of osmotic pressure in the kidney, acting as osmoprotective organic osmolyte in renal medullas.[42,43]

The tolerance of GPC is excellent and side effects are rare, never severe.[34,44] An oral NOAEL (No Observed Adverse Effect Level) of 150 mg/kg body weight/day following 26 weeks oral exposure has been reported.[25]

Although GPE and GPS do not have an application to date, some preliminary studies on their biological activities have been reported.

Tallan et al.[45] observed that GPE was present in normal liver tissue in higher levels compared to other organs, where it stimulated the growth of hepatocytes during liver regeneration.[46,47] This regeneration capacity was further confirmed in cultured hepatocytes where GPE could enhance the activity of epidermal growth factor (EGF).[47] Moreover, a recent study has demonstrated that GPE does not show any toxicity effect on human kidney-2 (HK-2) cells in concentrations up to 1000 μM, thus suggesting its possible use as a pharmaceutical candidate.[48]

In the human retina, GPE levels represent 22% of the total PE components. However, this relatively high content is not still completely understood, even if it has been demonstrated that GPE can form a direct adduct with bisretinoid called A2-GPE.[49] The formation and accumulation of these retinoid-derivatives in the retinal pigment epithelial (RPE) cells are observed in some inherited and age-related forms of macular degeneration. Investigations of potential treatments for macular degeneration include approaches that would reduce bisretinoid formation.

Although a plethora of biological activities of PS, an important membrane component, has been reported, a handful of information about GPS is available to date.

When PS is exposed on cell surface it acts as signaling triggering phagocytosis and subsequent apoptosis. Phagocytosis of apoptotic lymphocytes was inhibited in a dose-dependent manner when treated with liposomes containing PS but also by its derivatives structurally related such as GPS and phosphoserine.[50] In 1999, Tait et al.[51] have confirmed the activity of PS vesicle also on the monocytic leukemia cell lines THP-1 but no effects were observed for phosphoserine or GPS. By contrast, in recent years lyso-PS have emerged as signaling molecules in several biological processes, and deregulation of their metabolism has been linked to various human pathophysiological conditions.[52] Lyso-PS are involved in the release of histamine from Mast cells, macrophage-mediated efferocytosis, and inhibition of lymphocytes proliferation. Furthermore, lyso-PS stimulate intracellular calcium signaling and promote chemotactic migration in human cancer cells. Finally, lyso-PS have hormone-like signaling properties facilitating the transport and adsorption of glucose into muscle fibres and adipocytes.[52]

2. *sn*-Glycero-3-phosphoinositol (GPI)

Only one synthesis of GPI, specifically of '1-glyceryl-2-*myo*-inositol phosphate', as named in the original paper, has been reported so far. It dates back to 1959 when the position of the

phosphate group in *myo*-inositol moiety (whether it was 1 or 2) had not been elucidated, yet.[53] This synthesis starts from 3,4,5,6-tetracetyl-*myo*-inositol which was converted into 1,3,4,5,6-pentacetyl-*myo*-inositol and then treated with phenyl-phosphodichloride followed by addition of D,L-1,2-isopropily-dene glycerol, thus affording '1,2-isopropylidene-glyceryl-penta-cetyl-2-*myo*-inosityl-phenylphosphate'. Hydrogenolysis in the presence of Adam's catalyst, removal of the isopropylidene group, treatment with sodium methoxide in methanol, and isolation by passing the resulting mixture through an ion exchange resin led to the free acid derivative in an estimated 30% yield. Since the product was unsuitable for character-ization and handling, its sodium, potassium, and barium salts were isolated by further treating the product with an ion-exchange column in the appropriate basic form. Both final products and intermediates were characterized by elemental analysis and melting point determination, only. When D-isopropylidene glycerol was used as starting material, although various optically active intermediates were obtained, the final '1-glyceryl-2-*myo*-inosityl phosphate' was inactive, owing to racemization occurred during removal of the isopropylidene group. '1-Glyceryl-2-*myo*-inosityl phosphate' was compared with the product of the alkaline hydrolysis of liver PI as well as with the same compound prepared by an alternative synthesis based on the condensation of D,L-isopropylidene glycerol with 1,3,4,5,6-pentacetyl-*myo*-inositol-2-phosphate in the presence of dicyclohexylcarbodiimide.[53]

By contrast, numerous synthetic routes to PI and its various phosphorylated derivatives have been described.[54–59] This is not surprising, taking into account the crucial role played by inositol glycerophospholipids and phosphates in cellular signal-ing coupled to difficulties in isolating them from natural sources in sufficient amounts for biochemical studies. The need for chemical probes to profile PI metabolism and measure activity of PI metabolic enzymes has stimulated, indeed, the develop-ment of methods for the efficient chemical synthesis of naturally occurring phosphatidylinositols as well as unnatural derivatives such as those depicted in Figure 4.[55,60–62]

The key steps in the reported syntheses of PI are the choice of most suitable strategy for the selective protection/depro-tection of the six hydroxyl groups of the inositol moiety, the preparation of *myo*-inositol derivatives in enantiomerically pure form, and the formation of the phosphodiester bonds, *i.e.*, one to the glycerolipid portion and the other to the inositol head groups.

Recently, Greco and coworkers[63] reported the synthesis of the first fluorescent GPI probe (**6**) in 37% yield through a convergent approach involving two successive coupling reac-tions between the three key moieties: *myo*-inositol, glycerol, and the fluorophore (NBD-aminohexanoic acid, ((7-nitrobenzo [c][1,2,5]oxadiazol-4-yl) amino)hexanoic acid) (Scheme 1).

Using a well-established resolution strategy based on the use of camphor dimethyl acetal as a chiral auxiliary,[64,65] *myo*-inositol (**7**) was converted into the enantiomerically pure and regioselectively protected derivative D-3,4,5,6-tetra-*O*-benzyl-*myo*-inositol (**8**). Regioselective allylation of **8**, followed by insertion of a *N*-protected six-carbon spacer in 2 position afforded **9**. After selective removal of the allyl group, the resulting intermediate **10** was coupled with the phosphorami-dite (**11**) containing the glycerol moiety. Final steps include debenzylation and deprotection of **12** by hydrogenolysis,

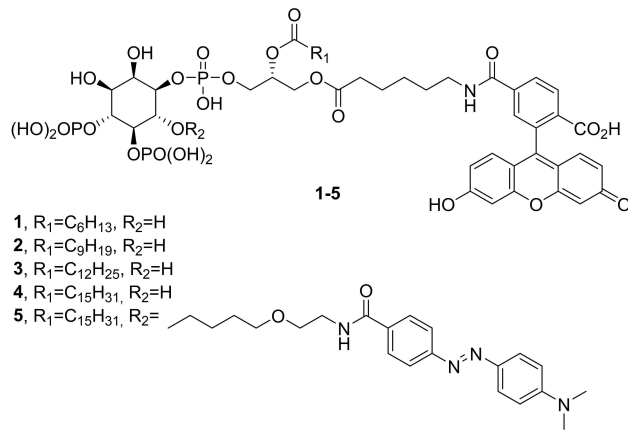
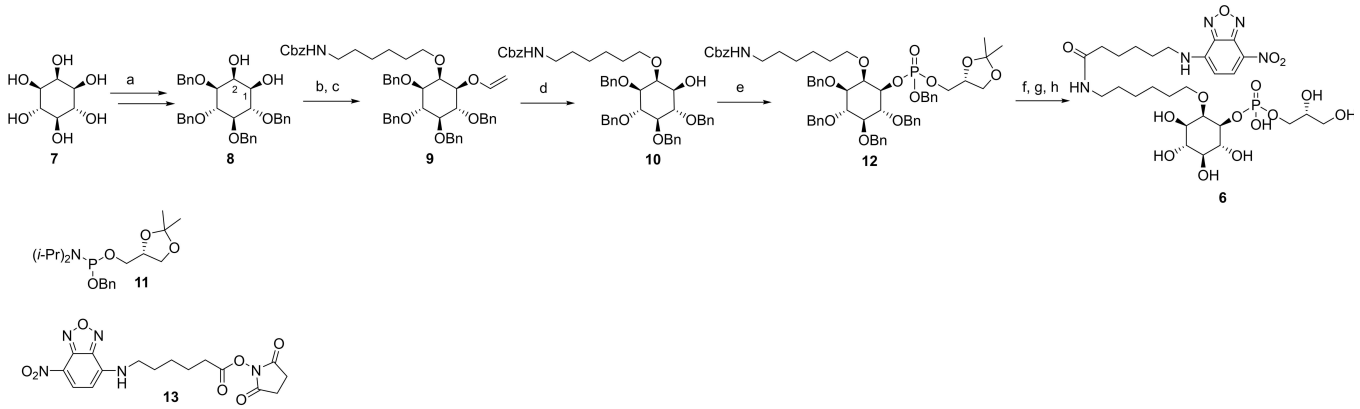


Figure 4. PI Analogues.[61,62]



Scheme 1. Synthesis of a GPI fluorescent derivative **6**.[63] Reagents and conditions: a) ref. [64,65]; b) Bu_2SnO, allyl bromide, CsF, dry MeOH, dry DMF, 24 h, 88%; c) $I(CH_2)_6NHCbz$, NaH, DMF, r.t., 16 h, 38%; d) PMHS, $ZnCl_2$, $Pd(PPh_3)_4$, THF, r.t., 4.5 h, 80%; e) **11**, 1*H*-tetrazole, *t*-BuOOH, dry MeCN, r.t., 2.5 h, 28%; f) H_2, $Pd(OH)_2$/C, EtOH, $NaHCO_3$, r.t., 24 h, 99%; g) **13**, Et_3N, DMF, r.t., 4 h, 60%; h) Dowex 50, H^+, MeOH, r.t, 12 h, 72%.

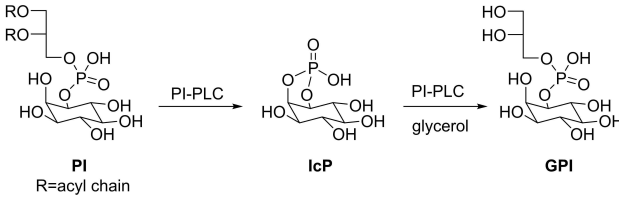
coupling between the resulting glycerol-protected derivative and the fluorophore activated as *N*-hydroxysuccinimidyl ester (NHS) (**13**), and acid-catalyzed methanolysis of the isopropylidene acetal to give **6**.

An alternative approach to the preparation of GPI and its organic (cyclohexylamine) and inorganic salts (calcium, magnesium, barium) was patented by Tronconi in 1994.[66] Deoiled soy lecithin, a low-cost commercial product, was hydrolyzed with sodium methoxide in methanol. After filtration, the residue was submitted to a number of tedious sequential steps of acidification, washing with methanol/water, elution through ion exchange resins, concentration, and precipitation by acetone and ethanol, to give GPI. Starting from 1.22 Kg of deoiled soy lecithin, 31 g of the target compound were obtained.

Corda et al.[22] have used this process to claim the preparation of a series of alkyl or acyl derivatives of GPI as modulators of activation or over-stimulation of cytosolic A_2 phospholipase.

2.1. Enzymatic synthesis

The enzymatic synthesis of GPI was reported by Bruzik et al.[67] within research focused on the transesterification of primary alcohols with inositol 1,2-cyclic phosphate (IcP), in the presence of a PI-specific phospholipase C (PI-PLC). IcP is in turn produced *via* a transesterification reaction involving the PI-PLC-catalyzed attack of the inositol 2-hydroxyl group on the phosphorus atom of PI with the release of 1,2-diacylglycerol (Scheme 2). Preparation of IcP was carried out starting either from PI-enriched soybean lecithin in a sodium deoxycholate dispersion or from crude soybean lecithin in a biphasic system water/diethyl ether. After purification, IcP was incubated in water with PI-PLC in the presence of glycerol (4.0 M) at room temperature. The resulting GPI was purified (81%) by ion-exchange chromatography by ammonium formate elution and characterized by ^{31}P NMR, ^{1}H NMR, and ES-MS. The reaction was shown to be regioselective as only the primary hydroxyl group of glycerol was phosphorylated. As for stereoselectivity, the reaction afforded two stereoisomers in 45:55 ratio arising from the phosphorylation of either the pro-*S* or pro-*R* primary hydroxyl group, as confirmed by the comparison with a single isomer of GPI obtained by deacylation of PI. To the best of our knowledge, this is the only enzymatic synthesis of GPI reported to date.



Scheme 2. Synthesis of GPI by PI-specific phospholipase C (PI-PLC)-catalyzed transesterification. IcP: inositol 1,2-cyclic phosphate.[67]

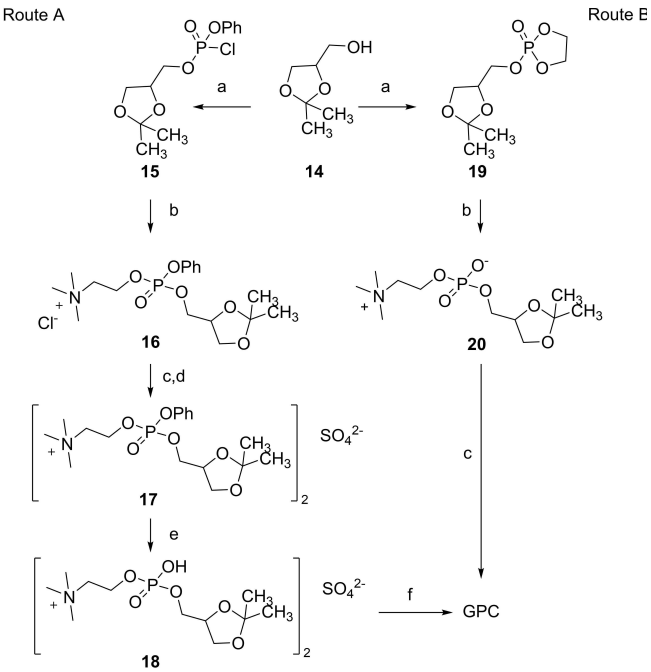
3. *sn*-Glycero-3-phosphocholine (GPC)

3.1. Chemical approaches

In 1945, Schmidt, Hershman, and Thannhauser[68] succeeded in isolating levorotatory GPC in fairly pure form from beef pancreas and elucidated its structure as the choline ester of GPA. Since isolation from natural sources hardly affords amounts exceeding a few grams, a number of chemical syntheses of GPC have been developed.

The first synthesis was reported by Baer and Kates (Scheme 3, route A).[69] Starting from 1,2-isopropylidene glycerol (**14**), GPC was obtained as a colorless, hygroscopic and viscous liquid in an overall 35–40% yield. Phenylphosphoryl chloride in the presence of quinoline was used for the phosphorylation of (**14**), followed by esterification with choline chloride and removal of the protecting groups.

Compound (**14**) was used as starting material also in the syntheses reported by other authors.[70–72] In a more straightforward preparation patented by Puricelli in 1992, the phosphorylation step was carried out by using 2-chloro-2-oxa-3,3,2-dioxophospholan to give isopropylidene 3-glyceryl-ethylenecyclic phosphate (**19**), which was treated with trimethylamine and finally deprotected to give GPC in 57% overall yield (Scheme 3, route B). It is noteworthy mentioning that GPC is obtained in optically active levorotatory, dextrorotatory or racemic form



Scheme 3. Route A: synthesis of GPC as described by Baer and Kates.[69] Reagents and conditions: a) $C_6H_5OPOCl_2$, dry quinoline, 10 min c.a., −10 °C→ r.t.; b) $HO(CH_2)_2N(CH_3)_3^+Cl^-$, dry pyridine, 4 h, r.t., c) $NH_4[Cr(NH_3)_2(NCS)_4]\cdot H_2O$, aq. Na_2CO_3, 45–55%; d) acetone-95% EtOH (2:3), Ag_2SO_4, r.t., 79%; e) H_2, PtO_2 (Adam's catalyst), EtOH, r.t.; f) H_2O/H^+ (pH 1.5), 15 h, r.t., 65–75%. Route B: synthesis of GPC as described by Puricelli.[72] Reagents and conditions: a) $C_2H_4O_2POCl$, Et_3N, Et_2O, 0 °C→10 °C, 18 h, 97%; b) $(Me)_3N$, DCM, r.t., 5 h; c) 0.1 N HCl, r.t., 3 h, 62%.

starting from D,L or racemic 1,2-isopropylidene glycerol (**14**), respectively.

Other authors reported the use of both (*R*)- and (*S*)-glycidol (**21** and **27**, respectively) as starting material to produce GPC (Scheme 4).[27,73] (*R*)-Glycidol (**21**, Scheme 4, route A) was reacted with benzyl alcohol, and the resulting protected diol (**22**) was acetylated (**23**) and debenzylated to give **24**. Diacetate **24** was phosphorylated with phosphorous oxychloride and treated with choline tosylate affording **25**. The final deacetylation of **25** in MeOH afforded the target compound in a 69% overall yield for the last two steps.

Phosphorylation, the introduction of choline moiety, and deacetylation can be carried out in a one-pot mode, although with a lower yield (58%).[27] On the other hand, the direct reaction of (*R*)-glycidol with phosphocholine chloride allows achieving **26** in about 80% yield.

A more straightforward route to GPC involves the direct phosphorylation of (*S*)-glycidol (**27**, Scheme 4, route B) followed by treatment with choline tosylate and *in situ* ring-opening of the intermediate **28** (34% overall yield).[27]

Recently, the use of racemic or optically pure (*S*) or (*R*)-3-halo-1,2-propanediol instead of glycidol has been patented in the reaction with choline phosphate to prepare racemic or optically active GPC in high yield (97%) and gram scale.[74]

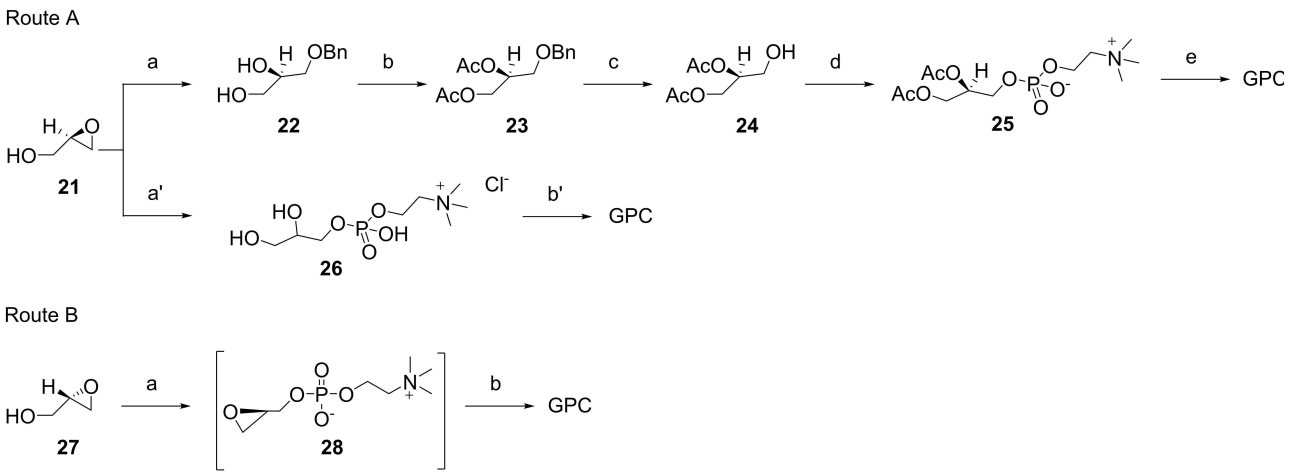
GPC can be obtained by deacylation of lecithin with mercuric chloride,[75] methanolic NaOH,[76] lithium aluminium hydride,[77] alkaline hydroxylamine,[78] or methanolic tetrabutylammonium hydroxide solution,[79,80] in 60–85% yields.

As for GPI, Tronconi disclosed a process for the preparation of GPC and GPE from crude or deoiled soy lecithin or from crude egg lecithin by deacylation with sodium methoxide in EtOH or MeOH.[81] After acidification to remove fatty acids, the methanolic solution was treated with zinc chloride or bromide and then with organic bases such as pyridine, to give a mixture of GPC and GPE that were separated by ion exchange resin: elution with water afforded GPC, followed by recovery of GPE by elution with dilute aqueous acetic acid. From 460 g of deoiled soy lecithin, 16.9 g of GPC were attained after crystallization from EtOH/Et_2O, whereas 4 g of GPE were recovered. In the following patent, isolation of GPC and GPE from mixtures obtained by deacylation of crude or deoiled soy or egg lecithin as well as from alcoholic extracts, thereof, is described.[82]

In another patent, mixtures of GPL obtained by treatment of deoiled soy lecithin with alcohols were eluted in a reactor containing a basic ion exchange resin, thus achieving deacylation and fractionation in a single step.[83] After elution from the ion exchange resins, GPC is purified from fatty acid esters and from other lipophilic impurities by extraction or treatment with apolar resins. GPC is finally crystallized from *n*-BuOH (yield: 13.5 g/100 g of ethanol-soluble soy lecithin). GPE and GPS are then recovered from the resin by eluting with solvents containing organic acids, such as acetic acid (1–10% v/v); GPE was obtained by crystallization from a 3:8 v/v H_2O/EtOH, whereas the eluted solution containing GPS is concentrated to dryness, and the residue is crystallized as calcium salt from a 1:0.4:0.7 v/v H_2O/EtOH/acetone mixture.

GPC can be also obtained by methanolysis of PC catalyzed by primary or secondary amines. The highest yields (>75%) were obtained with *iso*-propylamine, *tert*-butylamine, and 1,3-diaminopropane.[84]

Isolation and purification of GPC are often the major bottlenecks of all procedures described above. Crystallization,[85,86] resin column chromatography, and silica gel column chromatography[87] are the most frequently used techniques.



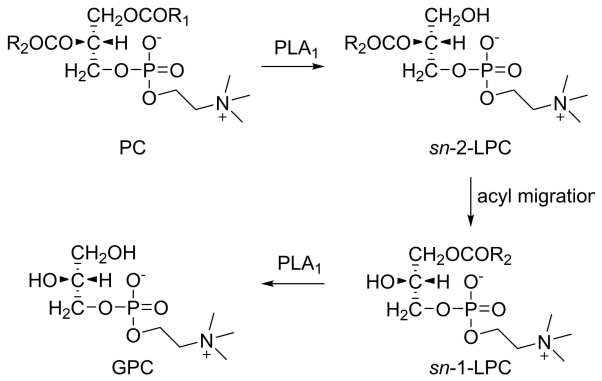
Scheme 4. Route A[73,27] synthesis of GPC starting from (*R*)-glycidol (**21**). Reagents and conditions: a) CsF, BnOH, 120 °C, 5 h, quant.; b) Et_3N, DMAP, CH_3COCl, DCM, 0 °C→r.t., 2.5 h, 92%; c) H_2, 10% Pd/C, *i*-PrOH, 40 °C, 9 h, 91%; d) i. $POCl_3$, Et_3N, DCM, 0 °C, 50 min.; ii. $HO(CH_2)_2N(CH_3)_3{}^+OTs^-$, pyridine, r.t., 12 h; iii. H_2O, r.t., 1 h, 76%; e) MeOH, K_2CO_3, r.t., 5 min., 91%. a') $(HO)_2(O)PO(CH_2)_2N(CH_3)_3{}^+Cl^-$, EtOH, 80 °C, 12 h, then *i*-$PrNH_2$ to pH 8, 3 h; b') H_2O, IONAC NM60 SG ion exchange resin, 60% c.a. (from **21**). Route B[27]: synthesis of GPC starting from (*S*)-glycidol (**27**). Reagents and conditions: a) i. $POCl_3$, Et_3N, DCM, 0 °C, 50 min.; ii. $HO(CH_2)_2N(CH_3)_3{}^+OTs^-$, pyridine, r.t., 12 h; iii. H_2O, r.t., 1 h, 80% (crude); b) K_2CO_3 aq., reflux, 5 h, 34% (from **27**).

3.2. Enzymatic approaches

The broad application of GPC as a cognitive enhancer and food ingredient makes its supply increasingly important in pharma and food sectors,[32,88] also considering that GPC is designated with the GRAS status by the US Food and Drug Administration.[89]

Enzymatic methods are a valuable alternative to chemical methods discussed above (see paragraph 3.1) which involve hydrolysis of PC or condensation of glycerol derivatives with phosphocholine donors. Even if chemical methods for lecithin modification are straightforward and inexpensive, enzymes can afford GPL and lysophospholipids with defined molecular structures due to their inherent selectivity.[90]

Several studies have reported the preparation of GPC through enzymatic hydrolysis of lecithin and/or PC-enriched lecithin under different reaction conditions, as summarized in Table 2. Due to the poor solubility of lecithin in water, most of the efforts have been devoted to the search of reaction media which could conjugate substrate solubility, enzyme stability, reaction yield, reaction time, and scalability. The most common strategies involved the use of biphasic systems, surfactants, or the control of substrate concentration by a fed-batch mode. Blasi et al. reported the preparation of GPC from egg yolk PC by using a combination of lipase from *Mucor miehei*, which is selective for the *sn*-1 position of PC and thus behaves like a PLA_1, and PLA_2 from hog pancreas. The highest conversion (94%) was achieved when the reaction was performed in a microemulsion system (isooctane/water) at 35 °C.[91] Lecitase Ultra is the most used PLA_1 as a biocatalyst to obtain GPC starting from PC under a wide range of experimental conditions. More in detail, Zhang et al.[92] reported the hydrolysis in aqueous medium starting from PC of food grade soy lecithin: GPC was obtained in 70–74% yield and up to 98.8% purity by silica or ion-exchange chromatography.[92,93] Hydrolysis of PC catalyzed by PLA_1 can lead to the formation of GPC as well as *sn*-1-LPC due to the migration of the acyl group from the *sn*-2 position to the *sn*-1 position in LPC.[94] Plückthun and Dennis[95] investigated acyl migration in LPC and reported that about 90% of thermodynamically stable 1-acyl LPC and 10% 2-acyl LPC were present in the mixture at equilibrium under the experimental conditions used. The presence of more *sn*-1 LPC results in an increased formation of GPC during the reaction because the acyl group at the *sn*-1 position of LPC is hydrolyzed by PLA_1 (Scheme 5).



Scheme 5. Synthesis of GPC from PC catalyzed by PLA_1. Mechanism of acyl migration.[90,92,95]

The acyl migration phenomenon can be intentionally induced to facilitate the formation of GPC, by tuning temperature, reaction time, water content, and enzyme loading.[96] As soybean accounts for more than 80% of the global supply of lecithin, it is not surprising that soy lecithin is the most widely used starting material for the enzymatic preparation of GPC.[97]

However, some challenges remain to be tackled, such as the poor water solubility of PC, which results in a low GPC productivity and could thus limit the industrial application of biotransformations. This evidence has prompted many authors to explore biphasic systems. Bang et al.[98] prepared GPC *via* Lecitase Ultra-catalyzed hydrolysis of soy PC in an *n*-hexane-water system, thus achieving enhanced productivity of GPC compared to other published enzymatic reaction systems.[91–93]

More recently, Kim et al.[26] have proposed the use of the same biphasic system but using Novozym 435 (an immobilized *Candida antarctica* lipase B) as the biocatalyst. In this case, both PC and the ethanol-soluble fraction of soy lecithin, which is very rich in PC, were used as starting material. After 6–8 hours, PC was completely converted into GPC; the target compound was recovered from the aqueous phase in 98.6% purity (ELSD-HPLC analysis). In addition, the immobilized lipase was easily removed from the reaction by filtration.

Surfactants are commonly used to enhance the solubility and dispersibility of non-polar chemicals in aqueous media. On this basis, aqueous solutions of lecithin have been supplemented with surfactants to increase the solubility of PC.[88] Among the six assayed surfactants, Tween 20 was the most

Table 2. Enzymatic synthesis of GPC by hydrolysis of lecithin.

Lecithin	Reaction system	Enzyme	Reaction time [h]	[Substrate] [$g \cdot L^{-1}$]	GPC yield [%]	GPC productivity [$g \cdot L^{-1} h^{-1}$]	References
Egg yolk[a]	Isooctane-water	Lipozyme IM and PLA_2	48	5.56	94[b]	–	[91]
Soybean	Water	Lecitase Ultra	3.5	6.67	73	0.65	[92]
Soybean	Hexane-water	Lecitase Ultra	30	240	85	15.74	[98]
Soybean	Hexane-water	Novozym 435	8	200	32	–	[26]
Egg yolk	Water-Tween 20	Lecitase Ultra	3	360	91[b]	37.52	[88]
Soybean	Soy oil-water	Lecitase Ultra	3	200	83	–	[99]

[a] PC and LPC from soybean lecithin; [b] HPLC conversion.

effective for enhancing GPC yield and productivity (see Table 2). In addition, to avoid substrate inhibition the enzymatic hydrolysis was carried out in a fed-batch mode. Under these conditions, this medium was found to be more tolerated by the enzyme (Lecitase Ultra) with respect to the biphasic system.

Cai et al.[99] developed a novel reaction medium consisting in solvent-free water-in-soybean oil for the hydrolysis of soy lecithin by using Lecitase Ultra as biocatalyst: 99.1% of the PC in soy lecithin was hydrolyzed with a GPC yield of 95.8% under optimized conditions (oil to PC ratio 4:1, water 30 wt%, enzyme loading 5 wt%, 55 °C, 3 h). The purity and recovery of GPC (by silica chromatography) were 94.8% and 83.0%, respectively.

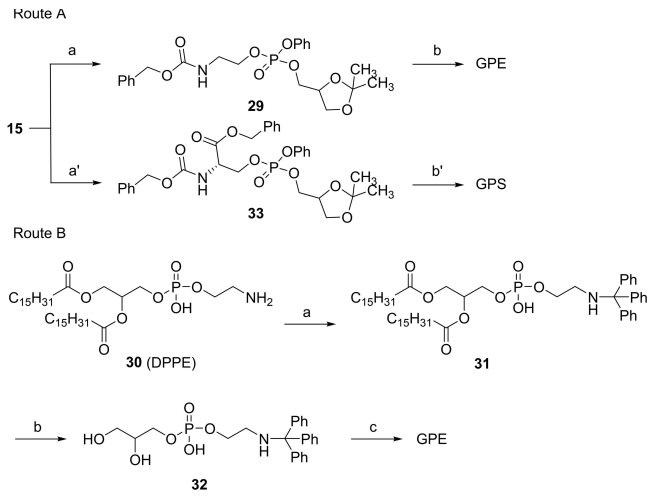
4. *sn*-Glycero-3-phosphoethanolamine (GPE)

4.1. Chemical approaches

The presence of GPE in extracts of animal tissues or cellular membranes (*e.g.* pig and rabbit liver,[100] rat and ox brain,[101] and boar spermatozoa[102]) has been described. However, such procedures to extract GPE are laborious, time-consuming, and, needless to say, are not suitable for preparative purposes. Due to the interest in GPE bioactivities, efficient synthetic methods to produce GPE have been developed afterward.

The first synthesis of GPE was reported by Baer and Stancer[103] by following the same approach developed for GPC, as previously depicted in Scheme 3. Thus, GPE was obtained in about 30% yield starting from **15** in two steps (Scheme 6, route A). Specifically, treatment of **15** with Cbz-ethanolamine in pyridine afforded **29**, which was submitted to catalytic hydrogenolysis and then to acid hydrolysis. In Scheme 6 (route B) a semi-synthetic approach to GPE is sketched[48] using the commercially available 1,2-dipalmitoyl *sn*-glycero-3-phosphoethanolamine (DPPE) as the starting material. As the effort of using alkaline hydrolysis of DPPE (**30**) was unsuccessful, the amino group was protected with an acid-labile trityl group. The resulting *N*-trityl-DPPE (**31**) was subsequently deacylated to *N*-trityl-GPE (**32**) under alkaline conditions. Final deprotection with trifluoroacetic acid/DCM (1:2) at 0 °C for 5 minutes gave GPE in an overall 66% yield.

Alternatively, as in the case of GPC (see paragraph 3.1), GPE can be prepared by deacylation of PE contained in lecithin.[81–83,104] To date, no enzymatic approaches have been reported for GPE synthesis.



Scheme 6. Route A: total synthesis of GPE[103] and GPS[105] (see paragraph 5.1), respectively. Reagents and conditions: a) $OH(CH_2)_2NHCbz$, dry pyridine, 15–20 °C, 2 h, 81.8% (from **15**, see Scheme 3); b) i. Pd, H_2, 99% EtOH, 1 h; ii. PtO_2, 5 N H_2SO_4, H_2, 2.5 h; iii. H_2O, pH 1.7–1.9, r.t., 6–7 h, followed by Amberlite IR 120 (H^+) and recrystallization (99% EtOH), 43.5% (from **29**); a') $CbzNHCH(CH_2OH)COOCH_2Ph$, dry pyridine, 15 °C→25 °C, 3 h, 91.5% (from **15**, see Scheme 3); b') i. Pd, H_2, CH_3COOH, 99% EtOH, 75 min; ii. PtO_2, H_2, CH_3COOH, 99% EtOH, 3 h, followed by Amberlite IR 120 (H^+), 75.5% (from **15**, see Scheme 3). Route B: semi-synthesis of GPE.[48] Reagents and conditions: a) trityl bromide, Et_3N, DCM, r.t., o/n, 91%; b) MeONa, $CHCl_3$:MeOH (2:3), r.t., 3 h, 90%; c) TFA:DCM (1:2), 0 °C, 5 min., 81%.

5. *sn*-Glycero-3-phosphoserine (GPS)

5.1. Chemical and enzymatic approaches

GPS was synthesized by the same approach used for GPC and GPE as depicted in Scheme 6 (route A): the reaction of glycerylphenylphosphoryl chloride **15** with *N*-carbobenzoxy-L-serine benzyl ester in the presence of quinoline gave the intermediate **33**. Removal of the protecting groups by two subsequent catalytic hydrogenolyses and acid hydrolysis afforded the target compound in 75% overall yield.[105] More recently, the use of *tert*-butoxycarbonyl (BOC) and ethyl ester as protecting groups of L-serine was reported, without affecting the final yield (74%).[106]

When GPS was prepared by using lecithin as the starting material, mixtures enriched with PS were obtained by transphosphatidylation reaction catalyzed by PLD; this enzyme is able to transfer the phosphatidyl moiety to an alcohol acceptor.[2,16,83,106] For example, starting from 7 g of a phospholipid mixture, containing up to 41% of PS, 0.65 g of GPS were obtained as a white crystalline solid (see paragraph 3.1).

6. Conclusions and Outlook

Esters of *sn*-glycero-3-phosphoric acid (GPAE) are highly polar, water-soluble molecules endowed with important biological and pharmacological activities. Particularly, *sn*-glycero-3-phosphocholine (GPC) has a well-established use as a food ingredient and a cognitive enhancer, whereas *sn*-glycero-3-phosphoinositol (GPI) acts as a natural anti-inflammatory agent. Generally speaking, methods for GPAE manufacturing, each with its own associated advantages and disadvantages, are based either on deacylation of glycerophospholipids by lipases/phospholipases, or on a multi-step total synthesis from commercially available building blocks such as 1,2-isopropylidene glycerol, glycidol, or 3-halo-1,2-propanediol, either racemic or in optically pure form. The use of lecithin as starting material does not require protection/deprotection steps, thus

resulting in a more straightforward approach, no matter if the deacylation is carried out by either alcoholysis or enzymatic hydrolysis. The main bottleneck of both approaches is the complexity of the starting material as well as reaction work-up and product isolation. Indeed, the presence of structurally related molecules strongly affects product downstream. Solvent extraction, precipitation, ion exchange and/or silica chromatography, and recrystallization are routinely all required techniques for product isolation and purification. On the other hand, using lecithin as a raw material allows upgrading this abundant oilseed industry by-product into high-added-value molecules. The increasing interest in the study and supply of glycerophospholipids as well as their deacylated derivatives (GPAE), requires the development of versatile and efficient synthetic routes to these compounds. This minireview was conceived with the aim to provide the reader with an overview of the current manufacturing routes to all GPAE derived from glycerophospholipids as well as their intricate biological functions.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Esters · Enzyme catalysis · Phosphoric acids · Phospholipids · Synthetic methods

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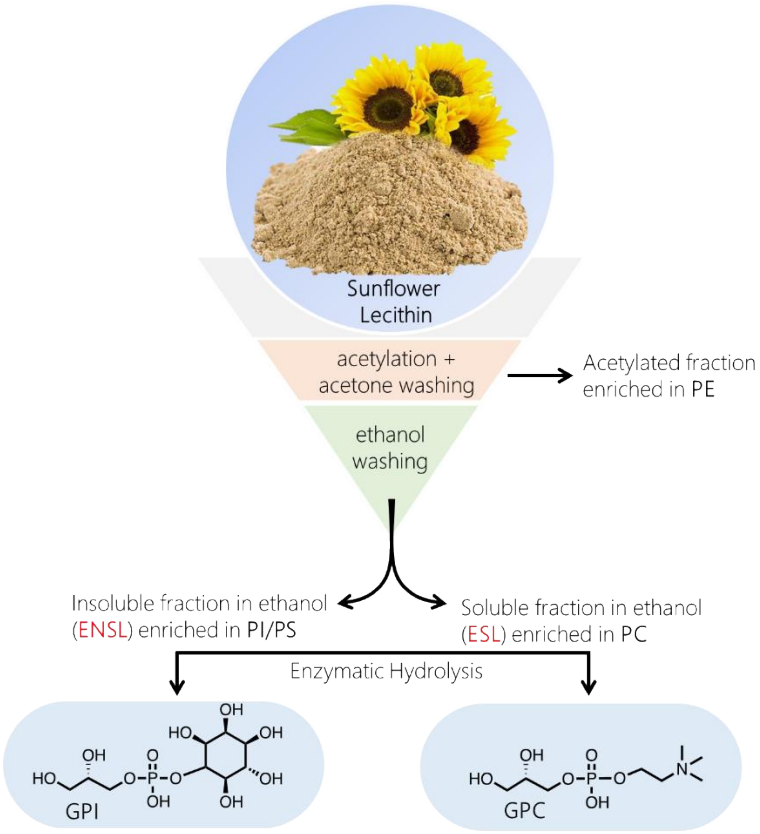
Chapter 2.2 Synthesis of glycerophosphoinositol and glycerophosphocholine by enzymatic hydrolysis of fractionated sunflower lecithin



2.2.1 Introduction

Lecithin is the commercial name for a natural mixture of phospholipids (PL). Phospholipids derive from natural sources, e.g., soybean, rapeseed, and sunflower seed, to name a few. Lecithin mainly contains phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylglycerol (PG), and phosphatidic acid (PA), and the corresponding lyso forms (bearing only one chain on the glycerol moiety) in addition to tri-, di- and monoacylglycerols and some vitamins.[1] Lecithin is a product of the vegetable oils refining by a "de-gumming" process. Water is added to crude oil to hydrate the phosphatides and the water–oil mixture is then heated. Afterwards, the oil-insoluble lecithin fraction (a wet gum known as lecithin hydrate) is separated by centrifugation. Lecithin is then dried to ensure a long-time shelf life and fluidity.[2]

Phospholipids contained in lecithin, due to their amphiphilic nature, are responsible for the high complexity of lecithin composition as well as its properties. From an applicative viewpoint, PL have a well-established use as emulsifiers for a vast range of food, feed, pharmaceutical and technical applications.

Beside their properties, PL can be used as precursors of biologically active molecules, thus making lecithin a renewable feedstock of high-added value products. This "upgrade" of phospholipids and, more in general, of lecithin can be performed by biocatalysis, which is advantageous over chemical catalysis in terms of selectivity and in the use of milder, eco-friendly conditions.

Phospholipases (EC 3.1.) form a large class of lipolytic enzymes that are well-distributed in most living organisms and play a crucial role in cellular regulation; in fact, they hydrolyze membrane PL, thus generating smaller molecules which are considered as second messengers or membrane signalling molecules. Phospholipases hydrolyze PL in different positions according to their specificities, as shown in Figure 1.

In particular, glycerophosphoric acid esters (GPAE) are PL derivatives in which both the fatty acid chains are removed. These deacylated derivatives find application in many industrial sectors.[3]

Glycerophosphocholine (GPC) has a well-established use as therapeutic agent in the field of cognitive disorders. It is the direct precursor of acetylcholine in the brain and has

shown significant advantages for increasing learning, memory abilities among stroke patients, and it has been demonstrated to be effective in the treatment of Alzheimer's disease. The production of GPC by enzymatic hydrolysis of lecithin under different reaction conditions has been recently investigated by several research groups.[4–6]

Glycerophosphoinositol (GPI) acts in the arachidonic acid (AA) cascade as a negative feedback inhibitor of cytosolic phospholipase A2 (cPLA2), the enzyme that catalyzes the release of AA from cell membranes upon inflammation stimuli and/or tissue damage. It is a natural occurring anti-inflammatory agent acting on the same molecular target of corticosteroid agents. Several papers described the mechanism and the biological application of GPI.[7–10] However, differently from GPC, there are very few reports on the synthesis of GPI. To the best of our knowledge, the only enzymatic approach to the preparation of GPI was reported by Bruzik et al. in 1996.[11] In this paper, GPI was prepared, besides many other derivatives, by a transesterification reaction of phosphatidylinositol with glycerol catalyzed by a specific PLC.

In this framework, glycerophosphoinositol (GPI) and glycerophosphocholine (GPC) were identified as target molecules to be obtained from sunflower lecithin by upgrading it through a biocatalytic approach. Sunflower lecithin was preferred over soy lecithin because it is reported to have a higher content of PI than soy lecithin. Moreover, sunflower lecithin has been generally less investigated than soy lecithin.

It is important to underline that lecithin is a complex mixture of chemically related molecules: any treatment which could afford a less "challenging" substrate would be highly desirable. Furthermore, in principle, a less complex mixture might assist in addressing the selectivity of the enzymes used for the planned biotransformations. Lecithin can be treated with organic solvents to perform the selective extraction of some of its components, thus obtaining fractions enriched in specific PL. This step is useful to reduce the high complexity of lecithin before using it for other reactions. Moreover, enriched lecithin can have different properties depending on its composition.[12,13] Specifically, after treatment of lecithin with EtOH, the resulting "soluble fraction" is reported to contain up to 70% PC and only 3% PI, based on the total PL content, whereas the so-called "insoluble fraction" can be considered as a "PI-enriched fraction" since it contains about 39% PI.

This pre-treatment was applied to sunflower lecithin herein. Then, two enzymatic processes were studied and developed to obtain GPC and GPI from the corresponding enriched fractions of lecithin. The target molecules were purified and fully characterized by NMR and MS spectroscopy. The downstream of the target molecules was in both cases the bottleneck of the processes because of the complexity of the starting material as well as solubility issues and the high hydrophilicity of the products.

2.2.2 Materials & Methods

Crude sunflower lecithin (SUN B) was supplied by Bict s.r.l. (Villanova del Sillaro, Lodi, Italy) and was characterized by TLC (**Figure S1**) and by ^{1}H and ^{31}P NMR spectroscopy as reported in the Supporting Information (**Figures S2** and **S3**), in collaboration with the University of Milano, Italy (Prof. G. Speranza). The composition of SUN B was compared with that of three different samples of soy lecithin, namely SOY B (refined soy lecithin provided by Bict s.r.l., Villanova del Sillaro, Lodi, Italy), SOY A SAMPLE (refined soy lecithin provided by Giusto Faravelli s.p.a., Milano, Italy), and FORTOM (raw soy lecithin enriched in PI provided by Fortom Chimica s.r.l., Lanzè di Quinto Vicentino, Vicenza, Italy). All reagents and solvents were purchased from Merck Life Science (Milano, Italy), unless stated otherwise. Phospholipase A1 (PLA1) from *Aspergillus oryzae*, Novozym 435, and lipase from *Pseudomonas cepacia* (PCL) were purchased from Merck Life Science (Milano, Italy). Lipase from *Pseudomonas stutzeri* (PSL) and immobilized acetylxylan esterase from *Bacillus pumilus* (AXE) were available from the Laboratory of Biocatalysis of the University of Pavia (Italy). Ion exchange resins were kindly supplied by Resindion s.r.l. (Binasco, Milano, Italy). The GPI standard (lysine salt) was supplied by Bict s.r.l. (Villanova del Sillaro, Lodi, Italy) and used without further purification.

^{1}H, ^{13}C and ^{31}P NMR spectra were recorded at 400.13, 100.61 and 161.96 Hz, respectively, on a Bruker AVANCE 400 spectrometer equipped with TOPSPIN software package (Bruker, Karlsruhe, Germany) at 300 K, unless stated otherwise. ^{1}H and ^{13}C chemical shifts (δ) are given in parts per million and were referenced to the solvent signals (δ_H 7.26 - δ_C 77.16, δ_H 2.50 - δ_C 39.52 and δ_H 4.79 ppm from tetramethylsilane (TMS) for $CDCl_3$, DMSO-*d*$_6$ and D_2O respectively). ^{31}P chemical shifts (δ) are given in parts per million, referenced to standard H_3PO_4 (aq) 85% (0 ppm). For GPI, ^{1}H NMR signals were assigned with the aid of ^{1}H-^{1}H correlation spectroscopy (^{1}H-^{1}H COSY). ^{13}C NMR

signal multiplicities were based on APT (Attached proton test) spectra. ^{13}C NMR signals were assigned with the aid of ^{1}H-^{13}C correlation experiments (Heteronuclear multiple quantum correlation spectroscopy, HSQC, and Heteronuclear multiple bond correlation spectroscopy, HMBC). Furthermore, electrospray ionization mass spectra (ESI-MS) were recorded on the Thermo Finnigan LCQ Advantage spectrometer (Hemel Hempstead, Hertfordshire, U.K.). Mass spectra were collected using a VG AUTOSPEC- M246 spectrometer (double-focusing magnetic sector instrument with EBE geometry) equipped with EI source. All NMR and ESI-MS analyses were performed at the University of Milano (Prof. G. Speranza).

HPLC-ELSD Analyses of the enzymatically hydrolyzed lecithin for the synthesis of GPI were performed on a HPLC Chromaster 600 bar System, Merck Hitachi VWR equipped with a Vydac 302IC ion column 250 x 4.6 mm 5 µm and a SEDEX 100 LT-ELSD detector with the following parameters: T= 80 °C, flow (N_2) = 2.7 bar; gain: Dynamic; filter= 8. Mobile phase: 4 mM CH_3COONH_4 pH 3 (adjusted with CH_3COOH)/ACN 90/10. Column oven was set at 35 °C. Retention time (GPI): 8.21 min.

Lecithin pre-treatment

The pre-treatment of lecithin was performed as reported in literature[12] with slight modifications. Lecithin (50 g) was dissolved in *n*-hexane (1 L). Acetic anhydride (3 mL, 0.03 mol) and triethylamine (8 mL, 0.057 mol) were added to the solution. The solution was stirred at room temperature for 1 h and then dried under reduced pressure to a glassy brown solid. The reaction was monitored by TLC (eluent: *n*-BuOH/H_2O/CH_3COOH 80:20:20). The residue was then de-oiled by suspending it in acetone (250 mL) and keeping the mixture under magnetic stirring for about 1 h. After filtration, the supernatant was discarded, and the solid residue was re-suspended again in acetone. This process was repeated three times. De-oiled lecithin was added to ethanol (1:3 w/v) and kept under magnetic stirring for 1 h. After filtration, the solid residue was re-suspended again in ethanol. The process was repeated three times and the solid residue was air-dried to yield a brownish solid, the so-called “ethanol non soluble lecithin” fraction (ENSL). The ethanol supernatants were collected and dried under vacuum, thus

obtaining an orange syrup, the "ethanol soluble lecithin" fraction (ESL). The pre-treatment outcome was evaluated by ^{31}P NMR as reported by Mackenzie et al.[14]

GPC preparation by enzymatic hydrolysis of ESL

The ESL (1 g) was solubilized in *t*-BuOH (10 mL) at 55 °C over 10 min, then Novozym 435 (0.5 g) was added and the mixture was kept under magnetic stirring till the complete consumption of PC (120 h, checked by TLC: *n*-BuOH/H_2O/CH_3COOH 80:20:20, PC R_f=0.3, detection by $Ce(SO_4)_2$/$(NH_4)_6Mo_7O_{24}$). The reaction was stopped and filtered to remove the immobilized enzyme. After drying the organic solvent under reduced pressure, water (10 mL) and *n*-hexane (20 mL) were added, and the mixture was poured in a separatory funnel. After phase separation, *n*-hexane was discarded, and water was washed twice with *n*-hexane (2 x 10 mL). Water was dried, and the residue was purified by flash chromatography (MeOH/H_2O 85:15). Yield: 37% (100 mg). The product was characterized by ^{1}H NMR and ^{31}P NMR (**Figures S4** and **S5**, Supporting Information).

GPI preparation by enzymatic hydrolysis of ENSL (bi-enzymatic process)

The ENSL (2 g) was submitted to a sequential bi-enzymatic hydrolysis catalyzed by commercially available enzymes, here referred to as E1 (a phospholipase A2) and E2 (a lipase) (these are sensitive technical details that will be left out of this report due to confidentiality reasons). The crude residue obtained from the work-up was suspended in acetone to remove the by-products (free fatty acids). Acetone was discarded and the remaining residue was suspended in water and purified by column chromatography using a weak anionic resin (Amberlyst A-21). The resin was firstly washed with water, then with a diluted solution of acetic acid (3% v/v). Thereafter, GPI was recovered by elution with a solution of acetic acid (8% v/v). Fractions containing GPI were collected, concentrated under reduced pressure, and freeze-dried, affording 56 mg of the title compound (yield: 25%), containing some impurities.

GPI preparation by enzymatic hydrolysis of ENSL: enzyme screening (lipases/esterases)

The ENSL (50 mg) was suspended in *t*-BuOH (1.5 mL) in Eppendorf tubes. Lipases from *Pseudomonas cepacia* (PCL, 51 mg) and *Pseudomonas stutzeri* (PSL, 11 mg), or immobilized acetylxylan esterase from *Bacillus pumilus* (AXE, 200 mg) were added to the

tubes. The mixtures were kept under stirring at 55 °C and the formation of GPI was checked by TLC (ACN/NH_4OH 30% 60:40; detection by $Ce(SO_4)_2$/$(NH_4)_6Mo_7O_{24}$, GPI R_f=0.28).

A further set of trials was performed by using a biphasic system. Briefly, the ENSL (1 g) was suspended in *n*-hexane (16 mL); distilled water (4 mL) was added followed by Novozym 435 (500 mg). The mixture was kept under stirring at 55 °C for 72 h and both the consumption of PI and the formation of GPI were checked by TLC as above. In this latter case, GPI formation was detected.

GPI preparation by enzymatic hydrolysis of ENSL: phospholipase A1

Phospholipase A1 (PLA1) was tested in the hydrolysis of ENSL both in a biphasic system and in a fully aqueous medium. For the former, ENSL (1 g) was dissolved in *n*-hexane (4 mL) and distilled water (0.700 mL) containing $CaCl_2$ (60 mg), followed by PLA1 (0.1 mL). The reaction was kept under shaking at 55 °C for 64 h. For the latter, ENSL (1 g) was suspended in distilled water (150 mL) containing $CaCl_2$ (30 mg). PLA1 (0.500 mL) was added, and the mixture was stirred at 55 °C for 6 h. In both cases, the consumption of PI and the formation of GPI were checked by TLC. According to the results obtained in these trials, the aqueous medium was selected for the scale-up and the product downstream, thereof.

GPI preparation by enzymatic hydrolysis of ENSL: scale-up and product downstream

The ENSL (2 g) was suspended in distilled water (150 mL) containing $CaCl_2$ (0.300 g, 0.08 M) under magnetic stirring at 55 °C till a homogeneous emulsified system was obtained (typically 20-30 min). Then, PLA1 (0.500 mL) was added and the mixture was kept under stirring at 55 °C for 6 h. The GPI formation was evaluated by TLC (ACN/NH_4OH 30% 60:40; detection by $Ce(SO_4)_2$/$(NH_4)_6Mo_7O_{24}$, GPI R_f=0.28) and HPLC (see Materials & Methods, GPI retention time= 8.4 min). At the endpoint, the reaction was cooled at room temperature and filtered under vacuum on a Celite pad, thus obtaining a clear filtrate which was concentrated under vacuum and partially purified by anion exchange chromatography on DIAION WA 55. The purification was performed as follows. The resin (23 g), supplied as OH form, was washed thoroughly with distilled water. For the activation, it was incubated with 3 M $HCOONH_4$ (50 mL) under rotary shaking in a falcon

for 30 min, filtered and then incubated with distilled water (50 mL) under the same conditions to remove the excess of salt. The activated resin was then used to fill a glass column (3 cm diameter) and the reaction crude was loaded on the column. The resin was washed with 5 bead volumes (BV) of distilled water followed by 5 BV of an aqueous solution containing 100 mM HCOOH and 10 mM $HCOONH_4$ (GPI was eluted in these fractions). The GPI containing fractions were collected and dried under vacuum. The residue was further purified by silica chromatography (eluent: ACN/NH_4OH 30% 60:40), thus obtaining pure GPI (98 mg). The product was characterized as reported in the Supporting Information (**Figures S6**-**S12**).

GPI purification by ion exchange chromatography: resins screening

An aqueous solution of standard GPI lysine salt (1 mg/mL; 1 mL) was incubated with different resins (DIAION WA 20, DIAION WA 30, DIAION WA 55, ReliSorb DA405/EB, Amberlyst A21; 0.100 g) under rotary shaking for 30 min. The resins were then separated from the supernatants and the solutions were analyzed by HPLC to evaluate the catch of GPI by the resin. Then, the resins which adsorbed GPI were incubated under different elution conditions (1 mL, see Results and Discussion) under rotary shaking for 30 min, filtered, and the supernatants were analyzed by HPLC to measure the release of GPI.

GPC preparation by chemical hydrolysis of ESL

The ESL (1 g) was solubilized in MeOH (15 mL) and K_2CO_3 (0.43 g, 0.0031 mol) was added. The reaction was kept under stirring a r.t. for 3 h. A solution of 1 M HCl was added, and the solution was dried under reduced pressure. The raw material was purified by flash chromatography (MeOH/H_2O 85:15) as reported in literature.[15,16] Pure GPC (0.205 g), as confirmed by ^{1}H NMR and ^{31}P NMR analyses (not reported) was obtained (yield: 88%).

GPI preparation by chemical hydrolysis of ENSL

The ENSL (2 g) was solubilized in CH_2Cl_2 (20 mL) and MeOH (20 mL) under magnetic stirring and an excess of MeONa was added. The reaction was immediate (TLC control; conditions as above reported). The reaction was quenched by adding 1 M HCl (3 drops). The solvent was removed under reduced pressure and the residue was purified by flash chromatography (gradient elution: ACN/NH_3 30%, 100:0-80:20-70:30-60:40). Fractions containing GPI were collected, concentrated and lyophilized. GPI was obtained as an

almost pure compound in 33% yield (22 mg). Analytical characterization was performed by ^{1}H NMR and ^{31}P NMR analyses (not reported).

2.2.3 Results & Discussion

Lecithin pre-treatment

The scheme of the sunflower lecithin pre-treatment is reported in **Figure 1**. Lecithin is a complex mixture of different phospholipids. The main components are phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidylethanolamine (PE). However, lecithin contains also other phospholipids such as phosphatidic acid (PA) and phosphatidylserine (PS), as well as non-polar lipids such as glycerides and free fatty acids. All these phospholipids are the precursors of high-value chemicals, such as GPI and GPC.[3]

We reasoned that the pre-treatment of lecithin aimed at the selective enrichment of this raw material in a specific phospholipid (i.e. PI and PC, in this case) might be convenient in order to maximize the conversion of lecithin into the target molecules. This strategy was addressed mainly by two considerations. Lecithin is a complex mixture of chemically related molecules (see **Figure S1**): any treatment which could afford a less "challenging" substrate would have been highly desirable. Furthermore, in principle, a less complex mixture might have assisted in addressing the selectivity of the enzymes used for the planned biotransformations. Lipases and phospholipases reported for PL modification (see **Chapter 2.1**) may display overlapping substrate specificities toward PL classes, thus resulting to be poorly efficient for the transformation of lecithin. For example, the phospholipase A1 from *Aspergillus oryzae* is active towards all the main components of lecithin, although to a different extent (PE>PI>PC).[17] Nonetheless, once that one or more PL classes have been removed from the starting mixture, the substrate "enriched" in PI, PC or PE etc. may be converted into a specific glycerophosphoric acid ester. Needless to say, a reduced number of by-products makes much easier the isolation and purification steps. Product downstream, in fact, appeared immediately to be one of the sore points of the route from lecithin to glycerophosphoric acid esters, both by biocatalysis and "conventional" chemical approaches. Complexity of the vegetable matrix, solubility issues (lecithins are natural emulsifiers), and the high polarity of glycerophosphoric acid esters were identified as the major bottlenecks.

We hypothesized that the removal of PE from lecithin might have been a key step before the hydrolysis. This experimental plan was corroborated by the evidence that PC and PI have a different solubility in short-chain alcohols (MeOH, EtOH or EtOH/water mixtures, and *iso*-PrOH).[12,18] Thus, alcohol treatment of lecithin allows to obtain a soluble fraction enriched in PC, and an insoluble fraction enriched in PI. Solubility of PC in alcohols is the method currently used to produce PC-enriched lecithin with a different degree of purity.[19]

The acetylation conditions used (see Materials & Methods) selectively acylated the primary amine group of PE, thus affording *N*-acetyl PE which was then removed from the mixture by exploiting its solubility in acetone (de-oiling), as sketched in **Figure 1**. As for the separation of PC and PI, among all the tested alcohols, fractionation with EtOH afforded the most enriched solution in PC (Ethanol Soluble Lecithin, ESL) and the most enriched residue in PI (Ethanol Non-Soluble Lecithin, ESL).

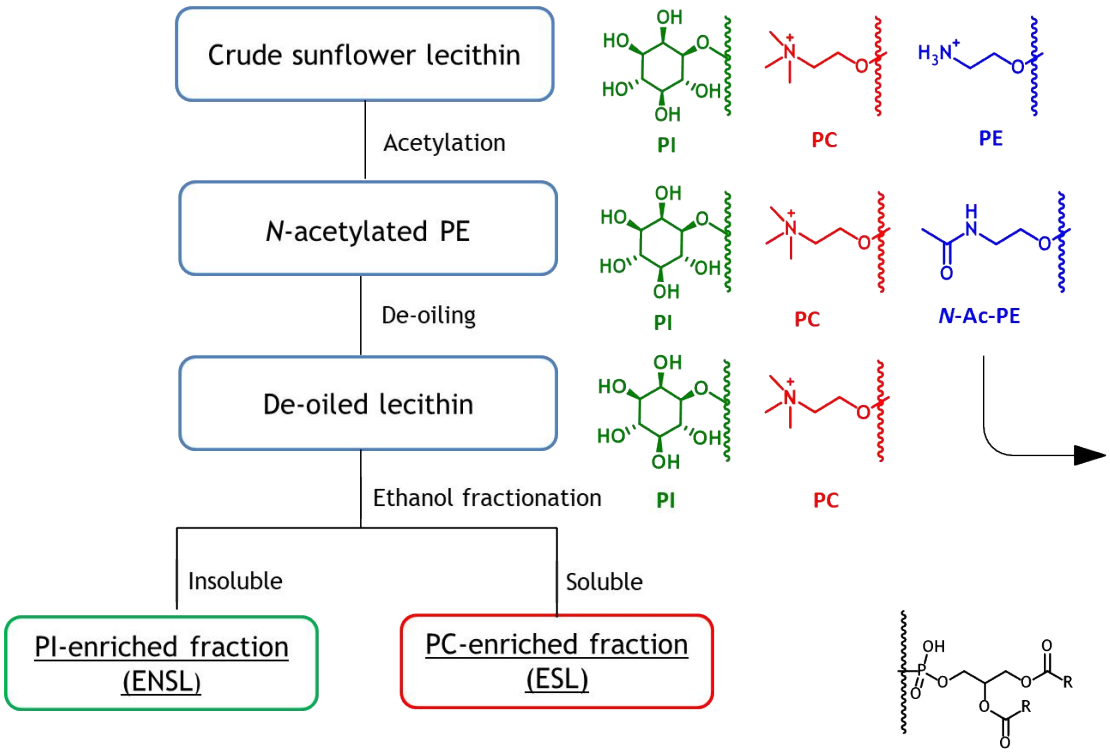


Figure 1: Work-flow of the fractionation of sunflower lecithin

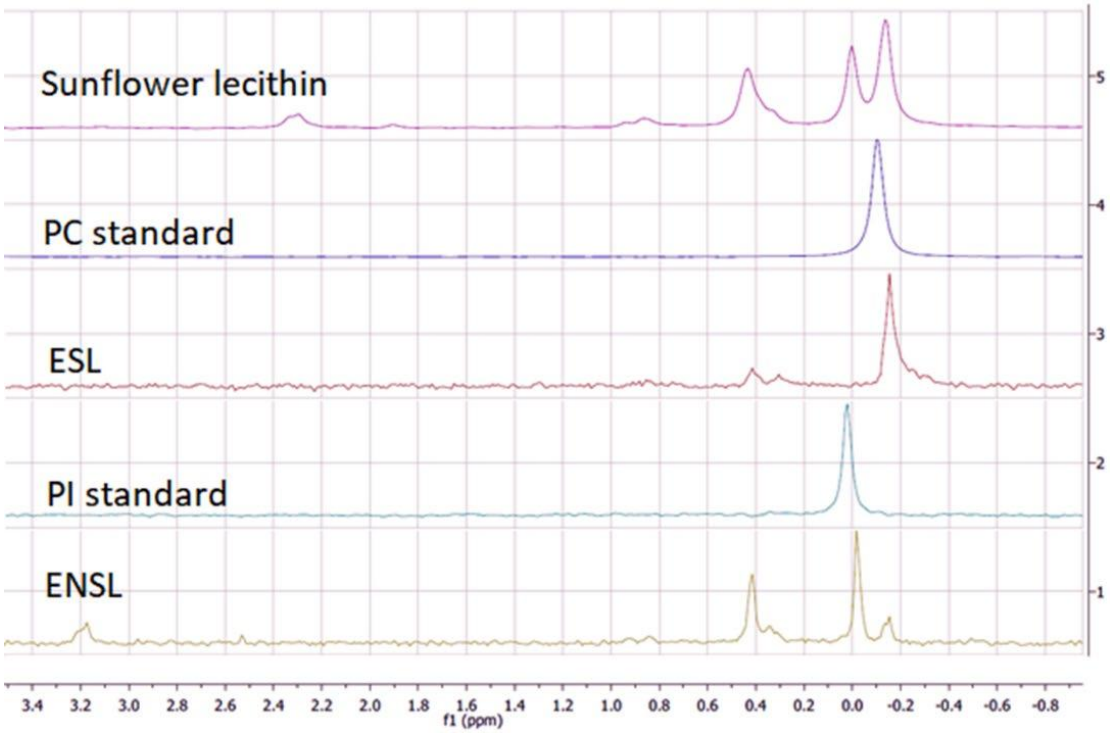


Figure 2: ^{31}P NMR spectra of sunflower lecithin fractions. Conditions: 10-15 mg of lecithin in D_2O 20% in H_2O, sodium cholate 10% (w/w), EDTA 1% (w/w), 1 M NaOH until pH 7.5

After three rounds of fractionation in EtOH, the composition of lecithin resulted to be suitable for the further step, that is the enzymatic hydrolysis. From the comparison of ^{31}P NMR spectra (**Figure 2**) of the starting lecithin with a reference standard of PC and PI, and the obtained ESL and ENSL samples, it clearly emerges that the ESL and ENSL fractions mostly contain PC and PI, respectively, and that can be further processed for the obtainment of GPC and GPI.

GPC preparation by enzymatic hydrolysis of ESL

The preparation of GPC by enzymatic hydrolysis of lecithin is a well-known process. The most common strategies, that have been recently reviewed,[3] involved the use of lipases or phospholipases in non-conventional media such as biphasic systems, aqueous media containing surfactants to improve the dispersibility of non-polar molecules, or systems adopting a fed-batch mode for the control of substrate concentration. Taking into account previous data from our laboratory about the controlled enzymatic hydrolysis of triglycerides in low-water *t*-BuOH,[20] the high tolerance of lipases to this solvent,[21] and that the PC-enriched fraction was obtained by alcohol fractionation, the enzymatic hydrolysis of lecithin (ESL) was carried out in *t*-BuOH. Besides being extensively used in lipase-catalyzed transesterifications and hydrolysis reactions,[21] *t*-BuOH has the status of

"preferred" solvent.[22] A homogeneous reaction medium, consisting of *t*-BuOH with a low-water concentration (12%), was used to dissolve the PC-enriched fraction at 55 °C. Water does not play the role of solvent itself, but it only acts as the necessary reagent to carry out the hydrolysis. Not less important, the controlled water content in the reaction medium leads to the control of by-product formation as well.

The enzyme CalB (lipase from *Candida antarctica,* commercially available as Novozym 435) was used as the biocatalyst owing to its high stability at high temperature and the advantage to have an immobilized enzyme. Moreover, while this work was in progress, a report[4] had described the synthesis of GPC from PC in water at 55 °C by using CAL B.

After filtration of the enzyme, the crude was purified by extraction (*n*-hexane/water) and flash chromatography, resulting in 37% yield (see Materials & Methods and Supporting Information, **Figures S4** and **S5**). The reaction yield was calculated assuming that, according to the literature,[12] the content of PC in ESL was 80%. The yield of the biotransformation resulted to be lower than that of the chemical hydrolysis (88%); moreover, the reaction time (5 days) appeared to be a still further shortcoming of this approach.

GPI preparation by enzymatic hydrolysis of ENSL

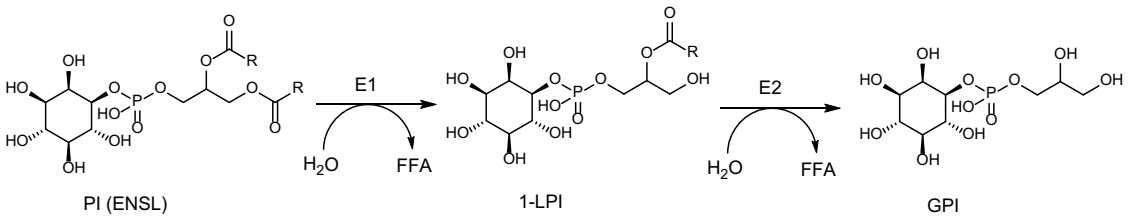
In contrast to GPC, very few reports have been published to date on the synthesis of GPI.[3] In fact, only one total synthesis of GPI, specifically of "1-glyceryl-2-*myo*-inosityl phosphate", as named in the original paper, has been reported so far. This report[23] dates back to 1959 when the position of the phosphate group in *myo*-inositol moiety (whether it was 1 or 2) had not been elucidated, yet.

The preparation of GPI and its organic (cyclohexylamine) and inorganic salts (calcium, magnesium, barium) by chemical hydrolysis of de-oiled soybean lecithin was patented by Tronconi in 1994.[24] De-oiled soy lecithin, a low-cost commercial product, was hydrolyzed with sodium methoxide in MeOH. After filtration, the residue was submitted to several tedious sequential steps of acidification, washings with MeOH/water, elution through ion exchange resins, concentration, and precipitation from acetone and EtOH, to give GPI. Starting from 1.22 Kg of de-oiled soy lecithin, 31 g of the target compound were obtained.

To the best of our knowledge, only one enzymatic synthesis of GPI has been reported to date within a research focused on the transesterification of primary alcohols with inositol 1,2-cyclic phosphate (IcP), in the presence of a PI-specific phospholipase C (PI-PLC).[11] The intermediate IcP is in turn produced *via* a transesterification reaction involving the PI-PLC-catalyzed attack of the inositol 2-hydroxyl group on the phosphorus atom of PI with the release of 1,2-diacylglycerol. Owing to the complexity of this approach (without going in detail about stereoselectivity issues and suitability of PLC for preparative syntheses) as well as the tricky downstream, as mentioned before, it is not surprising that pure GPI is available on the market at a very high cost (see, for example, https://www.echelon-inc.com/product/gropi/: $ 730,00/mg; data retrieved on December 2022).

Within this framework, it was planned to design a new process to GPI based on the use of lipases or phospholipases, taking into account the plenty of data available on the better-known GPC. As for the reaction conditions, both fully aqueous media and biphasic systems containing *n*-hexane as the organic solvent were assayed, being the latter able to achieve higher concentrations when working with substrates poorly soluble in water.

We started from a process in which two enzymes were used in a sequential mode (**Scheme 1**). These enzymes were selected from a screening performed by Bict s.r.l., one of the partners of the project BIOCOSM (see Acknowledgements).



Scheme 1: Synthesis of GPI by the bi-enzymatic hydrolysis of ENSL. E1: phospholipase A2; E2: lipase. 1-LPI: 1-acyl lysophosphatidylinositol

As expected, the product downstream was very challenging because the emulsion resulting from the suspension of the substrate in water was “over-stabilized” by the emulsifying properties of the lecithin itself and by the concomitant release of free fatty acids upon hydrolysis. Any attempts of work-up based on liquid-liquid extraction was discarded, because the phase separation was not even visible. Neither GPI precipitation with di-ciclohexylamine was successful due to a lack of selectivity. Dilution of the

mixture with acetone was also performed, taking into account that GPI is not soluble in acetone, whereas the FFA formed during the reaction, are soluble. Acetone was discarded, and the solid residue was then re-suspended in water and submitted to purification by ion-exchange chromatography by using a weak anionic resin, affording GPI as an almost pure product.

The use of ion exchange resins for the purification of GPI was suggested by the analysis of the patent by Tronconi,[24] previously mentioned about the preparation of GPI by chemical hydrolysis of lecithin. The choice of Amberlyst A-21 was addressed by data found in literature: i) weak anion resins are reported to be more efficient than strong anion resins in the separation of PL according to their polar head groups;[24] ii) a detailed description on how to prepare and use this resin was reported for the purification of ferulic acid from a wheat bran enzymatic hydrolysate.[25] This latter report was very useful to drive the experimental plan involving both the resin conditioning, adsorption and elution conditions.

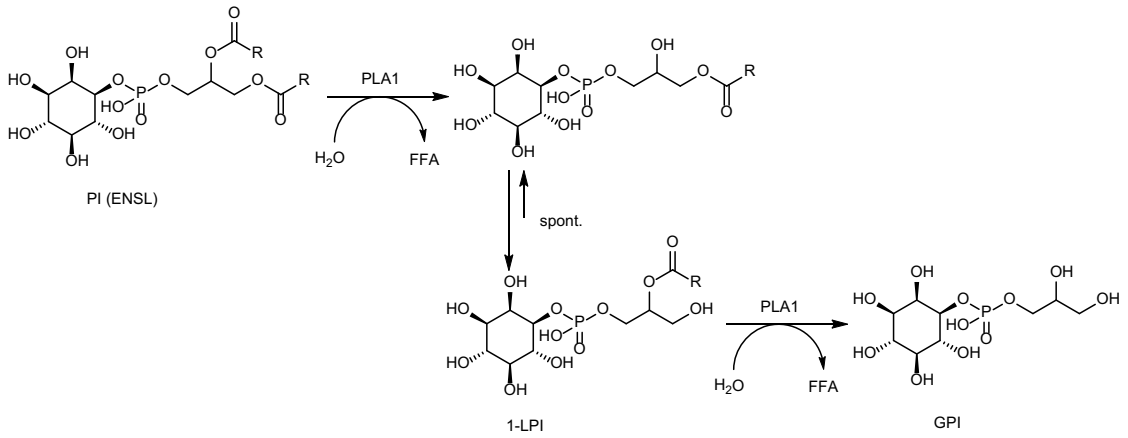
This result was the starting point to the development of a simpler process based on the use of a single enzyme. Moreover, the work-up requiring the acetone partitioning step was not sustainable because of the high consumption of solvent and the burden associated to GPI recovery.

For the explorative trials, we started from lipases. We selected Novozym 435, *Pseudomonas cepacia* lipase (PCL), *Pseudomonas stutzeri* lipase (PSL), and the immobilized acetylxylan esterase from *Bacillus pumilus* (AXE) immobilized on glyoxyl agarose.[26] The use of Novozym 435 is self-explaining since it is one of the most used enzymes in biocatalysis.[27] Acetylxylan esterase (AXE) is routinely used to perform the regioselective hydrolysis of sugar derivatives;[28] thus, it was included in the screening because xylan, the natural substrate of this enzyme, is an oligosaccharide and, indeed, the possibility that this enzyme might recognize PI as a substrate was not ruled out (inositol can somehow resemble a sugar). On the other hand, the enzymes PCL and PSL were previously used in our laboratory in the selective hydrolysis of triglycerides in *t*-BuOH.[20] From this screening, the GPI formation was detected only when Novozym 435 was used in the biphasic system (*n*-hexane/water 75:25).

Then, phospholipase A1 from *Aspergillus oryzae* (PLA1), commercially available as Lecitase Ultra, was assayed, taking into account the literature data about the hydrolysis

of PC to GPC.[6,16,29] Phospholipase A1 represents a very diverse sub-group of phospholipase isoenzymes with L-acyl hydrolytic activity.[30] Lecitase Ultra is obtained by the fusion of lipase genes from *T. lanuginosus* and phospholipase genes from *F. oxysporum*.[17] It has been reported that Lecitase Ultra displays a hydrolytic activity toward acyl glycerides and phospholipids; owing to these features, it is used in the oil chemical industries.[31] Several studies have been reported for the production of GPC through the hydrolysis of PC in aqueous media using PLA1.[29,32,33] The hydrolysis of PC catalyzed by PLA1 can lead to the formation of GPC as well as *sn*-1-LPC (lysophosphatidylcholine) due to the migration of the acyl group from the *sn*-2 position to the *sn*-1 position in LPC.[34] Plückthun and Dennis[35] investigated the acyl migration in LPC and reported that about 90% of the thermodynamically stable 1-acyl LPC and 10% 2-acyl LPC were present at the equilibrium under the conditions of their study. The presence of a higher amount of *sn*-1 LPC results in the increased formation of GPC during the reaction because the acyl group at the *sn*-1 position of LPC is hydrolyzed by PLA1. The acyl migration phenomenon can be intentionally induced to facilitate the formation of GPC, by tuning temperature, reaction time, water content, and enzyme loading. [36]

The biotransformation of ENSL to obtain GPI was thus performed in a fully aqueous medium supplemented with Ca^{2+} ions since PLA1 is a calcium dependent enzyme (**Scheme 2**). The absence of a buffer can be advantageous when ion exchange resins are used in the purification step because the risk of ion competition is avoided. As reported in the Materials & Methods, a pre-incubation of the ENSL in water at 55 °C before the addition of the enzyme is crucial to obtain a homogeneous emulsified system without solid aggregates which can negatively affect the mass transfer. The reaction was carried out up to 6 hours because a longer reaction time resulted in the formation of by-products derived from the hydrolysis of other PL, as PLA1 is not fully selective.



Scheme 2: Synthesis of GPI by the mono-enzymatic hydrolysis of ENSL. PLA1: phospholipase A1. 1-LPI: 1-acyl-lysophosphatidylinositol

Product downstream

In order to get rid off partitioning with acetone, the reaction mixture was filtered through a Celite pad. The partly clarified aqueous solution (**Figure 3**), containing GPI, unreacted PL, FFA, lyso-PL, and glycerides was submitted to purification by ion-exchange chromatography. Unexpectedly, Amberlyst A-21, that was previously used for the bi-enzymatic process, did not work out in these conditions. It was, indeed, necessary to set-up new conditions for the product downstream. To this aim, a panel of commercially available ion exchangers functionalized with tertiary amine groups and/or quaternary ammonium groups, were assayed (see Materials & Methods).

Figure 3: Synthesis of GPI by the mono-enzymatic hydrolysis of ENSL catalyzed by PLA1 in fully aqueous medium. Reaction mixture before (right) and after (left) filtration on the Celite pad

The screening of the resins for applying a “catch-and-release” strategy in the purification of GPI was hampered by the lack of a GPI standard in sufficient amounts, but an ethanolic solution of GPI lysine salt. From the screening, the adsorption of GPI was

almost quantitative in all cases but finding the elution conditions was troublesome. Elution with increasing concentrations of CH_3COOH,[24] 60 mM $HCOONH_4$, 5 mM $Na_2B_4O_7$ solution,[37] or NH_4OH resulted in a very low or an even negligible release of GPI from the resin. As a rule of thumb, the elution from weak ion exchange resins can be performed by changing the pH depending on the pKa of the target molecule and on the functional groups of the resin. However, as the pKa of GPI is around 1 (and thus GPI is generally in the anionic form), this could explain both its rapid adsorption and its poor release.

A different approach, which is generally used with strong ion exchange resins, is the elution driven by the ionic strength, which allows the elution of the analytes by competition with other ions. In order to test this strategy, DIAION WA55 was selected because of its quaternary ammonium portion. The use of the resin in the formate form instead of the free base form, and the elution with HCOOH/$HCOONH_4$ solutions were also suggested by literature,[38] describing the use of an anion exchange resin for the separation (and identification, thereof) of different inositol phosphates from the rat brain. These conditions were tested directly with the reaction mixture, which contained a mixture of phospholipid-derived ingredients able to interact with the resin. This step was crucial for the fine-tuning of the product downstream: in fact, it was soon realized that the set-up of adsorption and elution conditions by using the standard solution of GPI lysine salt was not a reliable model in the view of applying the purification protocol to the "real" GPI reaction mixture, owing to its much higher complexity.

Eventually, a 100 mM HCOOH and 10 mM $HCOONH_4$ solution was found to release GPI from the DIAION WA 55 in the $HCOO^-$ form. In parallel, the stability of GPI under these conditions was also evaluated (by using the GPI lysine salt standard; data not shown) before proceeding with the purification.

The crude GPI obtained by ion exchange chromatography was submitted to a further purification step by silica chromatography, according to protocols previously reported for GPC.[15] Also in this case, the stability of the GPI standard was evaluated under the elution conditions (Materials & Methods).

The GPI obtained by this procedure was fully characterized by ^1H, ^{13}C, ^{31}P NMR spectroscopy, HPLC-ELSD and ESI-MS (**Figures S6**-**S13**). To the best of our knowledge, this is the first report describing the synthesis of GPI through the enzymatic hydrolysis of lecithin in a fully aqueous medium.

2.2.4 Conclusions

Phospholipids contained in lecithin are responsible for the high complexity of lecithin composition as well as its properties. From an applicative viewpoint, PL have a well-established use as emulsifiers in many market sectors. In addition, PL can be used as precursors of biologically active molecules, thus making lecithin a renewable feedstock of high-added value products.

In this framework, GPC and GPI were identified as target molecules to be obtained from sunflower lecithin by upgrading it through a biocatalytic approach. Sunflower lecithin was preferred over soy lecithin because it is reported to have a higher content of PI than soy lecithin. Moreover, sunflower lecithin has been generally less investigated than soy lecithin.

The work-plan based on the use of lipases, phospholipases, or a combination of both, required first an in-depth analytical characterization of the starting material (in collaboration with the University of Milano), as well as the set-up of the analytical methods for reaction monitoring. In this context, ^{31}P NMR spectroscopy resulted to be the technique of choice for the analysis of PL and glycerophosphoric acid esters.

The complexity of the substrate and the evidence that PL are structurally related (but for the polar head group) make very hard to separate PL from lecithin as well as their recovery; this constraint was partly circumvented by submitting sunflower lecithin to a multi-step, sequential fractionation protocol in order to obtain a PC-enriched lecithin and a PI-enriched lecithin as reaction substrates. The pre-treatment of lecithin was thus a key step to reduce side-reactions involving PL other than PC or PI, and to maximize the formation of the target product.

In the case of GPC, this product was obtained by using the CalB lipase (Novozyme 435) as the biocatalyst in *t*-BuOH; this solvent has never been reported for this biotransformation. Besides being well-tolerated by lipases, *t*-BuOH is acknowledged as a “preferred” solvent for its “green” status.

More interestingly, a new enzymatic process was set-up for the obtainment of the high-value GPI by using PLA1 in a fully aqueous medium. Although we succeeded in obtaining pure GPI upon this enzymatic reaction, a greater effort is still needed to tune all the reaction parameters and to optimize the purification step. For GPI, the use of ion

exchange chromatography was crucial for the product recovery and purification, although to a modest yield so far (about 20%). Generally speaking, the natural feature of lecithin as emulsifier represented the main bottleneck in setting up the reaction conditions and the downstream step.

Finally, chemical routes to the synthesis of GPC and GPI were also used to provide the analytical standards; GPC and GPI were obtained with high to modest yield (88% and 22%, respectively). To date, whereas the chemical process to GPC appears still to be superior to the biocatalytic route described herein, in the case of GPI the enzymatic hydrolysis in a fully aqueous medium catalyzed by PLA1 represents an innovative synthetic scheme for the obtainment of this very high-added value product.

Acknowledgements

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2.2.6 Supporting Information

Characterization of lecithin

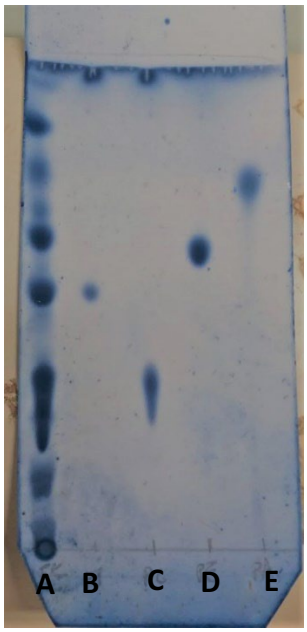

Figure S1: TLC of SUN B lecithin (A) and pure phospholipids isolated from the raw material: PI (B), PC (C), PE (D) and PA (E). Lecithin/PL samples were dissolved in $CHCl_3$. Eluent: *n*-BuOH/H_2O/CH_3COOH/ 80:20:20. Detection with $Ce(SO_4)_2$/$(NH_4)_6Mo_7O_{24}$

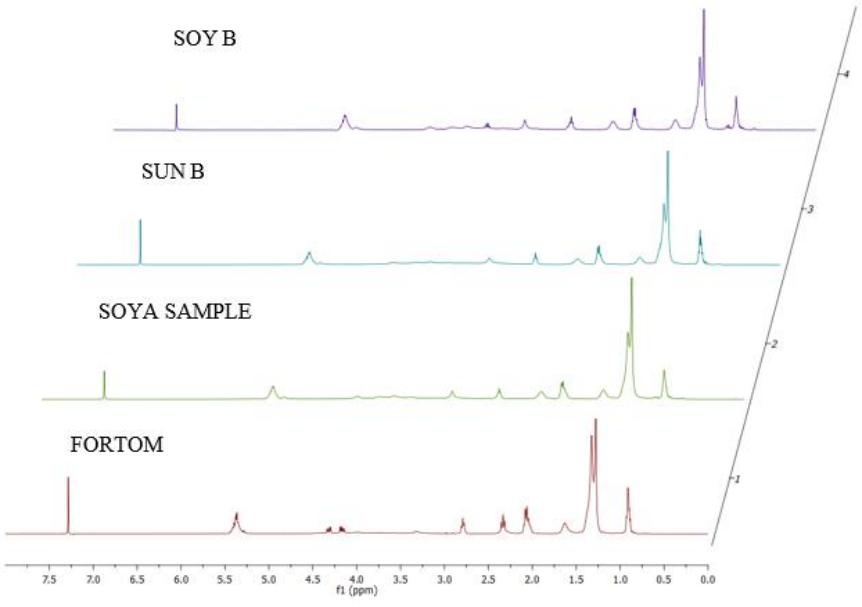

Figure S2: ^1H NMR ($CDCl_3$) of lecithin samples 1-4 (namely, SOY B, SUN B, SOY A, FORTOM). The sample SUN B corresponds to the sunflower lecithin used in this work

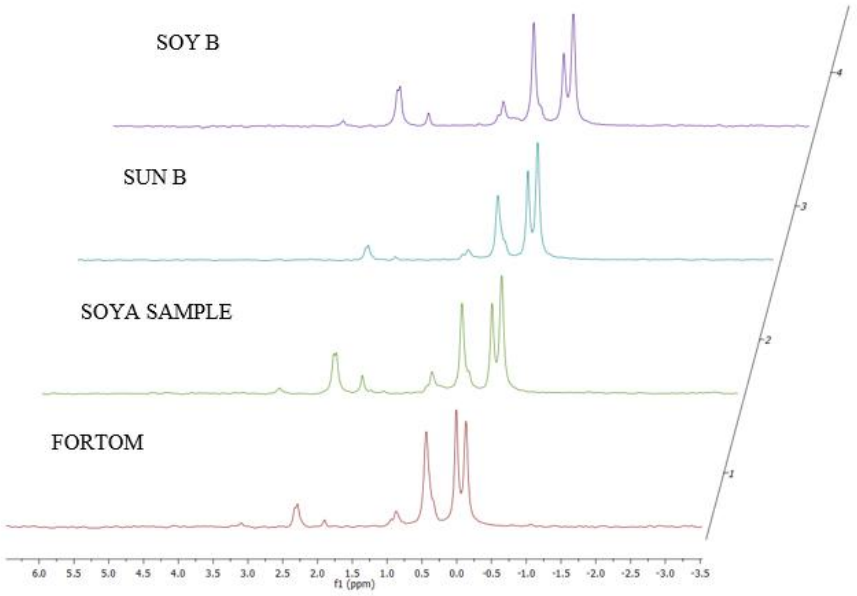

Figure S3: ^{31}P NMR of lecithin samples 1-4 (namely, SOY B, SUN B, SOY A, FORTOM). Conditions: 10-15 mg of lecithin in D_2O 20% in H_2O, sodium cholate 10% (w/w), EDTA 1% (w/w), 1 M NaOH until pH 7.5

NMR spectra of glycerophosphocholine (GPC)

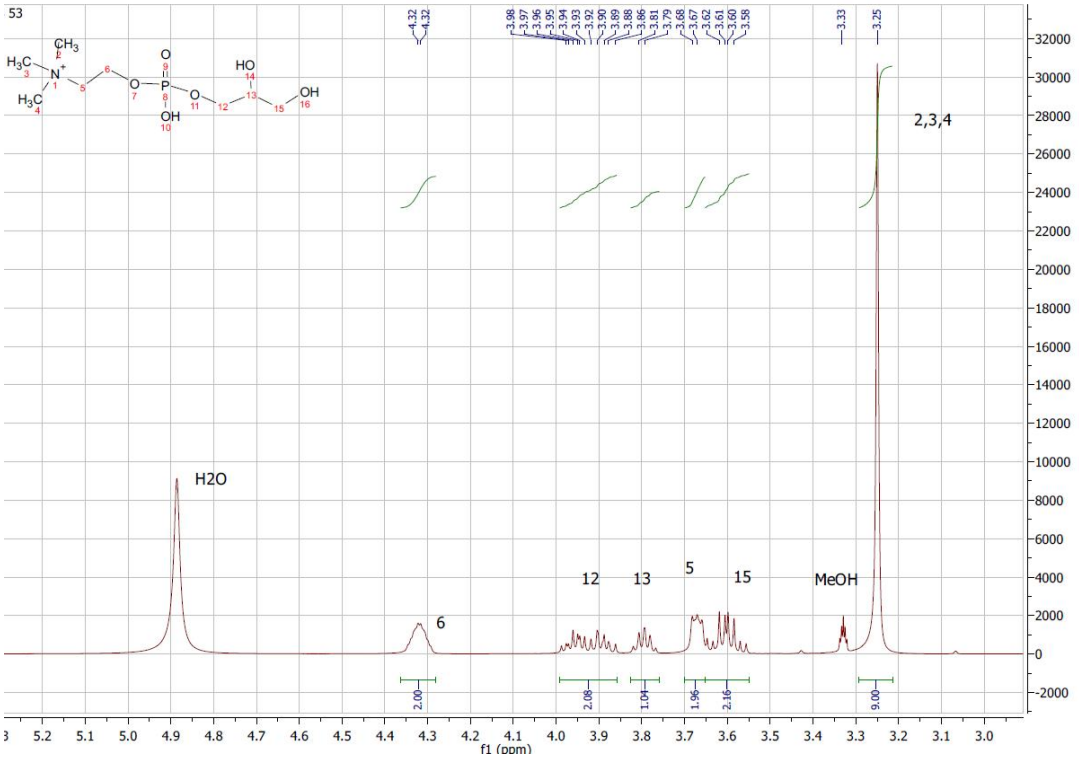

Figure S4: ^{1}H NMR (CD_3OD) of GPC obtained by chemical hydrolysis of the ESL fraction

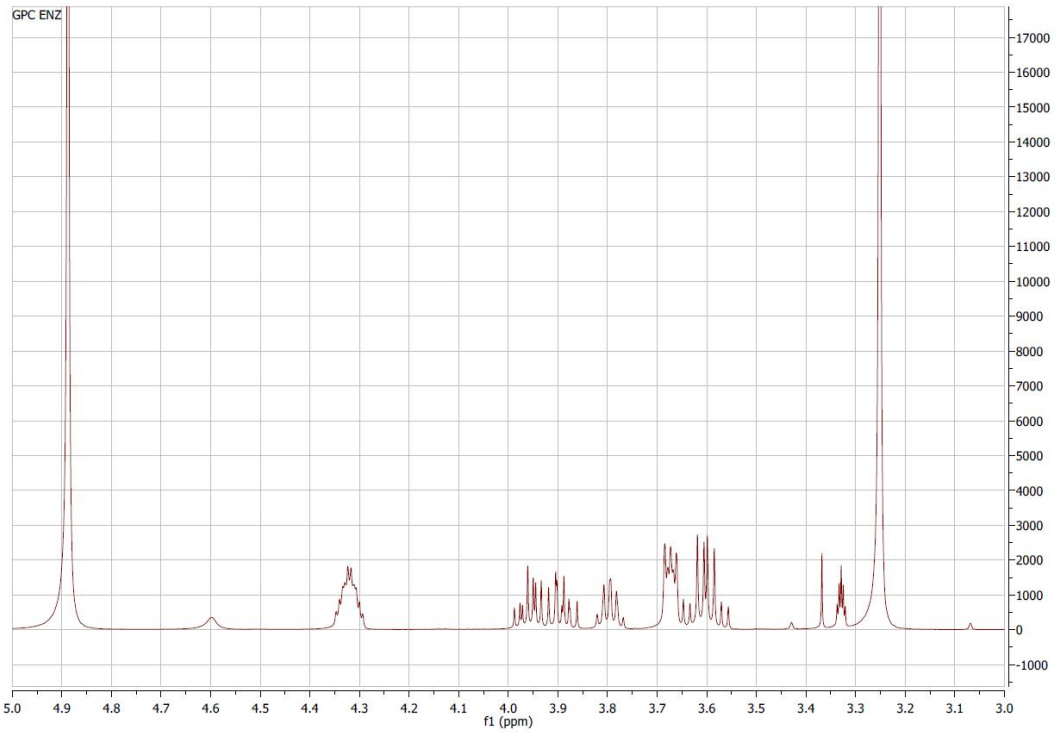

Figure S5: ^{1}H NMR (CD_3OD) of GPC obtained by enzymatic hydrolysis of the ESL fraction

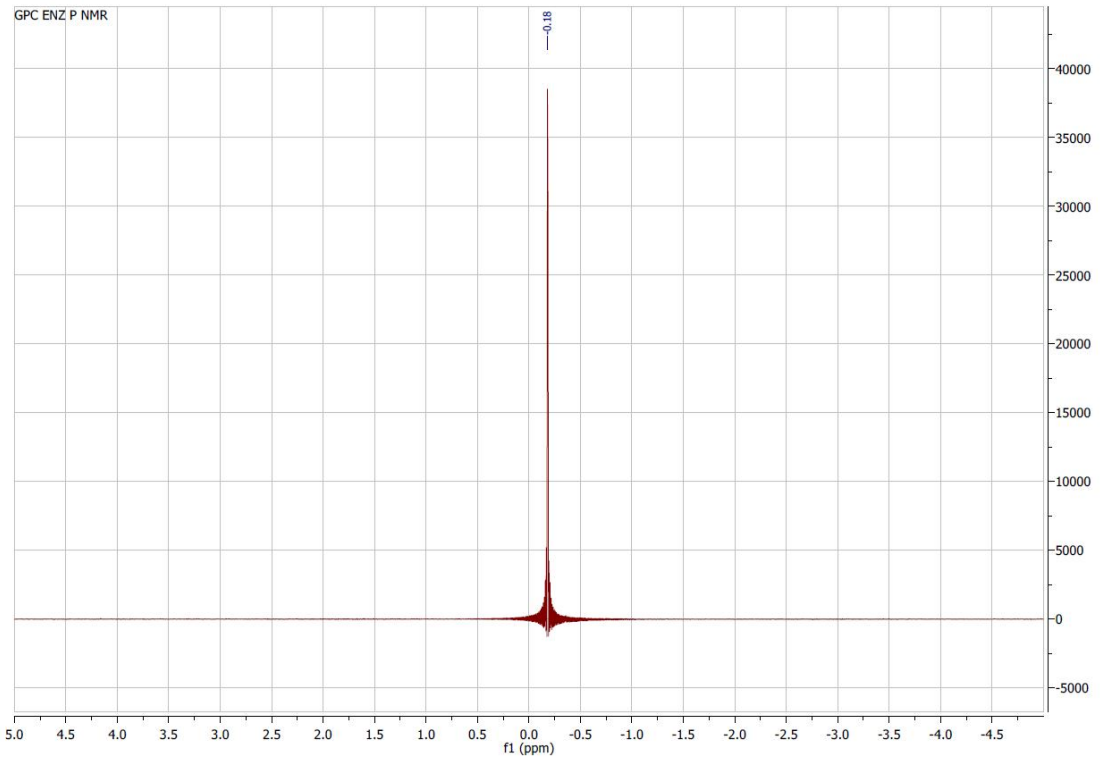

Figure S6: ^{31}P NMR (CD_3OD) of GPC obtained by enzymatic hydrolysis of the ESL fraction

NMR spectra of glycerophosphoinositol (GPI)

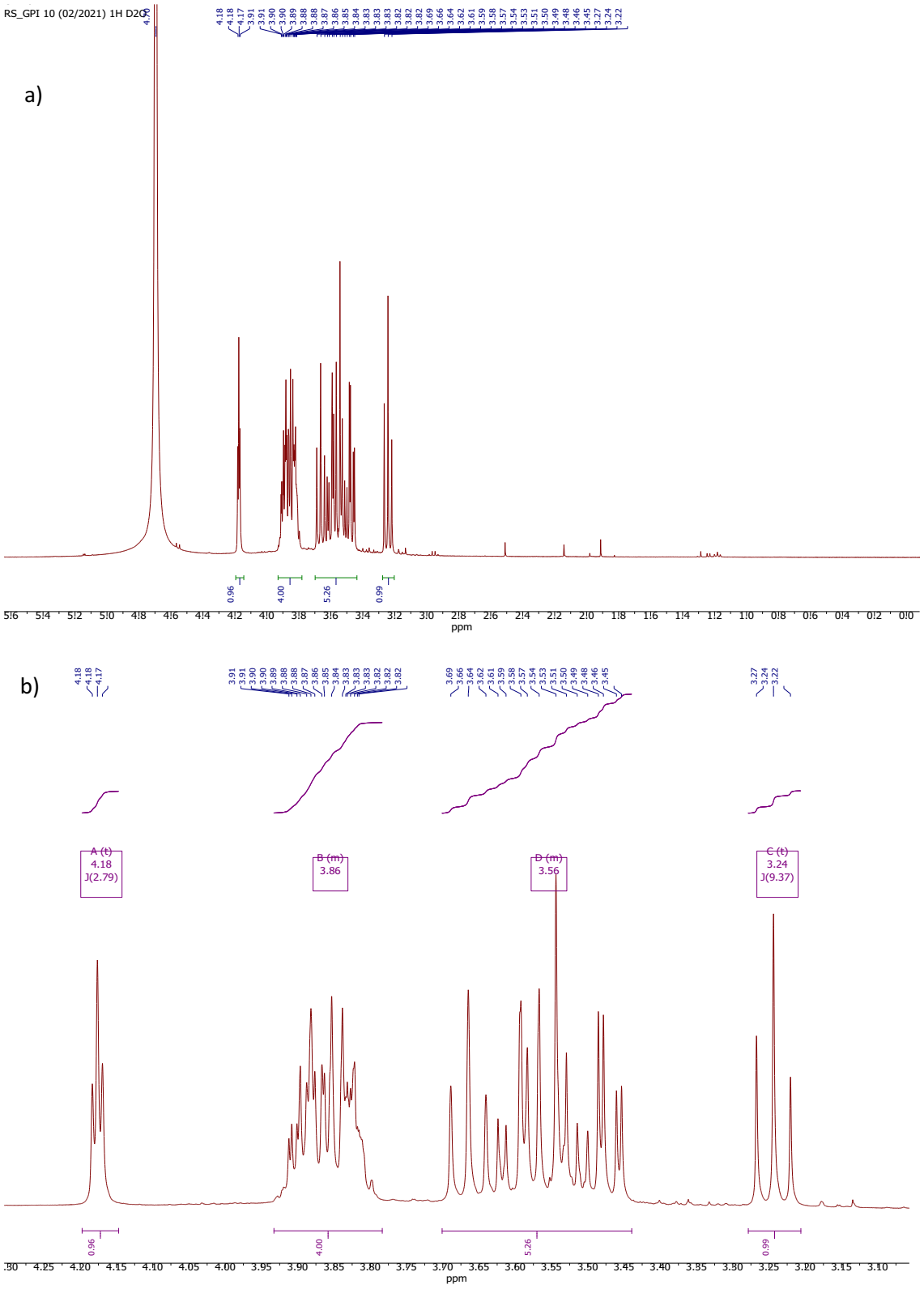

Figure S7: ^{1}H NMR (D_2O) of GPI obtained by the mono-enzymatic hydrolysis of the ENSL fraction. Full spectrum (a) and zoom (b)

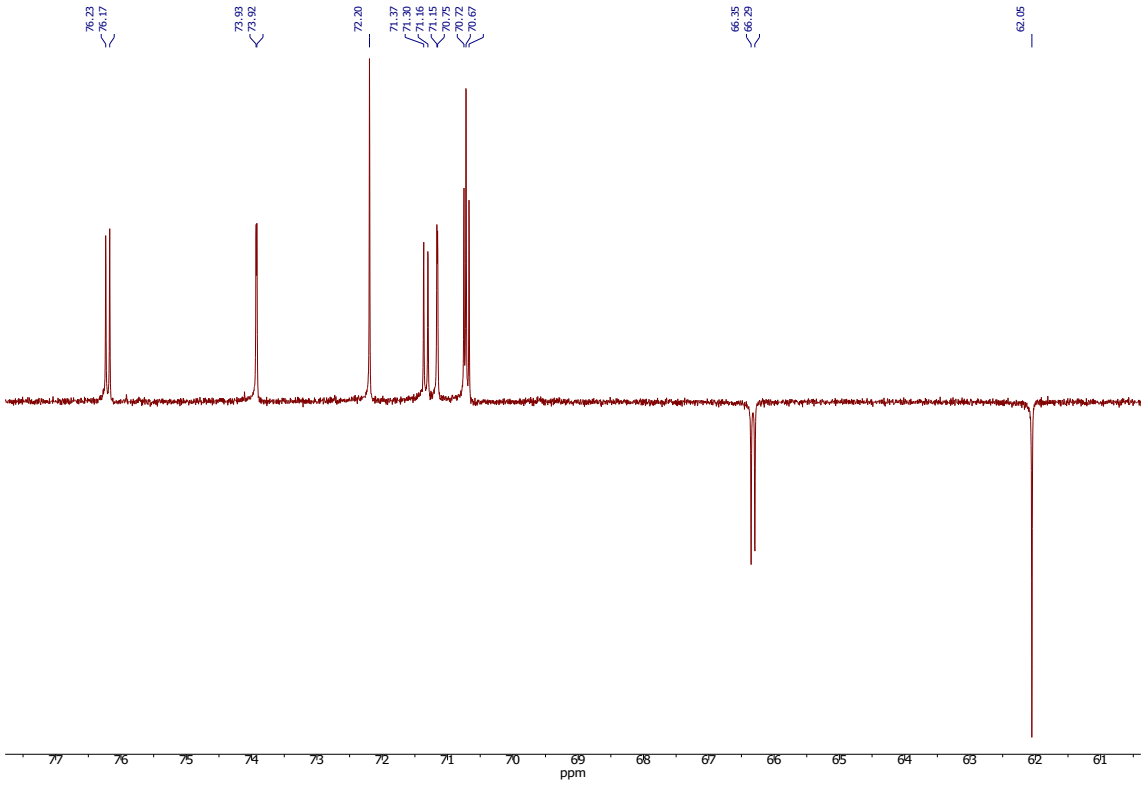

Figure S8: ^{13}C NMR (D_2O) of GPI obtained by the mono-enzymatic hydrolysis of the ENSL fraction

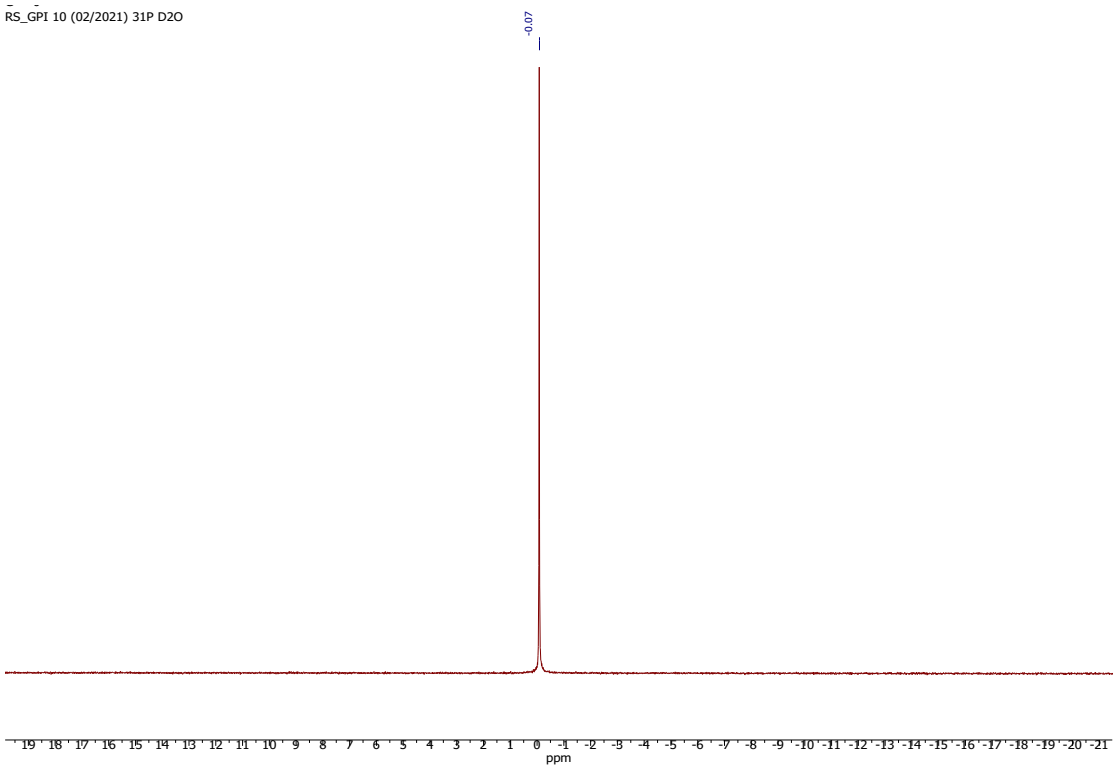

Figure S9: ^{31}P NMR (D_2O) of GPI obtained by the mono-enzymatic hydrolysis of the ENSL fraction

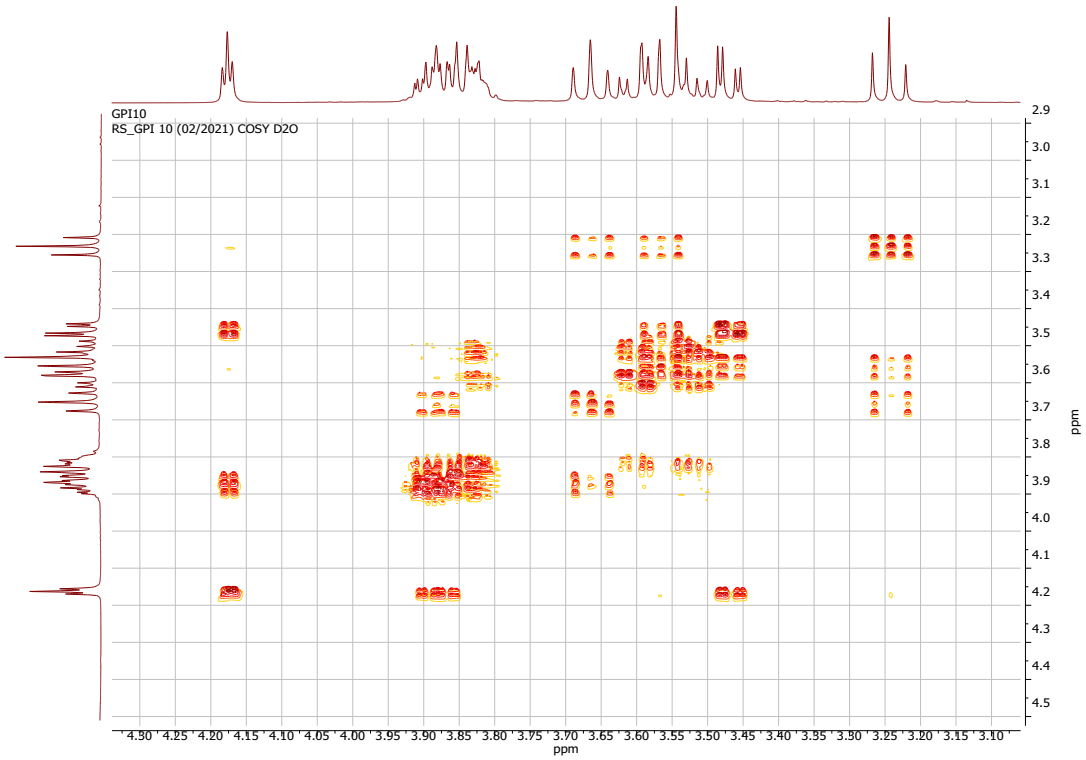

Figure S10: COSY of GPI obtained by the mono-enzymatic hydrolysis of the ENSL fraction

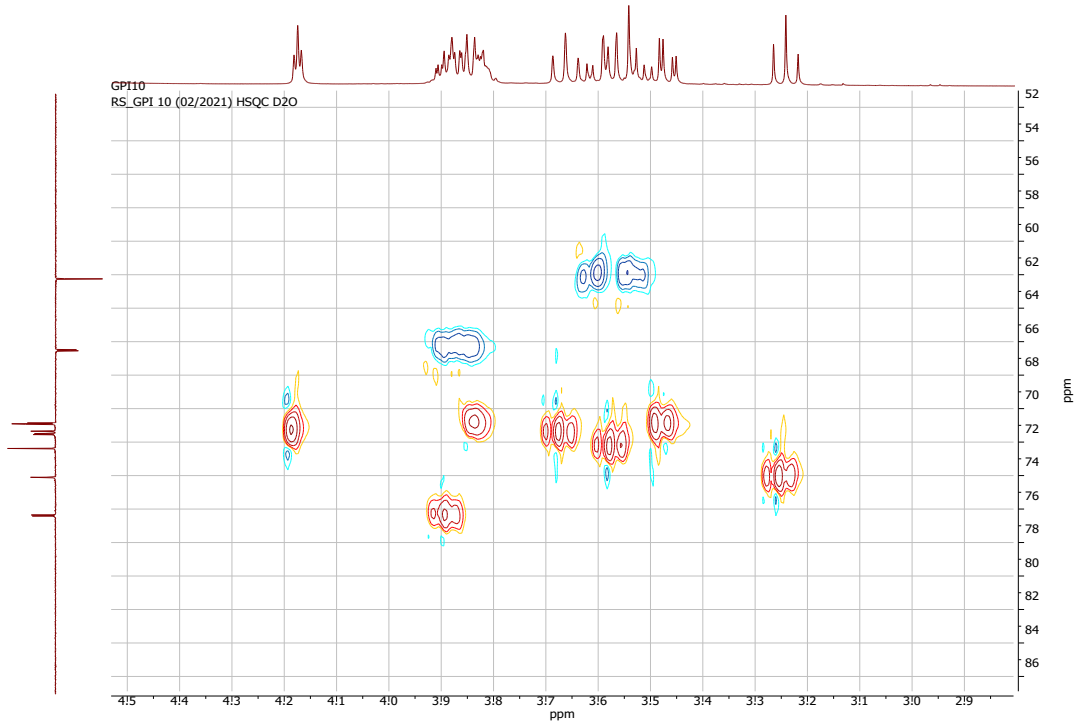

Figure S11: HSQC of GPI obtained by the mono-enzymatic hydrolysis of the ENSL fraction

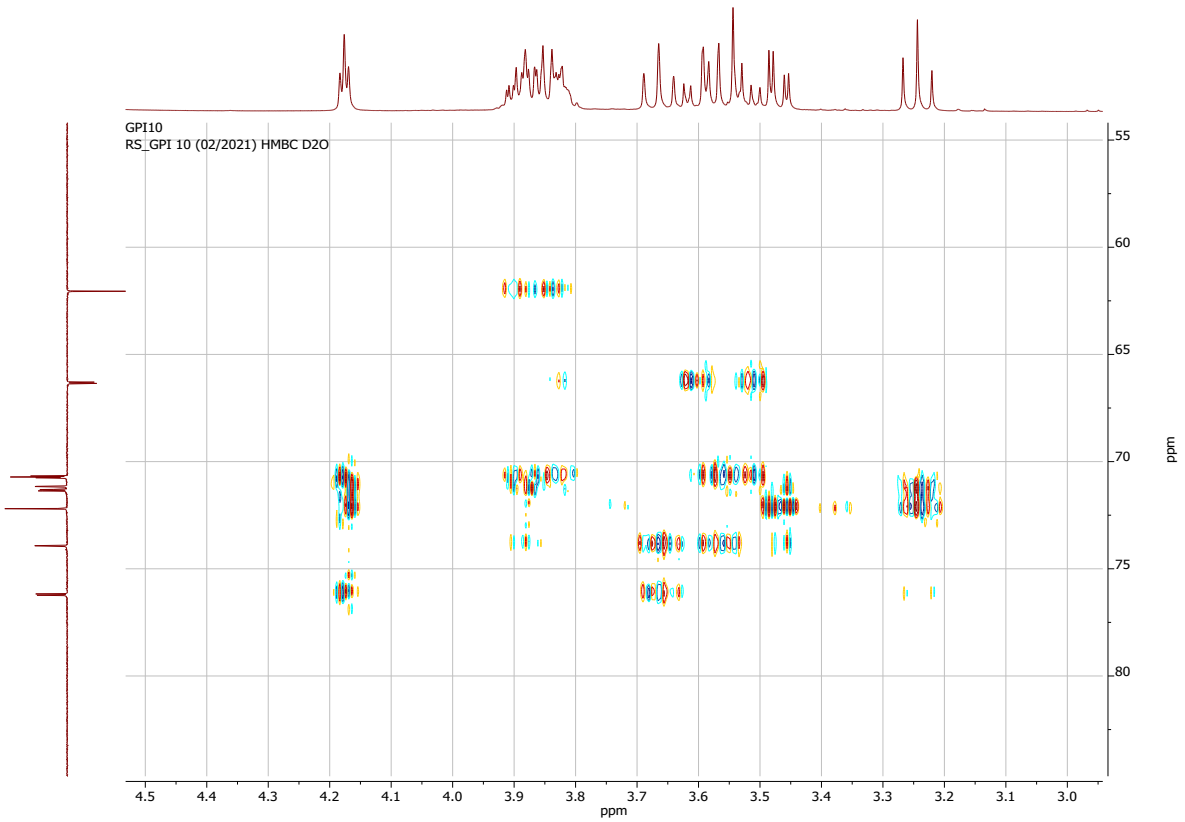

Figure S12: HMBC of GPI obtained by the mono-enzymatic hydrolysis of the ENSL fraction

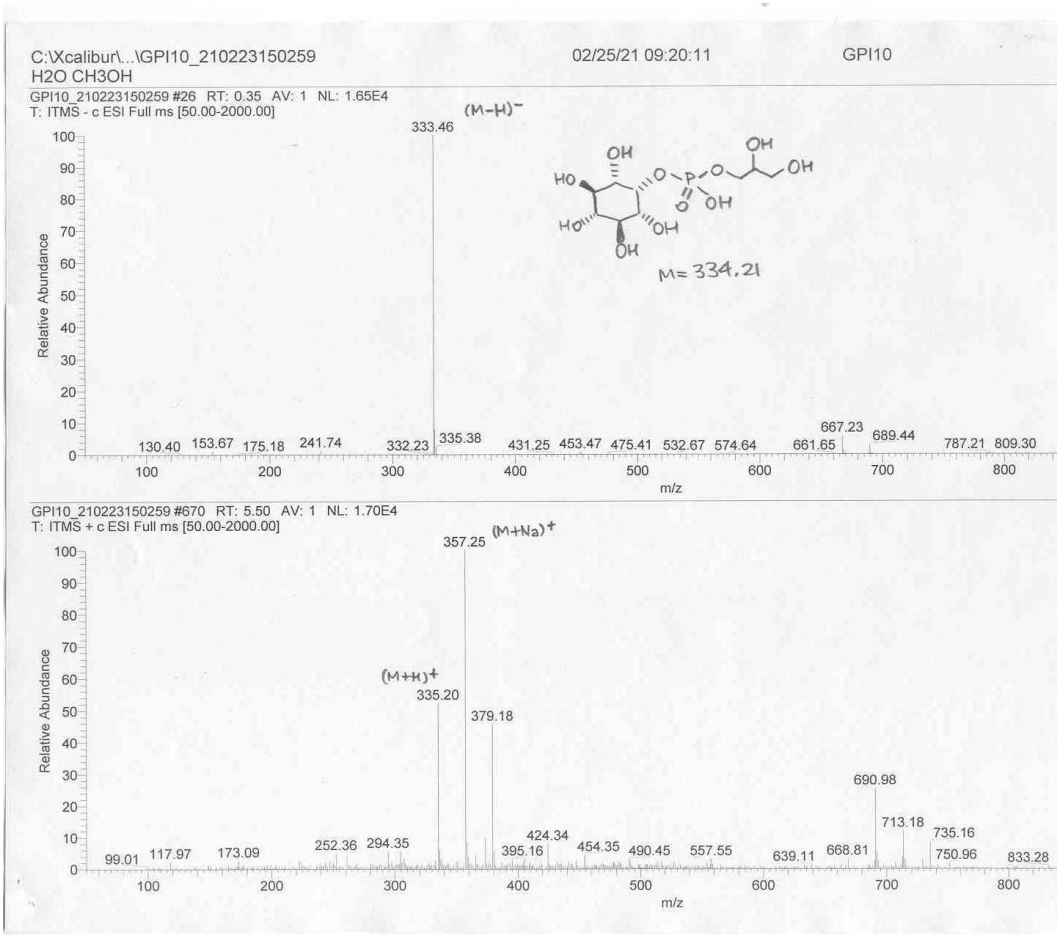

Figure S13: ESI-MS of GPI obtained by the mono-enzymatic hydrolysis of the ENSL fraction

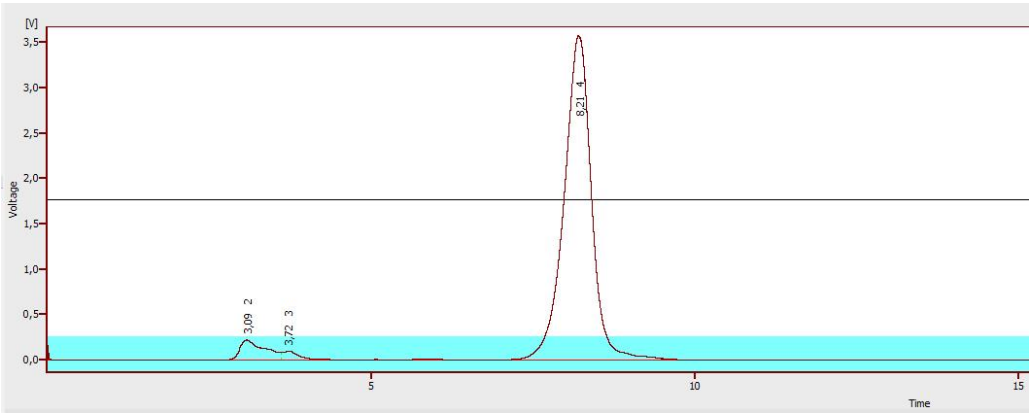

Figure S14: HPLC-ELSD of GPI obtained by the mono-enzymatic hydrolysis of the ENSL fraction

Chapter 2.3 2-*O*-Acetyl-3,4,5,6-tetra-*O*-benzyl-D-*myo*-inosityl diphenylphosphate: A new useful intermediate to inositol phosphate and phospholipids

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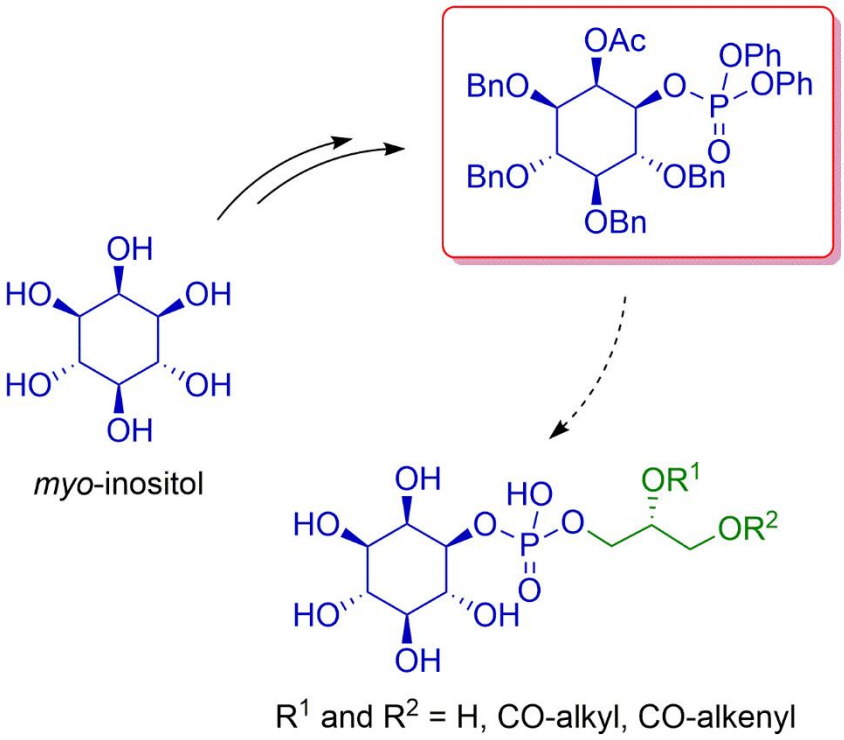

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SHORT COMMUNICATION

Chirality WILEY

2-*O*-Acetyl-3,4,5,6-tetra-*O*-benzyl-D-*myo*-inosityl diphenylphosphate: A new useful intermediate to inositol phosphate and phospholipids

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Abstract

Inositol phosphates and inositol phospholipids are ubiquitous in biochemistry and play a central role in cell signaling and regulation events. For this reason, their synthesis has attracted widespread interest. This paper describes the preparation of a new optically active inositol phosphate derivative, 2-*O*-acetyl-3,4,5,6-tetra-*O*-benzyl-D-*myo*-inosityl diphenylphosphate (**6**), and its characterization by spectroscopic methods. Compound (**6**) represents a useful intermediate for the preparation of inositol phosphate and phospholipids, in particular of glycerophosphoinositol (GPI), a natural anti-inflammatory agent.

KEYWORDS

anti-inflammatory activity, desymmetrization, L-camphor dimethyl acetal, *myo*-inositol phosphate, *myo*-inositol phospholipids

1 | INTRODUCTION

The inositols are the nine stereoisomeric forms of cyclohexanehexol belonging to the class of cyclitols, that is, cycloalkanes in which three or more ring atoms are each substituted with one hydroxyl group.[1,2] *myo*-Inositol, or *cis*-1,2,3,5-*trans*-4,6-cyclohexanehexol, is the most common isomeric form in nature that also uses at least five of the others (*scyllo*-, *epi*-, *neo*-, D-*chiro*-, and *muco*-inositols).[3] It constitutes the structural core of a group of important metabolites, that is, inositol phosphates and inositol phospholipids, that are involved in numerous important biological processes including cellular signal transduction, membrane transport, protein anchoring, and cytoskeletal regulation.[1–4] Specifically, phosphatidylinositols, which constitute approximately 1% of the phospholipids in cell membranes, are selectively phosphorylated by multiple kinases at the C-3, C-4 and C-5 positions to generate a number of endogenous phosphatidylinositol phosphates which are in turn converted into various inositol phosphates differing for the phosphorylation pattern of the inositol ring.[3,5]

Concentrations of inositol derivatives in biological systems are very low, thus strongly limiting their analytical detection and the isolation from natural sources in useful amounts to fully elucidate their physiological functions. For this reason, numerous synthetic efforts are still in progress to prepare biologically relevant inositol phosphates and inositol phospholipids as well as many of their analogs to be used as chemical probes in biological studies.[5–7]

Most of the synthetic routes to inositol phosphates and phospholipids commenced from *myo*-inositol, a

cheap and readily available starting material, and involve properly protected chiral derivatives of *myo*-inositol as key intermediates. Such derivatives have been prepared both by resolution of *myo*-inositol (a *meso* compound) and by stereoselective synthesis using chemical and/or enzymatic approaches and exploiting many selective protection and deprotection schemes of inositol hydroxyl groups.[8–12]

Among the known inositol phosphates, glycerophosphoinositols have recently attracted much attention due to their distinctive biological activity.[13] These water-soluble ubiquitous cellular metabolites, produced through the deacylation of the membrane phosphoinositides by receptor-activated cytosolic phospholipase A2α,[13] include non-phosphorylated *sn*-glycero-3-phosphoinositol (glycerophosphoinositol [GPI]) and its phosphorylated derivatives glycerophosphoinositol 4-phosphate and glycerophosphoinositol 4,5-bisphosphate. GPI has been found to play a role as an endogenous mediator in the inflammatory response, being part of a negative feedback loop that inhibits the de novo synthesis of pro-inflammatory and pro-thrombotic compounds. The anti-inflammatory activity of exogenous GPI has been investigated both in vitro and in an in vivo model in comparison with dexamethasone, showing that GPI parallels the anti-inflammatory effect of the corticosteroid drug.[13] Moreover, the anti-inflammatory effect of GPI in counteracting blood–brain barrier (BBB) failure has been found at lower doses than dexamethasone and without cytotoxic effects, thus suggesting the use of GPI as a natural anti-inflammatory agent and a "BBB enhancer" for neurodegenerative diseases such as multiple sclerosis and Alzheimer's dementia.[14]

As a part of our studies on the synthesis of GPI, here we report the chemical synthesis and the characterization of optically active 2-*O*-acetyl-3,4,5,6-tetra-*O*-benzyl-D-*myo*-inosityl diphenylphosphate (**6**), a new useful building block for the synthesis of inositol phosphates and phospholipids.

2 | MATERIALS AND METHODS

All solvents and reagents were purchased from Sigma-Aldrich and Scharlab and used without further purification. Analytical TLC was performed on silica gel F_{254} precoated aluminum sheets (0.2-mm layer, Merck). Silica gel 60, 40–63 μm (Merck, Darmstadt, Germany) was used for flash column chromatography. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded at 400.13, 100.61, and 161.96 Hz, respectively, on a Bruker AVANCE 400 (Bruker, Karlsruhe, Germany) spectrometer equipped with the TOPSPIN software package (Bruker, Karlsruhe, Germany) at 300 K, unless stated otherwise. MestReNova (v. 14.2) from Mestrelab Research was used for NMR processing. ^{1}H and ^{13}C chemical shifts (δ) are given in parts per million and are referenced to the solvent signal (δ_H 7.26–δ_C 77.16 ppm from tetramethylsilane [TMS] for $CDCl_3$). ^{31}P chemical shifts (δ) are given in parts per million and are referenced to standard $H_3PO_{4\ (aq)}$ 85% (0 ppm). ^{1}H NMR signals were assigned with the aid of ^{1}H–^{1}H correlation spectroscopy (^{1}H–^{1}H COSY). ^{13}C NMR APT (attached proton test) signals were assigned by ^{1}H–^{13}C correlation experiments (heteronuclear multiple quantum correlation spectroscopy [HSQC] and heteronuclear multiple bond correlation spectroscopy [HMBC]). Optical rotations were measured on a Jasco P-1030 polarimeter (LabX, Midland, Ontario, Canada). Electrospray ionization mass spectra (ESI-MS) were recorded on the Thermo Finnigan LCQ Advantage spectrometer (Hemel Hempstead, Hertfordshire, UK). For NMR and MS spectra, see Supporting Information.

3 | SYNTHESIS OF 2-*O*-ACETYL-3,4,5,6-TETRA-*O*-BENZYL-D-*MYO*-INOSITYL DIPHENYLPHOSPHATE (6) FROM *MYO*-INOSITOL (1)

D-1,2-*O*-(L-1,7,7-Trimethyl[2.2.1]bicyclohept-2-ylidene)-*myo*-inositol (**2**). L-Camphor dimethyl acetal (**7**) (220 mg, 1.11 mmol) and PTSA (8 mg, 0.04 mmol) were added to a dispersion of dry *myo*-inositol (**1**, 100 mg, 0.56 mmol) in anhydrous DMSO (2.0 mL) under inert atmosphere. The resulting mixture was stirred at 55°C for 4 h until complete dissolution of the substrate was observed. The reaction was cooled to room temperature, neutralized with Et_3N, and concentrated under reduced pressure. The residue was suspended in a mixture of $CHCl_3$/MeOH/H_2O (50:5:1, 10 mL), PTSA (5 mg) was added, and the resulting mixture was stirred at room temperature overnight. After neutralization with Et_3N, the resulting precipitate was filtered and washed with $CHCl_3$. The resulting crude was purified by flash chromatography ($CHCl_3$/MeOH 9:1, R*f* 0.22), and **2** was obtained as the major component of a mixture of diastereoisomers, which was used in the next reaction without further purification (90 mg, 0.29 mmol, 49%).

^{1}H NMR (400 MHz, DMSO-d_6, δ) *major isomer*: 4.81–4.74 (m, 3H, OH), 4.69 (d, $J = 4.1$ Hz, 1H, OH), 4.09 (dd, $J = 5.7$, 4.1 Hz, 1H, CH sugar), 3.66 (t, $J = 7.0$, 5.6 Hz, 1H, CH sugar), 3.52 (dt, $J = 8.9$, 4.3 Hz, 1H, CH sugar), 3.31–3.15 (m, 2H, 2 × CH sugar), 2.93 (td, $J = 9.4$, 3.9 Hz, 1H, CH sugar), 2.00–1.84 (m, 2H, 2 × CH_2 camphor), 1.72–1.61 (m, 2H, CH and CH_2 camphor), 1.40 (d,

J = 12.8 Hz, 1H, CH_2 camphor), 1.31 (td, J = 13.0, 12.4, 4.9 Hz, 1H, CH_2 camphor), 1.18–1.08 (m, 1H, CH_2 camphor), 0.97 (s, 3H, CH_3), 0.83 (2, 3H, CH_3), 0.77 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6, δ) 116.4 (Cq camphor), 77.1 (CH sugar), 76.7 (CH sugar), 76.1 (CH sugar), 74.4 (CH sugar), 72.3 (CH sugar), 70.2 (CH sugar), 51.6 (Cq camphor), 47.9 (Cq camphor), 45.7 (CH_2 camphor), 45.1 (CH camphor), 29.6 (CH_2 camphor), 27.3 (CH_2 camphor), 21.0 (CH_3 camphor), 20.8 (CH_3 camphor), 10.2 (CH_3 camphor); MS (ESI, *m/z*): $[M - H]^-$ calcd for $C_{16}H_{26}O_6$, 313.17; found, 313.04.

D-3,4,5,6-Tetra-*O*-benzyl-1,2-*O*-(L-1,7,7-trimethyl[2.2.1]bicyclohept-2-ylidene)-*myo*-inositol (**3**). NaH (60% dispersion in mineral oil, 2.46 g, 61.50 mmol) was added in one portion to a solution of **2** (1.20 g, 3.82 mmol) in dry DMF (50 mL) at 0°C under inert atmosphere. After stirring at 0°C for 30 min, BnBr (2.8 mL, 23.54 mmol) was added, and the mixture was stirred at room temperature for 24 h. The reaction was quenched first with MeOH and then with H_2O under vigorous stirring at 0°C. After evaporation of the solvent under reduced pressure, the residue was dissolved in AcOEt (30 mL) and washed with H_2O (2 × 15 mL) and brine (1 × 10 mL). The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. The resulting crude was purified by flash chromatography (*n*-hexane/AcOEt 9:1, R*f* 0.31) to get **3** as a light-yellow oil (2.17 g, 3.22 mmol, 84%).

^{1}H NMR (400 MHz, $CDCl_3$, δ) 7.45–7.28 (m, 20H, 4 × Ph), 4.95 (d, J = 11.4 Hz, 1H, CH_2Ph), 4.87–4.72 (m, 7H, CH_2Ph), 4.35 (dd, J = 6.2, 4.1 Hz, 1H, CH sugar), 4.01 (dd, J = 7.1, 6.2 Hz, 1H, CH sugar), 3.88 (t, J = 8.2 Hz, 1H, CH sugar), 3.84–3.75 (m, 2H, 2 × CH sugar), 3.48 (dd, J = 9.6, 7.9 Hz, 1H, CH sugar), 2.07–1.94 (m, 2H, 2 × CH_2 camphor), 1.83–1.73 (m, 2H, CH, and CH_2 camphor), 1.53 (d, J = 12.9 Hz, 1H, CH_2 camphor), 1.43 (td, J = 12.6, 4.9 Hz, 1H, CH_2 camphor), 1.37–1.25 (m, 1H, CH_2 camphor), 1.13 (s, 3H, CH_3 camphor), 0.92 (s, 3H, CH_3 camphor), 0.90 (s, 3H, CH_3 camphor); ^{13}C NMR (100 MHz, $CDCl_3$, δ) 138.8, 138.7, 138.5 (4 × Cq phenyl), 128.4, 128.3, 128.1, 128.0, 127.8, 127.7, 127.59, 127.55, 127.5 (20 × CH phenyl), 117.7 (Cq camphor), 83.3 (CH sugar), 82.2 (CH sugar), 80.8 (CH sugar), 77.4 (CH sugar), 76.3 (CH sugar), 75.2 (CH_2Ph), 75.0 (CH_2Ph), 74.0 (CH_2Ph), 73.7 (CH sugar), 72.5 (CH_2Ph), 51.6 (Cq camphor), 48.0 (Cq camphor), 45.2 (CH camphor), 45.0 (CH_2 camphor), 29.8 (CH_2 camphor), 27.1 (CH_2 camphor), 20.7 (CH_3 camphor), 20.4 (CH_3 camphor), 10.2 (CH_3 camphor); MS (ESI, *m/z*): $[M + Na]^+$ calcd for $C_{44}H_{50}O_6Na$, 697.35; found, 697.14.

D-3,4,5,6-Tetra-*O*-benzyl-*myo*-inositol (**4**). A solution of **3** (890 mg, 1.32 mmol) in AcOH (80% in H_2O v/v, 45 mL) was stirred at 100°C for 2 h. The solvent was removed under reduced pressure, and *n*-hexane (15 mL) was added to the residue to precipitate **4** as an off-white powder (503 mg, 0.93 mmol, 71%).

$[\alpha]_D^{20} = -0.23$ deg cm^3 g^{-1} dm^{-1} (c = 1.00 g cm^{-3} in $CHCl_3$); ^{1}H NMR (400 MHz, DMSO-d_6, δ) 7.41–7.22 (m, 20H, 4 × Ph), 5.00 (d, J = 4.0 Hz, 1H, OH), 4.89 (d, J = 2.4 Hz, 1H, OH), 4.87 (d, J = 6.8 Hz, 1H, CH_2Ph), 4.82 (d, J = 11.1 Hz, 1H, CH_2Ph), 4.76 (br s, 2H, CH_2Ph), 4.72 (br s, 2H, CH_2Ph), 4.71–4.68 (m, 1H, CH_2Ph), 4.54 (d, J = 11.9 Hz, 1H, CH_2Ph), 4.05 (q, J = 2.8 Hz, 1H, CH sugar), 3.81 (t, J = 9.5 Hz, 1H, CH sugar), 3.67 (t, J = 9.5 Hz, 1H, CH sugar), 3.49–3.44 (m, 2H, 2 × CH sugar), 3.40 (t, J = 9.3 Hz, 1H, CH sugar); ^{13}C NMR (100 MHz, DMSO-d_6, δ) 139.9, 139.52, 139.48, 139.4 (4 × Cq phenyl), 128.6, 128.53, 128.45, 127.98, 127.95, 127.8, 127.72, 127.69, 127.6 (20CH × phenyl), 83.2 (CH sugar), 82.3 (CH sugar), 81.6 (CH sugar), 80.5 (CH sugar), 75.1, 75.0, 74.6 (3 × CH_2Ph), 72.0 (CH sugar), 71.1 (CH_2Ph), 69.8 (CH sugar); MS (ESI, *m/z*): $[M + Na]^+$ calcd for $C_{34}H_{36}O_6Na$, 563.24; found, 563.15.

2-*O*-Acetyl-3,4,5,6-tetra-*O*-benzyl-D-*myo*-inositol (**5**). A solution of **4** (300 mg, 0.55 mmol), PTSA·H_2O (10 mg, 0.05 mmol) and trimethyl orthoacetate (0.30 mL, 2.74 mmol) in acetonitrile (20 mL) was stirred at room temperature for 2 h. The reaction was cooled to −40°C and H_2O (0.30 mL) was added; then the mixture was stirred at −40 °C for 4 h, neutralized with pyridine and concentrated under reduced pressure. The residue was dissolved in AcOEt (20 mL), washed with H_2O (2 × 10 mL), dried over Na_2SO_4 and evaporated under reduced pressure. The resulting crude was purified by flash chromatography (*n*-hexane/EtOAc 8:2, R*f* 0.30) to get **5** as a colorless oil (290 mg, 0.50 mmol, 91%).

$[\alpha]_D^{20} = -1.78$ deg cm^3 g^{-1} dm^{-1} (c = 2.00 g cm^{-3} in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$, δ) 7.41–7.28 (m, 20H, 4 × Ph), 5.75 (t, J = 2.8 Hz, 1H, CH sugar), 5.04–4.95 (m, 3H, 3 × CH_2Ph), 4.87 (d, J = 10.6 Hz, 1H, CH_2Ph), 4.83 (d, J = 10.7 Hz, 1H, CH_2Ph), 4.79 (d, J = 5.0 Hz, 1H, CH_2Ph), 4.76 (d, J = 5.1 Hz, 1H, CH_2Ph), 4.54 (d, J = 11.2 Hz, 1H, CH_2Ph), 3.92 (t, J = 9.5 Hz, 1H, CH sugar), 3.82 (t, J = 9.6 Hz, 1H, CH sugar), 3.64 (br d, J = 9.6 Hz, 1H, CH sugar), 3.60–3.54 (m, 2H, 2 × CH sugar), 2.34 (br s, 1H, OH), 2.18 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6, δ) 170.5 (C=O), 138.6, 138.3, 137.6 (4 × Cq phenyl), 128.7, 128.5, 128.4, 128.4, 128.3, 128.0, 128.0, 127.9, 127.8, 127.7 (4 × CH phenyl), 83.3 (CH sugar), 82.0 (CH sugar), 81.5 (CH sugar), 78.5 (CH sugar), 76.0, 76.0, 75.6, 72.2 (4 × CH_2Ph), 70.2 (CH sugar), 69.3 (CH sugar), 21.1 (CH_3); MS (ESI, *m/z*): $[M + Na]^+$ calcd for $C_{36}H_{38}O_7Na$, 605.25; found, 605.53.

2-*O*-Acetyl-3,4,5,6-tetra-*O*-benzyl-D-*myo*-inositol diphenylphosphate (**6**). DMAP (2.3 mg, 0.02 mmol), Et_3N (210 μL, 152 mg, 1.50 mmol), and DPC (160 μL, 207 mg, 0.77 mmol) were added to a solution of **5** (107 mg, 0.18 mmol) in CH_2Cl_2 (10 mL), and the resulting mixture was stirred at room temperature for 24 h. The reaction was washed with H_2O (20 mL) and brine (20 mL). The organic phase was dried over Na_2SO_4 and concentrated under reduced pressure to get a yellow oil crude, which was purified by flash chromatography (hexane/AcOEt 8:2, R*f* 0.31) to get **6** as a light-yellow oil (105 mg, 0.13 mmol, 70%).

$[\alpha]_D^{20} = +0.95$ deg cm^3 g^{-1} dm^{-1} ($c = 1.53$ g cm^{-3} in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$, δ) 7.43–7.06 (m, 30H, 2 × Ph and 4 × Bn), 5.98 (t, $J = 2.8$ Hz, 1H, H-2), 4.94–4.75 (m, 7H, CH_2Ph), 4.67 (ddd, $J = 10.1$, 8.4, 2.9 Hz, 1H, H-1), 4.47 (d, $J = 10.9$ Hz, 1H, CH_2Ph), 4.05 (t, $J = 9.6$ Hz, 1H, H-6), 3.87 (t, $J = 9.5$ Hz, 1H, H-4), 3.62–3.54 (m, 2H, H-3 and H-5), 2.11 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, δ) 169.4 (C=O), 150.5, 150.4 (2 × Ph), 138.5, 138.2, 138.1, 137.5 (4 × Bn), 129.8, 129.7, 128.5, 128.4, 128.4, 128.2, 128.0, 128.0, 127.9, 127.8, 127.7, 127.7, 127.5, 125.4, 125.3 (2 × Ph and 4 × Bn), 120.2, 120.0 (2 × Ph), 82.6 (C-5), 81.1 (C-4), 80.0 (C-6), 78.2 (C-3), 77.4 (C-1), 76.3, 76.0, 75.7, 72.4 (4 × CH_2Ph), 68.6 (C-2), 20.9 (CH_3); ^{31}P NMR (162 MHz, $CDCl_3$, δ) −12.30; MS (ESI, *m/z*): $[M + H]^+$ calcd for $C_{48}H_{48}O_{10}P$, 815.30; found, 815.24; $[M + Na]^+$ calcd for $C_{48}H_{47}O_{10}PNa$, 837.28; found, 837.45; Anal. calcd for $C_{48}H_{47}O_{10}P$: C 70.75, H 5.81; found: C 69.99, H 6.20.

L-Camphor dimethyl acetal (**7**). The title compound was synthesized in 68% yield according to a procedure already reported in literature, with some modifications.[15] In details, the crude was purified not by distillation but by flash chromatography (*n*-hexane-AcOEt 9:1, R*f* 0.86).

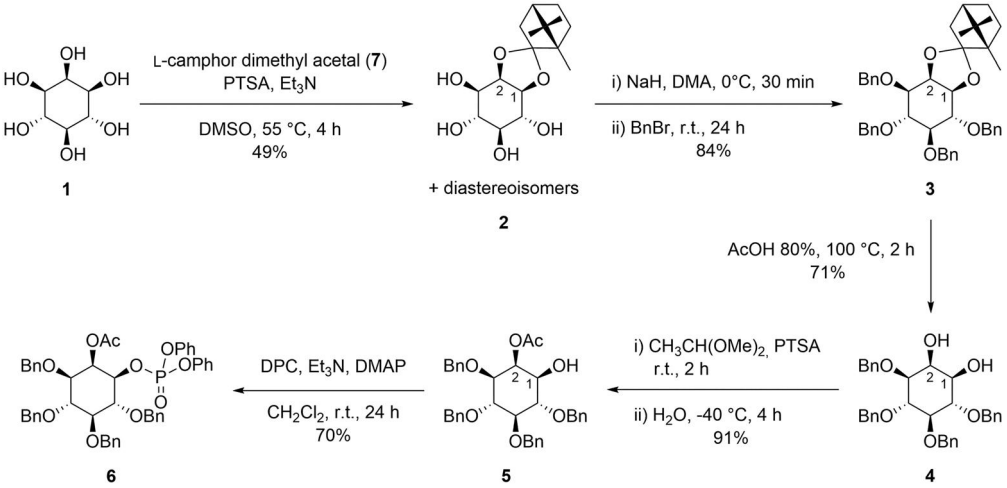
^{1}H NMR (400 MHz, $CDCl_3$, δ) 3.11 (s, 3H, OCH_3), 3.08 (s, 3H, OCH_3), 2.14 (ddd, $J = 12.9$, 4.6, 3.0 Hz, 1H, CH_2), 1.74–1.61 (m, 3H, CH, and 2 × CH_2), 1.36–1.27 (m, 1H, 2 × CH_2), 1.17–1.11 (m, 1H, 2 × CH_2), 1.08 (d, $J = 12.7$ Hz, 1H, CH_2), 0.91 (s, 3H, CH_3), 0.87 (s, 3H, CH_3), 0.79 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, δ): 109.1 (C [OCH_3]$_2$), 53.0 (Cq), 50.2 (OCH_3), 49.9 (Cq), 47.2 (OCH_3), 44.3 (CH), 41.0 (CH_2), 29.3 (CH_2), 27.3 (CH_2), 20.8 (CH_3), 20.5 (CH_3), 12.4 (CH_3); MS (ESI, *m/z*): $[M]^+$ calcd for $C_{12}H_{22}O_2$, 198.16; found, 198.07.

4 | RESULTS AND DISCUSSION

Several methods have been reported in the literature for the preparation of enantiopure *myo*-inositol derivatives by resolution of *myo*-inositol. Among them, we followed the methodology based on introduction of D- or L-camphor as chiral auxiliaries into the *myo*-inositol structure. Such an approach, first described by Bruzik et al,[8,9] results both in the desymmetrization of *myo*-inositol and in the regioselective protection of its hydroxyl groups in C-1 and C-2 positions.

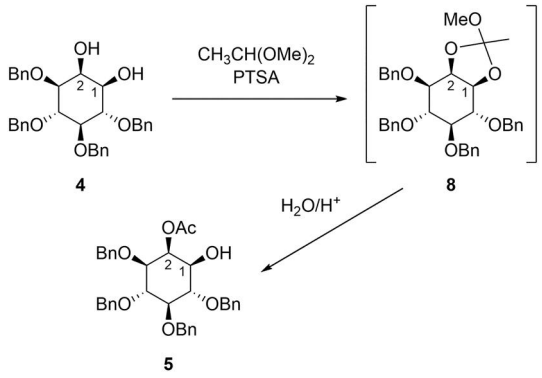
As illustrated in Scheme 1, we adopted the procedure reported by Nkambule, with some modifications,[10] to obtain 2-*O*-acetyl-3,4,5,6-tetra-*O*-benzyl-D-*myo*-inositol (**5**), which was then converted to enantiomerically pure 2-*O*-acetyl-3,4,5,6-tetra-*O*-benzyl-D-*myo*-inosityl diphenylphosphate (**6**). This strategy enabled us to synthesize the target molecule in five steps from *myo*-inositol (**1**) in an overall 19% yield.

More in details, **1** was transketalized by treatment with 2 equivalents of L-camphor dimethyl acetal (**7**, prepared by treatment of L-camphor with trimethyl orthoformate), and PTSA in dry DMSO at 55°C. The selectivity of the reaction led to the formation of *cis*



SCHEME 1 Synthesis of 2-*O*-acetyl-3,4,5,6-tetra-*O*-benzyl-D-*myo*-inosityl diphenylphosphate (**6**) from *myo*-inositol (**1**).

monoacetal **2** as the major product in a complex mixture of diastereoisomers. The PTSA-catalyzed equilibration of the crude mixture allowed to further increase the amount of the desired isomer **2**, protected at 1 and 2 positions and having the D configuration. After precipitation in dichloromethane, the mixture containing **2** was directly treated with benzyl bromide and NaH in dry DMF to protect the remaining four hydroxyl groups as benzyl ethers to give **3**. The latter intermediate was easily purified from the traces of diastereoisomers generated in the previous step by flash chromatography. The chiral auxiliary was then removed with concentrated acetic acid (80% v/v solution in water) at 100°C, and the resulting crude product **4** was used in the next step without further purification.



SCHEME 2 Regioselective hydrolysis and opening of **8**.

The regioselective acetylation of **4** was carried out by treatment with trimethyl orthoacetate and PTSA in acetonitrile, followed by hydrolysis of the intermediate mixed orthoacetate (**8**) and purification by flash chromatography to get the key intermediate **5**. The different reactivity of the equatorially and axially oriented oxygens in **8** directs the hydrolytic opening of the dioxolane ring selectively at the C-1 position, thus allowing the retention of the protection on the adjacent moiety at C-2 (Scheme 2).[16,17]

To complete the synthetic sequence, **5** was reacted with excess diphenyl phosphoryl chloride (DPC), a catalytic amount of 4-dimethylaminopyridine and triethylamine in dichloromethane, followed by simple aqueous workup and chromatographic purification to give the target product **6** in 70% yield.

2-*O*-Acetyl-3,4,5,6-tetra-*O*-benzyl-D-*myo*-inosityl diphenylphosphate (**6**) was fully characterized by mono- and bidimensional NMR spectroscopy (^{1}H-NMR, COSY, HMBC, HSQC, ^{13}C-NMR, ^{31}P-NMR), ESI-MS and $[\alpha]_D^{20}$.

The ^{1}H-NMR spectrum of **6** recorded in $CDCl_3$ (Figure 1) showed the expected pattern of signals. In details, the typical signal of the equatorial proton H-2, usually located slightly downfield with respect to the others, is further shifted to 5.98 ppm. Similarly, the axial proton H-1 was also shifted downfield to 4.66 ppm with respect to the corresponding signal in *myo*-inositol (**1**).[18]

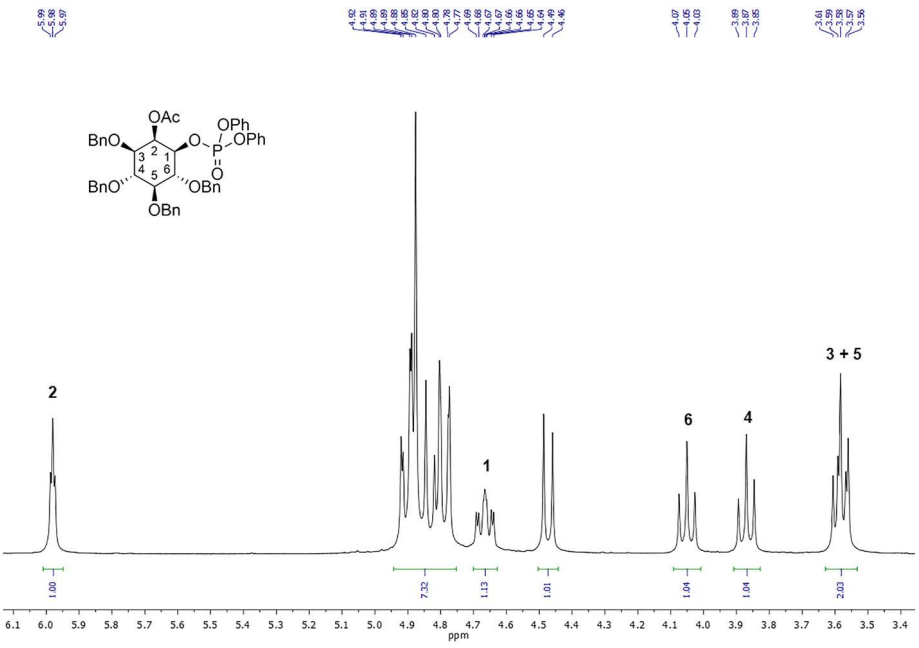


FIGURE 1 ^{1}H-NMR (400 MHz, $CDCl_3$) of **6** in the range 3.4–6.1 ppm

5 | CONCLUSION

To sum up, this short note reports the synthesis of 2-*O*-acetyl-3,4,5,6-tetra-*O*-benzyl-D-*myo*-inosityl diphenylphosphate (**6**), a new optically active inositol phosphate derivative that may be a useful building block for the synthesis of inositol phosphates and phospholipids such as the anti-inflammatory agent GPI. It is worth noting that, to the best of our knowledge, no chemical synthesis of GPI and its phosphorylated derivatives has been reported to date. Starting from **6**, GPI might be simply prepared by treatment with a properly protected glycerol synthon, followed by removal of all protective groups.[2]

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the Supporting Information of this article.

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SUPPORTING INFORMATION

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Chapter 3: (Chemo)enzymatic synthesis of carbohydrate-derived surfactants

The communication reported in **Chapter 3.1** describes the Fischer glycosidation of glucose in *n*-butanol in the presence of an acidic resin as catalyst (Amberlyst® 15). The isomeric mixture of *n*-butyl glucosides (α/β furanosides and α/β pyranosides) were submitted to the enzymatic esterification with lauric (C12), stearic (C16), and palmitic (C18) acids using Novozym® 435 as catalyst. The reaction was performed in a solvent-free system at 80 °C by exploiting the molten fatty acids as solvents. The physico-chemical properties of these surfactants (sunflower oil/water interfacial tension, W/O emulsifying capability, and emulsion stability over time) were investigated, highlighting that 6-*O*-palmitoyl- and 6-*O*-stearoyl-1-*O*-butyl glucopyranosides are the most performing molecules.

Derivatization of the sugar (the "polar head" of the surfactant) into a less polar precursor was shown to be effective in overcoming the striking different solubility profile of the "polar head" and the "fatty acid tail" of the surfactant as well as to fine-tune the HLB (hydrophilic lipophilic balance) of the resulting molecule.

The same approach to sugar derivatization was applied, indeed, to the synthesis of alkyl galactoside fatty acid esters (research paper, **Chapter 3.2**). In this case, the galactosides were prepared both by the Fischer glycosidation as above, and by transglycosylation of lactose with naturally occurring alcohols using the β-galactosidase from *Aspergillus oryzae* immobilized on glyoxyl Sepabeads. A library of alkyl galactosides was prepared and the *n*-butyl galactosides were esterified to give *n*-butyl galactoside fatty acid esters. Measurements of interfacial tension and emulsifying properties of the obtained esters revealed that 6-*O*-palmitoyl-1-*O*-butyl-β-D-galactopyranoside, which was prepared enzymatically from lactose, leads to the most stable emulsion. This two-step enzymatic approach allowed to use directly lactose as the raw material (and, indeed, cheese whey permeate) to achieve selectively alkyl β-galactopyranosides and the corresponding esters, thereof. All products and intermediates were analytically characterized by ^{1}H and ^{13}C NMR spectroscopy.

Chapter 3.3 (research paper, submitted) describes a combination of experimental and computational approaches to study the (trans)esterification of lactose with different

acyl donors (both free fatty acids and vinyl esters) in deep eutectic solvents (DES). More in details, several analytical scale reactions were performed in DES but the target molecule(s) (lactose fatty acid ester) was/were never detected. Thus, the non-conventional system resulted not suitable, especially if compared to the already published conventional organic mixture (THF/Py). The computational simulations (COSMO-RS and molecular dynamics) allowed a deeper insight into DES-substrate interactions, highlighting solubility issues of vinyl esters longer than C10 and a detrimental conformation of lactose in the DES system which may have negatively affected the reactivity.

As an alternative approach to the preparation of glycolipids, organocatalysis was also investigated (**Chapter 3.4**). This very preliminary and explorative study consisted of the screening of three organocatalysts (DL-lactic acid, L-tartaric acid and citric acid monohydrate) for the (trans)esterification of lactose both in a "solvent free" medium (neat acyl donor) and in a DES medium (ChCl:U). However, also in this case, the target molecule was not detected.

Chapter 3.5 describes the reductive amination of lactose and its hydrolysis products (glucose and galactose) with *N*-alkylamines to obtain the corresponding glycamines to be used as polar heads of new surfactants. Preliminary acylation trials of *N*-ethylglucamine with palmitic acid to obtain the corresponding fatty glycamide are also described. The amination of sugars by enzymatic transamination was also investigated by screening a set of ω-transaminases toward arabinose, but this biotransformation was not successful.

Chapter 3.1 Emulsifying properties of sugar-based surfactants prepared by chemoenzymatic synthesis

Sara Sangiorgio, Eleonora Pargoletti, Marco Rabuffetti, Marina S. Robescu, Riccardo Semproli, Daniela Ubiali, Giuseppe Cappelletti,* Giovanna Speranza*

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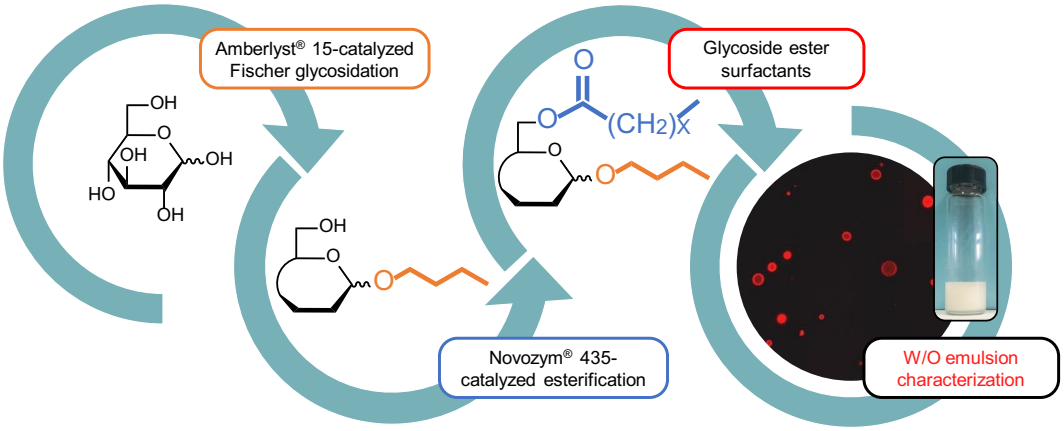

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Rapid Communication

Emulsifying properties of sugar-based surfactants prepared by chemoenzymatic synthesis

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W/O emulsifying properties
Interfacial features

ABSTRACT

Sugar Fatty Acid Esters (SFAEs) are a class of non-ionic surfactants that can be synthesized from inexpensive natural resources. Depending on carbon chain length and nature of the sugar head group, SFAEs cover a wide range of hydrophilic–lipophilic balance (HLB) values, which result in tunable tenside properties and in turn relevant for a wide variety of industrial applications. Three sugar-based surfactants (6-*O*-lauroyl-, 6-*O*-palmitoyl- and 6-*O*-stearoyl-1-*O*-butyl glucopyranosides) have been prepared by a lipase-catalyzed esterification of isomeric mixture of *n*-butyl glucosides. Specifically, their interfacial features together with W/O emulsifying properties and stability over time have been finely evaluated (interfacial tension (IFT) values, W/O emulsion turbidity and water droplet size distribution), resulting 6-*O*-palmitoyl- and 6-*O*-stearoyl-1-*O*-butyl glucopyranosides as the most efficient ones.

1. Introduction

Surfactants (or surface-active agents) are amphiphilic compounds widely used in almost every industrial field because of their ability to reduce surface and interfacial tensions, thus stabilizing dispersed systems [1]. In recent years, due to their high worldwide consumption volumes, surfactant residues are becoming an environmental risk because they are continuously discharged into treated and untreated wastewaters, thus entering in several environmental compartments, *i.e.*, surface and marine waters, sediments and sludge-amended soils, *etc.* [2]. Environmental concerns about effects of conventional tensides, mainly derived from petroleum precursors, have increased the market demand for more benign compounds [3].

Sugar fatty acid esters (SFAEs) are non-ionic surfactants that show excellent surface and interfacial tension reduction capability, along with low toxicity and easy biodegradability. For these reasons, they are extensively used as emulsifiers in many market sectors, *i.e.* cosmetic, food and pharmaceutical industry and they are becoming important commodity chemicals [4]. Moreover, these biosurfactants can be produced from renewable resources, *i.e.* fatty acids derived from vegetable oils and animal fats as well as sugars coming from industrial waste and biomasses [5]. Furthermore, according to the nature of the hydrophilic sugar head group and the hydrophobic carbon chain length and type, as well as the degree of esterification, SFAEs can be tuned with various hydrophilic-lipophilic balance (HLB). The resulting unique physico-chemical properties, displaced by this family of surfactants, translate into a wide range of possible applications, *i.e.* O/W or W/O emulsifiers, solubilizing agents, lubricants, penetrating enhancers and pore forming agents [6]. Today, the only sugar-based surfactants available on the market, used as emulsifiers, are sorbitan esters and ethoxylated sorbitan esters, known under the trade names of Span® and Tween®, as well as sucrose esters and alkyl polyglycosides [4].

The current industrial syntheses of SFAEs still rely on harsh reaction conditions (*i.e.*, high temperature and alkaline catalysts), which result, in most cases, in complex mixtures of products with different degrees of esterification and different acylation positions, as well as mixtures of undesired by-products, deriving from side reactions, such as sugar dehydration/caramelization [7]. Alternatively, the esterification reaction, which is crucial for the SFAEs synthesis, can be catalyzed by enzymes, specifically by lipases [8]. Because of the striking different polarity of the SFAE components, a key issue in the enzymatic synthesis is the selection of the proper solvent and/or co-solvent able to solubilize both the sugar and the fatty acid moieties without deactivating the enzyme. Polar aprotic solvents, solvents mixtures or hindered alcohols/

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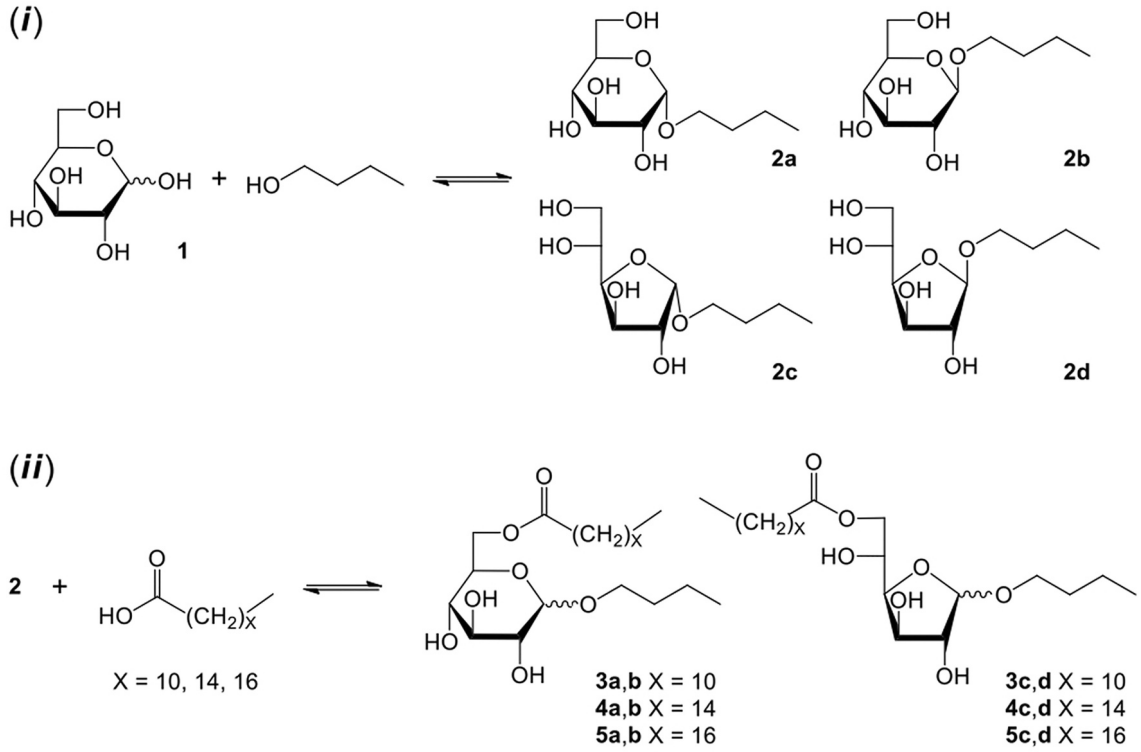

Fig. 1. *i*) D-(+)-glucose (**1**) derivatization with *n*-BuOH catalyzed by Amberlyst® 15. *ii*) Novozym® 435-catalyzed esterification of *n*-butyl-*O*-glucosides (**2**) with lauric, palmitic, and stearic acid in molten state.

ketones (*i.e.*, *N*,*N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), solution of DMSO/*tert*-butanol (*t*-BuOH); 9:1, 2-methyl-2-butanol (2M2B), methyl ethyl ketone (MEK)) have been reported [7,9]. However, to design a green process, solvents should generally be avoided, especially if hazardous to people and environment. Moreover, solvent can dramatically affect enzyme stability, thus the use of immobilized enzymes is generally preferred. The commercially available Novozym® 435 (Novozymes A/S, Denmark), a lipase type B from *Candida antarctica* (CALB) immobilized on Lewatit VP OC 1600 (Lanxess, Germany) polymethacrylate carrier, is the most used biocatalyst for SFAEs synthesis. However, in most reaction conditions reported so far, yields are generally poor [9].

A proper modification of the sugar moiety, namely its conversion into a less polar derivative, followed by solvent-free esterification could be an effective approach to overcome the above-mentioned drawbacks. Moreover, obeying to the Twelve Principles of the Green Chemistry [10], the sugar derivatization should be preferably performed by using safe, nontoxic, and environmentally friendly reagents, solvents, and catalysts under mild conditions. Among the various strategies to reduce the polarity of a sugar moiety, two reactions can be considered, *i.e.* acetalization and Fischer glycosidation. The former approach consists in the generation of sugar acetals: it is known that, in the presence of an acid catalyst, aldehydes and ketones react with suitably arranged diols of saccharide derivatives to give cyclic acetals [11]. These protected sugars can be enzymatically esterified, but the acetal groups have to be selectively hydrolyzed to restore the polar head of the surfactant. This experimental approach was not pursued because *i*) protection/deprotection steps have been demonstrated not to be economically feasible for the industrial production of tensides [12] and *ii*) glucose monoesters have been found to have poor surfactant properties [13].

Instead, the second strategy is based on the synthesis of glycosides, namely saccharide derivatives in which the sugar is bonded to a non-sugar moiety (aglycone) *via* a glycosidic bond. In the case of Fischer glycosidation, the reaction is performed in the presence of an alcohol and an acid catalyst, thus affording *O*-glycosides as products [11]. These sugar derivatives show an improved solubility in molten fatty acids with respect to unprotected sugars and the aglycone moiety of the glycoside esters does not need to be removed, thus being included in the final surfactant structure.

Although several studies on the enzymatic synthesis of glycoside esters have been carried out [12,14], to the best of our knowledge, only few data on the physicochemical performances of these compounds have been reported so far. Thus, the aim of this study is the enzymatic synthesis of some glycoside esters, namely 6-*O*-lauroyl-, 6-*O*-palmitoyl- and 6-*O*-stearoyl-1-*O*-butyl glucopyranosides, and the assessment of their surface properties, in particular the emulsifying capabilities.

2. Experimental

2.1. Materials

All solvents and reagents were purchased from Merck Life Science (Milano, Italy) and were used without further purification. Electrospray ionization mass spectra (ESI-MS) were recorded on a Thermo Finnigan LCQ Advantage spectrometer (Hemel Hempstead, Hertfordshire, UK). NMR experiments were performed at 298 K on 400 MHz Bruker NMR spectrometer (Bruker Corporation, Billerica, MA, USA) equipped with a z-gradient coil probe. Chemical shifts (δ) are given in parts per million (ppm) and were referenced to the solvent signal (DMSO-d_6 and deuterium oxide (D_2O), δ_H 2.50 ppm, δ_C 39.52 ppm and δ_H 4.79 ppm from TMS, respectively). The fatty acid profile of commercial sunflower oil (see Supplementary data) was determined by gas chromatography/mass spectrometry (GC/MS) analysis, after base-catalyzed transmethylation, using the protocol FIL-IDF 182:1999, reported elsewhere [15].

2.2. Synthesis of n-butyl-O-glucoside isomeric mixture

D-(+)-Glucose (**1**) was suspended in dry *n*-butanol (*n*-BuOH) (final

concentration 0.1 M) in the presence of the strongly acidic cation exchange resin Amberlyst® 15 (10%, w/w) and 3 Å molecular sieves (25%, w/w), at 120 °C (Fig. 1 (*i*)). After 2.5 h, the reaction was stopped by filtration of the solid catalyst, the alcohol was removed under reduced pressure and the reaction mixture was submitted to flash chromatography (dichloromethane (DCM)/methanol (MeOH); 9:1) to give *n*-butyl-*O*-glucosides (**2**) as a viscous syrup (Y = 91%). The isomeric mixture of glucosides (**2**) was characterized by TLC, ESI-MS and NMR analysis (See Supplementary data). NMR signals were identified by comparison with data reported in the literature [16]. D-(+)-Glucose, Amberlyst® 15 and molecular sieves were dried at 90 °C overnight prior to use.

2.3. Synthesis of 6-O-lauroyl, 6-O-palmitoyl and 6-O-stearoyl-1-O-butyl glucopyranosides

Isomeric mixture of *n*-butyl-*O*-glucosides (**2**), fatty acids (lauric, palmitic and stearic acids) in molar ratio 1:1 and Novozym® 435 (10%, *w*/w) were mixed together and charged into a round-bottom flask. The mixture was heated to 80 °C while rotating the flask by means of a glass oven B-585 Kugelrohr. After fatty acids melted, the reactions were performed under reduced pressure (30 mmHg) (Fig. 1 (*ii*)). After 8 h, reaction mixtures were taken up in ethyl acetate (EtOAc) and the immobilized enzyme was removed by filtration. Then, the esters were extracted in EtOAc (2 times) from 1 M NaOH, the organic phases were collected, dried over Na_2SO_4 and the solvent was removed under reduced pressure. The most abundant products were isolated by flash chromatography (*n*-hexane/EtOAc; 2:8), thus affording three SFAEs: 6-*O*-lauroyl-1-*O*-butyl glucopyranosides (**3a**,**b**) (Y = 29%), 6-*O*-palmitoyl-1-*O*-butyl glucopyranosides (**4a**,**b**) (Y = 56%), 6-*O*-stearoyl-1-*O*-butyl glucopyranosides (**5a**,**b**) (Y = 65%), which were fully characterized by TLC, ESI-MS and NMR analyses (See Supplementary data, Paragraphs 1 and 2).

2.4. Solubility measurements

To assess the solubility of the products, 6-*O*-lauroyl- (**3a**,**b**), 6-*O*-palmitoyl- (**4a**,**b**) and 6-*O*-stearoyl- (**5a**,**b**) 1-*O*-butyl glucopyranosides (5 mg) were added to different solvents (2 mL, see **Table S1** in Supplementary data, Paragraph 4) and further mixed using a hand-operated laboratory piston-type homogenizer (at 3000 rpm, 30 s; Vortex Mixer, VELP Scientifica, Usmate, Italy). Some of them were also submitted to a heating step (at 50 °C for 30 min) to improve the product solubility (see **Table S2** in Supplementary data).

2.5. Interfacial tension (IFT) measurements and emulsifying properties

The sunflower oil/water interfacial tension (IFT) values were measured at (25 ± 1) °C by means of Gibertini tensiometer following the Du Noüy ring method and by varying the amount (in the range between 0.004 and 0.350%wt in oil) of 6-*O*-lauroyl- (**3a**,**b**), 6-*O*-palmitoyl- (**4a**,**b**) and 6-*O*-stearoyl- (**5a**,**b**) 1-*O*-butyl glucopyranosides. Prior to tensiometric measurements, several parameters were introduced in the relative software to set up the method, such as liquid density, platinum ring and wire radii, required by the Harkins-Jordan correction. Data were reported as average values on three different replicates.

Water (milli-Q) in sunflower oil (W/O) emulsions were prepared using a Thermo Fisher Q700 sonicator equipped with a 3 mm-titanium alloy microtip. The operative conditions contemplate a frequency of 20 kHz in pulsed mode (3 s on and 3 s off) at 50% amplitude for 45 s. Preliminary tests regarding the W/O emulsion formation were carried out to select the best conditions: two different concentrations (4.5 and 6.0 mM solubilized at 80 °C) of 6-*O*-palmitoyl-1-*O*-butyl glucopyranoside (as model) and three phase volumes (Φ = 0.16, 0.14 and 0.13) were adopted. Once optimized the experimental conditions (4.5 mM, Φ = 0.13), stability tests were performed also with 6-*O*-lauroyl- and 6-*O*-stearoyl-1-*O*-butyl glucopyranosides by means of confocal microscopy and turbidimetric measurements over time (from 1 h to 1 month).

Droplets size distribution of both fresh and aged emulsions was evaluated by processing (by ImageJ software) the images (droplets number up to 150) acquired by Nikon A1 laser scanning confocal microscope (LSCM) working in oil immersion (NA1.4) equipped with a 60× objective. Before each analysis, emulsions were stained with Rhodamine B (a dye soluble only in the water phase) and samplings in the central part of each vial (where the emulsions showed whitish color typical of well-emulsified systems) were made. Images were acquired with an excitation wavelength of 561 nm and emitted signal was detected between 770 and 620 nm.

To evaluate the emulsions stability within 72 h, turbidimetric method [17] was adopted by following the absorbance value (Shimadzu UV–Vis spectrophotometer UV-2600) of the prepared samples at a fixed wavelength, in a 1 cm path length optical cell. Since the investigated systems showed a decreasing absorbance trend in the 400–700 nm range, a wavelength of 550 nm was chosen. Turbidity (τ) was calculated according to the equation reported elsewhere [17], together with the turbidity ratio (R) which is a useful, rapid and simple method to evaluate emulsions stability against sedimentation. R is defined as the ratio of turbidity at high and low wavelengths (in the present case 700 and 450 nm, $R = \tau_{700} / \tau_{450}$; see in the Supplementary data Paragraph 3). Then, the slope of turbidity ratio over time was calculated within 50 min. No samples dilution was performed according to Bai et al. [18].

3. Results and discussion

3.1. Green credentials of SFAEs

The building blocks used to synthesize the tensides here reported fulfil some of the Green Chemistry principles. In particular, glucose, which constitutes the sugar-based polar head of the surfactant, is readily available from starch and lignocellulose biomass [19], in compliance with the paradigm of Circular Economy.

n-Butyl glucoside was selected as sugar derivative because it seemed to be a protected sugar apolar enough to be soluble in molten fatty acids. In addition, according to Prat and co-workers [20], *n*-BuOH is classified as a green and recommended solvent because it meets criteria of safety, occupational health, environment, quality and risk of impurities, as well as general industrial constraints, *i.e.*, boiling point, freezing temperature, density, recyclability, and cost. Moreover, it is the shortest chain naturally occurring alcohol immiscible with water, thus favoring its removal from the reaction mixture. Amberlyst® 15, selected as acid catalyst for the Fischer glycosidation, is a commercially available macroreticular catalyst, prepared from cross-linking of styrene with divinyl benzene followed by sulfonation and it finds application as heterogeneous catalyst in a broad range of non-aqueous organic syntheses, even at industrial scale [21].

Finally, saturated fatty acids (lauric, palmitic and stearic acids) used as acyl donors in the enzymatic esterification are the main components of vegetable oils, which are historically and currently one of the most important renewable feedstocks of the chemical industry [22].

3.2. Synthesis of n-butyl glucoside esters and their characterization

The Fischer glycosidation, carried out with *n*-BuOH as a solvent in the presence of Amberlyst® 15 as an acid catalyst, led to an isomeric mixture of *n*-butyl-*O*-glucosides (**2**) with different ring sizes and configuration of the anomeric carbon (Fig. 1 (*i*)). It is known that the proportion of the various glycosidic forms (α-, β-pyranosides, **2a, 2b** and α-, β-furanosides, **2c, 2d**) present in the equilibrium mixtures at the completion of the reaction depends upon the relative thermodynamic stabilities of the isomers, which are governed by solvent, temperature, type of the sugar and chain length of the alcohol [11]. In our hands, the relative ratio between the glycoside isomers, namely **2a**/**2b**/**2c**/**2d**,

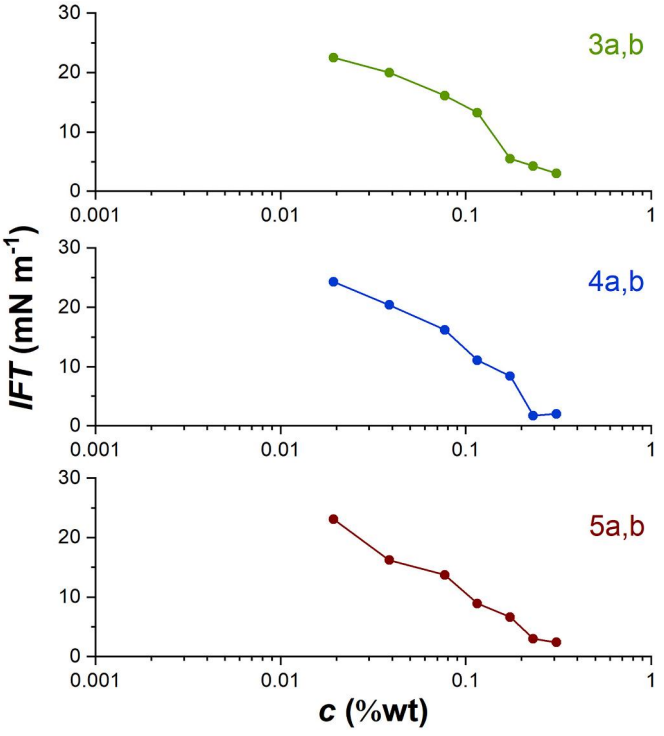

Fig. 2. Comparison of sunflower oil/water interfacial tension (IFT) data by varying the amount of 6-*O*-lauroyl- (**3a**,**b**), 6-*O*-palmitoyl- (**4a**,**b**) and 6-*O*-stearoyl- (**5a**,**b**) 1-*O*-butyl glucopyranosides in the range 0.004 and 0.350%wt in oil. Data were reported as average values on three different replicates at room temperature. The error bars are within the symbols and all below 1 mN m^{-1}.

resulted to be 46:38:6:10, by ^{1}H NMR analysis in D_2O (see Supplementary data, **Fig. S6**). Such values were estimated as the ratio of the areas of anomeric proton signals of each isomer present in the reaction mixture.

This isomeric mixture of *n*-butyl-*O*-glucosides (**2**) was submitted to a Novozym® 435-catalyzed esterification with lauric, palmitic, and stearic acid in a solvent-free system, at 80 °C and under reduced pressure (30 mmHg) (Fig. 1 (*ii*)). A glass oven B-585 Kugelrohr was used to carry out the reactions in place of magnetic stirring to avoid enzyme beads wrecking, thus favoring water removal and shifting the equilibrium towards the products formation.

^{1}H and ^{13}C NMR spectra, recorded in DMSO-d_6, of the reaction products (**3, 4, 5**) demonstrated that the enzymatic esterification is highly regioselective: only the acylation of the primary hydroxyl group was observed, with the α-anomer prevailing over the β- one (α/β ratio *ca.* 6:4).

Then, solubility tests in common polar, apolar and polar aprotic organic solvents were carried out (see Supplementary data). All the synthesized surfactants are soluble in almost all solvents tested (Table S1), except for water and alkanes (*n*-hexane and *n*-heptane). Particularly, in the latter cases, a slight increase of solubility occurred after a heating step at 50 °C for 30 min (Table S2). These behaviours can be explained by using the Hansen parameters (δ_D, δ_P and δ_H for dispersive, polar and hydrogen bonding components, respectively) of the solvents (Table S1), representing a powerful tool to understand the solubility features. As it can be seen from the Table S1, water and alkanes, being at the extremes of the polarity scale, are unable to solubilize the prepared sugar-based derivatives; instead, solvents characterized by multiple components (δ_D, δ_P and δ_H) allow the complete solubilization, as a result of their affinity towards the amphiphilic character of surfactants.

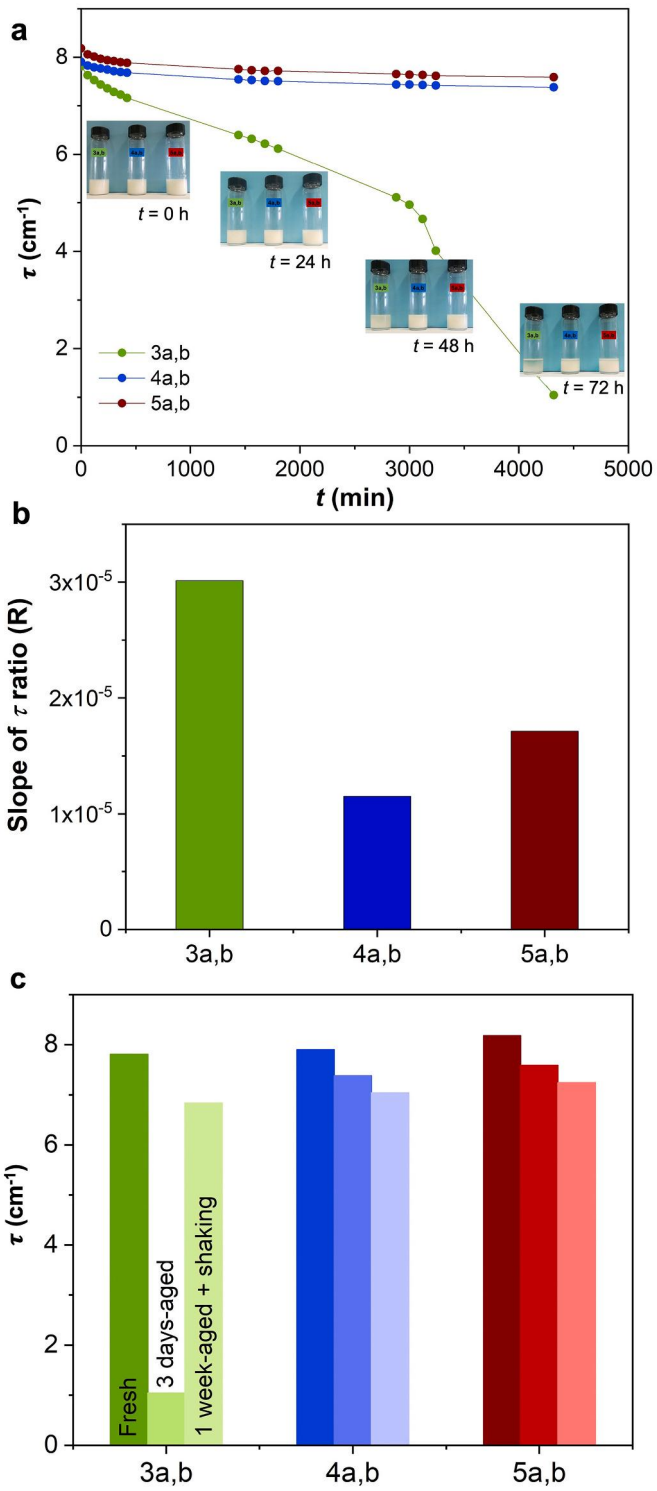

Fig. 3. *a*) Turbidity values (τ) of the prepared emulsions over time at fixed wavelength (550 nm). Insets: photos of the three samples at 0, 24, 48 and 72 h; *b*) slope of turbidity ratio (R) values as a function of the adopted surfactant; *c*) turbidity values of fresh and 3 days-aged emulsions (at fixed wavelength of 550 nm) alongside with τ data of the 1 week-aged samples subjected to a further agitation.

3.3. *Interfacial features and emulsions stability against sedimentation*

Sugar-based surfactants usually find application as emulsifiers in cosmetic, food, and pharmaceutical industries since they are odourless, tasteless and usually biodegradable, fulfilling physiological,

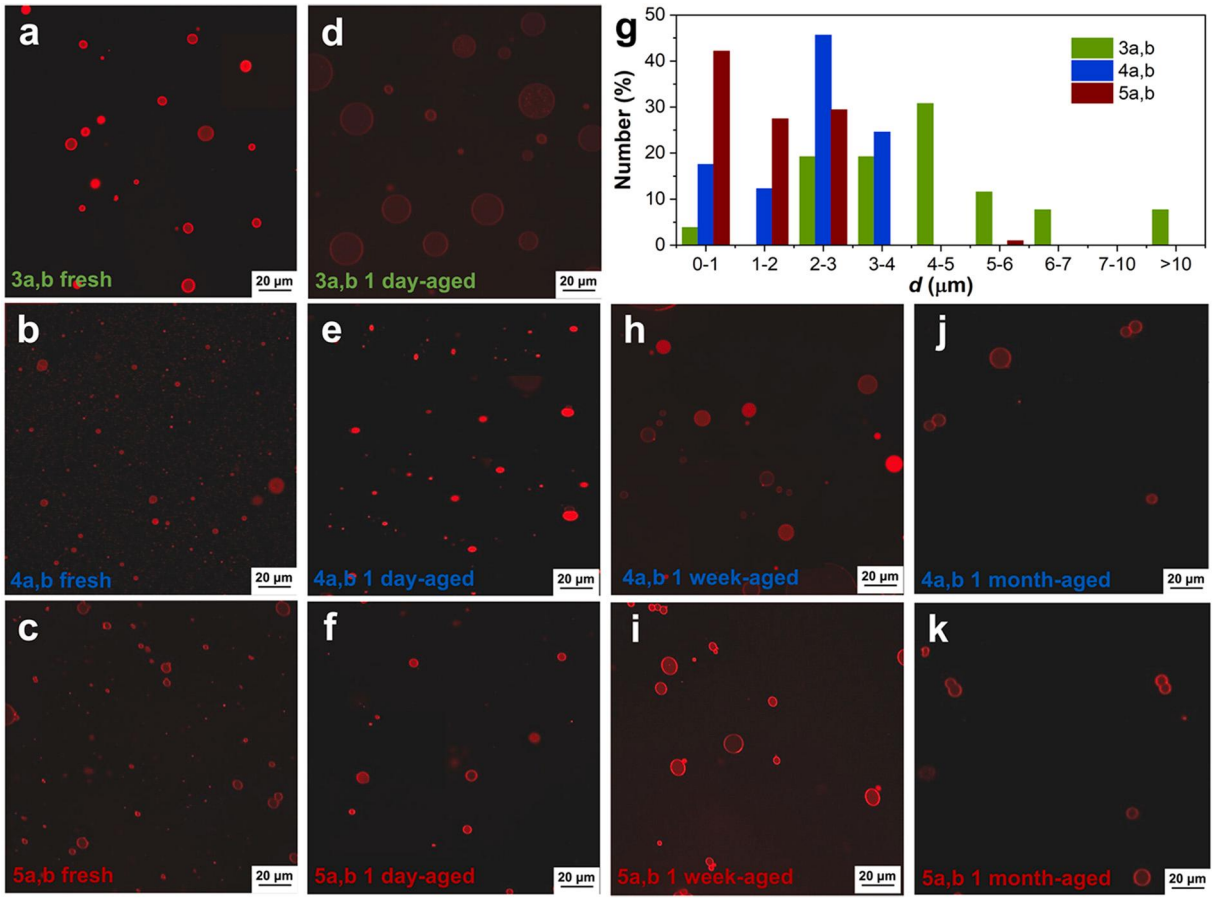

Fig. 4. LSCM images of *a-c*) freshly prepared, *d-f*) 1 day-aged, *h-i*) 1 week-aged and *j-k*) 1 month-aged 6-*O*-lauroyl- (**3a**,**b**), 6-*O*-palmitoyl- (**4a**,**b**) and 6-*O*-stearoyl- (**5a**,**b**) 1-*O*-butyl glucopyranosides emulsions; *g*) relative droplets size distribution (as number percentage) of the fresh samples.

dermatological and biological requirements.

To test the emulsifying properties of the three synthesized *n*-butyl glucopyranoside esters, IFT studies were performed according to the Du Noüy ring tensiometric method [23]. All the surfactants (Fig. 2) allowed the sunflower oil/water IFT reduction from 26 mN m^{-1} to a value lower than 3 mN m^{-1} at concentrations above 0.130%wt comparable to previously reported results with similar surfactants [18,24]. In order to corroborate these promising surface properties, emulsifying behaviour and stability over time were assessed by turbidity measurements [25] and microscopy images. Concerning the former, the change in turbidity over time is strictly related to the emulsion-breaking processes since τ decreases at any wavelength with the increase of destabilization phenomena [17]. Fig. 3 (*a*) exhibits gradually decreasing curves in the case of 6-*O*-palmitoyl- (**4a**,**b**) and 6-*O*-stearoyl- (**5a**,**b**) 1-*O*-butyl glucopyranosides, whereas a sharp τ reduction is appreciable for 6-*O*-lauyl-based (**3a**,**b**) surfactant. Notably, both **4a,b** and **5a,b** resulted sufficiently stable up to 72 h, whereas **3a,b** molecule caused the sedimentation of the water droplets already after 48 h. Furthermore, the magnitude variation of the emulsion instability was evaluated by determining the turbidity ratio (R) together with its slope within the first 50 min Fig. 3 (*b*). As reported by Song et al. [17], the less stable emulsion, the faster the turbidity ratio decrease; in the present case the slope was minimized adding **4a,b** and **5a,b** tensides, thus representing the most stable systems with respect to **3a**,**b** one. This is probably due to the shorter alkyl chain of the sugar derivatives thus limiting the affinity with the oil phase (characterized by traces of lauric acid [26], see Paragraph 5 in the Supplementary data), as also corroborated by both photos in insets of Fig. 3 (*a*) and confocal microscopy images (Fig. 4). Indeed, micrographs of the freshly prepared emulsions (Fig. 4 (*a-c*)) show very small droplets for all the three systems (1–20 μm) due to the ultrasound-assisted emulsification technique [27]. However, the relative distribution is quite different: the **3a,b**-based one exhibits a maximum centred around 4–5 μm, conversely the other two systems are characterized by an average diameter lower than 3 μm (Fig. 4 (*g*)). In addition, the obtained formulations were evaluated over time and in accordance with the turbidity results, after an ageing of 1 day (Fig. 4 (*d-f*)), a faster destabilization can be appreciated only in the case of the 6-*O*-lauryl-1-*O*-butyl glucopyranosides presence (average $d \geq 20$ μm, Fig. 4 (d)). After 1 week, a five-fold droplets size increase can be noticed for both **4a,b** and **5a,b**-based systems (Fig. 4 (*h,i*)) whereas the third molecule (*e.g.*, **3a,b**) leads to a faster concomitant droplets growth (by Ostwald ripening) and sedimentation. A further destabilization of **4a,b** and **5a,b**-based emulsions was achieved after 1 month, clearly observing the droplets aggregation into larger ones for **5a,b** and concomitantly a huge decrease of the droplets number (Fig. 4 (*k*)). On the contrary, the palmitoyl moiety seems to preserve the drops interlayer therefore resulting in a slightly greater stability against sedimentation (Fig. 4 (*j*)). Indeed, by analyzing the commercial sunflower oil composition through GC/MS analysis, a two-fold content of palmitic acid with respect to stearic acid is appreciable (See Supplementary data, Paragraph 5). Therefore, a moderately better stabilization of the emulsions generated in presence of **4a,b** with respect to **5a,b** could be due to the higher relative affinity of the palmitoyl derivative with the oil phase.

In order to confirm the previous outcomes, turbidimetric tests were carried out on the 1 week-aged emulsions after simple hand-agitation till the recovery of a milky system. Fig. 3 (*c*) shows the obtained results: all the adopted surfactants are able to partially restore the starting conditions, even if the **4a,b** and **5a,b** molecules seem to be the optimal ones.

4. Conclusion

Herein, three sugar-based surfactants have been enzymatically synthesized in good yields and high purity by means a solvent-free system, starting from a modified sugar to enhance its solubility in molten fatty acids. The physico-chemical properties of these surfactants, in terms of sunflower oil/water interfacial tension and W/O emulsifying capability, have been deeply investigated. For all surfactants a IFT reduction of around 20 mNm^{-1} allows the stabilization of W/O emulsion prepared by ultrasound-assisted method, that guarantees a very fine droplet size distribution (<d > around 3 μm). To the best of our knowledge, stability tests of the prepared glucoside esters-assisted emulsions, through turbidimetric data and confocal microscope images, have never been reported so far. Our results indicate that both 6-*O*-palmitoyl- (**4a**,**b**) and 6-*O*-stearoyl- (**5a**,**b**) 1-*O*-butyl glucopyranosides generate the most stable W/O emulsions, avoiding the sedimentation even after one month ageing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.colcom.2022.100630.

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Chapter 3.2 From lactose to alkyl galactoside fatty acid esters as non-ionic biosurfactants: a two-step enzymatic approach to cheese whey valorization

Riccardo Semproli, Marina S. Robescu, Sara Sangiorgio, Eleonora Pargoletti, Teodora Bavaro, Marco Rabuffetti, Giuseppe Cappelletti, Giovanna Speranza,* Daniela Ubiali*

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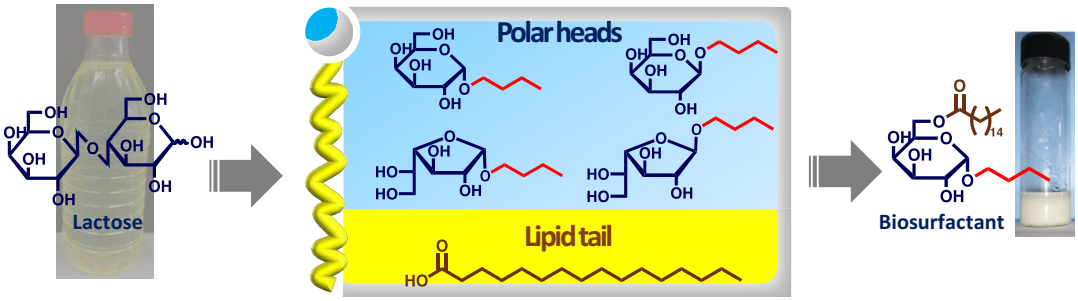

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From Lactose to Alkyl Galactoside Fatty Acid Esters as Non-Ionic Biosurfactants: A Two-Step Enzymatic Approach to Cheese Whey Valorization

Riccardo Semproli[+],[a] Marina Simona Robescu[+],[a] Sara Sangiorgio,[b] Eleonora Pargoletti,[b] Teodora Bavaro,[a] Marco Rabuffetti,[b] Giuseppe Cappelletti,[b] Giovanna Speranza,*[b] and Daniela Ubiali*[a]

A library of alkyl galactosides was synthesized to provide the "polar head" of sugar fatty acid esters to be tested as non-ionic surfactants. The enzymatic transglycosylation of lactose resulted in alkyl β-D-galactopyranosides, whereas the Fischer glycosylation of galactose afforded isomeric mixtures of α- and β-galactopyranosides and α- and β-galactofuranosides. *n*-Butyl galactosides from either routes were enzymatically esterified with palmitic acid, used as the fatty acid "tail" of the surfactant, giving the corresponding *n*-butyl 6-*O*-palmitoyl-galactosides. Measurements of interfacial tension and emulsifying properties of *n*-butyl 6-*O*-palmitoyl-galactosides revealed that the esters of galactopyranosides are superior to those of galactofuranosides, and that the enantiopure *n*-butyl 6-*O*-palmitoyl-β-D-galactoside, prepared by the fully enzymatic route, leads to the most stable emulsion. These results pave the way to the use of lactose-rich cheese whey as raw material for the obtainment of bio-based surfactants.

Introduction

Surfactants are amphiphilic compounds widely used in every industrial sector. Application of surfactants ranges from household detergents and personal care products to food processing, agricultural and oilfield chemicals, cleaners, paints and coatings, textile, plastics, adhesives, and others.[1] The growing interest of consumers and producers toward safer and more environmentally friendly products is driving the search for new bio-based and biodegradable surfactants.[2]

Sugar fatty acid esters (SFAE), usually called sugar esters, are non-ionic surfactants which, besides excellent emulsifying, stabilizing and detergency properties, overshadow petrochemical-derived surfactants in that they are tasteless, odorless, non-toxic, non-harmful to the environment, and fully biodegradable.[3] SFAE are constituted by a sugar moiety which acts as the "polar head" of the surfactant, and by a fatty acid "tail". The hydrophilic-lipophilic balances (HLB) of SFAE can be fine-tuned and thus customized for a specific application by controlling the degree of esterification and the nature of sugar and fatty acid residues. Moreover, the components of SFAE can be bio-derived from natural resources or from waste upgrading, thus producing bio-based surfactants.[4,5] Generally speaking, the chemical synthesis of SFAE requires harsh reaction conditions (hazardous solvents, high temperature, acid or base catalysts) resulting in high energy consumption, formation of undesirable by-products (*e.g.*, due to caramelization of sugars), and low regioselectivity. The regioselective chemical synthesis of SFAE can only be achieved through protection groups strategies, which are hardly acceptable for industrial processes.[6]

Enzyme-based synthesis can often circumvent the above-mentioned drawbacks: enzymatic reactions generally occur under milder conditions without the need for tedious protection/deprotection steps. Moreover, biotransformations of naturally occurring substrates provide more environmentally friendly synthetic routes allowing for the final product to be labelled as natural as well.[7] Sugar fatty acid esters can be obtained, indeed, through an esterification reaction between a sugar and a fatty acid catalyzed by a lipase.[6]

Tuning the reaction conditions of the enzymatic esterification between fatty acids and sugars for the SFAE preparation is a challenging task owing to the opposite solubility profiles of these reagents. A key issue is the quest for the solvent or co-solvent that can solubilize both the sugar and the fatty acid moieties without deactivating the enzyme.[8,9] To circumvent this constraint, the sugar can be indeed derivatized into a less polar precursor (*i.e.*, alkyl glycoside), followed by a solvent-free lipase-mediated esterification.[10–12] The SFAE that are currently available on the market include molecules derived from sucrose and sorbitan, as well as fatty acid glucamides and alkyl polyglycosides.[13] Amongst all sugar-based surfactants, sucrose

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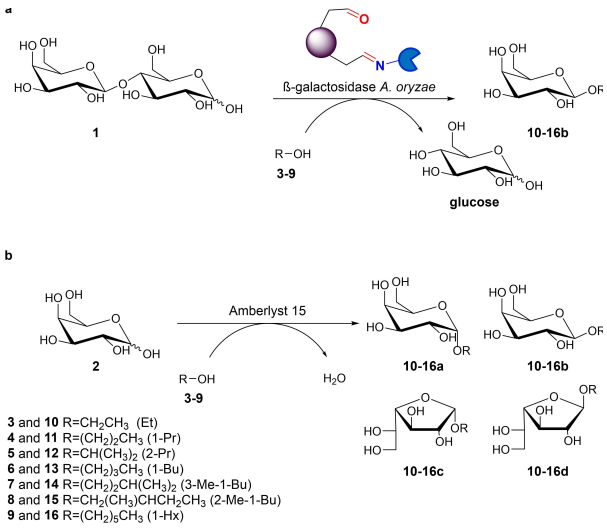
and glucose esters are the most studied and applied derivatives. The use of lactose to produce sugar esters has been scarcely reported to date,[14] despite recent studies have shown the high potential of lactose esters.[15–18] Galactose-based fatty acid esters have been poorly investigated, too.[14]

Lactose is a naturally occurring disaccharide, found in milk, composed of D-galactose and D-glucose. The world milk production was estimated to be nearly 906 million tons in 2020.[19] From the production of 1 kg of cheese, around 9–10 L of cheese whey (CW) are generated. CW is very rich in lactose, proteins, lipids, and mineral salts, but has also high disposal costs and environmental burden.[20]

In the frame of applying a bio-based circular economy approach, this study aims at exploring the use of lactose, which can be easily accessed through cheese whey permeate (CWP), resulting from protein recovery by ultrafiltration, as a cheap and abundant substrate for the production of SFAE. Lactose was indeed used as starting material in the enzymatic transglycosylation mediated by the immobilized β-galactosidase from *Aspergillus oryzae* in the presence of naturally occurring alcohols to synthesize a library of alkyl galactosides. 1-Butyl β-D-galactopyranoside (13b, Scheme 1a) was enzymatically esterified with palmitic acid producing the corresponding SFAE (17b, Scheme 2a). The emulsifying properties of 17b were evaluated and compared with those of the SFAE (17ad) obtained by a chemoenzymatic approach previously described for the obtainment of *n*-butyl glucoside fatty acid esters.[10] These latter compounds (*n*-butyl 6-*O*-palmitoyl-glucosides, labelled as SER herein) were used as reference standards.



Scheme 1. Synthesis of alkyl galactosides (**10–16**). a) Enzymatic transglycosylation of lactose (**1**) with aliphatic alcohols (**3–9**) catalyzed by immobilized β-galactosidase from *Aspergillus oryzae* in McIlvaine buffer pH 4.3/alcohol/(acetone), r.t., 6 h. b) Fischer glycosylation of galactose (**2**) with aliphatic alcohols (**3–9**) catalyzed by Amberlyst® 15, 90-120 °C, 1.5–6 h.

Results and Discussion

Immobilization of β-galactosidase from *A. oryzae*

β-Galactosidase used for the transglycosylation reactions was covalently immobilized on glyoxyl Sepabeads™ as a result of the screening of different carriers and conditions (see Supporting information, Table S1). Following previously reported protocols,[21,22] the enzyme was first immobilized by ionic interaction on an aminated carrier. Instead of using agarose activated with ethylendiamine (monoaminoethyl-*N*-aminoethyl-agarose or MANAE-agarose), the "ready-to-use" aminated Relizyme EA-112/S was used. The immobilization was fast (2 h) and high yielding (60 %), but protein leakage (40 %) detected in the reaction medium (McIlvaine buffer pH 4.3) after 2 h incubation discouraged the use of this immobilized biocatalyst in the transglycosylation reaction. Ionic immobilization is reversible and the enzyme can be desorbed depending on the conditions used. Post-immobilization cross-linking can assist in the stabilization of the enzyme-carrier interactions.[23] However, driven by a recent report[24] on the covalent immobilization of the β-galactosidase from *A. oryzae* on glyoxyl agarose and the use of the immobilized biocatalyst in the synthesis of **13 b** by transglycosylation of lactose, we also moved to aldehyde-activated carriers by using glyoxyl Sepabeads™ in this case. These carriers display the same activation group, but differ about the nature of the matrix, being the poly-hydroxylated agarose sharply hydrophilic as compared to the polymethacrylate Sepabeads™. Sepabeads™ was subjected to epoxide ring opening in sulphuric acid followed by oxidation of the resulting diols into aldehyde groups with sodium periodate.[25] Upon incubation of the enzyme with an aldehyde-activated carrier, imine bonds are formed as a result of the reaction between ε-amino groups of surface Lys and aldehydes. Chemical reduction of the Schiff's bases is recommended and usually performed to get C–N stable bonds. However, the reduction step is generally a sore point for enzyme activity retention and the β-galactosidase from *A. oryzae* was not an exception (see Supporting information, Table S1). Therefore, the non-reduced biocatalyst was used in the transglycosylation reactions. Owing to the poor stability of the β-galactosidase at the high pH required for the immobilization (pH 10),[24,26] the immobilization was performed at 4 °C with satisfactory results (immobilization yield = 58 %, activity recovery = 20 %).

Synthesis of alkyl β-D-galactopyranosides (10 b–16 b) by enzymatic transglycosylation of lactose

Although galactosidases are hydrolytic enzymes, they can be used also in synthesis through either a "direct glycosylation" ("reverse hydrolysis") or a transglycosylation approach. In this latter route, a preformed activated glycoside is used and the nucleophile (water in the "normal" hydrolytic pathway) can be replaced by other nucleophiles, such as an acceptor alcohol. Transglycosylation results in higher yields than reverse hydrolysis which requires high concentrations of both the carbohy-

drate and the alcohol, as well as to finely tune the medium engineering.[27] Following this strategy, lactose can be converted into valuable chemicals, such as prebiotics and sweeteners (*i.e.* galactooligosaccharides, lactulose, lactosucrose, and galactosylpolyhydroxyalcohols).[28] The transglycosylation of lactose here reported was carried out according to the method described by Ahumada[24] which was extended, with some modifications, to the preparation of the library of alkyl β-D-galactopyranosides **10b–16b** (Scheme 1a and Table 1).

The highest yield (45%) was obtained with 1-BuOH (**6**) as shown in Table 1. It is worth mentioning that the reaction system is affected by the type of alcohol used: a ternary homogeneous system was formed with the C4 alcohol (**6**), whereas C5 and C6 alcohols (**7–9**) generated a biphasic system, regardless the addition of acetone (up to 40% v/v). Low yields obtained for short-chain alcohols (**3–5**) can be ascribed to some precipitation of lactose as well as a partial enzyme inactivation. On the other hand, the low yields achieved with long-chain alcohols (**7–9**) are consistent with previous reports.[29–30] This trend might be a constraint for using glycosidases in the preparation of alkyl glycosides endowed with surfactant properties, which would require longer chains (typically C8–C14). Nevertheless, even "shorter" alkyl glycosides result to be sufficiently less hydrophilic derivatives than their sugar counterpart, thus allowing for the straightforward preparation of SFAE by a direct enzymatic esterification with fatty acids in a solvent-free system.[10] This approach answers the need to balance the opposite solubility profiles of the sugar "polar head" of the surfactant and the fatty acid "tail". In addition, at least for 1-butyl glucosides, the alkyl chain was shown to positively affect the HLB (hydrophilic-lipophilic balance).[10]

Alkyl galactosides can be also synthesized by chemical glycosylation of galactose with alcohols (see next paragraph and Scheme 1b). Enzymatic transglycosylation has the advantage of a higher selectivity (pure β-D-galactopyranosides are obtained) and a lower reaction temperature. Moreover, in the frame of upcycling lactose into new bio-based products, this approach allows to use directly lactose from CWP as feedstock. On the other hand, the enzymatic approach is characterized by lower yields, although not optimized yet. 1-Butyl β-D-galactopyranoside (**13b**) was selected out of the library of alkyl β-D-galactopyranosides (**10b–16b**) for the next esterification step, as a continuation of our previous work on the chemoenzymatic synthesis of 6-*O*-palmitoyl-*n*-butyl glucosides and the assessment of their surfactant properties.[10]

Table 1. Reaction yields (enzymatic transglycosylation and chemical glycosylation) and isomeric ratio of mixtures **10ad–16ad**.

R–OH (**3–9**)	Enzymatic transglycosylation (yield%)	Chemical glycosylation (yield%)	Isomeric ratio (a/b/c/d)
3 (R=Et)	**10b**[31] (23)	**10ad**[32] (73)	13/20/27/40
4 (R=1-Pr)	**11b**[33] (33)	**11ad**[34,35] (78)	24/27/19/30
5 (R=2-Pr)	**12b**[36,37] (25)	**12ad**[37,38] (36)	18/20/28/34
6 (R=1-Bu)	**13b**[36,39] (45)	**13ad**[38,39] (81)	46/38/6/10
7 (R=3-Me–1-Bu)	**14b** (29)	**14ad** (95)	37/29/14/20
8 (R=2-Me–1-Bu)	**15b**[40] (19)	**15ad** (88)	43/34/10/13
9 (R=1-Hx)	**16b**[41] (23)	**16ad** (32)	37/32/14/17

Synthesis of alkyl galactoside isomeric mixtures (10ad–16ad) by Fischer glycosylation of galactose

Another way to upgrade CWP is the use of the lactose constituents, *i.e.* glucose and galactose that can be both obtained by β-galactosidase-mediated hydrolysis of lactose.[42] Recently, due to the lactose price fluctuation, the demand for manufacturing monosaccharides and other "lactose-derived bioactives" from inexpensive whey permeate is growing.[28] Specifically, galactose can be isolated directly from whey permeate after protein removal, lactose enzymatic hydrolysis and several purification steps, including activated carbon treatment, electrodialysis, ion purification and simulated moving bed chromatography.[43]

Following the already established strategy to synthesize sugar-based surfactants,[10] namely the preparation of sugar derivatives apolar enough to allow the successive solvent-free enzymatic esterification, D-(+)-galactose (**2**) was submitted to Fischer glycosylation reactions with the selected naturally occurring alcohols (**3–9**). Similar experimental conditions (0.1 M final concentration of the reaction mixture, 10% w/w Amberlyst® 15, 25% w/w 3 Å molecular sieves, 90–120 °C and 1.5–6 h) were successfully applied to all the reactions, obtaining alkyl galactoside isomeric mixtures (**10ad–16ad**) in a variable yield depending on the alcohol used (Table 1). Modest isolated yields were obtained from Fischer glycosylation of galactose with 2-PrOH (**5**) and 1-HexOH (**9**) ($yield_{12ad}$ = 36%; $yield_{16ad}$ = 32%), probably because of steric hindrance effects in the case of **5**, and lower sugar solubility in the case of **9**. On the contrary, high yields (73–95%) were achieved for all the other alcohols, both branched and linear.

It is important to underline that this industrially relevant reaction is usually performed in presence of a homogenous acid catalyst, *i.e. p*-toluenesulfonic acid or sulphamic acid, which requires a neutralization step.[44] We employed an alternative and more sustainable method based on the use of Amberlyst® 15, which is a strongly acidic cation exchange resin that can be eventually filtered and recovered.[45]

Unlike the enzymatic transglycosylation, which produces only the β-galactopyranoside isomer, the Fischer glycosylation leads to an isomeric mixture of α-/β-pyranosides and α-/β-furanosides (Table 1), whose relative ratio strictly depends on the sugar, the alcohol and the experimental conditions in which the reaction is carried out.[46] According to ^{1}H NMR analysis in D_2O, the shorter is the chain length of the alcohol (for EtOH, 1-PrOH and 2-PrOH), the higher is the amount of furanoside derivatives produced, till a plateau is reached of the relative ratio between the two structural isomeric forms of about 1:1 for longer chain alcohols (1-BuOH, 3-Me–1-BuOH, 2-Me–1-BuOH and 1-HexOH).

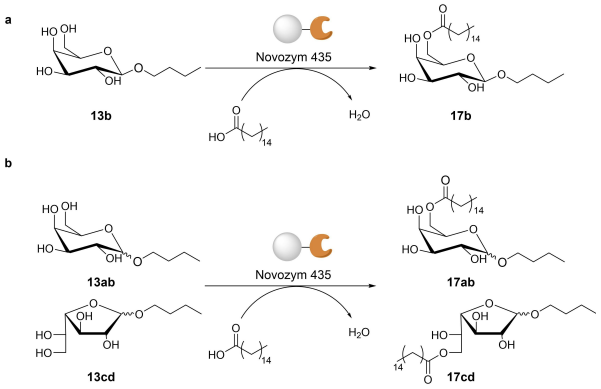
Synthesis of *n*-butyl 6-*O*-palmitoyl-galactosides (17 ad) *via* solvent-free enzymatic esterification

Galactosides, obtained both through enzymatic and chemical approaches, were used as "polar heads" for the preparation of biosurfactants. Thus, focusing on the alkyl galactoside obtained with the highest yield from the enzymatic transglycosylation, namely 1-butyl β-D-galactopyranoside (**13 b**), and in connection with our previous studies on alkyl glucosides,[10] this compound was submitted to the known protocol[10] of solvent-free Novozym® 435-catalyzed esterification with palmitic acid to give *n*-butyl 6-*O*-palmitoyl-*β*-D-galactopyranoside (**17 b**, $yield_{17b}$ = 15 %) (Scheme 2 and Table 2).

As in the synthesis of alkyl glucoside fatty acid esters,[10] the reaction was carried out in a glass oven B-585 Kugelrohr at the optimal working temperature of the immobilized lipase (80 °C), under rotation (400 rpm) instead of magnetic stirring to avoid enzyme beads wrecking, and under reduced pressure conditions (30 mmHg) to favor water removal and to shift the equilibrium towards product formation. Even in the case of **17 b**, Novozym® 435 catalyzes regioselectively the esterification of the primary hydroxyl group, as confirmed by the Heteronuclear Multiple Bond Correlation (HMBC) ^{1}H-^{13}C NMR experiment, carried out in DMSO-d_6 (see Supporting information, Figure S39).

In parallel, the same experimental conditions were applied to the isomeric mixture of 1-butyl galactosides (**13 ad**), obtained through the chemical route, to achieve an isomeric mixture of *n*-butyl 6-*O*-palmitoyl-galactosides. The remarkable difference in polarity between pyranoside and furanoside ester isomers allowed the separation of the two species, which was performed by flash chromatography (*n*-hexane/EtOAc, 2:8), affording *n*-butyl 6-*O*-palmitoyl-galactopyranosides (**17 ab**, $yield_{17ab}$ = 21 %) and *n*-butyl 6-*O*-palmitoyl-galactofuranosides (**17 cd**, $yield_{17cd}$ = 14 %). Regarding the former, the Heteronuclear Single Quantum Coherence (HSQC) ^{1}H-^{13}C NMR correlation spectrum (see Supporting information, Figure S85) showed the presence of the β-anomer only in traces (about 6 %), as further confirmed by the purity assessment through quantitative TLC and image analysis (see Supporting information, Figure S94). On the contrary, in the case of furanosides, the β-anomer seemed to be prevailing (α/β furanosides; 20:80). Beside the anomeric effect, the low yield of the *n*-butyl β-D-galactopyranoside ester, which ranges from 38 % in the mixture of 1-butyl galactopyranosides to 6 % in the corresponding *n*-butyl 6-*O*-palmitoyl-galactopyranosides (**17 ab**), might depend on the different interaction of these substrates in the catalytic site.

The two-step chemoenzymatic process for the synthesis of *n*-butyl 6-*O*-palmitoyl-galactosides (**17 ad**) was carried out also by cutting off intermediate and final chromatographic steps. The crude from the Fischer glycosylation (containing 1-butyl galactoside isomeric mixture **13 ad** and traces of unreacted **2**) was directly reacted with palmitic acid according to the solvent-free enzymatic esterification protocol. The reaction mixture was taken up in EtOAc, filtered to remove the biocatalyst and the esters were extracted in EtOAc from 1 M NaOH solution, thus affording the isomeric mixture of *n*-butyl 6-*O*-palmitoyl-galactosides (**17 ad**, $yield_{17ad}$ = 35 %) after EtOAc evaporation. The mixture **17 ad** was obtained in comparable yield with respect to the reported above methods involving chromatographic separations, resulting in 90 ± 2 % purity (about 10 % of unreacted 1-butyl galactoside isomeric mixture, **13 ad**) and a 52/4/11/33 relative ratio between α-/β-pyranosides and α-/β-furanosides, which were assessed by quantitative TLC and image analysis (Figure S94).



Scheme 2. Synthesis of *n*-butyl 6-*O*-palmitoyl-galactosides **17 ad** by enzymatic esterification of **13 ad** with palmitic acid catalyzed by Novozym® 435 in a solvent-free system, 80 °C, 8 h, *s. v.*

Table 2. Reaction yields (enzymatic esterification) and isomeric ratio of mixtures **17 ad**.

Substrate	Product	Yield (%)	Isomeric ratio (α/β)
13 b	**17 b**	15	0/100
13 ad	**17 ab**	21	94/6
	17 cd	14	20/80

Interfacial features study and emulsifying properties evaluation

For a deeper insight in the surfactant properties of the isomeric mixture of α-/β-pyranosides and α-/β-furanosides of *n*-butyl 6-*O*-palmitoyl-galactosides (**17 ad**), it is mandatory to study the contribute of each isomer.

The stabilization of a disperse system, *i.e.* emulsion, can be achieved by reducing the interfacial tension (IFT) between two immiscible phases. Surfactants, due to their amphiphilic nature, are adsorbed at interphases, thus modifying the interfacial tension.[47] Therefore, in the present case, the IFT reduction (Figure 1a) between water (milli-Q) and sunflower oil was evaluated by adding the isomeric mixture of *n*-butyl 6-*O*-palmitoyl-galactosides (**17 ad**), as well as the single isomeric components (*n*-butyl 6-*O*-palmitoyl-galactopyranoside **17 ab** containing 6 % of **17 b**, *n*-butyl 6-*O*-palmitoyl-β-D-galactopyranoside **17 b**, *n*-butyl 6-*O*-palmitoyl-galactofuranoside **17 cd** containing about 6 % of *n*-butyl 6-*O*-palmitoyl-galactopyranoside isomers **17 a** and **17 b**), at increasing concentration (0.1, 1.5 and 3.0 mM in sunflower oil), up to the solubility limit of some of them.

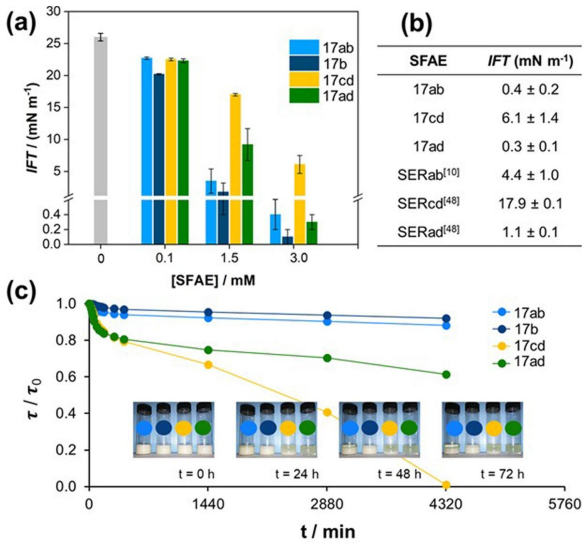


Figure 1. a) Sunflower oil/water interfacial tension (IFT) data for the synthesized surfactants (**17ab**, **17b**, **17cd**, **17ad**). b) Table: comparison of IFT values at 3 mM with other sugar-based esters used as references (**SERab**,[10] **SERcd**,[48] and **SERad**[48]) c) Normalized turbidity values (τ) of the prepared emulsions over time at fixed wavelength (550 nm). Insets: photos of the four samples at 0, 24, 48 and 72 h.

At 0.1 mM, all the tensides are comparable, showing a negligible IFT reduction with respect to the starting IFT value between sunflower oil and water in absence of a surfactant (26 mN m^{-1}; grey histogram in Figure 1a). On the contrary, at 3.0 mM, except for the furanoside derivatives (**17cd**), all the prepared tensides (**17ab**, **17b**, **17ad**) resulted to be able to reduce the IFT to a value lower than 2 mM m^{-1}, thus indicating extremely promising interfacial features. However, at an intermediate surfactant concentration (1.5 mM), some differences can be easily appreciated: particularly, only *n*-butyl 6-*O*-palmitoyl-β-D-galactopyranoside (**17b**) seems to reach an IFT value close to 2 mN m^{-1}. This result may be due to a better surfactant chain orientation, which favors the packing capacity of the molecules adsorbed at the interface, thus reducing the IFT significantly. Concomitantly, it is worth noting that the *n*-butyl 6-*O*-palmitoyl-galactoside isomeric mixture (**17ad**) showed intermediate interfacial features with respect to pure **17ab** and **17b**, due to the presence of furanosides (**17cd**) that hinder the IFT reduction at this concentration. These results are fully in agreement with those reported in the case of other sugar-based esters (**SER** family in the Table of Figure 1)[10,48] bearing *n*-butyl glucoside as the "polar head" and palmitic acid as the fatty acid "tail".

The ability of reducing the IFT was also observed in the cases of *n*-butyl 6-*O*-palmitoyl-glucopyranoside (**SERab**), *n*-butyl 6-*O*-palmitoyl-glucofuranoside (**SERcd**), and *n*-butyl 6-*O*-palmitoyl-glucoside isomeric mixture (**SERad**). Generally, the IFT reduction is strongly driven by the degree of disorder of the interfacial film, which is related to the interfacial entropic contributions of water/oil/surfactant molecules. When no surfactant is present, oil and water molecules at the interface lose their degrees of freedom, since parallel alignments occur, due to strong short-range van der Waals interactions between the two phases. Conversely, the addition of a surfactant pushes away the oily molecules from the water surface, making them more disordered, by increasing their rotational and translational modes.[49] As reported in the Table of Figure 1, all the galacto-side-based esters (**17ab**, **17cd** and **17ad**) led to a strong IFT reduction, much more than the corresponding glucoside-based isomers (**SERab**, **SERcd** and **SERad**). These results could be explained by looking into the chemical structures: **17** and **SER** compounds are epimers in the C-4 position, being the orientation of the OH(4) group the only difference. According to the literature,[50] this behavior is strictly connected to a different ability of matching the tetrahedral hydrogen bond network of water. Sugars with an axial OH(4) group, such as galactose, match the three-dimensional water structure much worse than sugars with an equatorially-oriented OH(4), as in the case of glucose. The mismatch causes a distortion of the water structure in the primary hydration shell, which is also transmitted to the more distant layers, inducing a more disordered structure in the water configuration and, consequently, the increase in the total entropy of the system.[49]

Finally, water in sunflower oil (W/O) emulsions ($\Phi_V = 0.13$) were prepared by ultrasounds using a micro-tip sonicator in the presence of the biosurfactant, and their stability was evaluated within 72 h. Figure 1c shows the decrease of turbidity over time, strictly related to the emulsion destabilization phenomena:[10] compound **17b** leads to the most stable emulsion after 72 h ageing (see also the pictures in the inset of Figure 1c), whereas the other tensides were not able to stabilize the disperse systems over time, undergoing water droplets sedimentation processes and irreversible coalescence mechanisms. Hence, these results confirm the IFT data previously obtained.

Conclusion

Sugar fatty acid esters find application as emulsifiers in the cosmetic and food industry.[51–53] In this work, a general procedure for the preparation of some SFAE starting from lactose through a two-step enzymatic approach catalyzed by the immobilized β-galactosidase from *Aspergillus oryzae* and the immobilized CalB (Novozym® 435), or from galactose through a two-step chemoenzymatic strategy was set-up. The exploitation of lactose as a raw material, either as such or as a source of galactose, for the production of surfactants answers the need for tackling the issue of cheese whey disposal in dairy industry, while upcycling this abundant and cheap feedstock, which is independent from season and climate and is not in competition with food. Starting from the results achieved herein, we aim indeed at using lactose-rich CWP as raw material both for the synthesis of the "polar head" of the surfactant, and as fermentation medium for the obtainment of microbial lipids to be used as fatty acid "tail".[54]

According to the preliminary physicochemical study here reported, *n*-butyl galactoside fatty acid esters were shown to generally possess promising interfacial features, although to a

different extent; in particular, the enantiopure *n*-butyl 6-*O*-palmitoyl-β-D-galactopyranoside (**17 b**), which was prepared enzymatically from lactose, leads to the most stable emulsion.

Experimental Section

General

All chemicals were from Sigma-Aldrich (Milano, Italy), if not stated otherwise. Anhydrous lactose was purchased from Honeywell Fluka (Rodano, Italy). All solvents were from Merck Life Science (Milano, Italy) and were used without further purification. β-Galactosidase from *Aspergillus oryzae* was purchased from Sigma-Aldrich (Milano, Italy). Lipase B from *Candida antarctica* immobilized on an acrylic carrier (Novozym® 435) was kindly supplied by Novozymes (Denmark). Sepabeads™ EC-EP/S and Relizyme™ EA-112/S were a gift of Resindion s.r.l. (Binasco, Italy).

Electrospray ionization mass spectra (ESI-MS) were recorded on a Thermo Finnigan LCQ Advantage spectrometer (Hemel Hempstead, Hertfordshire, UK). ^{1}H and ^{13}C NMR spectra were recorded at 400.13 and 100.61 MHz, respectively, on a Bruker AVANCE 400 spectrometer (Bruker, Karlsruhe, Germany) interfaced with a workstation running a Windows operating system TOPSPIN software package, at 300 K. Chemical shifts (δ) are given in parts per million (ppm) and were referenced to the solvent signal (DMSO-d_6, δ_H 2.50 ppm and δ_C 39.52 ppm, and D_2O, δ_H 4.79 ppm, from TMS, respectively). The ^{13}C NMR signal multiplicities were based on APT (attached proton test) spectra. The ^{13}C NMR signals were assigned with the aid of ^{1}H-^{13}C correlation experiments (heteronuclear single quantum correlation spectroscopy, HSQC, and heteronuclear multiple bond correlation spectroscopy, HMBC). The fatty acid profile of commercial sunflower oil was determined by gas chromatography/mass spectrometry (GC/MS) analysis, after base-catalyzed transmethylation, using the protocol FIL-IDF 182:1999 reported elsewhere.[10]

Immobilization of β-galactosidase from *A. oryzae* on glyoxyl Sepabeads™

Glyoxyl Sepabeads™ was prepared as previously reported.[25] Briefly, Sepabeads™ EC-EP/S (20 g) was suspended in 0.5 M H_2SO_4 (260 mL) under mechanical shaking for 2 h at r.t., then filtered under vacuum and washed thoroughly with distilled H_2O until neutral pH. The resin was then oxidized by incubation with 0.1 M $NaIO_4$ (80 mL) for 2 h at r.t., filtered, washed thoroughly with distilled H_2O and stored at 4 °C till use. β-Galactosidase from *A. oryzae* (17 mg; 5 IU) was dissolved in 50 mM $NaHCO_3$ buffer pH 10 (10 mL) containing 1% D-(+)-galactose (w/v) under stirring at 4 °C till a clear solution was obtained. Glyoxyl Sepabeads™ (1 g) was added to the mixture to start the immobilization. At the endpoint (24 h), the immobilized enzyme was filtered, washed thoroughly with distilled water, and stored at 4 °C till use.

Synthesis of alkyl β-D-galactopyranosides (10 b–16 b) by enzymatic transglycosylation of lactose (general protocol)

Enzymatic transglycosylation reactions (Scheme 1a) were performed as reported by Ahumada et al.[24] with slight modifications. Anhydrous lactose (**1**, 0.342 g, 1 mmol) was dissolved in McIlvaine buffer pH 4.3 (20 mL) and the desired alcohol (**3–9**, 50 mL) was added under magnetic stirring. Acetone (30 mL or 40 mL) was added to the mixture when 1-BuOH (**6**) or C5/C6 alcohols (**7–9**) were used, respectively, thus generating a ternary homogeneous system or a biphasic system. For water-miscible alcohols (**3–5**), no acetone was used. The immobilized β-galactosidase (2 g, 1.6 IU) was added to the reaction and the mixture was stirred at r.t. After 6 h (TLC monitoring, DCM/MeOH, 85:15, detection by H_2SO_4 5% v/v in EtOH), the suspension was filtered under vacuum and the immobilized enzyme was washed with the same reaction mixture (20 mL) by replacing the buffer with distilled water. The filtrate was evaporated under reduced pressure and purified by flash chromatography (DCM/MeOH, 85:15). The crude was added with silica and MeOH, dried under reduced pressure and loaded on the silica column. Purified alkyl β-D-galactopyranosides (**10 b–16 b**) were characterized by TLC, ESI-MS, and NMR analysis (see Supporting information). Yields are listed in Table 1.

Synthesis of alkyl galactoside isomeric mixtures (10 ad–16 ad) by Fischer glycosylation of galactose (general protocol)

D-(+)-Galactose (**2**, 0.900 g, 5 mmol) was suspended in dry naturally occurring alcohols (**3-9**, 50 mL) in the presence of the strongly acidic cation exchange resin Amberlyst® 15 (10%, w/w) and 3 Å molecular sieves (25%, w/w), under reflux or at 120 °C, according to the alcohol boiling points (Scheme 1b). After 1.5–6 h, depending on the alcohol, the reactions were stopped by filtration of the solid catalyst, the alcohols were removed under reduced pressure, and the reaction mixtures were submitted to flash chromatography (DCM/MeOH, 9:1) to give alkyl galactosides **10 ad–16 ad** as viscous syrups (yields are listed in Table 1). The isomeric mixtures of galactosides (**10 ad–16 ad**) were characterized by TLC, ESI-MS and NMR analysis (see Supporting information). NMR signals were identified by comparison with data reported in literature.[55] D-(+)-Galactose (**2**), Amberlyst® 15 and 3 Å molecular sieves were dried at 90 °C overnight prior to use.

Enzymatic synthesis of *n*-butyl 6-*O*-palmitoyl-galactosides (17 b, 17 ab, 17 cd) (general protocol)

1-Butyl β-D-galactopyranoside (**13 b**, 0.708 g, 3 mmol), palmitic acid (0.768 g, 3 mmol) and Novozym® 435 (10%, w/w) were mixed and poured into a round-bottom flask. The mixture was heated to 80 °C while rotating the flask by means of a glass oven B-585 Kugelrohr (Büchi, Cornaredo, Italy). After fatty acid melting, the reaction was performed under reduced pressure (30 mmHg) (Scheme 2a and Table 2). After 8 h, the reaction mixture was taken up in EtOAc and the immobilized enzyme was removed by filtration. Then, the ester was extracted in EtOAc (2x) from 1 M NaOH, the organic phases were collected, dried over Na_2SO_4 and the solvent was removed under reduced pressure. The product was isolated by flash chromatography (*n*-hexane/EtOAc, 2:8), thus affording *n*-butyl 6-*O*-palmitoyl-β-D-galactopyranoside (**17 b**) (yield = 15%). The same experimental conditions were used for reacting the isomeric mixture of 1-butyl galactosides (**13 ad**). *n*-Butyl 6-*O*-palmitoyl-galactopyranoside (**17 ab**) (yield = 21%) and *n*-butyl 6-*O*-palmitoyl-galactofuranosides (**17 cd**) (yield = 14%) were isolated by flash chromatography (Scheme 2b and Table 2). The products were characterized by TLC, ESI-MS and NMR analysis (see Supporting information).

Chemoenzymatic synthesis of *n*-butyl 6-*O*-palmitoyl-galactosides (17 ad)

D-(+)-Galactose (**2**, 0.900 g, 5 mmol) was suspended in dry 1-BuOH (**6**, 50 mL, 546 mmol) in the presence of the strongly acidic cation exchange resin Amberlyst® 15 (10%, w/w) and 3 Å molecular sieves (25% w/w) at 120 °C. After 3 h, the solid catalyst was filtered off and the alcohol was removed under reduced pressure. Then, the resulting isomeric mixture of 1-butyl galactosides (**13 ad**), palmitic

acid (0.768 g, 3 mmol) and Novozym® 435 (10% w/w) were mixed together and poured into a round-bottom flask. The mixture was heated to 80 °C while rotating the flask by means of a glass oven B-585 Kugelrohr (Büchi, Cornaredo, Italy). After fatty acid melting, the reaction was performed under reduced pressure (30 mmHg). After 8 h, the reaction mixture was taken up in EtOAc and the immobilized enzyme was removed by filtration. The organic phase was further diluted with EtOAc, washed with 0.1 M NaOH (3x), dried over Na_2SO_4, and the solvent was removed under reduced pressure, thus affording *n*-butyl 6-*O*-palmitoyl-galactosides (**17 ad**) (yield = 35%).

Interfacial tension (IFT) measurements and emulsifying properties

The sunflower oil/water interfacial tension (IFT) values at 0.1, 1.5 and 3.0 mM of *n*-butyl 6-*O*-palmitoyl-α-D-galactopyranoside (**17 ab**), *n*-butyl 6-*O*-palmitoyl-β-D-galactopyranoside (**17 b**), *n*-butyl 6-*O*-palmitoyl-galactofuranoside isomeric mixture (**17 cd**), and *n*-butyl 6-*O*-palmitoyl- galactoside isomeric mixture (**17 ad**) were measured at (25 ± 1) °C by means of a Gibertini tensiometer (Du Noüy ring method, Harkins-Jordan corrections).

IFT values at 3 mM relative to other already reported sugar-based esters [10,48] *n*-butyl 6-*O*-palmitoyl-glucopyranoside labelled as **SER-ab**, *n*-butyl 6-*O*-palmitoyl-glucofuranoside (**SERcd**), and *n*-butyl 6-*O*-palmitoyl-glucoside isomeric mixture (**SERad**) were compared to the data of the herein investigated surfactants.

Water (milli-Q) in sunflower oil (W/O) emulsions (phase volume of $\Phi_V = 0.13$) were prepared by ultrasound homogenizer, using a Thermo Fisher Q700 sonicator equipped with a 3 mm-titanium alloy microtip, following the operative conditions previously reported.[10] The synthesized surfactants were solubilized at 80 °C in oil (3.0 mM) and added in a fixed amount (0.06% wt) to form the final emulsions. Their stability was evaluated within 72 h by means of turbidimetric measurements as reported elsewhere.[10,56]

Author Contributions

Conceptualization: GS, DU; data curation: RS, MSR, SS, EP, TB, MR; funding acquisition and project administration: GS, DU; investigation: RS, MSR, SS, EP; supervision: GS, DU, GC; visualization: RS, MSR, SS; writing-original draft: RS, MSR, SS; writing-review & editing: all authors.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: biocatalysis · biosurfactants · emulsifiers · lactose · sugar fatty acid esters

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Chapter 3.3: Deep Eutectic Solvents for the Enzymatic Synthesis of Sugar Esters: A Generalizable Strategy?

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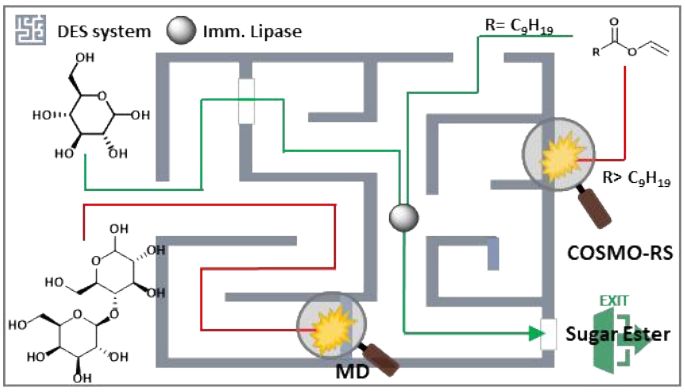
Submitted



3.3.1 Abstract

Sugar (fatty acid) esters are industrially relevant compounds, with a cumbersome production process due to the solubility issues of the substrates, what forces the use of environmentally unfriendly reaction media. Herein, Deep Eutectic Solvents (DES) are considered a promising solution, and several literature examples use glucose and different acyl donors to illustrate the efficient synthesis of sugar esters in classic DES like choline chloride:urea (ChCl:U). However, this paper discloses that when sugars like lactose or other disaccharides are used, enzymes cannot efficiently perform (trans)esterifications in DES, while the same reaction can proceed in mixtures like pyridine:tetrahydrofuran (Py:THF). This could be explained by computational solubility studies, and molecular dynamic simulations of both reaction media, showing two effects: i) on one hand, large acyl donors (more than C10) display poor solubility in DES; ii) on the other hand, disaccharides interact with DES components. Thus, the DES affects the conformation of lactose (compared to the conformation observed in Py:THF mixture), in such a way that the enzymatic reaction results impaired. Despite classic DES (e.g. ChCl:U) may not be useful for generalizing their use in saccharide ester syntheses, the achieved theoretical understanding of the reaction may enable the design of future DES that can combine enzyme compatibility with eco-friendliness and efficiency in sugar chemistry.

3.3.2 Introduction

Surfactants are amphiphilic compounds that partition between liquid interphases reducing surface tensions. Because of this, they constitute a widely used class of chemicals, across many sectors and markets.[1-2] In particular, sugar fatty acid esters (SFAEs or just “sugar esters”), are non-ionic surfactants with excellent emulsifying, stabilizing, and detergent properties. Furthermore, SFAEs are non-toxic, benign to the environment, tasteless, odorless, and fully biodegradable. On the other hand, they exhibit antimicrobial activity and are skin-compatible.[3] Interestingly, these molecules, as well as other carbohydrate-based surfactants like alkyl (poly)glycosides, can be derived from renewable sources.[4-5] The chemical synthesis of SFAEs typically requires harsh reaction conditions (high temperatures, reduced pressure, metal or alkaline catalysts) which result in high energy consumption, formation of undesirable by-

products, and low regioselectivity.[6-7] Furthermore, volatile organic solvents are frequently used, even though sugar solubility is limited.[8-12] In this context, using other non-conventional (benign) media, and an enzyme-based synthesis are strategies that can overcome the above-mentioned drawbacks.[13]

In this area, glucose and saccharose esters have been widely studied,[3, 14-15] but research on other important sugars has been scarce hitherto. As a relevant example, the disaccharide lactose has been under-investigated despite its large availability, being the most abundant component of cheese whey, the main waste stream of the dairy industry. This feedstock can be used as a cheap and renewable source for lactose to develop circular processes directed towards the obtainment of high-value compounds, including SFAEs.[16-19] The same may apply for many other examples of saccharides, which can be obtained from renewable resources.

The major issue in the preparation of sugar esters is the opposite solubility profile of the polar head (sugar) and the hydrophobic tail (fatty acid).[20-22] Therefore, SFAEs have been previously obtained in organic solvents, such as dimethylsulfoxide (DMSO), dimethylformamide (DMF), and pyridine (Py), in which both sugars and fats are soluble.[6, 23-24] These processes are efficient, but the use of toxic and non-volatile organic solvents do not match at all with the green chemistry principles.[25] The use of other, more benign, organic solvents like tertiary alcohols or ketones has been reported as well, but it generally results in low yields, and/or very long reaction times due to the very low solubility of sugars (especially disaccharides as lactose) in these solvents.[11-12, 26] Sugar derivatizations can be performed to enhance carbohydrate solubility in organic media (e.g. for lactose oleate[16]), yet at the cost of establishing multi-step and cumbersome processes. In this context, the use of non-conventional media like ionic liquids and deep eutectic solvents (DES) has been proposed as a promising alternative to organic solvents.[27-29]

DES are straightforwardly prepared by mixing hydrogen bond donors (HBD), such as polyols, with hydrogen bond acceptors (HBA), such as ammonium salts.[30] Because of the formation of intermolecular hydrogen bonds, the melting point of the mixture decreases sharply, and DES are liquid and stable at room temperature. Owing to the vast variety of possible HBD and HBA, the possibility of changing the components' ratio, and the amount of external water added, the potential number of combinations is immense,

which confers them a high degree of tunability. Moreover, it is possible to appropriately select the components to prepare non-toxic, and highly biodegradable DES.[31-32] Remaining challenges for DES are mainly associated to their high viscosity, which affects the mass transfer in the reaction, and adds complexity to the downstream processing. Moreover, a deep understanding about the relationship between the structure of its constituents, and the solvent properties of DES is still in its infancy.[33] For instance, it is hard to predict how DES interact with substrates, and with the tertiary structure of the enzyme. We have recently shown that the use of computational simulation tools can shed light on the complex interactions that may occur during the process.[34]

Several articles disclosed the use of glucose and several acyl donors for enzymatic transesterifications in DES. Pöhnlein et al.[35] reported the transesterification of glucose with vinyl hexanoate using immobilized CalB (Novozym 435®) in different DES. The product was detected when ChCl:U and ChCl:glucose DES were used. More recently,[27] the same two hydrophilic DES were reported for the transesterification of glucose with vinyl decanoate. The same group also explored, for the first time, the use of a hydrophobic DES (menthol:decanoic acid) as a suitable reaction medium for sugar esters production.[28] The use of fatty acid methyl esters (FAME) derived from single cell oil as acyl donor, for the production of glucose fatty acid esters was also reported in a circular, integrated process fully based on lignocellulose.[4] The aforementioned examples show that, as a matter of fact, these emerging reaction media can be used for these processes. Given the industrial relevance of other SFAE, the possibility of using DES for disaccharides was considered in this work. To understand the system, experimental results were combined with molecular dynamics (MD) simulations that can assess the interactions between the enzyme and the reaction media. As a result, a new approach is here presented, which can assist in establishing structure-reactivity relationships between DES, substrates, and enzymes, thus driving the selection and/or the design of novel benign solvents for sugar chemistry and, particularly, for biocatalytic applications.

3.3.3 Materials and Methods

Materials

The immobilized *Candida antarctica* lipase B under the trade name of Novozym® 435 was kindly supplied by Novozymes A/S (Denmark), vinyl palmitate (VP) was purchased

from TCI Europe N.V. (Belgium), and anhydrous lactose was purchased from Fluka (Italy). All other reagents and chemicals were purchased from Merck Life Science and used as received.

Preparation of DES

All DES used in this work were prepared by mixing the components under magnetic stirring at 80-95 °C in a thermostated vessel, until a clear liquid was obtained. In some cases, the addition of water was necessary to form the DES or to obtain less viscous liquids.

Ultrasound pretreatment

The corresponding fatty acid was suspended in the DES composed of choline chloride (ChCl):urea (U) (1:2 mol/mol + 5% w/w MilliQ H_2O) (from now onwards, ChCl:U (1:2) (5% w/w water) (10 mL), and the mixture was sonicated by a SONOPULS Ultrasonic homogenizer for 5 min (20 s pulse and 30 s pause), thus obtaining white foamy suspensions (final concentration: 100 mM). An analogous treatment was performed to prepare 10 mL suspensions containing decanoic, lauric and palmitic acid vinyl esters, all with a concentration of 500 mM. The mixtures were kept in an ice bath during sonication.

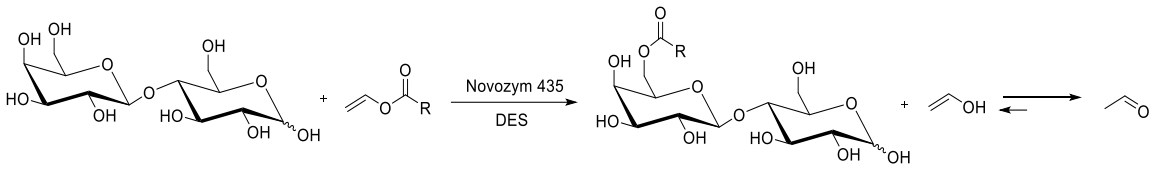
Screening of extraction solvents on commercial saccharose laurate

Saccharose laurate (SL, 10 mg) was incubated in 0.5 mL DES-water mixture ChCl:U (1:2) (5% w/w water) at 55 °C and 800 rpm for 10 min. Then, the respective extraction solvent, such as ethyl acetate (EtOAc), dichloromethane (DCM), 2-methyl-2-butanol (2M2B), or 1-octanol (0.5 mL), was added and the extraction was performed at 1200 rpm for 30 min using a termoshaker (Hettich Benelux, ProfiLab24 GmbH, Berlin, Germany). Temperature was adjusted depending on the boiling point of each studied solvent. At the endpoint, the mixtures were centrifuged (13400 rpm, 2 min) and the organic phase was analyzed by HPLC-RID. The peak area of the extracted samples was compared with the area of a solution of SL (20 mg/mL in CH_3OH), taken as a reference value of 100%.

General procedure for the biocatalytic transesterification of sugars in DES ChCl:U (1:2) (5% w/w water) using fatty acids and vinyl esters (C10-C16)

Reactions were performed in a total volume of 0.5 mL (using either glass vials, or microcentrifuge tubes). The corresponding sugar (glucose, lactose or saccharose) was suspended in the corresponding DES, followed by addition of the acyl donor. The

following acyl donors were evaluated: vinyl decanoate (VD, C10), lauric acid (LA, C12), vinyl laurate (VL, C12), palmitic acid (PA, C16), and vinyl palmitate (VP, C16). When ultrasound-pretreated reactions were performed, the sugar was suspended in the sonicated acyl donor suspension in a DES. These mixtures were incubated at 55, 70 or 80 °C for 10-15 min before adding the enzyme. Several sugar concentrations, acyl acceptor: acyl donor ratios, temperatures, reaction times were analyzed. The conditions are summarized in **Table 3**.



Scheme 1. General scheme of transesterification of lactose with vinyl esters.

At the endpoint, reaction mixtures were extracted with an organic solvent at 1200 rpm for 30 min. When glucose was used as acyl acceptor, the extraction was performed with ethyl acetate (EtOAc) at 55 °C, following a previous report.[27] When disaccharides were used as acyl acceptors, the extraction was performed with 2-methyl-2-butanol (2M2B) at 80 °C. Afterwards, the samples were centrifuged (13400 rpm, 2 min) and the organic phase analyzed either by HPLC-RID, HPLC-ELSD or HPLC-MS. Reactions were performed in duplicates, and in all cases control and blank reactions were performed.

Biocatalytic transesterification in "2-in-1" DES system

For the reaction running in sugar-based DES, in which acyl acceptor acts as substrate and as part of the solvent, the acyl donor VP (0.0167 mL; corresponding to a final concentration of 0.1 M) was added to the sugar-based DES (0.5 mL) and incubated at 55 °C for 10-15 min before adding the enzyme (25 mg, corresponding to 50 mg/mL). At the endpoint (48 h), the reaction mixture was extracted with 2M2B at 80 °C, 1200 rpm for 30 min before being centrifuged (13400 rpm, 2 min) and analyzed by HPLC-RID.

For the reactions in hydrophobic DES, in which acyl donor acts as substrate and as part of the solvent, the sugar (final concentration: 0.1 M) was suspended in the hydrophobic DES (0.5 mL) and incubated at 60 °C for 10-15 min before adding the enzyme (10 mg, corresponding to 20 mg/mL). For the transesterifications, reaction mixtures were supplemented with VP (0.0167 mL; corresponding to 0.1 M) and reactions were performed at 60 °C for 96 h. At the endpoint, the mixtures were diluted with dioxane

(dilution factor of 1:3) and stirred for further 30 min at 80 °C, 1200 rpm before being centrifuged (13400 rpm, 2 min) and analyzed by HPLC-RID.

Preparative synthesis of sugar fatty acid esters in organic media

The reference standard of glucose decanoate was prepared according to literature protocol[24] with some modifications. D-glucose (0.600 g; 3.33 mmol), vinyl decanoate (2.24 mL; 10 mmol) and Novozym® 435 (0.6 g) were suspended in anhydrous Py/THF (1:1 v/v; 20 mL) and the mixture was stirred at 55 °C and 500 rpm. The round bottom flask was equipped with a condenser to prevent the evaporation of THF. At the endpoint, the mixture was filtered under vacuum and the immobilized enzyme was washed with CH_2Cl_2 (2 x 10 mL) and then with CH_3OH (2 x 10 mL). The filtrate was dried under vacuum, obtaining a viscous yellow crude which was purified by flash chromatography (eluent: CH_2Cl_2:CH_3OH, 90:10 v/v). Since the reaction mixture resulted insoluble in the eluent, a solid phase sample loading was performed using CH_3OH as solvent. The product was characterized by MS; ^{1}H-NMR and HBMC. The NMR signals are consistent with literature.[29] The reference standard of lactose decanoate was prepared in the same way by using immobilized lipase from *Thermomyces lanuginosus* (TLL).

HPLC methods

All analyses were performed using a Waters C8 Symmetry column 150 x 4.6 mm, 5 µm at a flow rate of 0.5 mL/min. The injection volume was 2 µL. For HPLC-RID analyses, a 1260 Agilent Infinity, equipped with RID thermostated at 40 °C was used, whereas in the case of the HPLC-ELSD analyses, a Chromaster 600 bar System, Merck Hitachi VWR, equipped with a Sedex 100 LT-ELSD was the equipment of choice. To perform HPLC-MS analyses, an Agilent 1290 infinity instrument was used. Elution conditions changed depending on the reactions and are described in Supporting Information (SI).

Calculation of infinite dilution activity coefficients and solubility using COSMO-RS

The calculations of infinite dilution activity coefficients γ_i^{∞} (IDAC) and the solubility $x_{i,S}$ of the sugars and fatty acid vinyl esters in different solvent mixtures have been performed using BIOVIA COSMOtherm 2020.[36-39] The conformer sets of all molecules have been generated using COSMOconf v3.0 and the COSMO calculations have been performed with Turbomole v6.6 using TZVPD-FINE parameter set. The following equation was used for calculating the solid-liquid equilibrium (SLE).[40]

$$\ln x_{i,\mathrm{S}}\gamma_{i,\mathrm{S}} = \frac{\Delta h_{\mathrm{f}}^{0}}{RT}\cdot\left(\frac{T}{T_{\mathrm{m}}}-1\right)+\Delta c_{\mathrm{p}}^{\mathrm{LS}}\left(\frac{T_{\mathrm{m}}}{T}-\ln\frac{T_{\mathrm{m}}}{T}-1\right)$$

(1)

with $x_{i,\mathrm{S}}$ as solubility in mole fractions, $\gamma_{i,\mathrm{S}}$ as activity coefficient at the solubility, T_{m} as melting temperature, $\Delta h_{\mathrm{f}}^{0}$ as standard state enthalpy of fusion at the temperature T_{m} and $\Delta c_{\mathrm{p}}^{\mathrm{LS}}$ as difference in the standard state heat capacity between the solid and liquid state. To solve equation 1, solid-state data of the component of interest must be known. However, if a reference solubility $x_{i,\mathrm{S}}^{\mathrm{ref}}$ of component i is known, e.g., in water, equation 1 using the reference solubility $x_{i,\mathrm{S}}^{\mathrm{ref}}$ and the solubility of interest $x_{i,\mathrm{S}}^{\mathrm{L}}$ can be combined to:

$$\ln x_{i,\mathrm{S}}^{\mathrm{L}}\gamma_{i,\mathrm{S}}^{\mathrm{L}} = \ln x_{i,\mathrm{S}}^{\mathrm{ref}}\gamma_{i,\mathrm{S}}^{\mathrm{ref}}.$$

(2)

The reference solubility of lactose and glucose in water, the temperature of the reference solubility and the melting temperatures of both components that were used within this work can be found in **Table 1**.

Table 1: Input data (reference solubility $x_{i,\mathrm{S}}^{\mathrm{ref}}$ in water and melting temperature T_{m}) for COSMOtherm calculations of the solubility of glucose and lactose in different solvents.

Sugar	Reference solubility in H_2O $x_{i,\mathrm{S}}^{\mathrm{ref}}$ (% mol/mol)	Temperature of reference solubility (°C)	Melting temperature T_{m} (°C)
Glucose	58.18 [41]	35	174.85 [42]
Lactose	25.15 [43]	40	223 [43]

The pure component state of the fatty acid vinyl esters is liquid. In these cases, the solubility of the vinyl esters in different solvents is calculated via the following equation for a liquid-liquid equilibrium (LLE).

$$x_{i,\mathrm{S}}^{\mathrm{I}}\gamma_{i,\mathrm{S}}^{\mathrm{I}} = x_{i,\mathrm{S}}^{\mathrm{II}}\gamma_{i,\mathrm{S}}^{\mathrm{II}},$$

(3)

whereby $x_{i,\mathrm{S}}$ is the solubility and $\gamma_{i,\mathrm{S}}$ the activity coefficient of component i in phase I or II. In these cases, no reference solubility data is necessary.

Molecular dynamics (MD) simulations of sugars

The MD simulations of lactose and glucose in the DES ChCl:U (1:2) with and without water (5% w/w) and in the mixture of Py/THF have been performed using GROMACS *version 2019.4.*[44-45] The interactions between sugars and solvent molecules have been modeled using parameters from *CHARMM General Force Field* (CGenFF) version 4.X[46] taken from the CHARMM-GUI[47]. In case of water molecules, the CHARMM-TIP3P variant[48] was used. A cut-off radius of 1.2 nm has been introduced for the electrostatics and LJ-interactions, whereby the forces were smoothly switched between 1.0 and 1.2 nm. Long-range electrostatics were modelled with the smooth particle-mesh Ewald (PME) method[49] using a Fourier spacing of 0.18 nm and a PME order of 4. The bonds with hydrogen atoms were set using SETTLE[50] for water and the LINCS[51] algorithm for all other molecules using a LINCS order of 4.

Cubic boxes including one sugar molecule – either β -glucose or α -lactose – in the respective solvent mixture have been prepared using packmol.[52] In case of the pyridine/THF mixture, 1005 molecules of pyridine and 995 molecules of THF have been added. For the DES/water mixture, 500 molecules of choline chloride, 1000 molecules of urea and 380 molecules of water were added. An energy minimization for 5000 steps using the steepest decent algorithm has been performed. In case of the pyridine/THF mixture, this is followed by an equilibration phase in the NPT ensemble for 10 ns. Newton's equations of motion were numerically integrated using the leapfrog algorithm[53] and a time step of 2 fs. The temperature of the system was set to 328.15 K and controlled using the velocity rescale thermostat[54] with a time constant of $\tau_{\mathrm{T}} = 1\ \mathrm{ps}$. The pressure was adjusted to 1 bar using the berendsen barostat[55] and a time constant of $\tau_{\mathrm{P}} = 2\ \mathrm{ps}$. In case of the highly viscous ChCl:U (1:2) (5% w/w water), additional equilibration steps at elevated temperatures were necessary.[34, 56] The equilibration is started with an NVT simulation at 328.15 K for 1 ns using a time step of 1 fs. Afterwards, the temperature was increased to 500 K and was kept at 500 K for 20 ns to improve the equilibration of the viscous system. This is followed by a 2 ns simulation in the NPT ensemble using the berendsen barostat ($\tau_{\mathrm{P}} = 5\ \mathrm{ps}$) to equilibrate the pressure to 1 bar. The equilibration phases are followed by the sampling in the NPT ensemble for 50 ns switching the pressure control to Parrinello-Rahman.[57] Two replica simulations from different initial configurations and initial velocities have been prepared. The last 30 ns

of the trajectory have been used to calculate spatial distribution functions of the solvent molecules within 5 Å of the sugar molecule using GROma*ρ*s[58] and a spacing of 0.05 nm. Moreover, characteristic angles and dihedrals of the sugar molecules in the solvent mixtures have been determined. The MD simulations have been visualized using VMD.[59]

3.3.4 Results and Discussion

Preparation of DES

Different DES were prepared, all of them resulting clear, viscous solutions. Some of them contained choline chloride (ChCl), which was combined with urea (U) or hydrated lactose as hydrogen bond donors (HBD). Another DES was formed by mixing lidocaine and palmitic acid as HBD. In some cases, water (up to 20% w/w) as cosolvent was added[60] (**Table 2**). ChCl:U was used as the model DES, since it is one of the most common DES used for biocatalysis. To complement that, a DES comprising lactose was included, as an example of "2-in-1" DES, concept reported for other processes as well.[27-28, 61-63] Herein, it was observed that the DES was obtained only if lactose monohydrate was used whereas with anhydrous lactose the mixture remained heterogeneous. It may be presumed that the water of crystallization of lactose monohydrate participates in the formation of the hydrogen bond network, as observed with other DES (e.g., ChCl:levulinic acid).[64] To further explore this "2-in-1" DES concept, an hydrophobic DES containing lidocaine and the acyl donor as components was included.

Table 2. List of DES that were prepared using different HBD and HBA, molar ratios and the preparation conditions.

HBA, DES Component 1	HBD, DES component 2	Molar ratio (HBA:HBD)	H_2O (% w/w)	T (°C)	Time (min)
ChCl	U	1:2	5	90	< 30
ChCl	U	1:2	0	90	40-60
ChCl	Lac.H_2O	2.5:1	20	90	90
Lidocaine	PA	1:1	0	90	60

Experimental enzymatic reactions with sugars in DES using different acyl donors

Following previous successful results reported in literature using glucose as a sugar for transesterifications,[27-28] we aimed to extrapolate this methodology. Therefore, the first

set of experiments focused on the esterification of lactose using different acyl donors, either free acids (LA, C12; PA, C16) or vinyl esters (VD, C10; VL, C12; VP, C16). To perform these experiments, the commonly used DES composed of ChCl:U (1:2) (5% w/w water) was selected, since the preparation of glucose decanoate by enzymatic transesterification with CAL-B was previously reported in this solvent.[27] Results obtained are depicted in **Table 3**.

Table 3: Reaction conditions: DES-water mixture ChCl:U (1:2) (5% w/w water) with Novozym® 435, and at 800 rpm. After the stated endtime points, all reaction mixtures were extracted with 2M2B at 80 °C for 30 min at 1200 rpm and centrifuged before the analysis.

Sugar (M)	Acyl donor (M)	Rxn type[b]	Enzyme loading (mg/mL)	Temperature (°C)	Time (h)	Outcome
Lac (0.1)	LA (0.1)	E	20	55	48	By-product
Lac (0.1)	VL (0.1)	T	20	55	48	Hydrolysis and by-product
Lac (0.1)	VP (0.1)	T	20	55	48	Hydrolysis and by-product
Lac (0.1)[a]	VP (1.25)	T	100	80	96	Hydrolysis and by-product
Lac (0.01)	PA (0.01)	E	50	55	48	By-product
Lac (0.01)	VP (0.01)	T	50	55	48	Hydrolysis and by-product
Lac (0.01)	PA (0.01)	E	100	55	48	By-product
Lac (0.01)	VP (0.01)	T	100	55	48	Hydrolysis and by-product
Lac (0.5)	VL (0.5)	T	20	55	48	Hydrolysis and by-product
Lac (0.5)	VP (0.5)	T	100	70	72	Hydrolysis and by-product
Sac (0.5)	VP (0.5)	T	100	70	72	Hydrolysis and by-product
Sac (0.1)	VP (0.1)	T	20	55	48	Hydrolysis and by-product

[a]In this experiment, DES without the addition of water was used.
[b]E: Direct esterification with fatty acid, T: Transesterification with vinyl ester.

As can be observed in **Table 3**, the lactose ester formation was not detected in any of the reaction conditions. Hydrolysis of the acyl donor was observed when vinyl esters were used. Besides hydrolysis, another product was observed (see SI, **Figure S1-S4**) which resulted to be the fatty acid amide. The use of urea as source of ammonia for the chemical and enzymatic synthesis of amides has been reported.[65-67] Several attempts to

increase product formation were carried out, including the use of vinyl esters large excess of acyl donor – to perform irreversible reactions –, and very high enzyme loadings and/or prolonged reaction time. Unfortunately, they were unsuccessful. The ultrasound pretreatment of the reaction mixture was reported to be a key step to improve the availability of fatty acid acyl donor and to increase conversions;[27] but, in this case, it did not have any effect on the reaction outcome. Since no conversions were observed with lactose, glucose was incorporated in the reaction conditions, to benchmark with literature results (**Table 4**).

Table 4: Transesterification experiments using glucose as substrate,in previously reported reaction conditions.[27] DES-water mixture ChCl:U (1:2) (5% w/w water); Novozym® 435 (20 mg/mL), 50 °C and 24 h. Samples were extracted with EtOAc at 55 °C (glucose) or 2M2B at 80 °C (disaccharides) and analyzed by HPLC-RID/HPLC-ELSD

Sugar	Acyl donor	Outcome
Glucose	VD (C10)	Glucose ester detected
Glucose	VL (C12)	Hydrolysis and by-product
Glucose	VP (C16)	Hydrolysis and by-product
Saccharose	VD (C10)	Hydrolysis and by-product
Lactose	VD (C10)	Hydrolysis and by-product

Interestingly, the sugar ester was detected only with glucose and vinyl decanoate, C10, consistently with literature,[27] but not with larger acyl donors, namely C12 and C16 (with or without ultrasound pretreatment). This outcome might be ascribed to the poor solubility of large acyl donors in DES, what hampers its "accessibility" to the enzyme. In fact, it was observed in literature[27] that the distribution of vinyl decanoate in ChCl:U was a limiting factor in the synthesis of glucose decanoate when using this hydrophilic DES. In that case, the authors demonstrated that the ultrasound pre-treatment reduced the mean droplet size of the vinyl ester/DES mixture, thus significantly improving the conversion. Our results suggest, however, that this pretreatment is not efficient when longer chain vinyl esters are used.

Interestingly, when disaccharides were used (saccharose or lactose), the products were not observed with C10 acyl donors either. These results suggest a different behavior of disaccharides in the DES, compared to glucose, which is not correlated to the acyl donor. Both saccharose and lactose are recognized as substrates by Novozym® 435 in other solvents, as demonstrated elsewhere.[11, 14, 18, 26] Moreover, CALB is active in diverse DES,

including ChCl:U (1:2 mol/mol), for other (trans)esterification-like reactions.[68] To confirm this, the enzyme was incubated in ChCl:U (1:2) (5% w/w water) containing lactose and vinyl palmitate (0.1 M each) for 24 h and then 0.1 M glycerol was added, thus observing the formation of glyceride monopalmitate (see SI, **Figure S5**).

Considering all data, the results obtained cannot be due to enzyme inactivation, but should be attributed to the interactions between substrates (acyl acceptors and donors), enzyme, and the DES components. Furthermore, effects related to sugars as acyl acceptors (glucose *vs*. disaccharides) and to acyl donors (C10 *vs*. C12-16) seem to play an important role. To further understand the results, the same reaction was performed in Py:THF (1:1 v/v), as it has been reported for the high-yield synthesis of several lactose esters by transesterification with vinyl esters.[24] Likewise, a commercial lipase from *Thermomyces lanuginosus* immobilized on Immobead 150 (TLL), similar to the one used in that paper, was included in the study as well (**Table 5**).

Table 5. Transesterification of lactose with vinyl decanoate: comparison between DES and organic medium. Reactions were performed at 55 °C and for 24h.

Lactose (M)	Vinyl decanoate (M)	Enzyme loading (mg/mL)	Reaction medium	Outcome
0.2	0.6	68 (Novozym® 435)	ChCl:U (1:2) (5% w/w water)	Hydrolysis and by-product
0.2	0.6	68 (TLL)	ChCl:U (1:2) (5% w/w water)	Hydrolysis
0.2	0.6	68 (Novozym® 435)	Py:THF (1:1 v/v)	Lactose ester detected
0.2	0.6	68 (TLL)	Py:THF (1:1 v/v)	Lactose ester detected

Remarkably, lactose ester formation was detected with both enzymes in the organic medium, (**Table 5**). Conversely, no sugar esters were observed with none of the two enzymes.

In a final attempt to find an alternative DES for lactose esterification, experiments were performed in two other DES namely, ChCl:Lac (2.5:1 mol/mol) (20% w/w water) and lidocaine (Lid):PA (1:1 mol/mol), as shown in **Table 6**. In both solvents, one of the substrates of the reaction acts simultaneously as substrate and as solvent component.

This “2-in-1” approach enables the dissolution of solid substrates making them available for an enzymatic reaction without additional solvents and has been reported for the enzymatic esterification of sugars and sugar alcohols. [27, 32, 35, 61] Moreover, by using this “2-in-1” system, undesirable side reactions with the other DES components, as we observed with urea (formation of the fatty amide), could be minimized.

Table 6. Trials of lactose (trans)esterification in “2-in-1” system. Lactose-based DES contained 20% w/w water, and any attempt to reduce this amount resulted in no DES formation. The last two entries summarize literature data.[28]

Acceptor (M)	Donor (M)/ reaction type	Enzyme loading (mg/mL)	Reaction medium	T (°C)	Outcome
Lactose (excess)	VP (0.1)/ Transesterification	50	[a]ChCl:Lac (2.5:1 mol/mol) (20% w/w H_2O)	55	Hydrolysis
Lactose (0.1)	PA (excess)/ Esterification	20	[b]Lid:PA (1:1 mol/mol)	55	No conversion
Lactose (0.1)	VP (0.1)/ Transesterification	20	[b]Lid:PA (1:1 mol/mol)	60	Hydrolysis
Glucose (0.5)	VD/ Transesterification	30	(-)-menthol: decanoic acid 1:1	50	Glucose ester detected[28]
Glucose (1.5)	DA/ Esterification	20	(-)-menthol: decanoic acid 1:1	50	Glucose ester detected[28]

[a]Lactose monohydrate was used to prepare the DES; reaction time: 48h, mixture was extracted with 2M2B at 80 °C.
[b]Reactions diluted with dioxane (dilution factor of 3) and centrifuged before the analysis.

Fatty acids are also commonly used to prepare hydrophobic DES, which are currently receiving increased attention thanks to their advantageous properties.[28, 69] Nevertheless, also in these cases the formation of lactose ester was not detected.

As observed (**Table 6**) no sugar esters were detected in any of the reaction conditions applied. Importantly, hydrophobic DES like Lid:PA, in which the acyl donor may act both as solvent and substrate, was also tested. In fact, hydrophobic DES (menthol: decanoic acid (1:1 mol/mol)) was also reported for the synthesis of glucose decanoate.[28-29] The viscosity of this DES is lower, and it is known that lipases work very well in lipophilic media. However, no sugar esters were detected, presumably due to the low solubility of lactose. Moreover, this DES is not stable at temperatures below 60 °C and tends to

solidify. In the same DES, a transesterification trial was also performed by adding vinyl palmitate (VP), but also in this case the target product was not detected.

Computational solvent assessment. Overall, the experimental results suggest that the reaction between disaccharides and different acyl donors is negatively affected by the DES. Reasons related to interactions between the substrates and the DES components may be addressed, analogous to findings reported in the literature for DES.[68] Other reasons may be found in the modification of the enzyme structure and flexibility moving from an organic mixture to a DES. In any case, the assistance of computational methods became crucial to shed light on the system and find an explanation for the obtained results. For a better understanding of the substrate behavior in different reaction environments (Py:THF (1:1 v/v) and ChCl:U (1:2) (5% w/w water)) infinite dilution activity coefficients $\ln \gamma^{\infty}$ and the solubility of the sugars and fatty acid vinyl esters were calculated using the highly predictive BIOVIA COSMOtherm 2020 software.[36-39] In addition, molecular dynamics (MD) simulations of a sugar molecule in these solvent mixtures were performed on the atomistic level.

Figure 1 shows the infinite dilution activity coefficients $\ln \gamma^{\infty}$ (IDAC) of α- and β-glucose/lactose as well as of vinyl decanoate, vinyl laurate, vinyl myristate and vinyl palmitate in ChCl:U, ChCl:U (1:2) (5% w/w water), Py, THF and Py:THF (1:1 v/v). The IDAC provide only information about the interaction of the substrate molecule with the solvent mixture. An $\ln \gamma^{\infty} < 0$ signify thereby attractive interaction between the substrate and solvent mixture, while an $\ln \gamma^{\infty} > 0$ implies repulsive interactions. These values are straightforward to compute, widely used in computational solvent screening and give reasonable solubility trends, although they are calculated a substrate concentration of $x_{\mathrm{Substrate}} = 0$ (**Figure 1**)

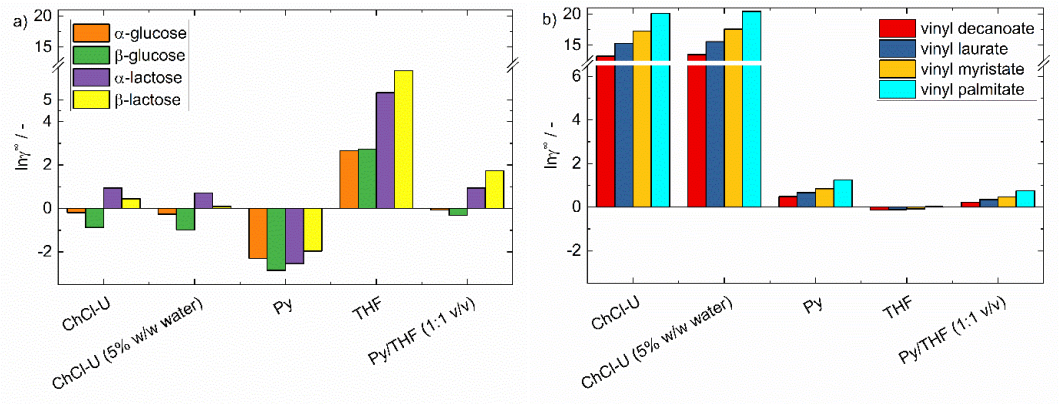


Figure 1: Infinite dilution activity coefficients $\ln \gamma^{\infty}$ of α-/ β -glucose and α-/ β -lactose (a) as well as vinyl decanoate, vinyl laurate, vinyl myristate and vinyl palmitate (b) in the DES ChCl:U (1:2) with and without water (5% w/w) as well as in mixtures of Py and THF. All values have been calculated using BIOVIA COSMOtherm 2020.[36-39]

For glucose and lactose, the IDAC are close to zero and between -1 and 1 for the DES and DES/water mixture, whereby there is no significant influence of the water content of the DES mixtures. Moreover, the data suggest that the β -sugar variants are more soluble (smaller IDAC) than their -α-sugars. It was important to analyze both the anomers of lactose because, in the experiments with ChCl:U (1:2) (5% w/w water), it was used as isomeric mixture. Moreover, lactose is a reducing sugar, so it presents mutarotation in solution regardless of the starting powder form. The final anomer equilibrium of lactose in aqueous solutions is reported to be 40% α-lactose and 60% β-lactose.[70] To the best of our knowledge, there are no information on the mutarotation of lactose in DES. Nevertheless, the IDAC do not confirm that the sugars are incorporated in the DES of ChCl:U (1:2) (5% w/w water), for which a strong negative deviation from ideality ($\ln \gamma^{\infty} = 0$) would be necessary. In case of the Py:THF (1:1 v/v) mixture, a huge difference between the interaction of the sugars with Py and THF can be observed. The IDAC suggest strong attractive interactions with Py, while the large values in THF suggest a poor solubility behavior of the sugars for lactose. In the mixture of Py and THF (1:1 v/v) the attractive and repulsive interactions balance out resulting in IDAC between -0.5 and 2, which is in the range of ChCl:U (1:2) (5% w/w water). In case of the fatty acid vinyl esters, very high IDAC in the range of $\ln \gamma^{\infty}$ = 13-8 can be observed in ChCl:U (1:2) with and without water. This signifies a very poor solubility behavior, which even increases for longer alkyl chains. In Py, THF and their mixture, the activity coefficients are close to zero indicating a reasonable solubility of the vinyl esters.

Moreover, the solubility of the sugars and vinyl esters in the tested solvent mixtures can be calculated *via* BIOVIA COSMOtherm 2020. However, in this case additional information about the solid state of the sugars is necessary. Since this data is difficult to retrieve, only the solubility of the α-anomer of glucose and lactose are shown. As the pure component state of the fatty acid vinyl esters at 328 K is a liquid, no melting properties of the vinyl esters are needed for the solubility calculation. The solubility of α-glucose, α-lactose, vinyl decanoate, vinyl laurate, vinyl myristate and vinyl palmitate in the tested solvent mixtures are summarized in **Figure 2**. It can be observed that α-glucose shows a good solubility in all tested solvents ranging from 18 to 34 mol% except for THF (solubility of ~1 mol%). In the two experimentally tested solvent mixtures (Py:THF (1:1 v/v) and ChCl:U (1:2) (5% w/w water), the solubility of α-glucose is ~21 mol%. On the other hand, α-lactose shows in general a much lower solubility in all tested solvents compared to α-glucose with its highest solubility in pure Py and its lowest in pure THF. In the mixture of both this evens out to ~0.05 mol%, which is equal to the lactose solubility in the ChCl:U (1:2) (5% w/w water) DES. On the other hand, the fatty acid vinyl esters are completely miscible with Py, THF and their 1:1 v/v mixture. They have conversely a very low solubility in the DES mixtures (10^{-9} – 10^{-6} mol mol^{-1}), which becomes even lower for longer alkyl chains.

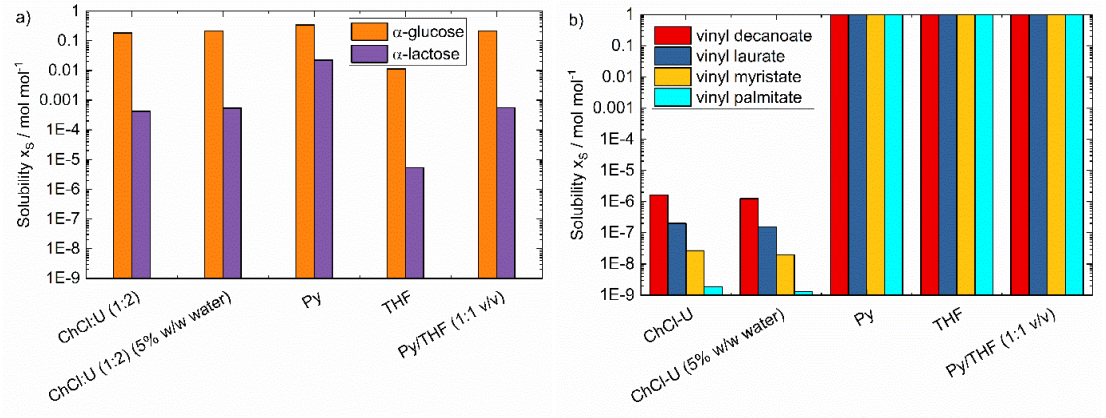


Figure 2: Solubility x_S of α-glucose and α-lactose (a) as well as vinyl decanoate, vinyl laurate, vinyl myristate and vinyl palmitate (b) in the DES ChCl:U (1:2) with and without water as well as in mixtures of Py and THF. All values have been calculated using BIOVIA COSMOtherm 2020.[36-39] For α-glucose and α-lactose a reference solubility and melting temperature (**Table 1**) have been used for the calculations of the solid state.

To further understand the behavior of glucose and lactose, and their interactions with the solvent mixture, molecular dynamics simulations of one α-glucose/lactose molecule in ChCl:U (1:2) (5% w/w water) as well as in a mixture of Py and THF (1:1 v/v) were

performed in replicas. **Figure 3** shows the configuration of α-lactose in ChCl:U (1:2) (5% w/w water) (a,b) and Py:THF (1:1 v/v) (c,d) with the oxygen atom that is part of the esterification reaction highlighted as van-der-Waals sphere. Spatial distribution function of the solvent molecules around α-lactose are added, which allows the identification of specific interaction sites of the solvents. As already suggested by the low IDAC of the sugars in pure pyridine, α-lactose preferably interacts with pyridine in the solvent mixtures, hence, very few interactions sites of THF around the sugars can be found (**Figure 3, c,d**). In case of ChCl:U (1:2) (5% w/w water) (**Figure 3, a,b**), the interaction with α-lactose is dominated by choline (blue) with chloride (pink) forming salt bridges between choline and the OH-groups of α-lactose. Nevertheless, some parts of α-lactose are also solvated by urea molecules (green) around the glucose part. Although 5% w/w of water are present in the DES mixture, they do not show any effect on the sugar solvation as already indicated by the IDAC (**Figure 1**). A similar pattern can also be found for α-glucose in the tested solvent mixtures (**Figure S11**).

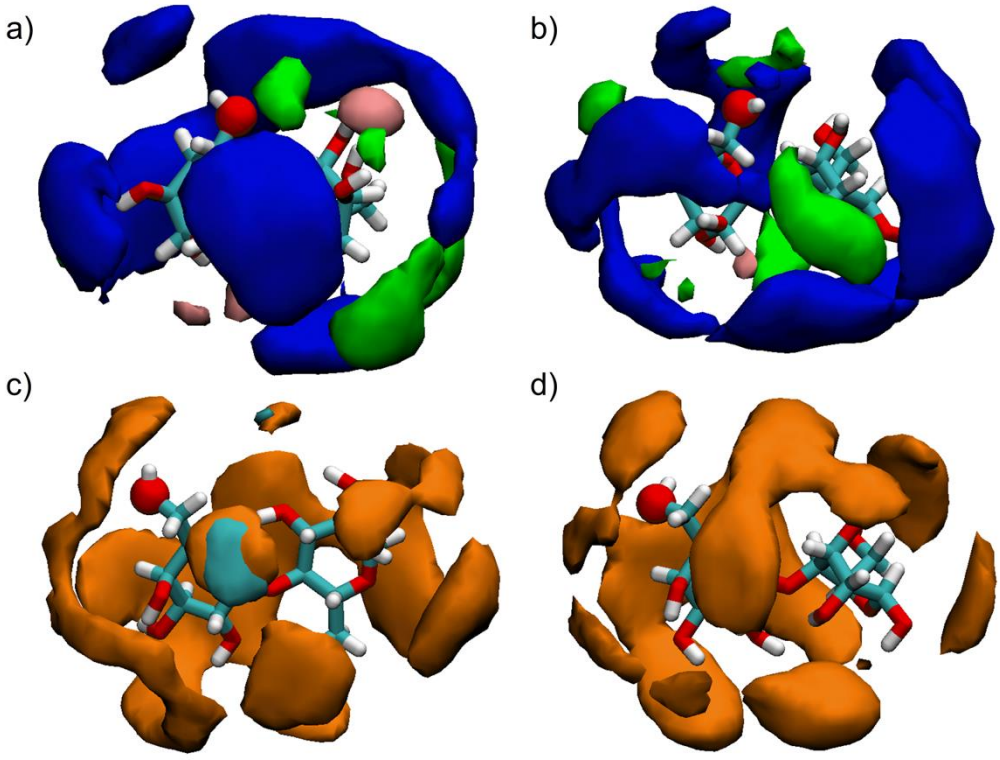


Figure 3: Snapshots of the configuration of α-lactose in the DES ChCl:U (1:2) (5% w/w water) (a,b) and in Py:THF (1:1 v/v) (c,d) from the MD simulations and two replica simulations each. α-lactose is displayed as licorice, while the oxygen atom that is part of the esterification, is highlighted as red van der Waals sphere with a radius scaled by 0.5. Spatial distribution function of the solvents around α-lactose from the MD simulations is added to the figures. Iso-surfaces of region with high probability of a specific surface are shown in orange (Py), cyan (THF), green (urea), pink (chloride) and blue (choline).

As lactose has a larger conformational flexibility caused by the glycoside bond and the enzymatic catalysis of the sugar esters was not successful in the DES mixtures, its configurational changes in different solvent mixtures are studied in detail. A reference structure of α-lactose with highlighted atoms is displayed in **Figure S10**. In the MD simulations, α-lactose appears to be in a flat configuration with the reactive OH-group pointing outwards in Py:THF (1:1 v/v), while its conformation in ChCl:U (1:2) (5% w/w water) is folded with the reactive OH-group pointing inwards (**Figure 4**).

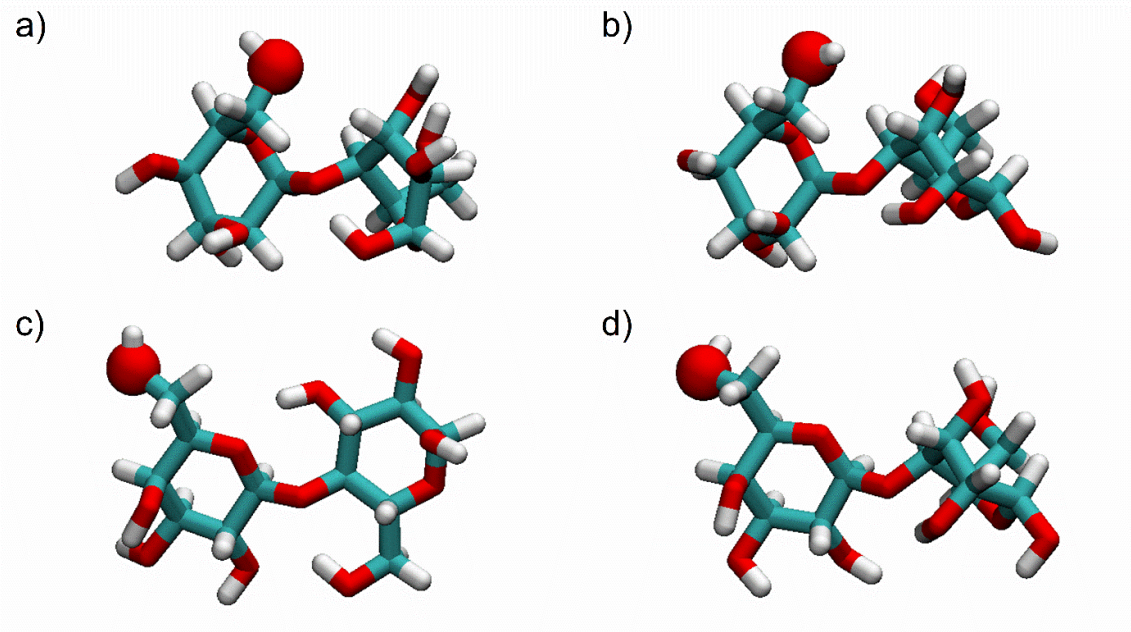


Figure 4: Conformation of α-lactose in the tested solvents ChCl:U (1:2) (5% w/w water) (a,b) and Py:THF (1:1 v/v) (c,d) from the MD simulations and two replica simulations each starting from independent initial configurations and velocities. The oxygen atom that is part of the esterification reactions is highlighted as van der Waals sphere with a scaled radius of 0.5.

Interestingly, the galactose molecules are in the chair conformation in Py:THF (1:1 v/v), which causes the reactive OH-group to be pointing outwards, while galactose is in the boat conformation in the DES mixtures resulting in the folded conformation. To quantify the different configurations of α-lactose in ChCl:U (1:2) (5% w/w water) and Py:THF (1:1 v/v), characteristic angles and dihedrals within the sugar molecule are monitored from the MD simulations. **Figures 5a,c** display the distance between two carbon atoms at the end of both sugar parts (C3-C9) as well as the angle between the two opposing carbons of galactose and glucose within lactose (**Figure 5b,d**). It can be seen that the distance C3-C9 is much larger in Py:THF (1:1 v/v) compared to the DES mixtures. While the

orientation of both monosaccharides in pyridine is around 30°, α-lactose is present in a folded state in ChCl:U with the angle between both monosaccharides around 70°. **Figure S12** shows a shift in the dihedrals of the glycosidic bond within α-lactose, which further highlights configurational changes between both tested solvents. Moreover, the angle between the carbons C1-C2 and the glycosidic bond (C6-O2) is much larger in Py:THF (1:1 v/v) compared to the DES mixtures quantifying the chair *vs*. boat conformation in the different environments. In the folded state in ChCl:U α-lactose is more likely to have intramolecular hydrogens bonds ($d_{\mathrm{Don-Acc}} < 0.35nm$ and $\phi_{\mathrm{H-Don-Acc}} < 30°$) between the oxygen of the glycosidic bond and the OH-groups O6-H15 and O5-H14 (**Figures S12 and S13**), however, the active OH-group is not involved in these hydrogen bonds. Nevertheless, such an intramolecular hydrogen bond is also present in one of the simulations in Py:THF (1:1 v/v) (**Figure S13**).

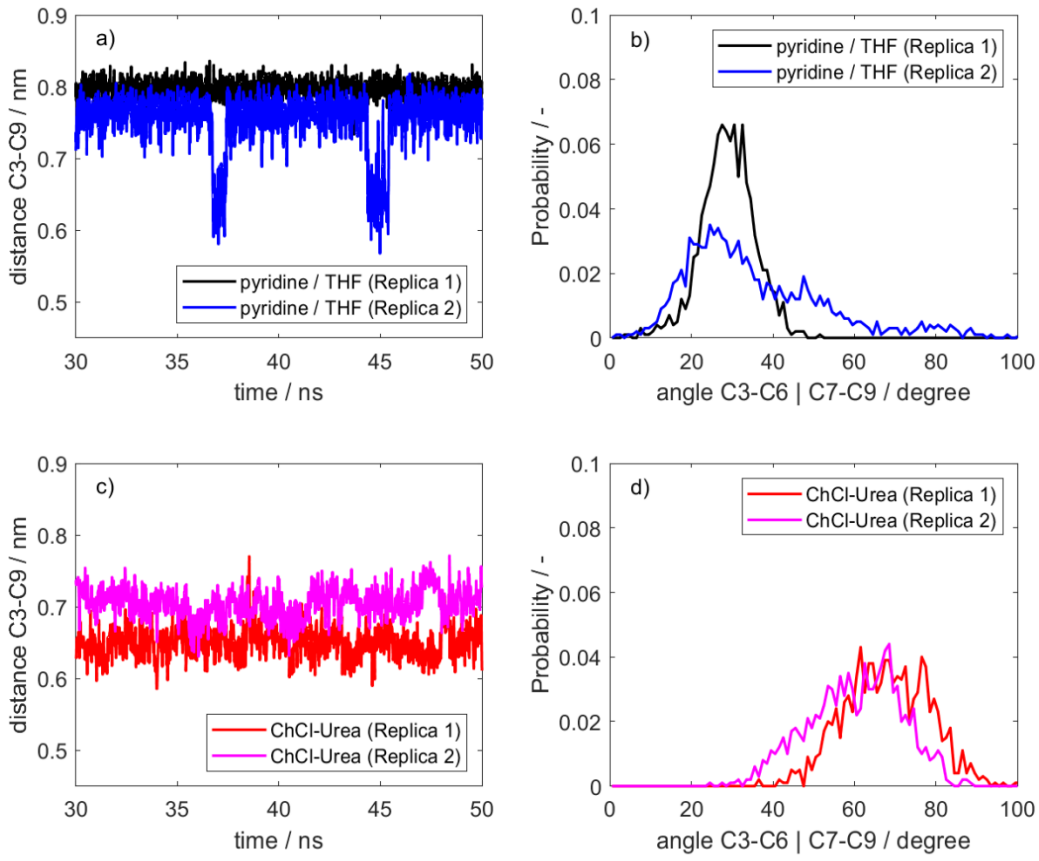


Figure 5: Characteristic angles and distances within α-lactose in the last 30 ns of the MD simulations. Distance between two opposing carbon atoms in both monosaccharides (C3-C9) in Py:THF (1:1 v/v) (a) and ChCl:U (1:2) (5% w/w water) (c) from two replica simulations each. Distribution of the angle between the two monosaccharides described by the vector of the opposing carbons of both monosaccharides (C3-C6 and C7-C9) in Py:THF 1:1 v/v (b) and ChCl:U (1:2) (5% w/w water) (d) from two replica simulations each starting from independent configurations and velocities.

Overall, the BIOVIA COSMOtherm calculations show that the reaction performed in ChCl:U (1:2) (5% w/w water) is solubility-limited for the vinyl esters, which becomes even worse for longer alkyl chains. On the contrary, no such limitation exists in Py, THF and their mixture. However, this cannot explain the difference in enzyme activity between glucose and lactose in reaction with vinyl decanoate. To this end, the MD simulations revealed completely different conformations of lactose in a mixture of Py and THF and in ChCl:U (1:2) (5% w/w water), which may affect the catalytic reaction in these media.

3.3.5 Conclusions and Outlook

Sugar (fatty acid) esters are an important group of industrially-relevant molecules. In order to address their synthetic challenges, the use of DES as reaction media for enzymatic (trans)esterifications of disaccharides was studied. Starting from results reported in literature for glucose, this paper shows that the use of DES cannot be generalized from some substrates to others. Acyl donors (fatty acids or vinyl esters) larger than C10 are not adequate substrates for the reaction with glucose, due to their poor solubility in the analyzed DES. Likewise, none of the studied acyl donors results an effective substrate for the reaction with lactose. Differently from glucose, performing an ultrasound pretreatment did not lead to increased activity for lactose either. Computational methods have shown that besides the low solubility of acyl donors, there is also an orientation effect of DES on the lactose, that is not present in the mixture Py:THF in which only product formation was detected. Thus, a different conformation of lactose in ChCl:U (1:2) (5% w/w water), compared to Py:THF (1:1 v/v) is observed, and this may hamper the reaction, regardless of which acyl donor used. This finding is substantiated by the fact that the formation of the fatty acid amide (from urea) was observed as a side reaction, showing that the enzyme remained active, and that the esterification of lactose was largely unfavourable. The synergy of experimental results with computational methods has shed light on these findings, and we expect that it will pave the way to design novel DES that can broaden (and hopefully generalize) the use of sustainable solvents in sugar chemistry.

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3.3.7 Supporting Information

HPLC Method 1

This method was used to monitor the transesterification of sugars with a C12 (LA or VL) or C16 (PA or VP) acyl donors.

HPLC-RID:

Eluent: CH_3OH:H_2O 85:15 vol., containing 0.1% v/v HCOOH (isocratic); T (column and RID)= 40 °C. run time= 45 min.

HPLC-ELSD

Eluent: CH_3OH:H_2O 85:15 vol., containing 0.1% v/v. HCOOH (isocratic); T (column)= 40 °C;

ELSD parameters: T= 38 °C, gain= Dynamic, filter= 4, P (N_2)= 2.8 bar; run time= 45 min.

Retention times (min): saccharose laurate (SL): 5.6 min; lauric acid amide: 7.1 min; lauric acid (LA): 9.2 min; vinyl laurate (VL): 15.7 min; palmitic acid amide: 13.7 min; palmitic acid (PA): 19.5 min; vinyl palmitate (VP): 36.5 min.

HPLC Method 2

This method was used to monitor the transesterification of disaccharides with vinyl decanoate (VD).

HPLC-ELSD

Eluent: CH_3OH 0.1% v/v. HCOOH:H_2O 0.1% v/v. HCOOH; gradient showed in **Table S1**. T (column)= 50 °C; ELSD parameters: T= 55 °C, gain= Dynamic, filter=10, P (N_2)= 2.8 bar; run time= 60 min.

Retention times (min): Lactose decanoate (LD): 10.9 min; decanoic acid (DA): 50.2 min; vinyl decanoate: 56.1 min (slightly detected by ELSD).

Table S1. Gradient of method 2.

Time (min)	CH_3OH (% vol.)	H_2O (% vol.)
0	65	35
20	65	35
50	100	0
50.1	65	35
60	65	35

HPLC Method 3

This method was used to monitor the transesterification of glucose with vinyl decanoate (VD).

HPLC-ELSD

Eluent: CH_3CN:H_2O; gradient showed in **Table S2**. T (column)= 50 °C.

ELSD parameters: T= 55 °C, gain= Dynamic, filter=10, P (N_2)= 2.8 bar; run time= 45 min.

Retention times (min): glucose decanoate (GD): 8.4 min; decanoic acid: 18.1 min.

Table S2. Gradient of method 3.

Time (min)	CH_3CN (% vol.)	H_2O (% vol.)
0	50	50
10	60	40
20	75	25
25	75	25
25.1	50	50
40	50	50

Extraction of commercial saccharose laurate from DES

Table S3. Evaluation of solvents and temperature for the extraction of saccharose laurate from ChCl:U (1:2) (5% w/w water) DES.

Extraction Solvent	Extraction T (°C)	Recovery (%)[a]	Notes
EtOAc	55	1	
EtOAc	55	1	NaCl was added to DES before the extraction.
EtOAc	55	4	DES was diluted with H_2O (0.250 mL) before the extraction.
CH_2Cl_2	30	0	
$CHCl_3/CH_3OH$ 2/1	30	2	
1-octanol	80	N.D.	1-octanol interference in HPLC.
2M2B	80	67	
2M2B	80	100	DES was diluted with H_2O (0.250 mL) before the extraction.
Dioxane	80	35	
Dioxane	80	100	DES was diluted with H_2O (0.250 mL) before the extraction.

[a]The peak area (HPLC-RID, method 1) in the extracted sample was compared with the area of a solution of SL 20 mg/mL in CH_3OH, taken as 100%.

Reaction in DES ChCl:U (1:2) (5% w/w water). Typical chromatograms

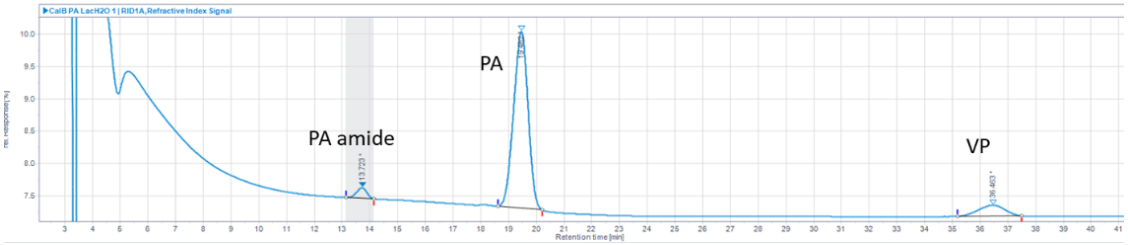


Figure S1. HPLC-RID analysis (Method 1) of transesterification of lactose with PA in ChCl:U (1:2) (5% w/w water) (t= 48 h).

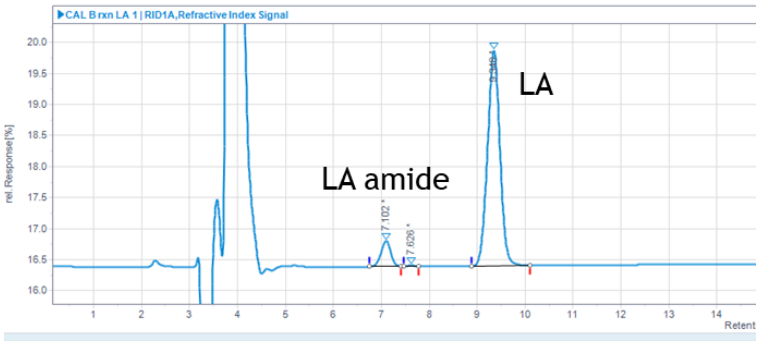
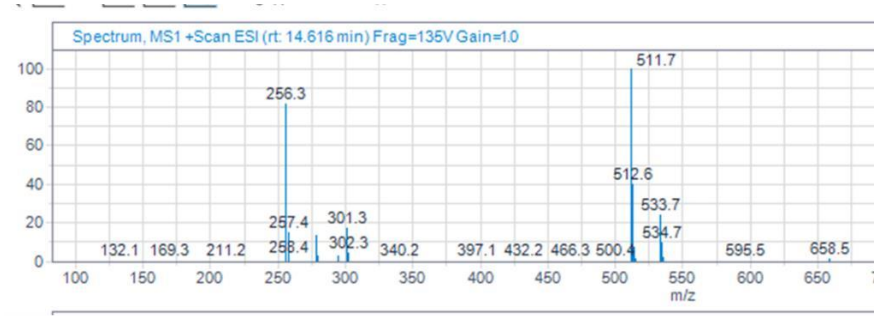
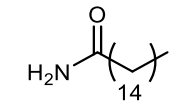


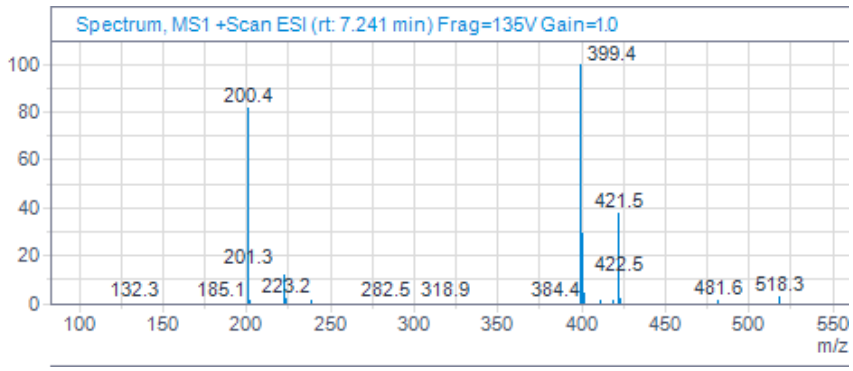
Figure S2. HPLC-RID analysis (Method 1) of direct esterification of lactose with LA in ChCl:U (1:2) (5% w/w water) (t= 48 h).

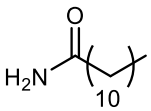




palmitic acid amide
MW: 255.5 g/mol

Figure S3. MS of PA amide from HPLC-MS.





lauric acid amide
MW: 199.2 g/mol

Figure S4: MS of LA amide from HPLC-MS.

Inactivation test with glycerol

This test was performed to demonstrate that Novozym® 435 is active in ChCl:U DES. Briefly, the enzyme was incubated with lactose and VP in ChCl:U (1:2) (5% w/w water) for 24 h. Then, glycerol (0.05 mmol) was added, and the mixture was stirred for further 24 h. The formation of glyceryl palmitate, beside the PA amide, was highlighted by HPLC-RID and HPLC-MS analyses (**Figure S5**).

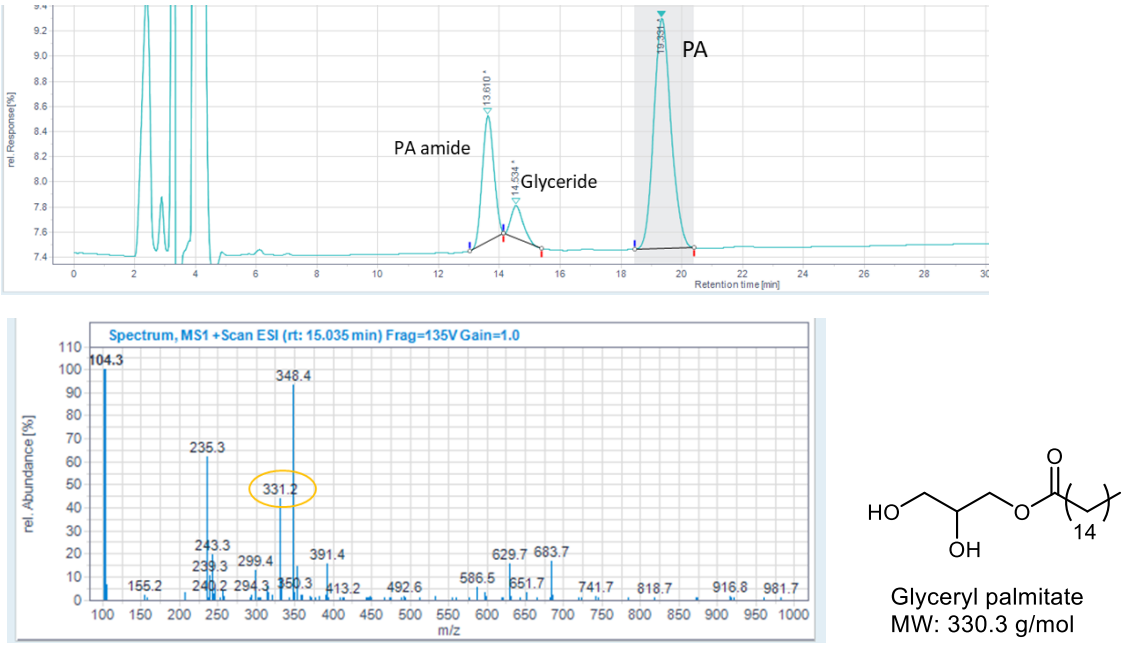


Figure S5. HPLC-RID of the inactivation test (above) and MS spectrum extracted from the peak at 14.5 (below).

Transesterification of lactose and glucose with vinyl decanoate: Comparison between DES and organic solvents

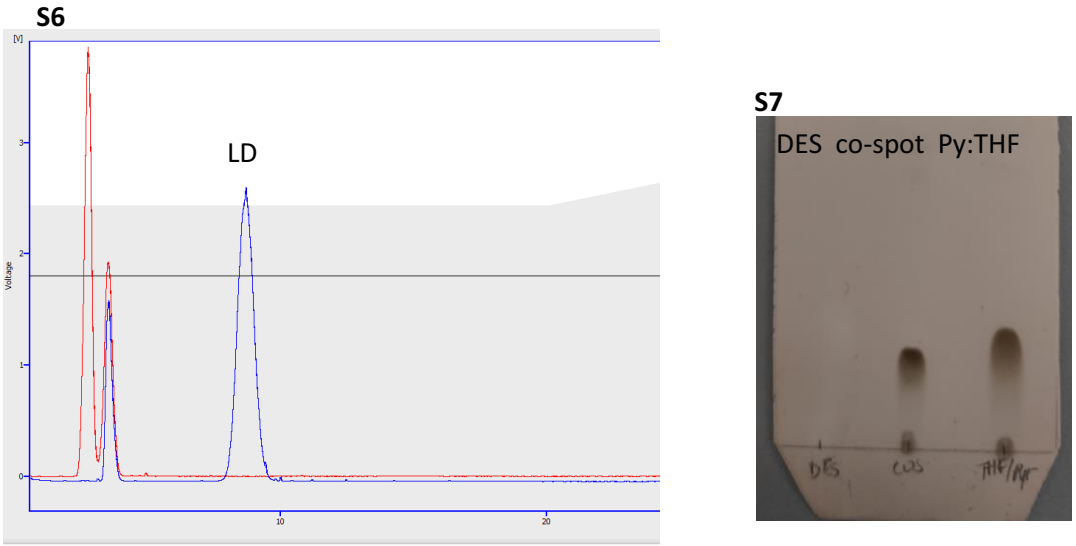


Figure S6-S7. Transesterificafion of lactose with vinyl decanoate: comparison (HPLC-ELSD and TLC) between reaction in ChCl:U (1:2) (5% w/w water) DES (red chromatogram) and in Py:THF (1:1 v/v) (blue chromatogram). HPLC conditions: see method 2. TLC conditions: CH_2Cl_2/CH_3OH 85/15 v/v, H_2SO_4 5% in C_2H_5OH. LD: lactose decanoate.

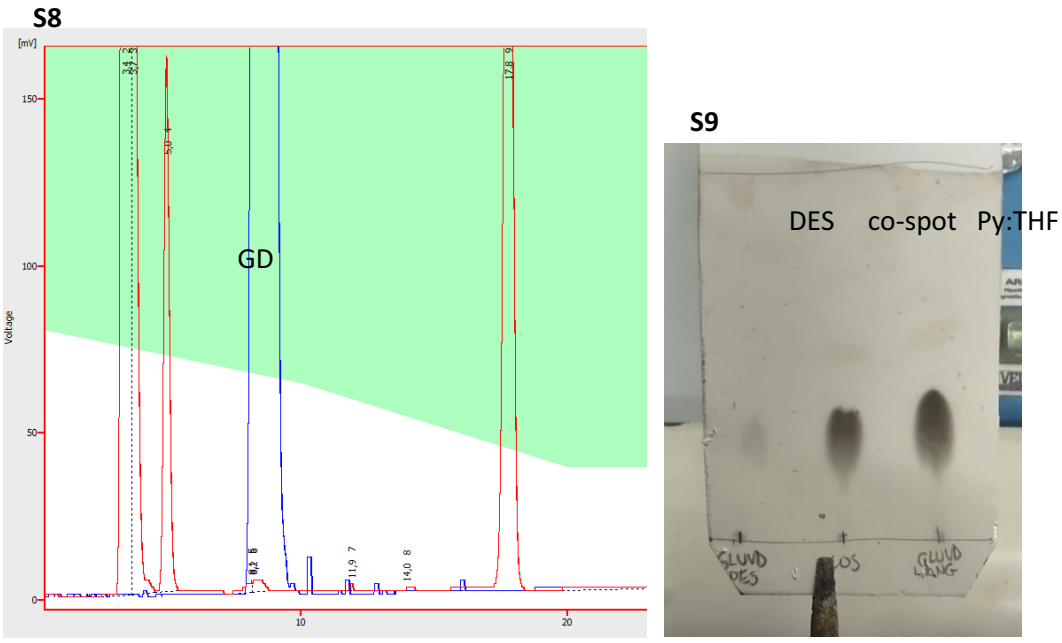


Figure S8-S9. Transesterification of glucose with vinyl decanoate: comparison (HPLC-ELSD and TLC) between reaction in ChCl:U (1:2) (5% w/w water) DES (red chromatogram) and in Py:THF (1:1 v/v) (blue chromatogram). HPLC conditions: see Method 3. TLC conditions: CH_2Cl_2/CH_3OH 85/15, H_2SO_4 5% in C_2H_5OH. GD: glucose decanoate.

Structural analysis of glucose and lactose from the MD simulations

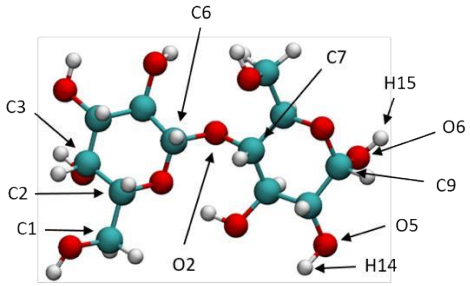


Figure S10. Structure of α-lactose used in the MD simulations with labeled atoms as used in the CGenFF force field.

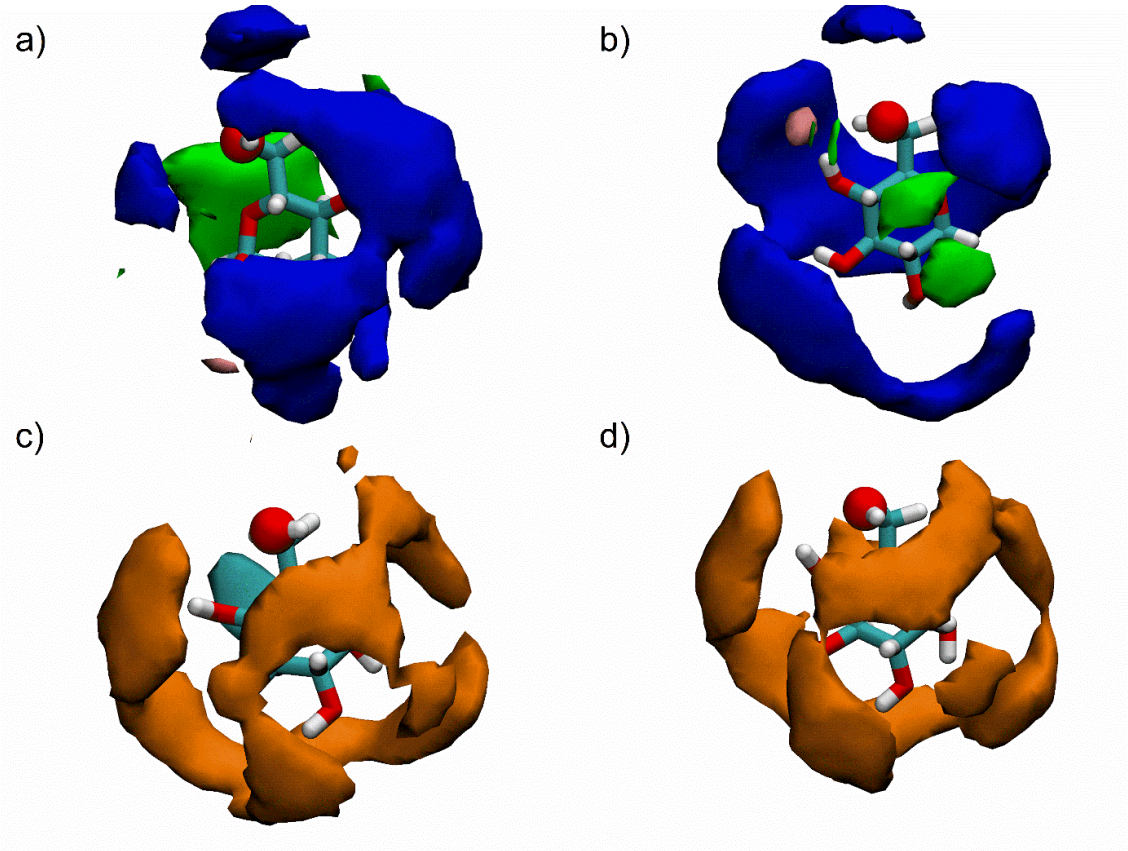


Figure S11. Snapshots of the configuration of α-glucose in the DES ChCl:U (1:2) (5% w/w water) (a,b) and Py:THF (1:1 v/v) (c,d) from the MD simulations and two replica simulations each. α-glucose is displayed as licorice, while the oxygen atom that is part of the esterification, is highlighted as red van-der-Waals sphere with a radius scaled by 0.5. Spatial distribution function of the solvents around α-glucose from the MD simulations are added to the figures. Iso-surfaces of region with high probability of a specific surface are shown in orange (pyridine), cyan (THF), green (urea), pink (chloride) and blue (choline).

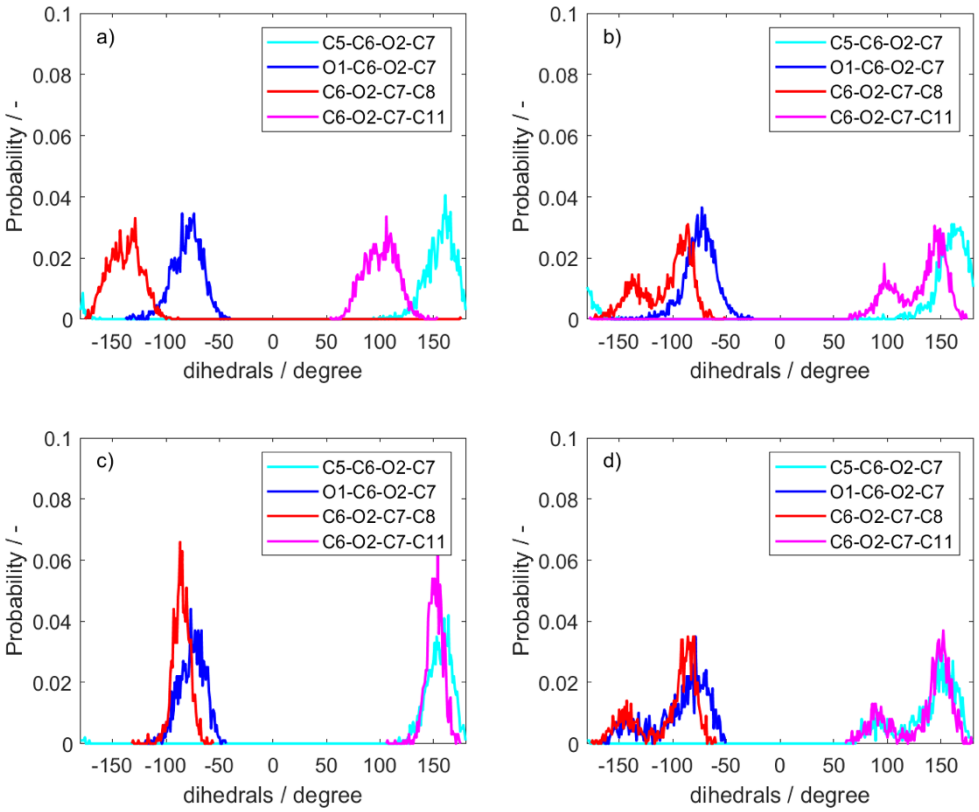


Figure S12. Distribution of characteristic dihedrals of the glycoside bond around oxygen O2 in Py:THF (1:1 v/v) from different replica simulations (a,b) and in in ChCl:U (1:2) (5% w/w water) from different replica simulations (c,d).

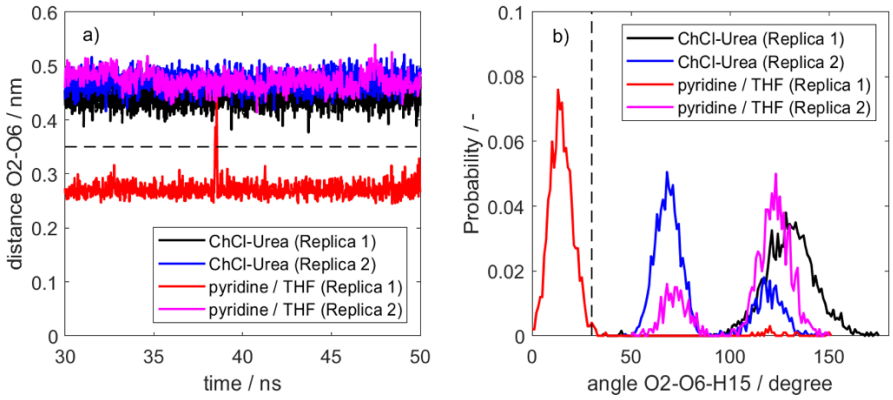


Figure S13. Distance between the oxygen of the glycoside bond (O2) and the oxygen O6 in Py:THF (1:1 v/v) and in ChCl:U (1:2) (5% w/w water) from different replica simulations each (a). Distribution of angle between the oxygen of the glycoside bond (O_2) and the OH group O6-H15 in Py:THF (1:1 v/v) and in ChCl:U (1:2) (5% w/w water) from different replica simulations each (b).

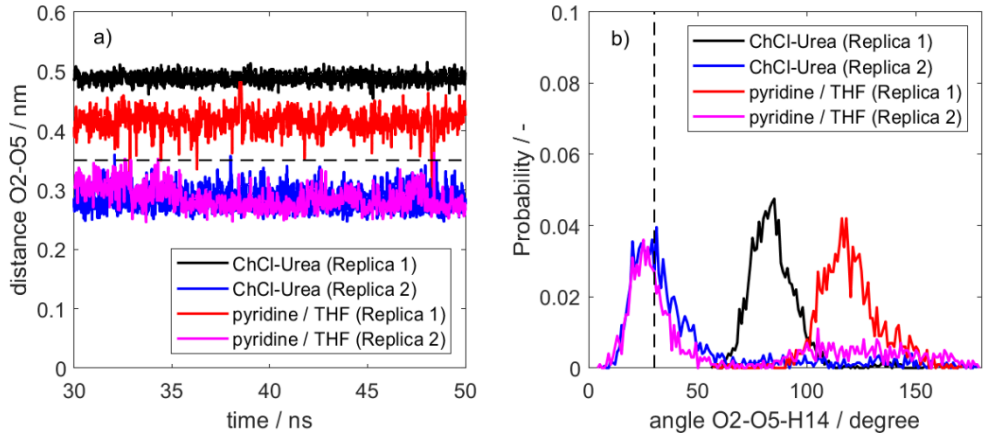


Figure S14. Distance between the oxygen of the glycoside bond (O2) and the oxygen O5 in Py:THF (1:1 v/v) and in ChCl:U (1:2) (5% w/w water) from different replica simulations each (a). Distribution of angle between the oxygen of the glycoside bond (O2) and the OH group O5-H14 in Py:THF (1:1 v/v) and in in ChCl:U (1:2) (5% w/w water) from different replica simulations each (b).

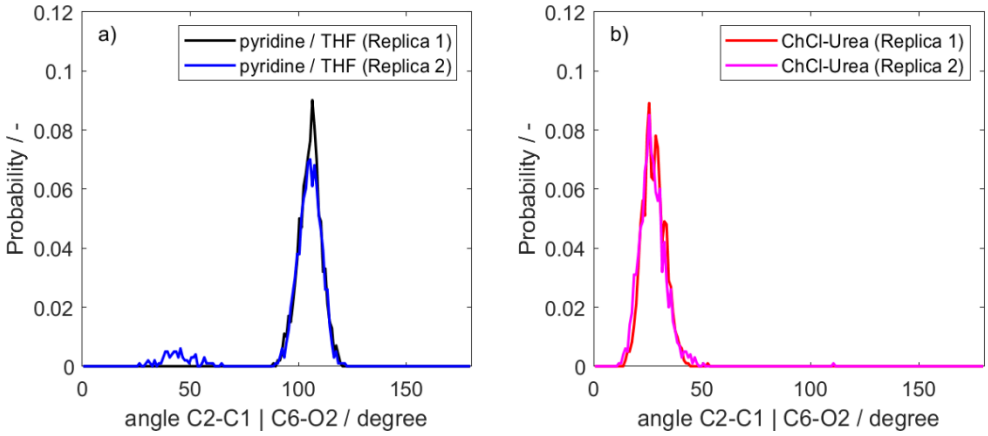
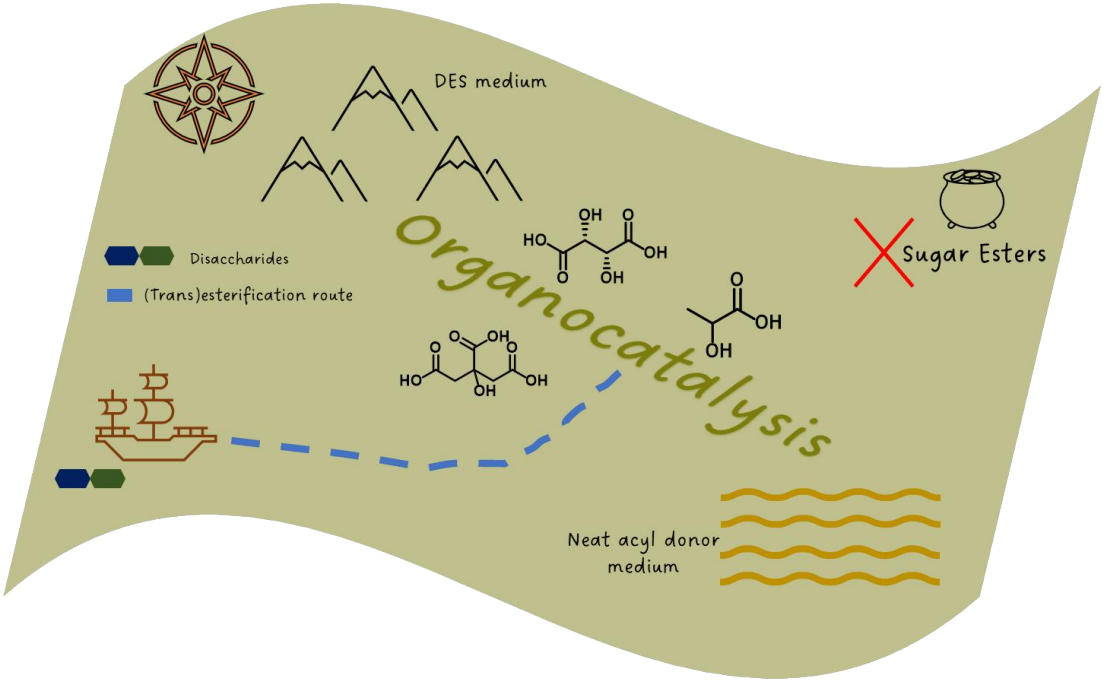


Figure S15. Distribution of angle between the carbons C2-C1 and the glycoside bond (C6-O2) in Py:THF (1:1 v/v) (a) and in ChCl:U (1:2) (5% w/w water) (b) from different replica simulations each.


DES medium
Disaccharides
(Trans)esterification route
Organocatalysis
Sugar Esters
Neat acyl donor medium

3.4.1 Introduction

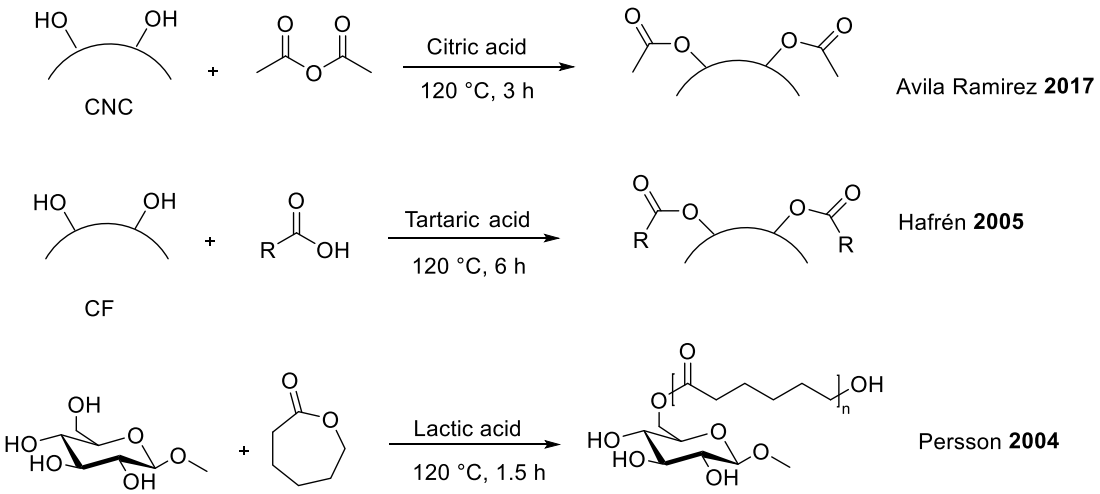
In the framework of the synthesis of sugar fatty acid esters (see **Chapter 3**), it clearly emerged that the obtainment of lactose-based SFAE through a (chemo)enzymatic approach was a true challenge, beyond all expectations. Organocatalysis, that has recently emerged as a concept with a number of synthetic applications,[1,2] despite being still underexplored in the synthesis of SFAE, was considered to this aim. High selectivities and reduced waste formation are some of the features claimed for organocatalysis that share, indeed, some of the well-recognized strengthpoints of biocatalysis.[2–4] Specifically, our attention was drawn by organocatalytic examples reported for ring-opening polymerization of lactones and (trans)esterification reactions catalyzed by α-hydroxycarboxylic acids (e.g. lactic acid, tartaric acid, citric acid among others). These organocatalysts catalyze reactions in a similar fashion to hydrolases, such as lipases or esterases [1,5] and, because of that, these organocatalysts are referred to as "minimal hydrolases". Not less important, an attractive characteristic of α-hydroxycarboxylic acids is their quite easy access by biotechnological routes (i.e. fermentation) as well as the safety associated to their use and disposal.

As for the catalytic mechanism of α-hydroxy acids, it has been postulated that the hydroxy group from the organocatalyst can mimic that of serine hydrolase-catalyzed reactions.[1]

Some examples of organocatalysis applied to the esterification of sugars and polysaccharides are reported in **Scheme 1** and **Table 1**. These examples were the starting point for the explorative experiments described herein.

Table 1: Organocatalysis applied to the esterification of carbohydrates: some examples

Acyl acceptor	Acyl donor	Catalyst	Reference
Cellulose nanocrystals (CNC)(4.32 mmol of anhydroglucose)	Acetic anhydride (530 mmol)	Citric acid (0.26-2.6 mmol)	Avila Ramirez **2017**[6]
Cellulose fibers (20 mg)	Caprolactone (2.5 mmol) Palmitic acid (0.25 mmol)	Tartaric acid (0.25 mmol)	Hafren **2005**[7]
Methylglucopyranoside Sucrose Raffinose (0.82 mmol each)	Caprolactone (7.1 mmol)	Lactic acid (0.25 mmol)	Persson **2004**[8]



Scheme 1: Examples of esterification of sugars and polysaccharides catalyzed by α-hydroxy acids. CNC= cellulose nanocrystals, CF: cellulose fibers

3.4.2 Materials & Methods

General

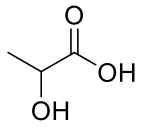
Vinyl palmitate (VP) was purchased from TCI Europe N.V. (Belgium), and anhydrous lactose was purchased from Fluka (Italy). All other reagents and chemicals were purchased from Merck Life Science (Denmark) and used as received.

All the HPLC analyses were performed using a Waters C8 Symmetry column 150 x 4.6 mm, 5 µm at a flow rate of 0.5 mL/min. The injection volume was 2 µL. HPLC-RID Analyses of the analytical scale reactions in neat donor and DES (see **Table 2**) were performed on a 1260 Agilent Infinity, equipped with a RID thermostated at 40 °C. HPLC-ELSD Analyses of the preparative synthesis of dodecanamide were performed on a HPLC Chromaster 600 bar System, Merck Hitachi VWR equipped with a SEDEX 100 LT-ELSD detector with the following parameters: T= 38 °C, flow (N_2) = 2.8 bar; gain: Dynamic; filter= 4. Mobile phase: CH_3OH: H_2O 85:15 vol., containing 0.1% v/v. HCOOH (isocratic); T (column)= 50 °C;. Retention time (dodecanamide): 6.87 min.

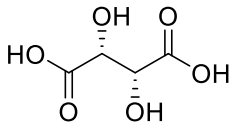
^{1}H NMR spectra were recorded at 298 K on a AVIII 400 MHz Bruker NMR spectrometer (Bruker Corporation, Billerica, MA, USA) equipped with a z-gradient coil probe, available at the Centro Grandi Strumenti (CGS) of the University of Pavia (Italy). All 1D and 2D NMR spectra were acquired using the standard pulse sequences available with Bruker Topspin 3.6. Solvent DMSO-d_6.

The FT-IR analysis was performed on Perkin Elmer Spectrum One (Perkin Elmer Italy) equipped with an ATR accessory (MIRacle™ device, Pike Technologies, Madison, WI, USA) with a ZnSe crystal cell. The analysis was recorded in medium IR (650-4000 cm^{-1}) with a resolution of 4 cm^{-1} and 32 number of scans (Prof. M. Sorrenti, Department of Drug Sciences, University of Pavia, Italy).

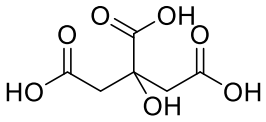
Organocatalysis in neat donor



Lactic acid



Tartaric acid



Citric acid

Figure 1: α-Hydroxycarboxylic acids tested as organocatalysts for the (trans)esterification of sugars

For the trials with citric acid as catalyst, lactose or saccharose (0.017 g; 0.05 mmol) were suspended in neat acyl donor (0.2 g) at 95 °C for 15 min. Then, citric acid monohydrate (0.09 mmol) was added and the mixtures were stirred at the same temperature and 800 rpm for 48 h. At the endpoint, reaction mixtures were diluted with 2M2B (1 mL) and stirred at 1200 rpm for further 30 min, then centrifuged and analyzed by HPLC-RID.

For tartaric and lactic acid, lactose or saccharose (0.027 g; 0.08 mmol) were suspended in neat acyl donor (LA, PA or VP; 0.71 mmol) at 95 °C, 800 rpm for 15 min. Then, the organocatalyst (lactic acid or tartaric acid; 0.05 mmol) was added and the mixtures were stirred at the same temperature and rpm for 48 h. At the endpoint, reaction mixtures were diluted with 2M2B (1 mL) and stirred at 1200 rpm for further 30 min, then centrifuged and analyzed by HPLC-RID. A trial at 120 °C for 6 h was also performed with lactic acid as catalyst. Controls without the sugar and without the catalyst were also performed.

Organocatalysis in DES

The reactions were performed in DES (0.5 mL) at 90-95 °C, 800 rpm for 18-24 h. If the DES contained the acceptor and the catalyst (e.g. sac:lactic acid), the donor (0.1 M) was added to the mixtures. For the trials in ChCl:U DES, the acyl donor and acceptor were added in a 1:1 ratio followed by the organocatalyst. At the endpoint, the reactions were extracted with 2M2B (0.5 mL) for 30 min at 1200 rpm, then centrifuged and analyzed by HPLC-RID. Results are summarized in **Table 2**. To evaluate the interference of DES components, choline chloride and urea (0.08 mmol) were incubated in neat VP (0.71 mmol) with lactic acid (0.05 mmol) as catalyst.

Synthesis of lauramide (dodecanamide)

Urea (0.240 g; 4 mmol) was suspended in vinyl laurate (VL; 9.1 mL; 35 mmol) and lactic acid (0.186 mL; 2.5 mmol) was added. The mixture was stirred at 120 °C, 600 rpm for 4 h. 2M2B (10 mL) was added and the mixture was stirred for further 30 min then left to cool at room temperature; the formation of a white precipitate was observed. The mixture was cooled to 4 °C to enhance the precipitation. The white solid was filtered and washed with cold 2M2B. The HPLC-ELSD analysis showed the presence of a major peak with traces of lauric acid (LA) and VL in the precipitate. The solid was further purified by silica chromatography. After loading the sample, the column was washed with CH_2Cl_2/MeOH 90/10 to remove VL and LA. The amide was then recovered, as a white solid, by washing the column with MeOH, and the product was characterized by ^{1}H NMR, ^{13}C NMR, FT-IR and HPLC-ELSD (**Figures S1-S7**).

3.4.3 Results & Discussion

Organocatalysis in neat donor

Saccharose and lactose were reacted with neat acyl donors (PA and VP) in the presence of the organocatalysts reported in **Figure 1** (lactic acid, tartaric acid and citric acid). At the endpoint (see Materials & Methods), only the peak of the donors was observed in HPLC-RID (in the case of VP, the partial hydrolysis of this reagent was observed). This result was ascribed to the insolubility of both the sugars and the organocatalysts (citric acid and tartaric acid) in the neat donor. On the other hand, lactic acid was soluble in the donor and a higher temperature (120 °C vs 90-95 °C) was used in this case according to literature examples (see **Table 1**). However, also in this case, the target molecules were not detected. Moreover, the caramelization of saccharose (**Figure 1**) was observed.

Figure 1: Transesterification of saccharose (sac) and lactose (lac) with vinyl palmitate (VP) using lactic acid as catalyst at 120 °C

Organocatalysis in DES

To circumvent the solubility issue observed in the neat donors, the transesterification of lactose and saccharose was performed by using different DES as solvents (**Table 2**). Nevertheless, once again, the desired products were not detected.

Two considerations were drawn: due to the high viscosity of all DES tested, a mass transfer issue is highly probable. Secondly, quenching of the catalyst reactivity exerted by the H-bond network of the DES itself cannot be ruled out.

In the case of ChCl:U, the formation of a by-product was detected as previously observed in the lipase-biocatalyzed transformation of disaccharides carried out in this DES (**Chapter 3.3**). The by-product was finally identified as the fatty acid amide upon running, as a control, the preparative reaction using urea and vinyl laurate. The use of urea as ammonia source for the synthesis of fatty amides was found in literature after a focused bibliographic research.[9–11]

Table 2: Organocatalytic transesterifications of sugars in DES

Acceptor (mmol)	Donor (mmol)	Catalyst (mmol)	medium	T (°C)	Time (h)
Lac (0.05)	VP (0.05)	Citric acid (0.04)	ChCl:U 1:2 + 5% H_2O	90	24
Sac (0.05)	VP (0.05)	Citric acid (0.04)	ChCl:U 1:2 + 5% H_2O	90	24
Sac (DES)	VP (0.05)	Citric acid (DES)	Sac:CitrAc 1:1+20% H_2O	95	16
Sac (DES)	VP (0.05)	Lactic acid (DES)	Sac:LactAc 1:3 + 5% H_2O	90	18
Lac (0.05)	VP (0.05)	Lactic acid (DES)	Sac:LactAc 1:3 + 5% H_2O	90	18
Sac (DES)	VP (0.05)	Lactic acid (DES)	Sac:LactAc 1:10	90	24
Lac (0.08)	VP (0.08)	Tartaric acid (0.05)	ChCl:U 1:2 + 5% H_2O	95	24
Sac (0.08)	VP (0.08)	Tartaric acid (0.05)	ChCl:U 1:2 + 5% H_2O	95	24
Lac (0.08)	VP (0.08)	Lactic acid (0.05)	ChCl:U 1:2 + 5% H_2O	95	24
Sac (0.08)	VP (0.08)	Lactic acid (0.05)	ChCl:U 1:2 + 5% H_2O	95	24

3.4.4 Conclusion & Outlook

Organocatalysis in neat donor gave no conversion of the sugar in the corresponding ester. This result was ascribed to the low solubility of both disaccharides (saccharose and lactose) in this system. Even when organocatalysis was performed in DES containing the α-hydroxycarboxylic acids, no conversion of the sugar in the corresponding ester was observed as well. This result was preliminarly ascribed to the high viscosity of the system and/or the quenching of the catalyst caused by DES hydrogen bond network. In the case of ChCl:U, the formation of the fatty acid amide was detected as a competitive reaction. The data reported herein have to be intended as explorative trials.

3.4.5 References

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3.4.6 Supporting Information

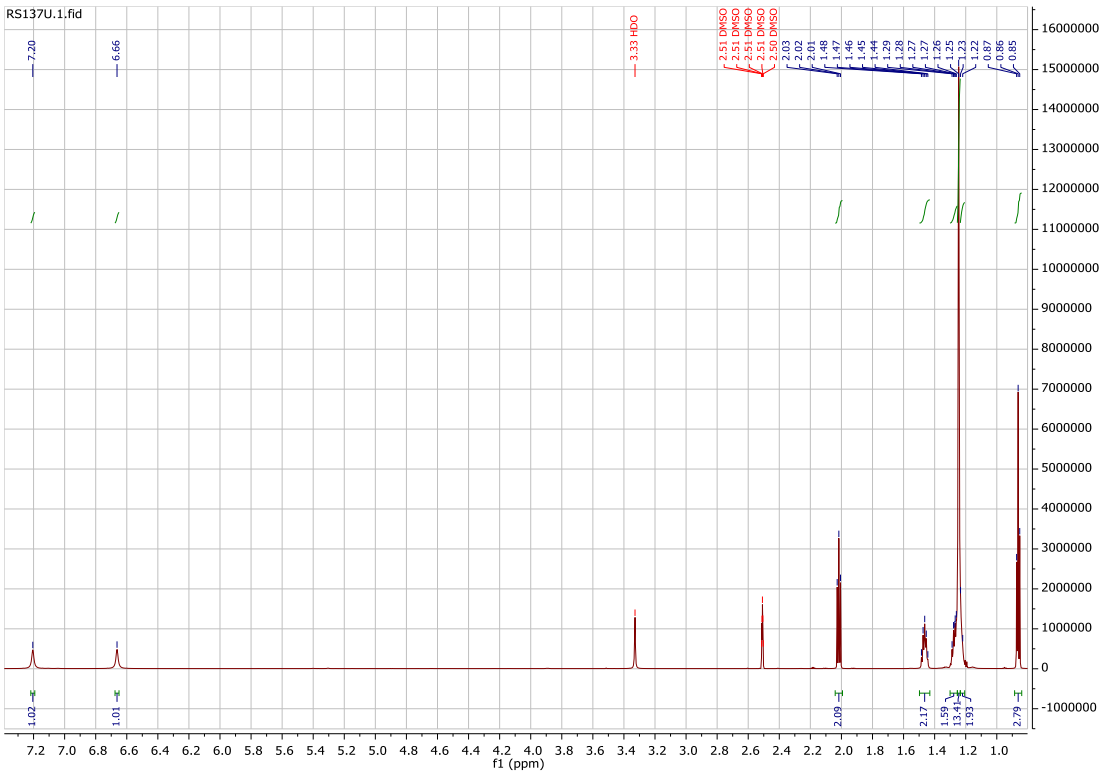


Figure S1: ^{1}H NMR (DMSO-*d*) of lauramide (dodecanamide)

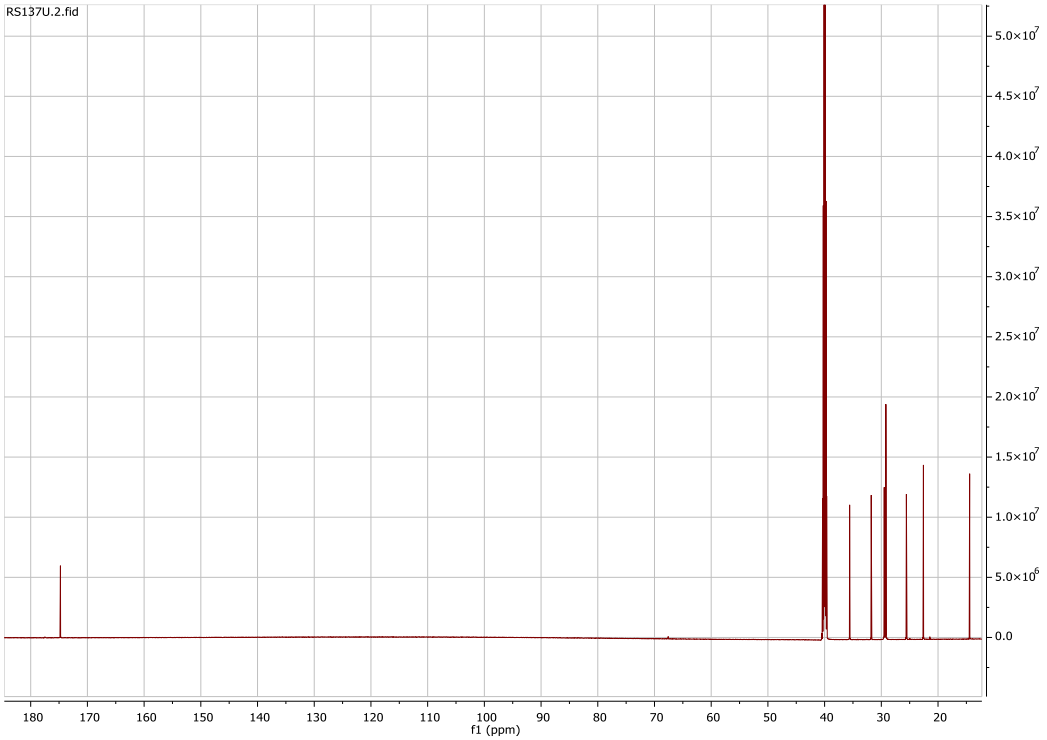


Figure S2: ^{13}C NMR (DMSO-*d*) of lauramide (dodecanamide)

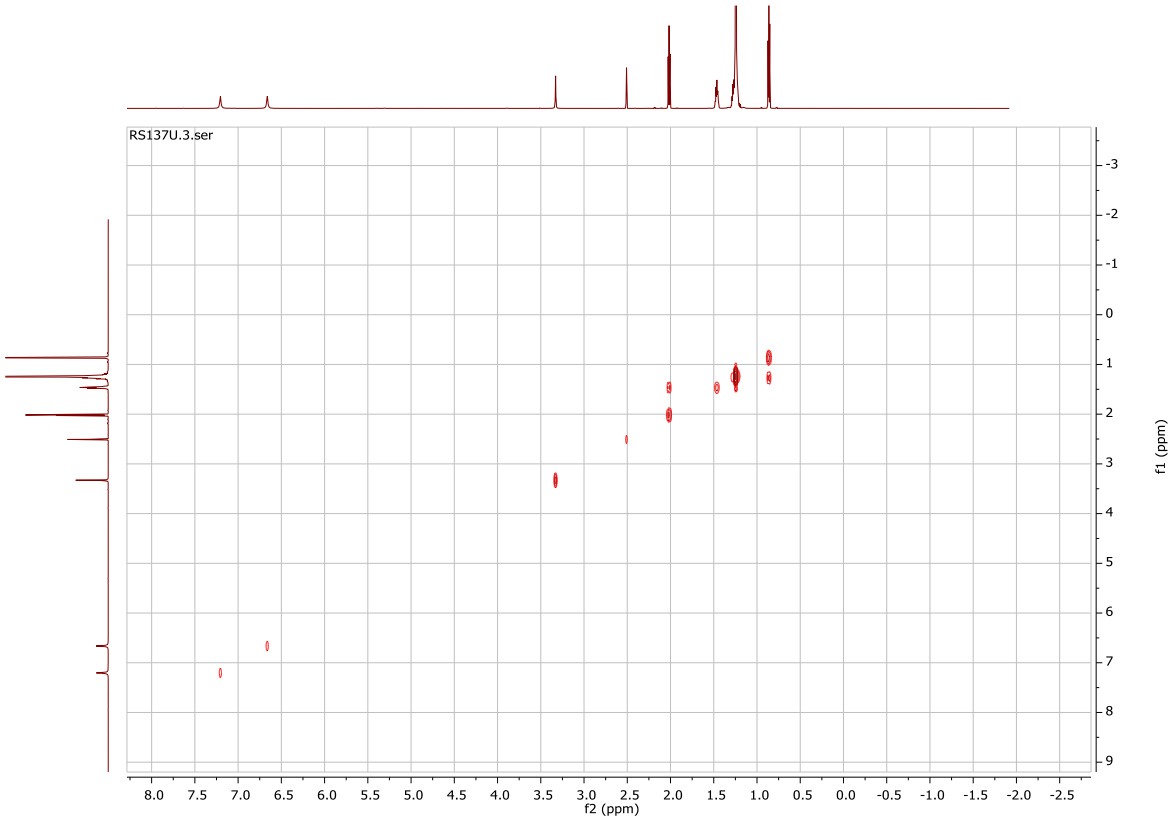


Figure S3: ^{1}H-^{1}H COSY (DMSO-*d*) of lauramide (dodecanamide)

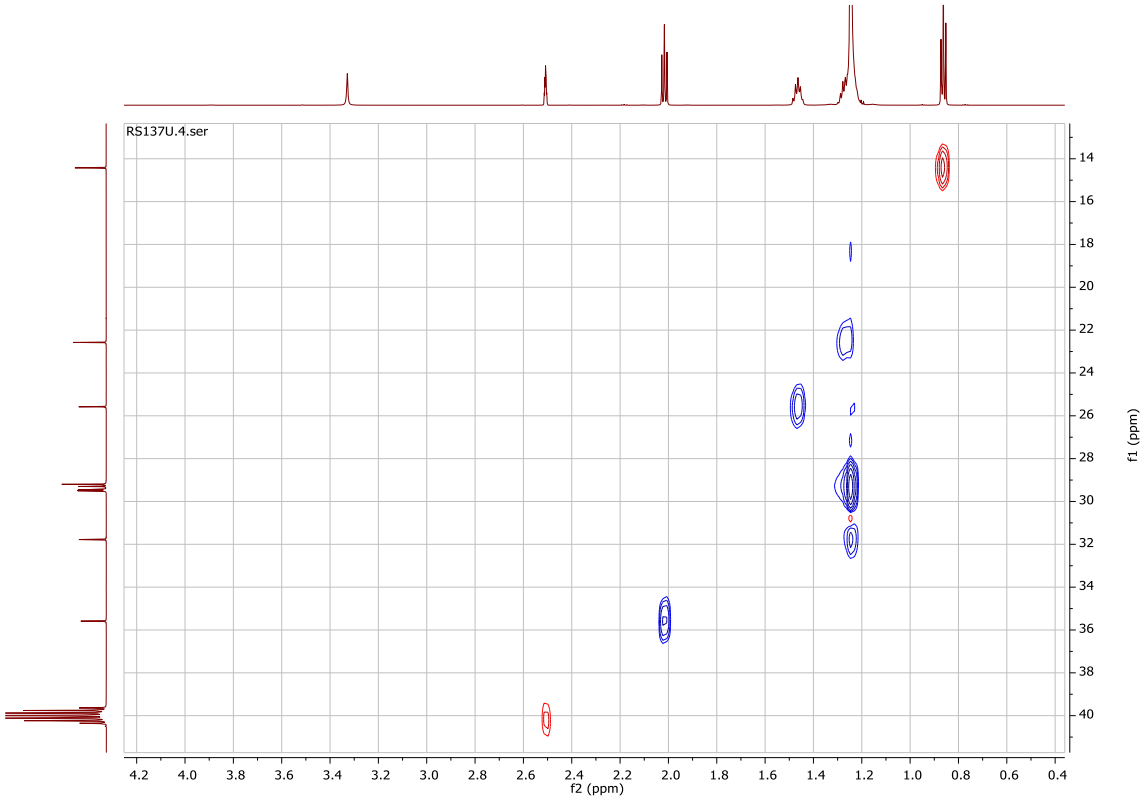


Figure S4: HSQC (DMSO-*d*) of lauramide (dodecanamide)

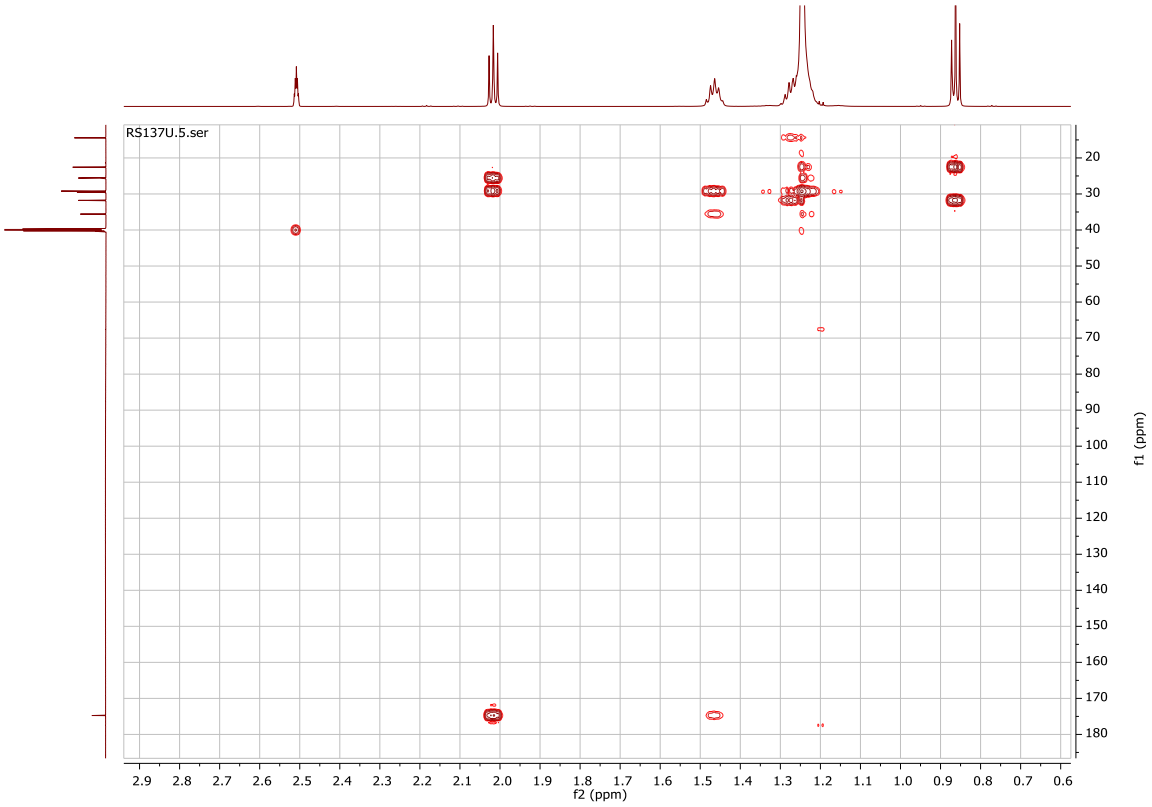


Figure S5: HMBC (DMSO-*d*) of lauramide (dodecanamide)

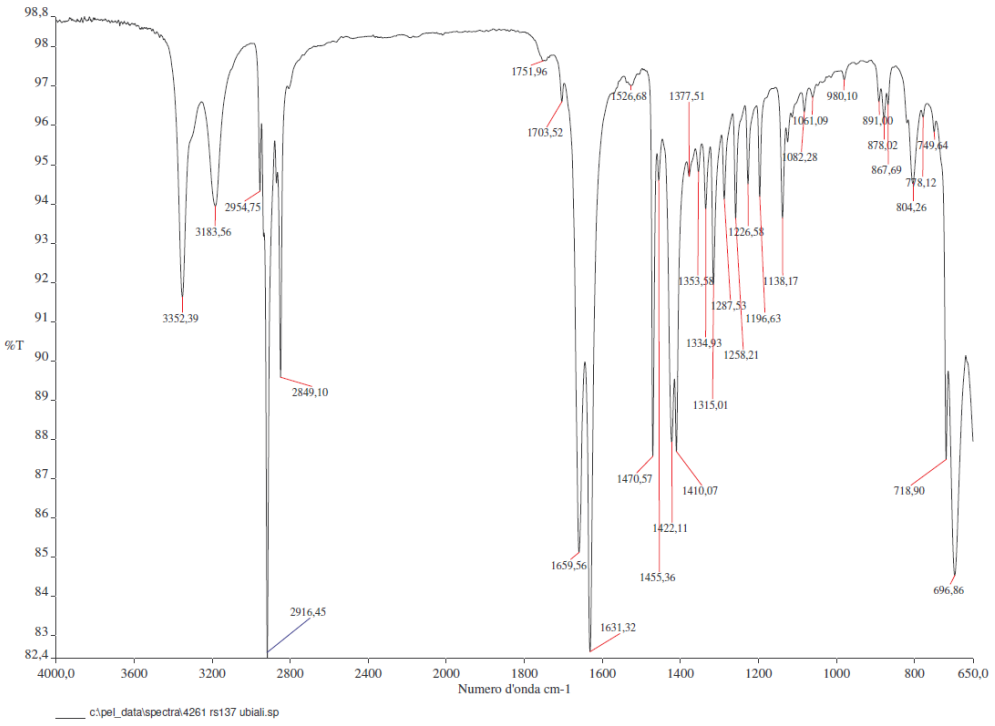


Figure S6: FT-IR of lauric acid amide

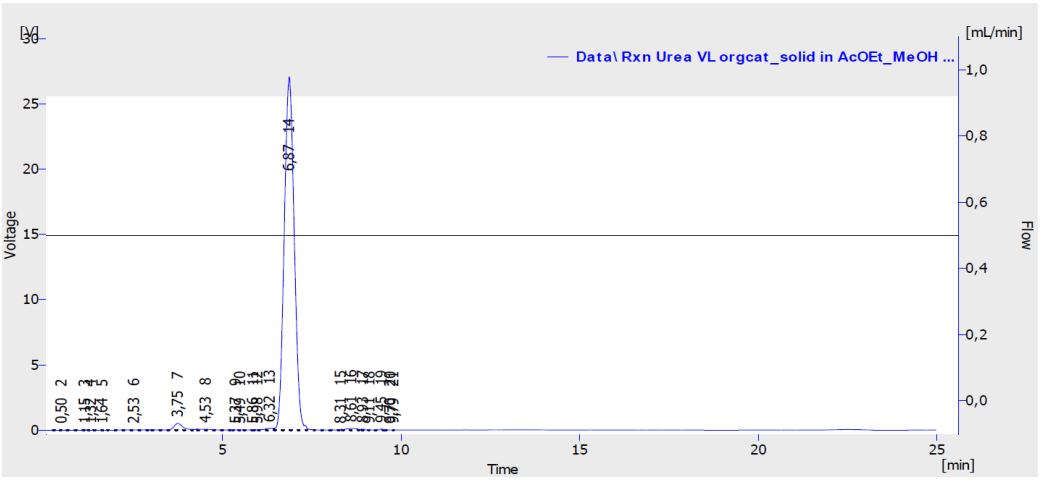
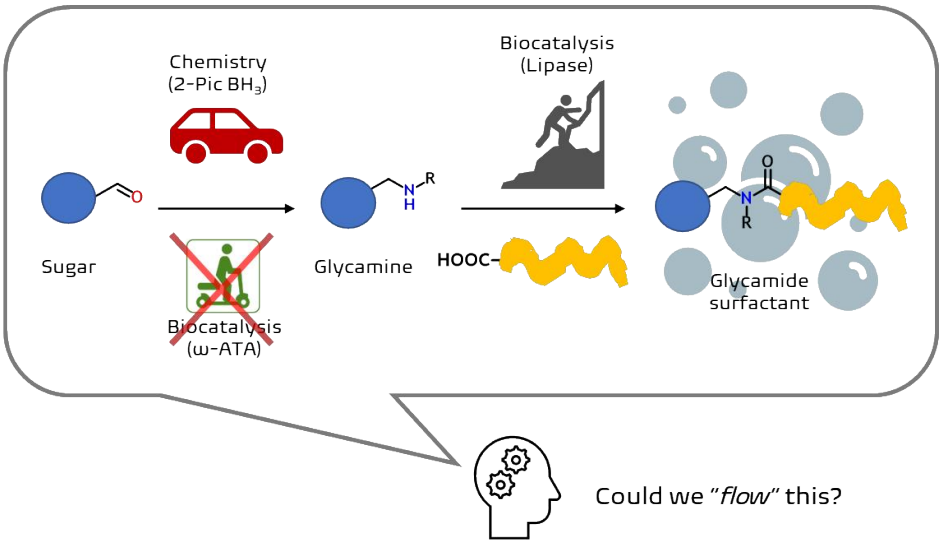


Figure S7: HPLC-ELSD of lauramide (dodecanamide)

Chapter 3.5 Synthesis of *N*-alkyl glycamines as precursors of carbohydrate-based surfactants



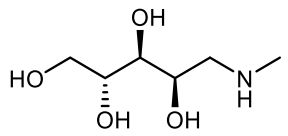
3.5.1 Introduction

Surfactants are amphiphilic molecules that reduce surface and interfacial tension by accumulating at the interface of immiscible fluids. Because of these properties, surfactants modify the way other molecules behave in a two-phase system. Either of biological origin or produced by chemical synthesis, surfactants find application in various sectors, from personal care to detergency, from food processing to textiles, etc. (see **Chapter 1**).[1]

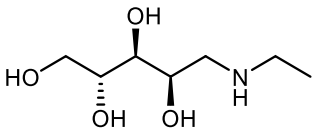
As previously described (see **Chapters 3.1** and **3.2**), sugar fatty acid esters (SFAE) represent an important class of non-ionic surfactants which can be obtained from renewable carbohydrate-rich biomass by (chemo)enzymatic approaches.[2,3] In connection with the research reported in **Chapter 3**, lactose and some monosaccharides (glucose, galactose, and arabinose) were herein submitted to the reductive amination with methylamine and ethylamine to obtain the corresponding glycamines (1-deoxy-1-(alkylamino)-alditols, **Figure 1**) to be used as "polar heads" of a new class of surfactants. Specifically, this research project was inspired by a new sugar-based surfactant from renewable sources recently launched on the market by Clariant. GlucoPure® (*N*-cocoyl-*N*-methyl glucamide, **Figure 10**, **Chapter 1**) is an amide in which the "polar head" is derived from glucose and the "non-polar tail" is made up of a fatty acid.

As a first trial, the synthesis of primary glycamines (1-deoxy-1-(amino)-alditols, see compound **1c**, **Scheme 2**) was carried out through a biocatalytic approach based on the use of ω-transaminases (ω-ATA), as recently reported by Subrizi et al.[4] Thus, arabinose was selected as the starting material, in agreement with this report. The first step was to assay the substrate specificity of the panel of ω-ATA in our hands (three (*S*) ω-ATA and one (*R*) ω-ATA).

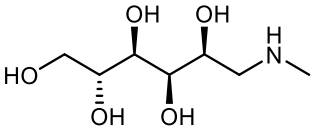
In parallel, the chemical reductive amination was carried out with all the substrates in order to obtain directly the library of "polar heads" (**1-4 ab**). The following acylation step was carried out by using the same protocol previously set-up for the synthesis of SFAE (see **Chapters 3.1** and **3.2**).



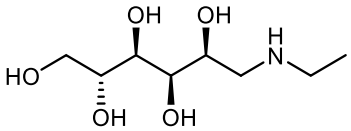
1-deoxy-1-(methylamino)-DL-arabinitol (**1a**)



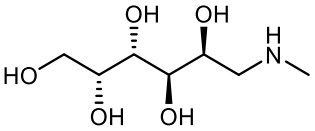
1-deoxy-1-(ethylamino)-DL-arabinitol (**1b**)



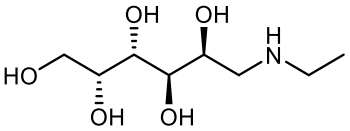
1-deoxy-1-(methylamino)-D-glucitol (**2a**)



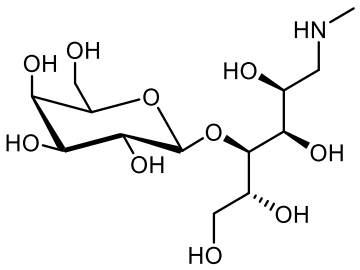
1-deoxy-1-(ethylamino)-D-glucitol (**2b**)



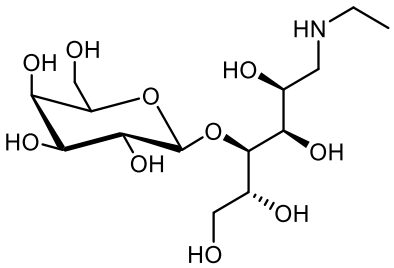
1-deoxy-1-(methylamino)-D-galactitol (**3a**)



1-deoxy-1-(ethylamino)-D-galactitol (**3b**)



1-deoxy-1-(methylamino)-D-lactitol (**4a**)



1-deoxy-1-(ethylamino)-D-lactitol (**4b**)

Figure 1: *N*-alkyl glycamines obtained from arabinose (**1a**-**1b**), glucose (**2a**-**2b**), galactose (**3a**-**3b**), lactose (**4a**-**4b**)

3.5.2 Materials & Methods

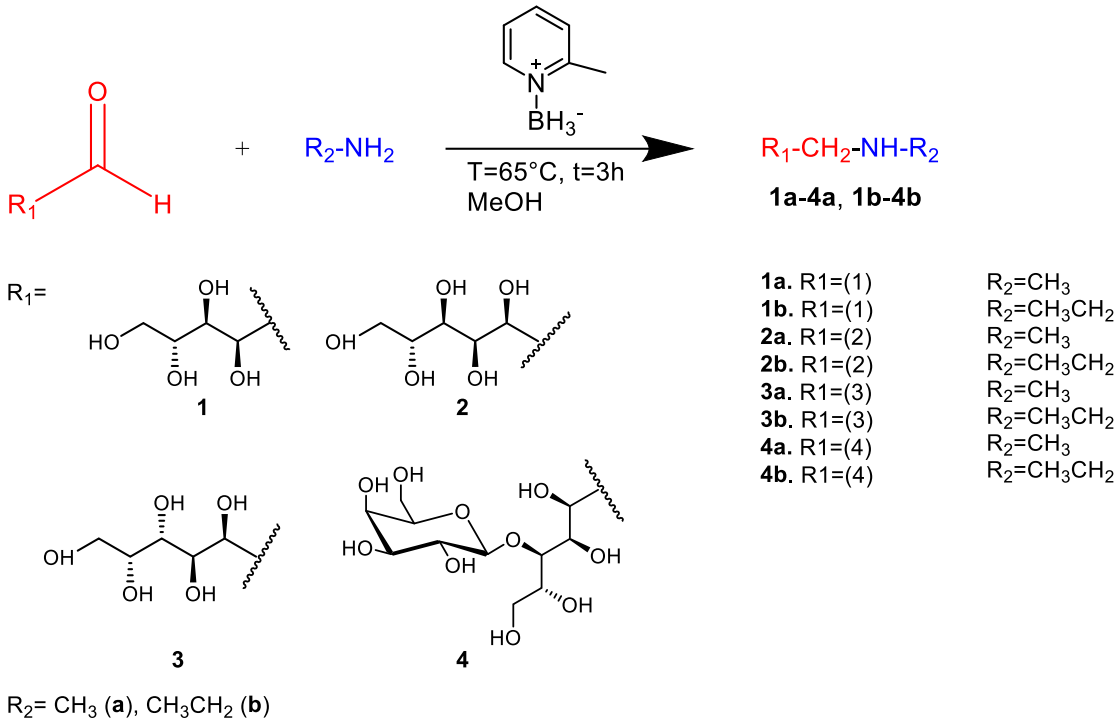
All reagents and solvents were purchased from Merck Life Science (Milano, Italy), unless otherwise stated. ω-Transaminases (ω-ATA) from *Vibrio fluvialis* (*S*)-*Vf*ATA, *Chromobacterium violaceum* (*S*)-*Cv*ATA, *Streptomyces* sp. (*S*)-BV333ATA, (*S*)-IS-3ATA, *Aspergillus terreus* (*R*)-ATA117 were produced and kindly supplied in a purified form by Dr. Erica E. Ferrandi (SCITEC-CNR, Istituto di Scienze e Tecnologie Chimiche "G. Natta", CNR, Milano).

Reactions were monitored by Thin Layer Chromatography (TLC) using *n*-PrOH/CH_3COOH/H_2O 8/2/2 as eluent. For the detection, ninhydrin 1% w/v in EtOH or H_2SO_4 5% v/v in EtOH were used in a sequential mode.

^1H and ^{13}C spectra were recorded at 400.13 and 100.61 Hz, respectively, on a Bruker AVANCE 400 spectrometer equipped with TOPSPIN software package (Bruker, Karlsruhe, Germany) at 300 K, unless stated otherwise. ^1H and ^{13}C chemical shifts (δ) are given in parts per million and were referenced to the solvent signals (δ_H 7.26 - δ_C 77.16, δ_H 2.50 - δ_C 39.52 and δ_H 4.79 ppm from tetramethylsilane (TMS) for $CDCl_3$, DMSO-d_6 and D_2O respectively). For compounds **4b** and **6**, ^1H NMR signals were assigned with the aid of ^1H-^1H correlation spectroscopy (^1H-^1H COSY). ^{13}C NMR signal multiplicities were based on APT (Attached proton test) spectra. ^{13}C NMR signals were assigned with the aid of ^1H-^{13}C correlation experiments (Heteronuclear multiple quantum correlation spectroscopy, HSQC, and Heteronuclear multiple bond correlation spectroscopy, HMBC). Electrospray ionization mass spectra (ESI-MS) were recorded on a Thermo Finnigan LCQ Advantage spectrometer (Hemel Hempstead, Hertfordshire, UK). All NMR and MS analyses were performed at the University of Milano (Prof. G. Speranza).

HPLC-ELSD Analyses of the enzymatic reactions catalyzed by ω-transaminases were performed on a HPLC Chromaster 600 bar System, Merck Hitachi VWR equipped with a Phenomenex Luna NH_2 150 x 4.6 mm and a SEDEX 100 LT-ELSD detector with the following parameters: T= 50 °C, flow (N_2) = 2.7 bar; gain: Dynamic; filter= 8. Mobile phase: ACN/$HCOONH_4$ 8 mM pH 6.60/ACN 20/80. Column oven was set at 40 °C. Retention time (arabinose): 4.61 min. The samples from the enzymatic reactions were analyzed after filtering off the enzyme through centrifugal filter devices (10 kDa MWCO, VWR International, Milano, Italy).

Reductive amination of sugars: general protocol (1a-4a and 1b-4b)

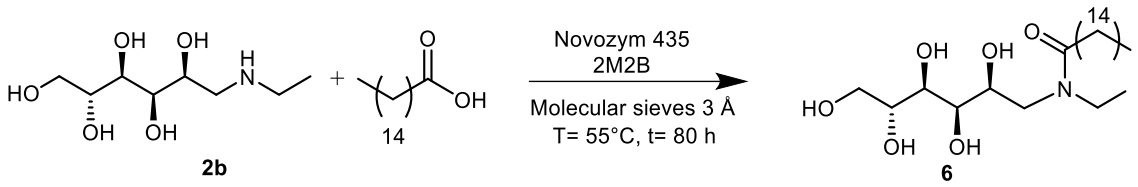


Scheme 1: General scheme of reductive amination of sugars

The reaction was performed on arabinose (**1**), glucose (**2**), galactose (**3**), and lactose (**4**) as previously reported[5] with slight modifications (**Scheme 1**). The sugar (1.33 mmol) was suspended in MeOH (16 mL) under magnetic stirring at r.t.; the amine donor CH_3NH_2HCl (**a**) or $C_2H_5NH_2$ 70% in water (6.65 mmol) and the reducing agent (2-picolin borane) were added. The mixture was refluxed at 65 °C for 3 h. At the endpoint, the solvent and the excess of amine were removed under reduced pressure and purified by cation exchange chromatography using Amberlite IR 120 H^+. Briefly, the resin (15 mL) was washed by incubation in H_2O (30 mL) at 70 rpm, 20 min (x2). Then, the resin was activated by incubation in 1 M HCl (30 mL) at 70 rpm, 20 min (x2) and then it was transferred into a glass column and washed with H_2O (4 BV) to remove the excess of HCl. The reaction mixture was dissolved in H_2O and loaded on the stationary phase. The column was eluted with H_2O (200 mL) to remove the unreacted sugar and then with 1 M NH_4OH (800 mL) to elute the glycamines. The collected fractions were dried under vacuum, thus obtaining the products as yellow oils. Toluene was added during the evaporation step to speed up the procedure.

The reductive amination of glucose and lactose with ethylamine was performed also on a gram scale (2.4 g of glucose and 1 g of lactose, respectively), obtaining comparable yields. In this case, the elution of the products from Amberlite was performed with 16 M NH_4OH (200 mL). All the products were characterized by ^1H NMR and MS spectroscopy. Product **4b** was characterized by ^1H NMR, ^{13}C NMR and MS analyses (see **3.3.4**).

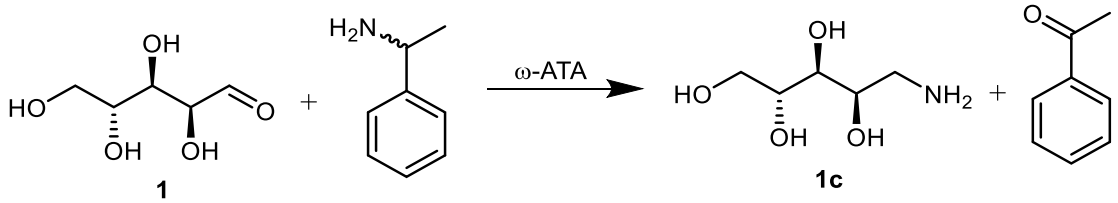
Synthesis of *N*-palmitoyl-*N*-ethyl glucamide (6)



Scheme 2: Synthesis of *N*-palmitoyl-*N*-ethyl glucamide

N-Ethyl glucamine (**2b**, 0.366 g, 1.75 mmol), palmitic acid (0.448 g, 1.75 mmol), molecular sieves 3 Å (0.188 g), and Novozym® 435 (0.101 g) were suspended in 2-methyl-2-butanol (10 mL) at 55 °C (**Scheme 2**). At the endpoint (80 h), the solvent was evaporated under reduced pressure and the residue was purified by flash chromatography (CH_2Cl_2/MeOH 9/1), thus obtaining the amide **6** as a yellow solid (yield: 17%), still impure of palmitic acid. The product was characterized by ^1H NMR, ^{13}C NMR, and ESI-MS (see **3.3.6**).

Enzymatic amination of arabinose: enzyme screening (ω-transaminases)



Scheme 3: Amination of arabinose catalyzed by ω-transaminases (ω-ATA) using MBA as amine donor

The screening was performed according to literature conditions,[4] with some modifications. Reactions were performed in a solution (final volume=1.5 mL) containing arabinose (5 mM), (*S*)- or (*R*)-MBA depending on the selectivity of the ω-ATA (6 mM),

PLP (1 mM), 50 mM pH 8 phosphate buffer containing DMSO (2% v/v). These solutions were prepared from a stock solution which was prepared by dissolving arabinose (0.015 g, 0.1 mmol) in the buffer (19.6 mL) followed by addition of MBA (15.4 µL, 0.12 mmol, dissolved in 0.4 mL DMSO), and 20 mM aqueous solution of PLP (7.5 µL). The enzyme ω-ATA (1 IU) was added to each solution and the mixtures were magnetically stirred at 30 °C for 48 h. At the endpoint, the reactions were centrifuged at 13000 rpm for 3 min to remove the protein (see Materials and Methods) and the supernatant was analyzed by HPLC-ELSD.

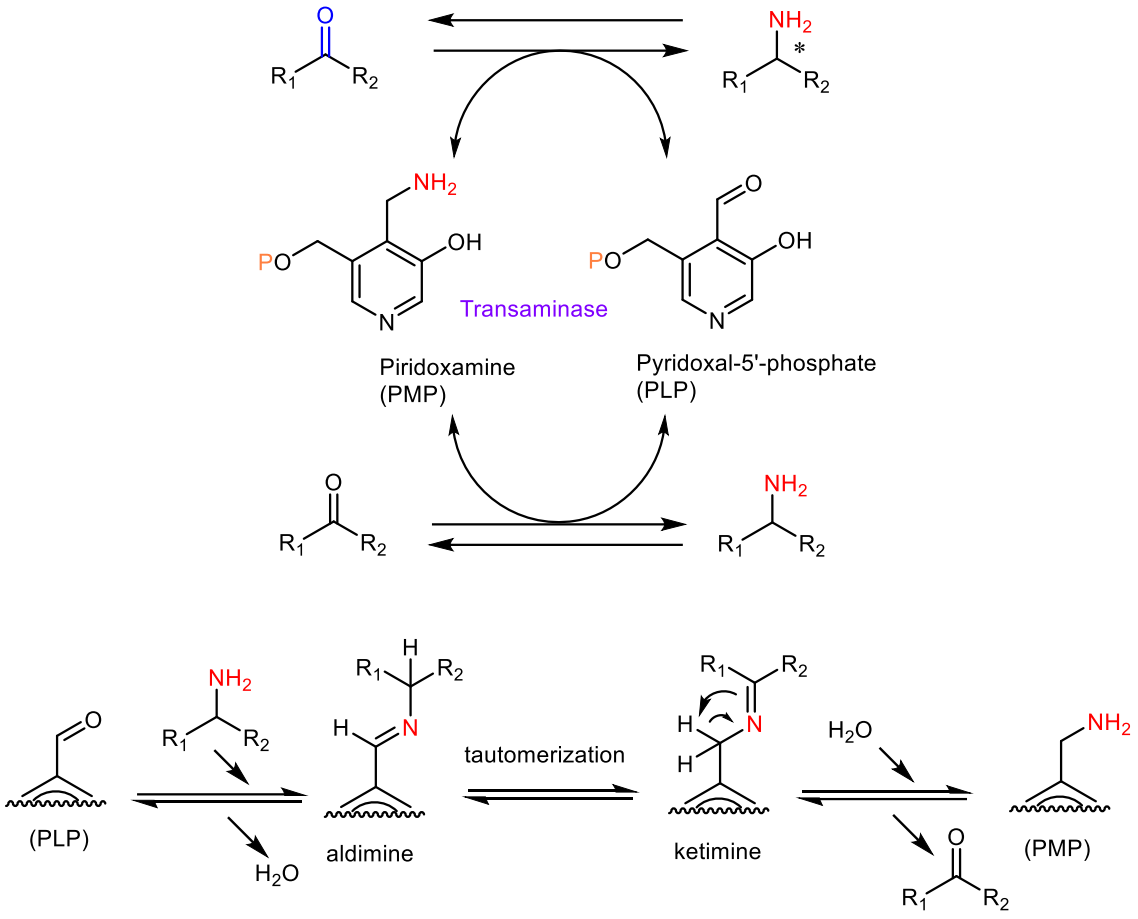
3.5.3 Results & Discussion

Enzymatic amination of arabinose catalyzed by ω-transaminase: enzyme screening

ω-Transaminases (ω-ATA, amino transferases EC 2.6.1) can be employed for the synthesis of enantiopure amines by three different approaches: kinetic resolution of racemic amines, deracemization of amines by using two stereocomplementary ω-ATA, and asymmetric amination of prochiral ketones; this latter strategy is generally the preferred approach, since it can generate a theoretical yield of 100%. In a two-step mechanism (**Scheme 4**), a primary amine group is transferred from an amine donor to a carbonyl amino acceptor with the participation of the "activated benzaldehyde" pyridoxal-5'-phosphate (PLP, B_6 vitamin) which acts as a molecular shuttle for the transfer of the amine group.[6] In the first step, an aldimine is formed between the aldehyde of PLP and the amine donor followed by the tautomerization of the aldimine to ketimine which is then hydrolyzed, thus giving the aminated form of the cofactor (PMP) and releasing the carbonyl form of the amine donor. Pyridoxamine reacts with the amino acceptor, thus regenerating the cofactor, which is ready for the successive cycle, and the aminated acceptor, which is the desired chiral amine. It is worth pointing out that the tightly bound cofactor does not need external regeneration system.

Transaminases are classified in six classes and ω-ATA belong to the II class; their nomenclature derives from their physiological substrates that are ω-amino-carboxylic acids like ornithine, lysine, β-alanine and ω-aminobutyrate. These transaminases are more interesting from an applicative viewpoint than α-transaminases: in fact, the latter can transform only α-amino acids, while the former can be used to aminate aldehyde and ketones.

The use of ω-ATA for the valorization of biomass-derived carbohydrates has been recently reported. Dunbabin and co-workers have described the transamination of furfurals, renewable feedstock for the production of biofuels and chemicals derived from the dehydration of sugars from cellulosic biomasses, to furfurylamines.[7] Aumala et al. have reported enzymatic cascades involving oxidoreductases (e.g. galactose oxidase from *Fusarium graminearum*) and transaminases for the production of amino carbohydrates from galactose.[8]



Scheme 4: Catalytic mechanism of transaminases. Modified from Faber K. **2011**.[6] P=Phosphate group; *new stereocenter

However, sugars are challenging substrates for the transamination with ω-ATA. In fact, few transaminases accept polyhydroxylated ketones or aldehydes. Moreover, sugars are present mostly in the cyclic hemiacetal form in solution, with few carbonyl groups readily available for the amination.[4] Besides the examples cited above, the synthesis of (2*R*,3*S*)-5-aminopentane-1,2,3-triol (yield=69%) was catalyzed by (*S*)-ATA256, a

commercially available enzyme, starting from 2-deoxy-D-ribose in the presence of isopropylamine (IPA) as the amine donor. Furthermore, D-xylose, L-arabinose, and D-fructose have been used as substrates of (*S*)-*Rh*ATA (from *Rhodobacter sphaeroides*) and (*R*)-*Mv*ATA (from *Mycobacterium vanbaalenii*) by using IPA or (*S*)- o (*R*)-α-methylbenzylamine. L-Arabinose and D-xylose were converted into the corresponding aminopolyols by using IPA and (*S*)-*Rh*ATA as catalyst (yields=42% and 79%, respectively). As for the conversion of D-fructose into the aminoalditol counterpart, the yield was 40% (amine donor=(*R*)-MBA; biocatalyst=(*R*)-*Mv*ATA).[4,9]

Starting from these results, we screened some ω-ATA (kindly supplied by Dr. E. Ferrandi, SCITEC-CNR, Milano) for the transamination of arabinose by using MBA as the amine donor (see Materials & Methods), with the aim to apply the same strategy to the sugars contained in/derived from cheese whey permeate (lactose, glucose and galactose). All the transaminases were characterized about their protein content and activity in the 7-9 pH range (data not reported), according to standard assays previously reported.[10] However, none of the ω-ATA tested gave the formation of the expected glycamine (**1c**, **Scheme 3**) under the tested conditions, thus prompting us to move toward a "conventional" reductive amination reaction (see the next paragraph).

Reductive amination of sugars

Lactose and its hydrolysis products (glucose and galactose) were used as starting material (**Scheme 1**). Arabinose, which was previously assayed for the enzymatic amination with ω-ATA,[4] was included in the sugar library, too. Starting from literature data,[5] 2-picoline borane was used as the reducing agent as a safer alternative to $NaCNBH_3$. The glycamines **1a**-**4a** and **1b**-**4b** were obtained in good to high yield, as reported in **Table 1**, being **4a** the only compound obtained in a very poor yield.

Table 1: Yields of the reductive amination reactions (**1a**-**4a** and **1b**-**4b**). See also **Scheme 3**

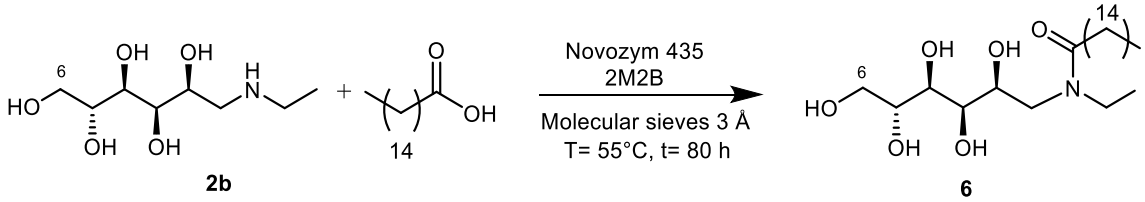
Product	Yield (%)	SI
1a	74	**Figure S1**
1b	77	**Figure S2**
2a	60	**Figure S3**
2b	89	**Figure S4**
3a	63	**Figure S5**
3b	92	**Figure S6**
4a	10	**Figure S7**
4b	97	**Figure S8-S12**

Interestingly, **4b** was obtained in 97% yield. This result might be related to lactose solubility. In fact, lactose is almost insoluble in alcohols and, even at high temperature, the reaction mixture was a suspension when methylamine hydrochloride was used. On the other hand, a clear yellow solution was obtained after 30 min with ethylamine 70% in water. The water contained in the ethylamine solution might have served as a "co-solvent" for lactose, thus generating a homogeneous system and, ultimately, resulting in a higher yield. The reductive amination allows to obtain aminopolyols from different sugars, either monosaccharides or disaccharides, thus providing new "polar heads" other than alkyl glycosides (see **Chapters 3.1** and **3.2**) for the synthesis of surfactants containing fatty acid-based "tails". Lactose-based fatty acid esters/amides are very difficult to obtain. The synthesis of alkyl lactosides by chemical glycosidation is hampered by the low solubility of lactose in alcohols (**Chapters 3.1** and **3.2**). On the other hand, the enzymatic transglycosylation mediated by the β-galactosidase results in the formation of modified monosaccharides (alkyl galactosides) due to the cleavage of the β-1,4-glycosidic bond. The use of cellulases (belonging to glycosidases) for the synthesis of alkyl lactosides has been described,[11,12] but the reports are still scarce and yields reported are scarce as well. Latge and co-workers[13] reported the synthesis of a library of long chain 1-deoxy-1-(alkylamino)-D-lactitols (named "*N*-alkyllactylamines" by the authors) tested as a new class of surfactants, prepared by a two-step process using $NaBH_4$. Later on, a whole chemical process was reported for the synthesis of fatty amides of compound **4a** (named *N*-alkanoyl-*N*-methyllactitolamines by the authors).[14]

The reductive amination of lactose was performed with Ni Raney as catalyst and then the amine was acylated by the fatty acid chlorides. To the best of our knowledge, no enzymatic acylation of 1-deoxy-1-(alkylamino)-D-lactitols has been reported to date.
The glycamines (1-deoxy-alkylamino-alditols) **1a**-**4a** and **1b**-**4b** were generally obtained in good yields with both the amine donors. Starting from these results, *N*-ethyl glucamine (**2a**) was selected for the following enzymatic acylation step with the aim to obtain a surfactant structurally related to the commercial GlucoPure®.

Synthesis of *N*-palmitoyl-*N*-ethylglucamide (6)

The use of a solvent-free system is reported for the enzymatic amidation of glucamines, such as *N*-methylglucamine, catalyzed by Novozym® 435 to give *N*-lauroyl-*N*-methylglucamide.[15] Thus, the conditions applied to the esterification of alkyl glycosides with fatty acids reported in the **Chapters 3.1** and **3.2** (molten fatty acid, 80 °C, 300 mbar) were used for the synthesis of **6**. However, the high reaction temperature caused the degradation of the glycamine, thus suggesting to switch to a lower temperature. This modification required to use a solvent because palmitic acid cannot serve as a solvent at a temperature <80° C. The reaction was run at 55 °C in 2M2B, one of the most used solvents for the esterification of sugars[16] and reported also for the synthesis of fatty acid glucamides.[17,18] Under these conditions, the target molecule (**6**) was obtained in a poor yield (17%) and very long reaction time (80 h). The ester formation in 6 position as a by-product was observed, in agreement with the literature.[17]



2b **6**

Scheme 3: Synthesis of *N*-palmitoyl-*N*-ethylglucamide (**6**)

This biotransformation is currently under investigation, also assisted by the application of flow conditions (in collaboration with the University of Milano, Prof. L. Tamborini). Flow chemistry is an increasingly popular technology in biocatalysis.[19,20] The principle of flow chemistry is simple: two or more solutions containing the reactants are pumped by

HPLC or syringe pumps into a (bio)reactor and mixed together to allow for the reaction to occur. Advantages of flow chemistry over "traditional" batch chemistry account for higher efficiency and safety, while reducing time, resources consumption, and the need for the equipment scale-up. In fact, flow processing has the potential to accelerate biotransformations due to enhanced mass transfer, making large-scale production more economically feasible in significantly smaller equipments with a substantial decrease in reaction time, from hours to a few minutes, and improvement in space-time yield.[19,20] Flow chemistry was recently applied to the enzymatic synthesis of carbohydrate-based surfactants.[21]

3.5.4 Conclusions & Outlook

Lactose and its hydrolysis products were successfully converted into the corresponding *N*-alkyl glycamines through a chemical reductive amination using 2-picolin borane as the reducing agent. *N*-Ethyl glucamine (**2b**) was acylated with palmitic acid into *N*-palmitoyl-*N*-ethylglucamide (**6**) using Novozym 435® as the biocatalyst. Both this biotranformation and the chemical reductive amination are currently under investigation in a continuous mode system. The enzymatic amination of arabinose catalyzed by a panel of four ω-ATA was not successful.

Acknowledgements

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3.5.5 References

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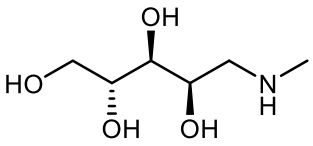
3.5.6 Supporting information

^{1}H-NMR and ESI-MS spectra of compounds 1a-4a and 1b-3b



1-deoxy-1-(methylamino)-DL-arabinitol (**1a**)

^{1}H NMR (400 MHz, D_2O): δ 3.99 (ddd, J = 8.9, 3.9, 2.0 Hz, 1H), 3.75 (dd, J = 11.6, 2.8 Hz, 1H), 3.65 (ddd, J = 8.7, 6.1, 2.8 Hz, 1H), 3.57 (dd, J = 11.6, 6.1 Hz, 1H), 3.41 (dd, J = 3.7, 7.4 Hz, 1H), 2.86 (dd, J = 12.7, 9.1 Hz, 1H), 2.75 (dd, J = 3.9, 12.7 Hz, 1H), 2.43 (s, 3H).

MS (ESI$^+$): *m/z* theoretical $[C_6H_{15}NO_4]^+$: 166.10; observed: 166.21 $[M+H^+]$, 188.17 $[M+Na^+]$, 353.05 $[2M+Na^+]$.

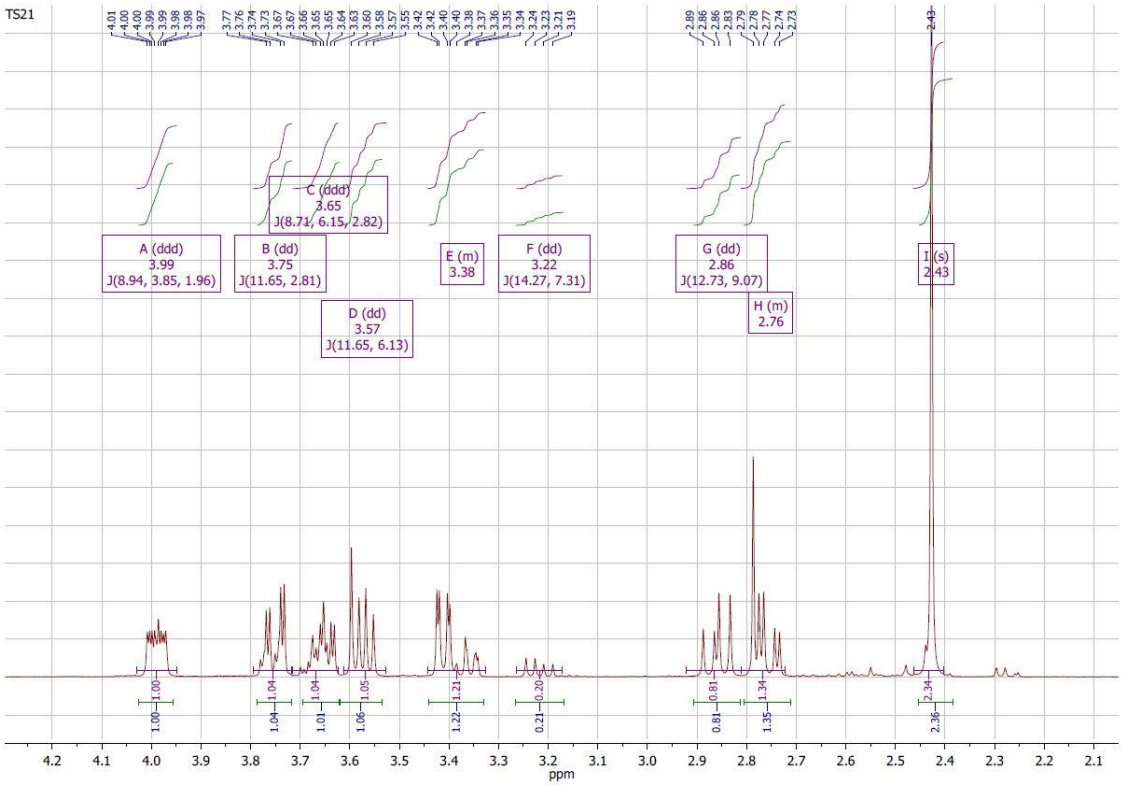
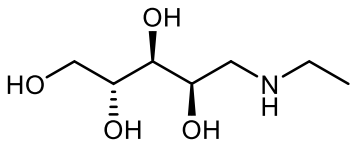


Figure S1: ^{1}H-NMR (D_2O) of 1-deoxy-1-(methylamino)-DL-arabinitol (**1a**)



1-deoxy-1-(ethylamino)-DL-arabinitol (**1b**)

^{1}H NMR (400 MHz, D_2O): δ 3.96 (ddd, J = 8.9, 3.9, 2.1 Hz, 1H), 3.75 (dd, J = 11.6, 2.8 Hz, 1H), 3.65 (ddd, J = 8.7, 6.2, 2.8 Hz, 1H), 3.57 (dd, J = 11.6, 6.2 Hz, 1H), 3.41 (dd, J = 8.4, 2.0 Hz, 1H), 2.90 - 2.62 (m, 4H), 1.07 (t, J = 7.2 Hz, 3H).

MS (ESI$^+$): m/z theoretical $[C_7H_{17}NO_4]^+$: 180.12; observed: 180.23 [M+H$^+$], 202.21 [M+Na$^+$], 381.06 [2M+Na$^+$].

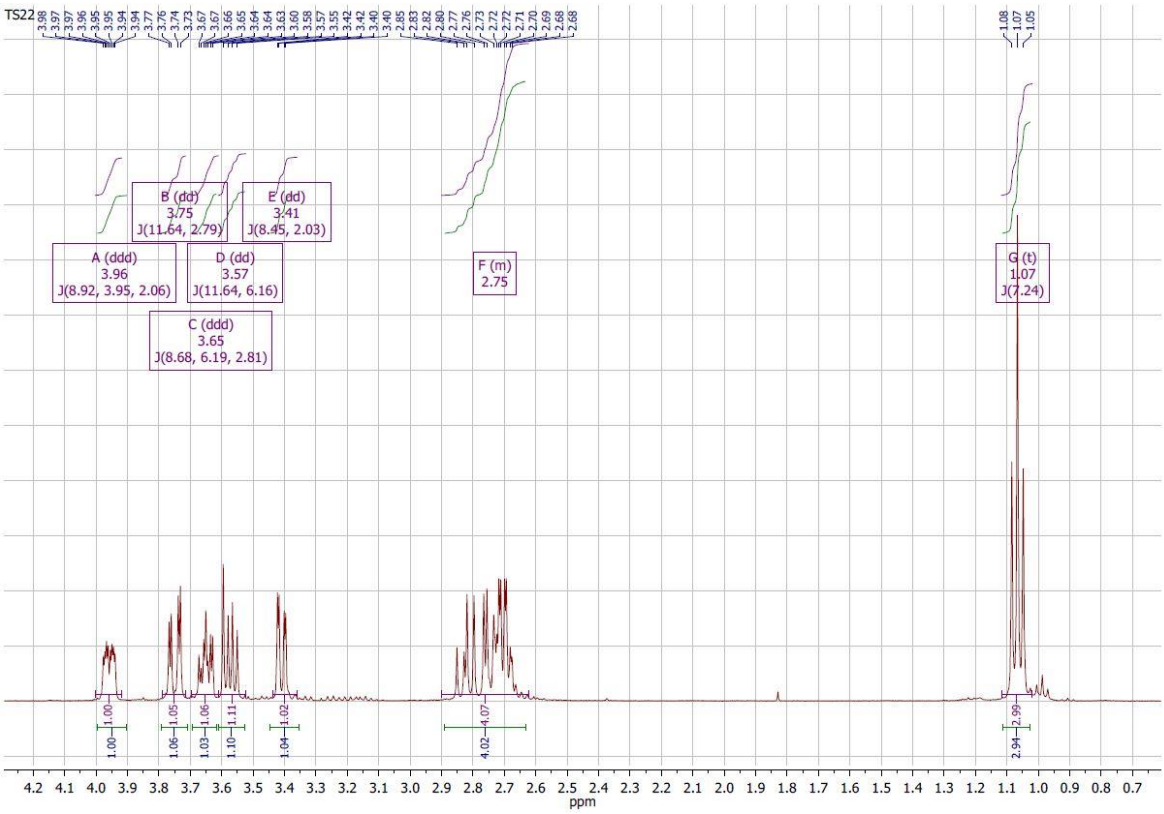
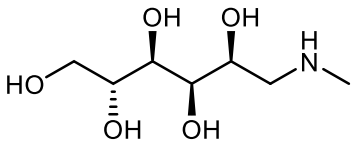


Figure S2: ^{1}H-NMR (D_2O) of 1-deoxy-1-(ethylamino)-DL-arabinitol (**1b**)



1-deoxy-1-(methylamino)-D-glucitol (**2a**)

^{1}H NMR (400 MHz, D_2O): δ 3.90 - 3.81 (m, 1H), 3.74 (dd, J = 11.7, 2.9 Hz, 1H), 3.69 (ddd, J = 9.3, 5.8, 3.2 Hz, 2H), 3.60 - 3.52 (m, 2H), 2.82 - 2.62 (m, 2H), 2.36 (s, 3H).

MS (ESI$^+$): *m/z* theoretical $[C_7H_{17}NO_5]^+$: 196.12; observed 196.32 [M+H$^+$], 218.30 [M+Na$^+$], 413.19 [2M+Na$^+$].

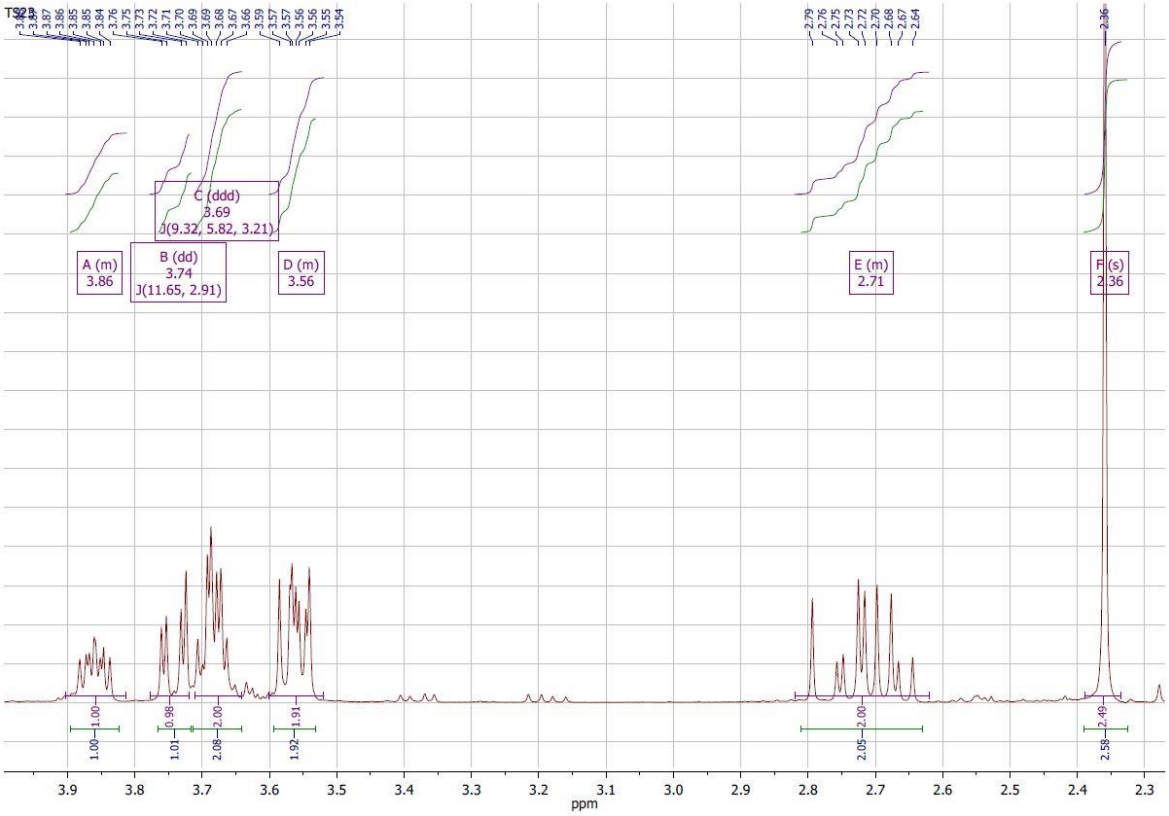
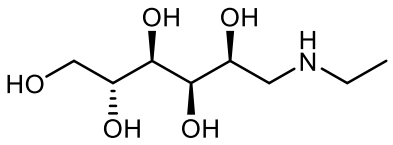


Figure S3: ^{1}H-NMR (D_2O) of 1-deoxy-1-(methylamino)-D-glucitol (**2a**)



1-deoxy-1-(ethylamino)-D-glucitol (**2b**)

^{1}H NMR (400 MHz, D_2O): δ 3.89 (ddd, J = 9.0, 5.6, 3.5 Hz, 1H), 3.78 - 3.65 (m, 3H), 3.62 - 3.52 (m, 2H), 2.86 (dd, J = 12.8, 3.5 Hz, 1H), 2.76 (ddd, J = 11.8, 8.0, 4.5 Hz, 3H), 1.09 (t, J = 7.2 Hz, 3H).

MS (ESI$^+$): m/z theoretical $[C_8H_{19}NO_5]^+$: 210.13; observed: 210.36 [M+H$^+$], 232.42 [M+Na$^+$], 441.44 [2M+Na$^+$].

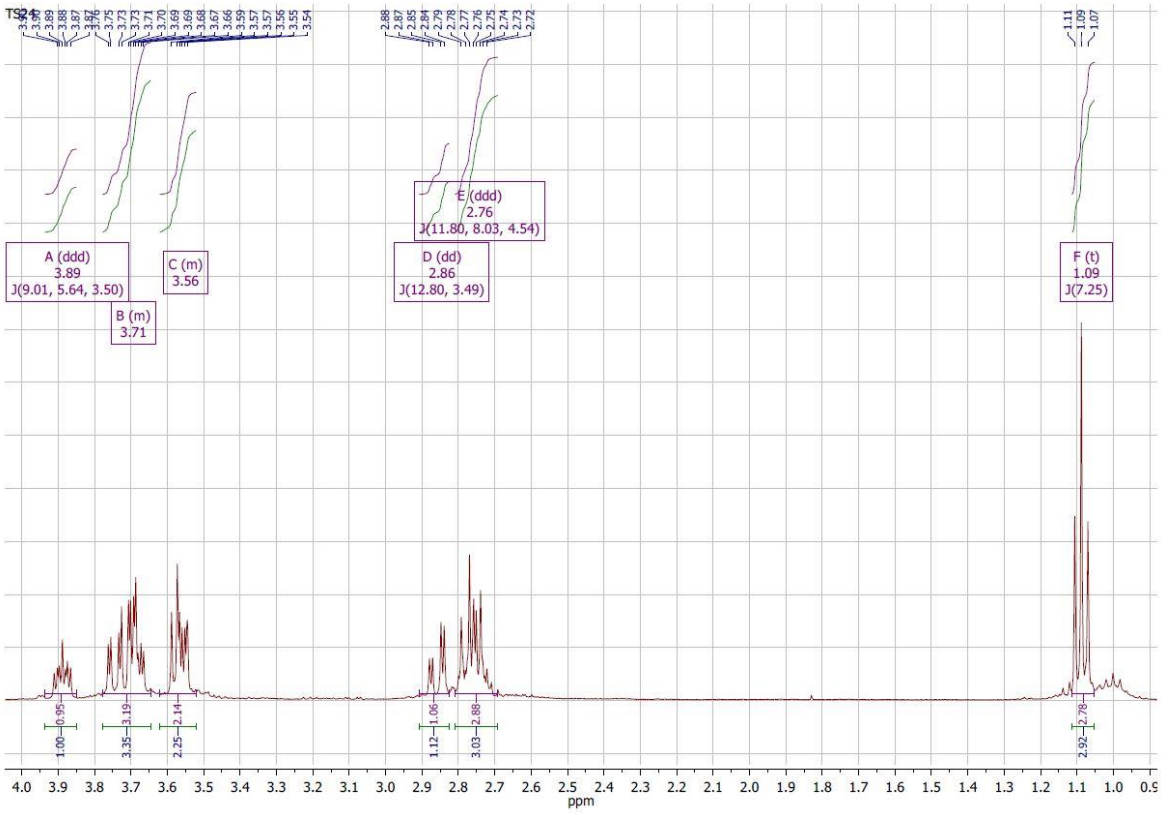
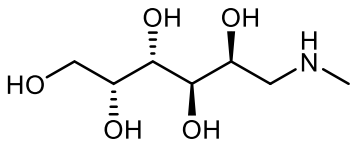


Figure S4: ^{1}H-NMR (D_2O) of 1-deoxy-1-(ethylamino)-D-glucitol (**2b**)



1-deoxy-1-(methylamino)-D-galactitol (**3a**)

^{1}H NMR (400 MHz, D_2O): δ 4.08 (dd, J = 9.3, 2.4 Hz, 1H), 3.92 - 3.85 (m, 1H), 3.63 - 3.56 (m, 4H), 3.02 (dd, J = 12.7, 9.4 Hz, 1H), 2.91 (dd, J = 12.8, 3.4 Hz, 1H), 2.54 (s, 3H).

MS (ESI$^+$): *m/z* theoretical $[C_7H_{17}NO_5]^+$: 196.12; obtained: 196.16 $[M+H^+]$, 218.16 $[M+Na^+]$, 412.93 $[2M+Na^+]$.

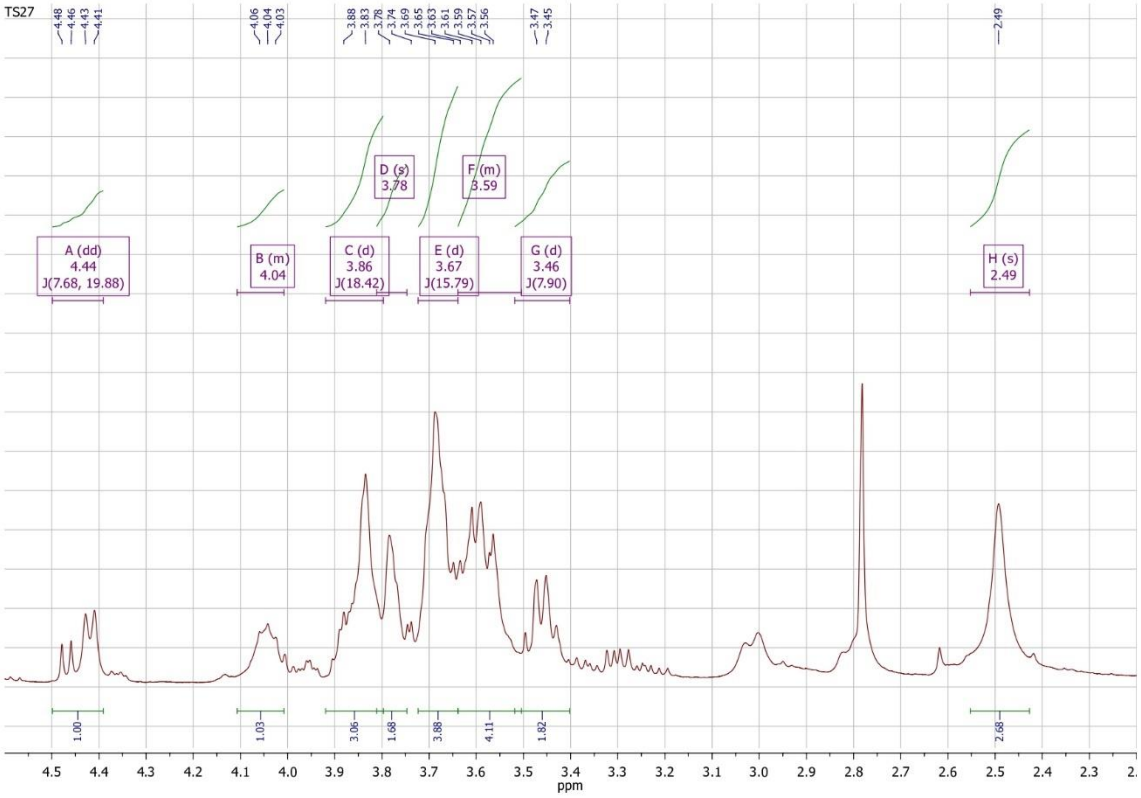
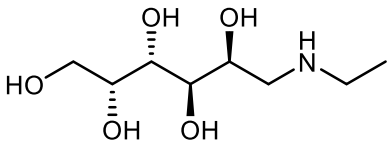


Figure S5: ^{1}H-NMR (D_2O) of 1-deoxy-1-(methylamino)-D-galactitol (**3a**)



1-deoxy-1-(ethylamino)-D-galactitol (**3b**)

^{1}H NMR (400 MHz, D_2O): δ 4.07 - 3.98 (m, 1H), 3.92 - 3.82 (m, 1H), 3.65 - 3.46 (m, 4H), 2.98 - 2.70 (m, 3H), 1.15 - 1.01 (m, 3H).

MS (ESI$^+$): *m/z* theoretical $[C_8H_{19}NO_5]^+$: 210.13; obtained: 210.29 [M+H$^+$], 441.14 [2M+Na$^+$].

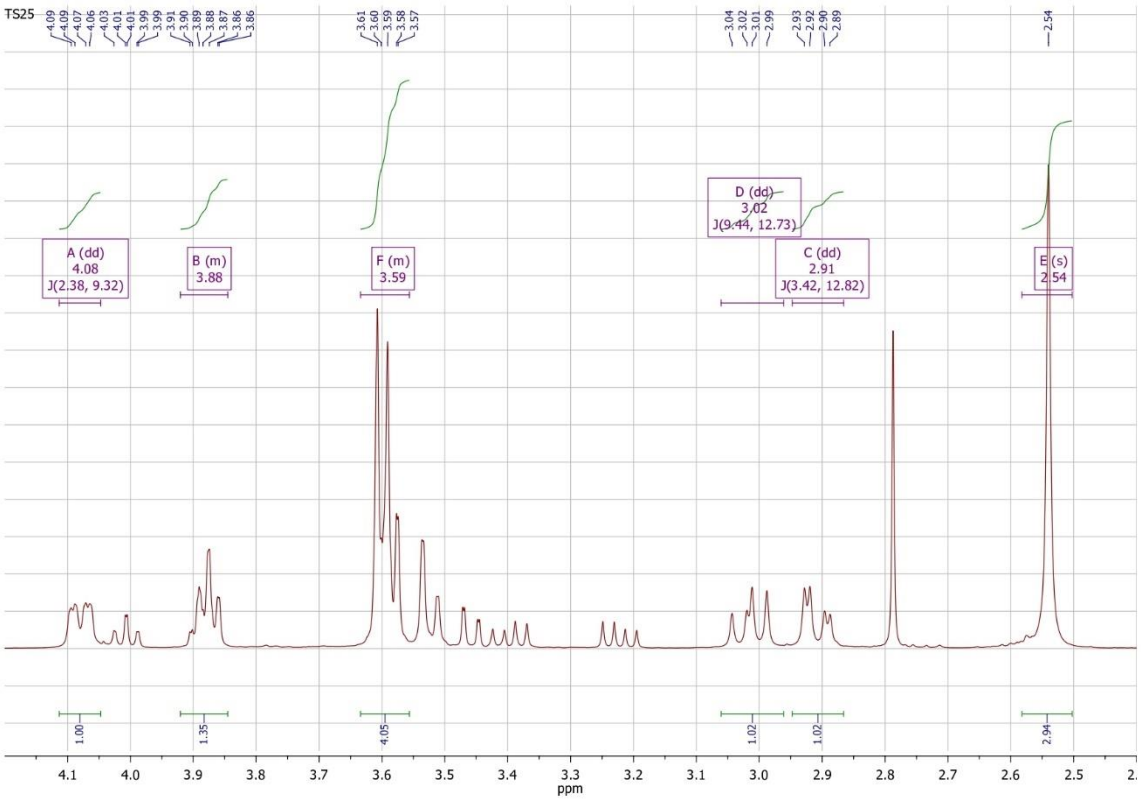
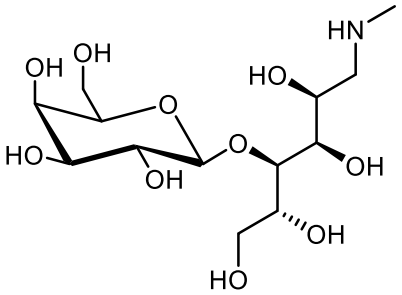


Figure S6: ^{1}H-NMR (D_2O) of 1-deoxy-1-(ethylamino)-D-galactitol (**3b**)



1-deoxy-1-(methylamino)-D-lactitol (**4a**)

^{1}H NMR (400 MHz, D_2O): δ 4.44 (dd, J = 19.9, 7.7 Hz, 1H), 4.11 - 4.01 (m, 1H), 3.86 (m, 3H), 3.78 (m, 2H), 3.67 (m, 3H), 3.64 - 3.50 (m, 3H), 3.46 (m, 2H), 2.49 (s, 3H).

MS (ESI$^+$): *teorico* $[C_{13}H_{27}NO_{10}]^+$: 358.13; ottenuto: 358.31 [M+H$^+$].

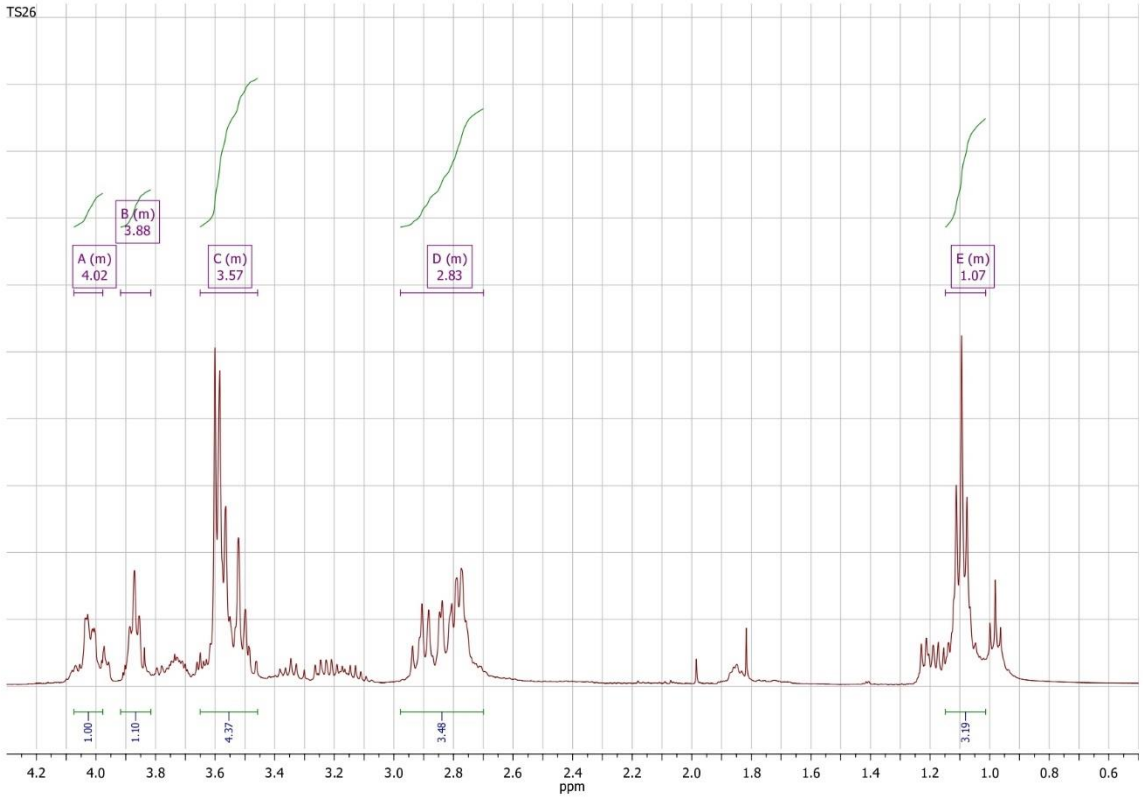
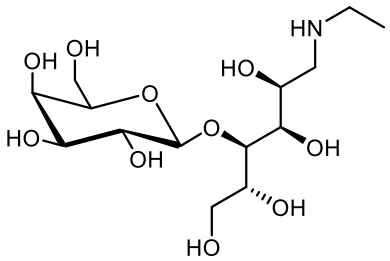


Figure S7: ^{1}H-NMR (D_2O) of 1-deoxy-1-(methylamino)-D-lactitol (**4a**)

Spectra of compound 4b: ^{1}H and ^{13}C NMR; ESI-MS; COSY, HSQC and HMBC NMR



1-deoxy-1-(ethylamino)-D-lactitol (**4b**)

^{1}H NMR (400 MHz, D_2O): δ 4.45 (d, J = 7.8 Hz, 1H, **H$^{1''}$**), 4.07 (m, 1H, **H$^{2'}$**), 3.88 (m, 2H, **H$^{5'}$**, **H$^{4''}$**), 3.84 – 3.77 (m, 2H, **H$^{4'}$**, **H$^{6'a}$**), 3.77 – 3.70 (m, 4H, **H$^{3'}$**, **H$^{6'b}$**, **H$^{6''}$**), 3.64 (m, 1H, **H$^{5''}$**), 3.58 (m, 1H, **H$^{3''}$**), 3.49 (dd, J = 9.9, 7.8 Hz, 1H, **H$^{2''}$**), 3.04 (dd, J = 12.6, 3.0 Hz, 1H, **H$_2$$^{1'a}$**), 2.84 (dt, J = 12.5, 8.5 Hz, 3H, **H$_2$$^{1'b}$**, NHC**H$_2$**CH$_3$), 1.24 – 1.09 (m, 3H, C**H$_3$**).

^{13}C NMR (100 MHz, D_2O): δ 102.83 (**C$^{1''}$**), 78.65 (**C$^{4'}$**), 75.29 (**C$^{5''}$**), 72.42 (**C$^{3''}$**), 70.97 (**C$^{5'}$**), 70.95 (**C$^{2''}$**), 70.63 (**C$^{3'}$**), 69.09 (**C$^{2'}$**), 68.72 (**C$^{4''}$**), 61.97 (**C$^{6'}$**), 61.09 (**C$^{6''}$**), 49.82 (**C$^{1'}$**), 43.12 (NH**C**H$_2$CH$_3$), 11.77 (**C**H$_3$).

MS (ESI$^+$): *m/z* theoretical $[C_{14}H_{29}NO_{10}]^+$: 372.18; obtained: 372.34 [M+H$^+$].

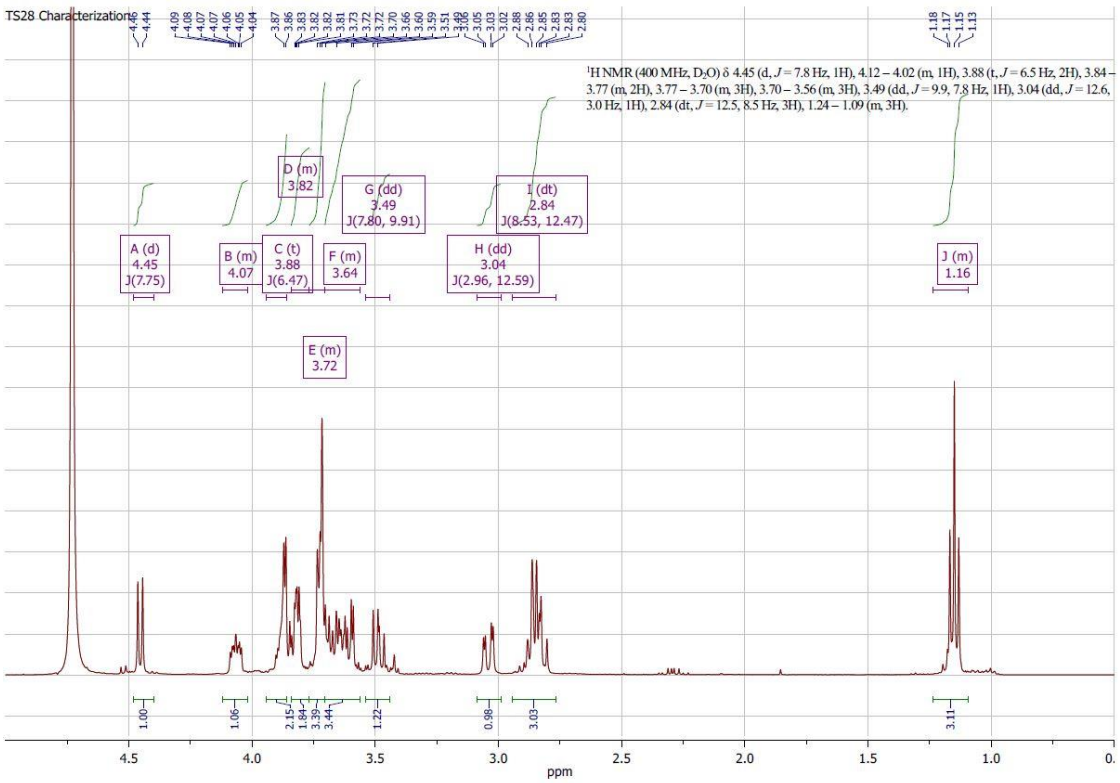


Figure S8: ^{1}H-NMR (D_2O) of 1-deoxy-1-(ethylamino)-D-lactitol (**4b**)

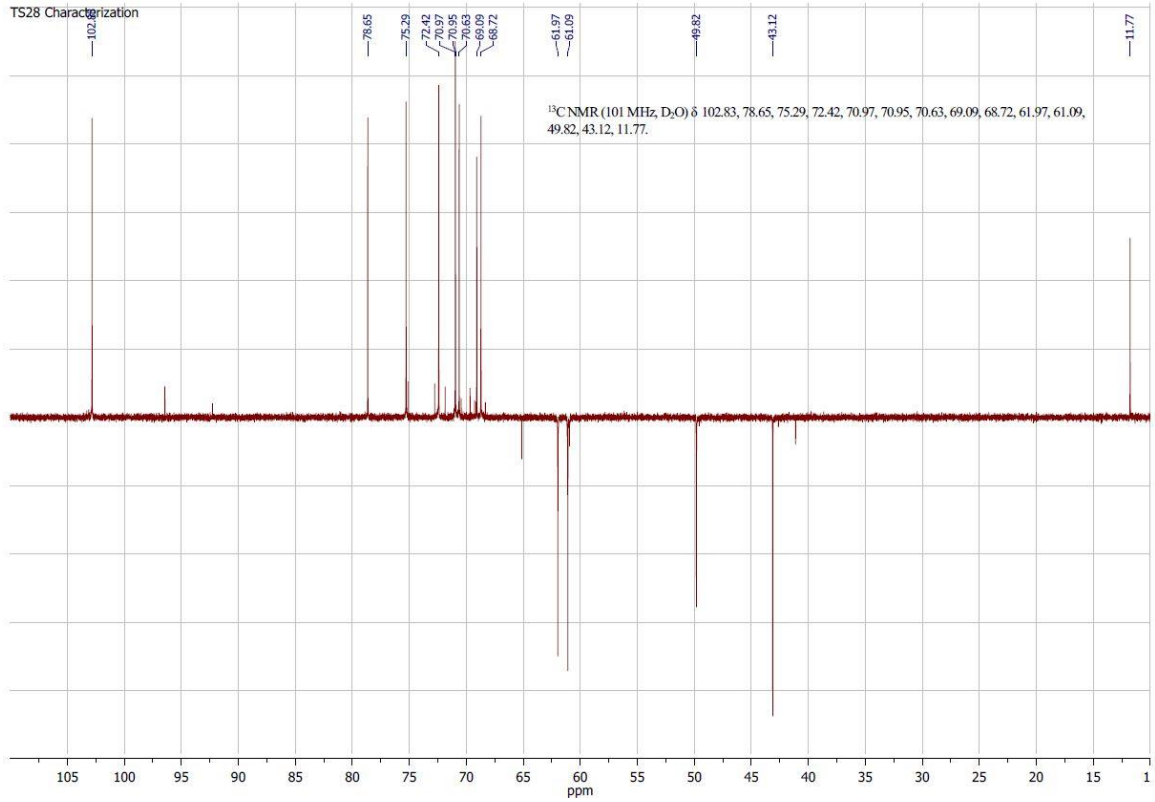


Figure S9: ^{13}C (D_2O) of 1-deoxy-1-(ethylamino)-D-lactitol (4b)

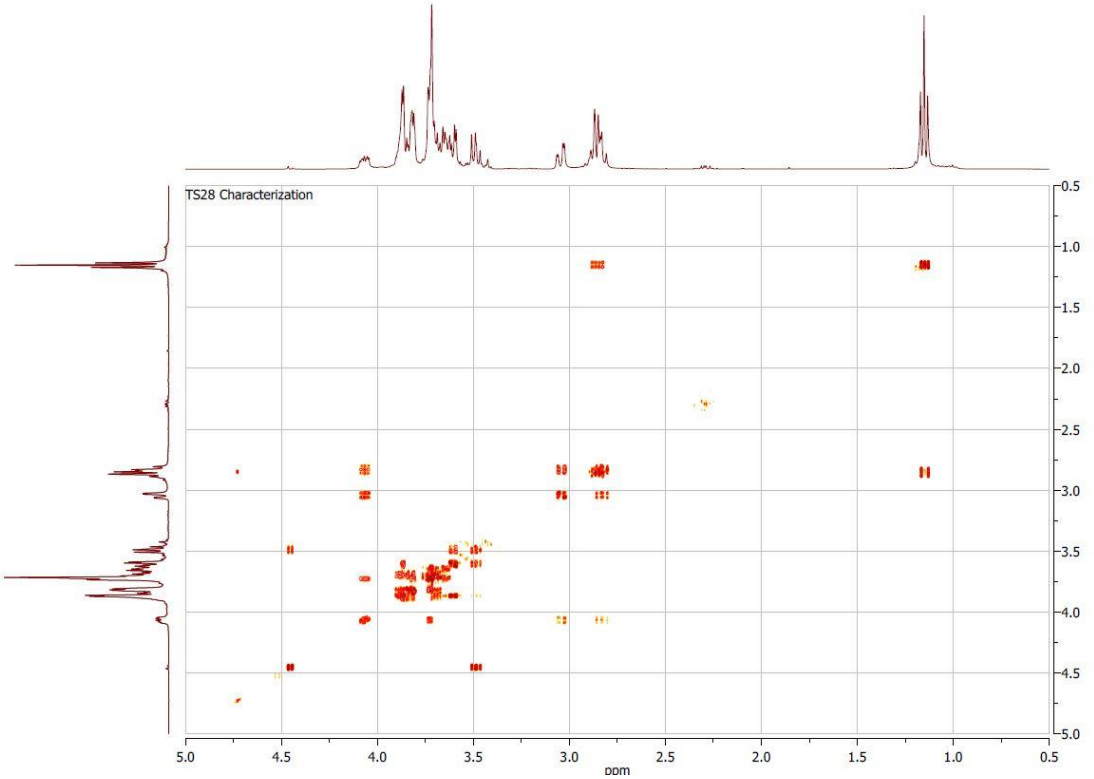


Figure S10: COSY (D_2O) of 1-deoxy-1-(ethylamino)-D-lactitol (**4b**)

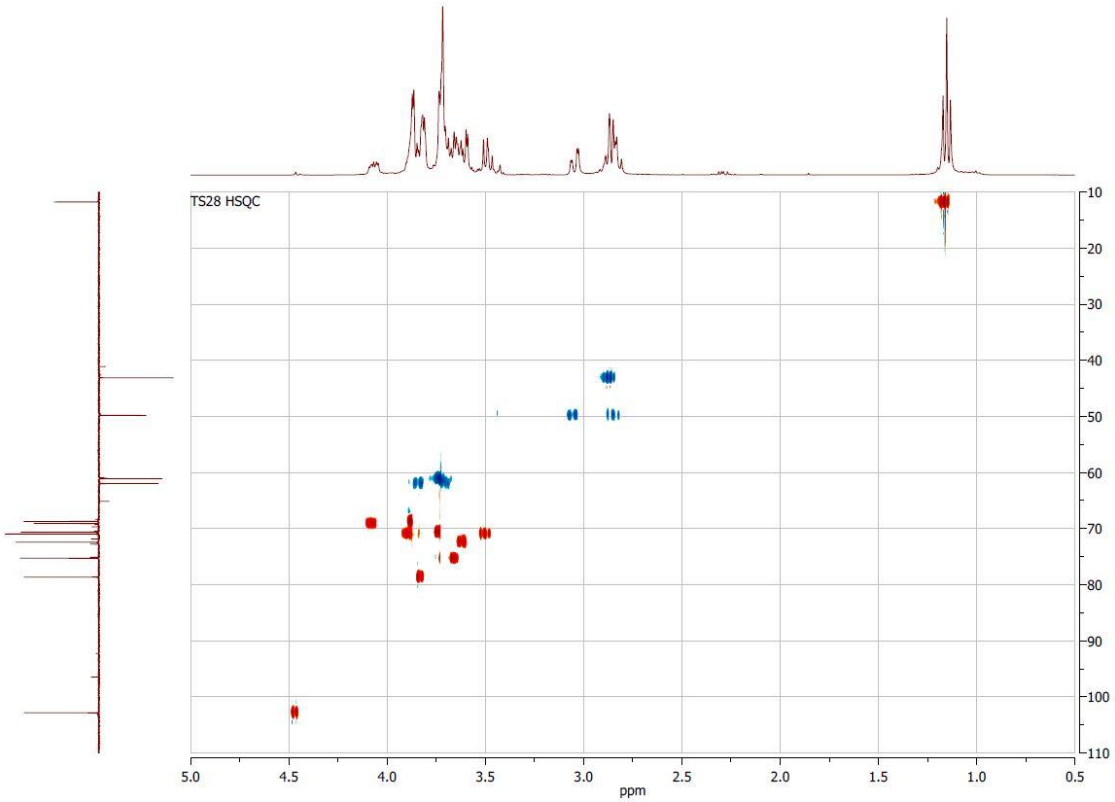


Figure S11: HSQC (D_2O) of 1-deoxy-1-(ethylamino)-D-lactitol (**4b**)

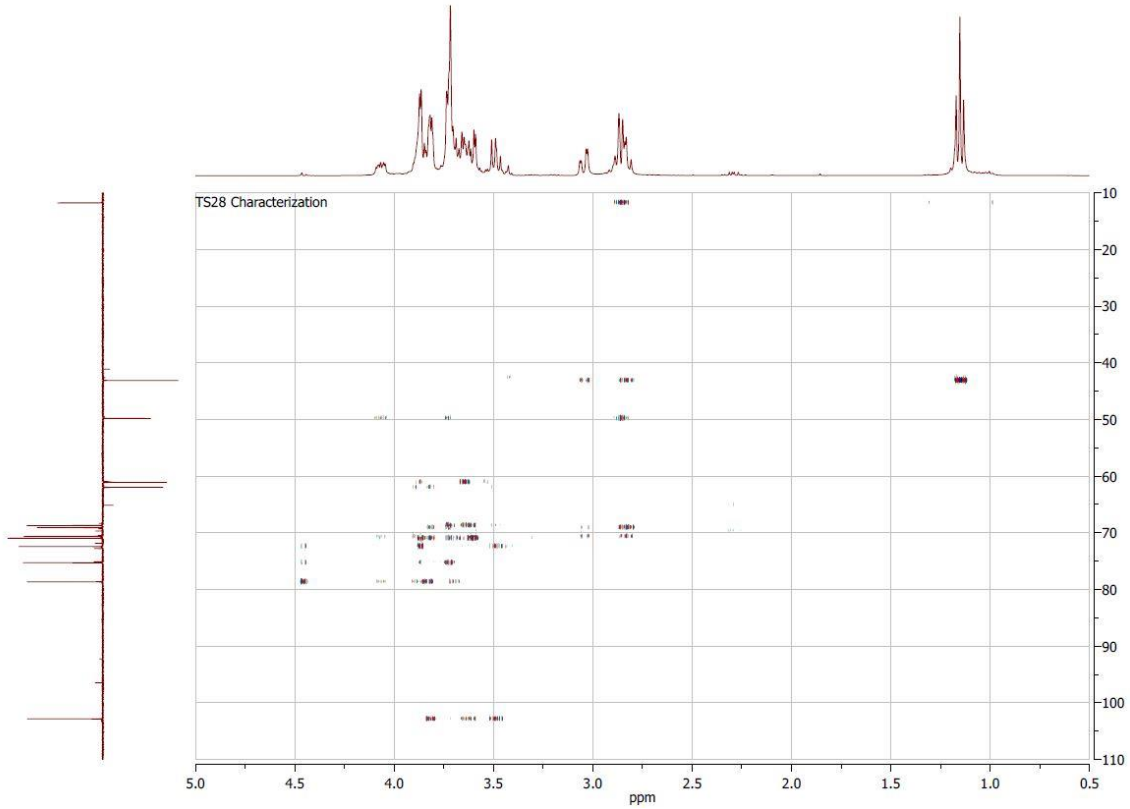
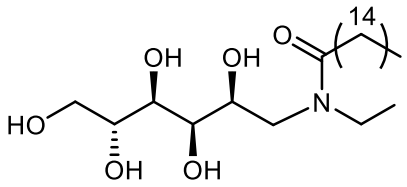


Figure S12: HMBC (D_2O) of 1-deoxy-1-(ethylamino)-D-lactitol (**4b**)

Spectra of compound 6: ^{1}H and ^{13}C NMR; ESI-MS; COSY, HMBC and HSQC NMR



N-palmitoyl-*N*-ethyl glucamide (**6**)

^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ 3.73 ((m, 1H, C**H**OH), 3.62 - 3.56 (m, 2H, C**H**OH, C**H**$_2^a$OH), 3.51 (br s, 1H, C**H**OH), 3.48 - 3.43 (m, 2H, C**H**OH, CONC**H**$_2^a$CHOH), 3.42 - 3.37 (m, 1H, C**H**$_2^b$OH), 3.36 - 3.28 (m, 2H, CONC**H$_2$**CH$_3$), 3.19 (m, 1H, CONC**H**$_2^b$CHOH), 2.34 - 2.23 (m, 2H, C**H$_2$**CONR$_2$), 1.53 - 1.42 (br t, 6H, C**H$_2$**CH$_2$CONR$_2$), 1.24 (s, 71H, (C**H$_2$**)$_{12}$), 1.09 (t, J = 6.9 Hz, 3H, CONCH$_2$C**H$_3$**), 0.86 (t, J = 6.6 Hz, 9H, (CH$_2$)$_{12}$C**H$_3$**).

^{13}C NMR (100 MHz, DMSO-*d*$_6$): δ 172.37 (**C**ONR$_2$), 72.26 (**C**HOH), 71.87(**C**HOH), 70.34 (**C**HOH), 69.68 (**C**HOH), 63.80 (**C**H$_2$OH), 49.66 (N**C**H$_2$CH$_3$), 48.78 (**N**CH$_2$CHOH), 32.80 (**C**H$_2$CONR$_2$), 31.76 - 29.02 ((**C**H$_2$)$_{12}$), 25.45 (**C**H$_2$CH$_2$CONR$_2$), 14.41 (CONCH$_2$**C**H$_3$), 14.29 ((CH$_2$)$_{12}$**C**H$_3$).

Residual impurity of palmitic acid:

^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ 2.18 (t, J = 7.4 Hz, 4H, C**H$_2$**COOH), 1.53 - 1.42 (br t, 6H, C**H$_2$**CH$_2$COOH), 1.24 (s, 71H, (C**H$_2$**)$_{12}$), 0.86 (t, J = 6.6 Hz, 9H, (CH$_2$)$_{12}$C**H$_3$**).

^{13}C NMR (100 MHz, DMSO-*d*$_6$): δ 174.93 (**C**OOH), 34.13 (**C**H$_2$COOH), 31.76 - 29.02 ((**C**H$_2$)$_{12}$), 24.96 (**C**H$_2$CH$_2$COOH), 14.29 ((CH$_2$)$_{12}$**C**H$_3$).

MS (ESI$^+$):*m/z* theoretical [C$_{24}$H$_{50}$NO$_6$]$^+$: 448.36; obtained: 470.87 [M+Na$^+$], 917.59 [2M+Na$^+$].

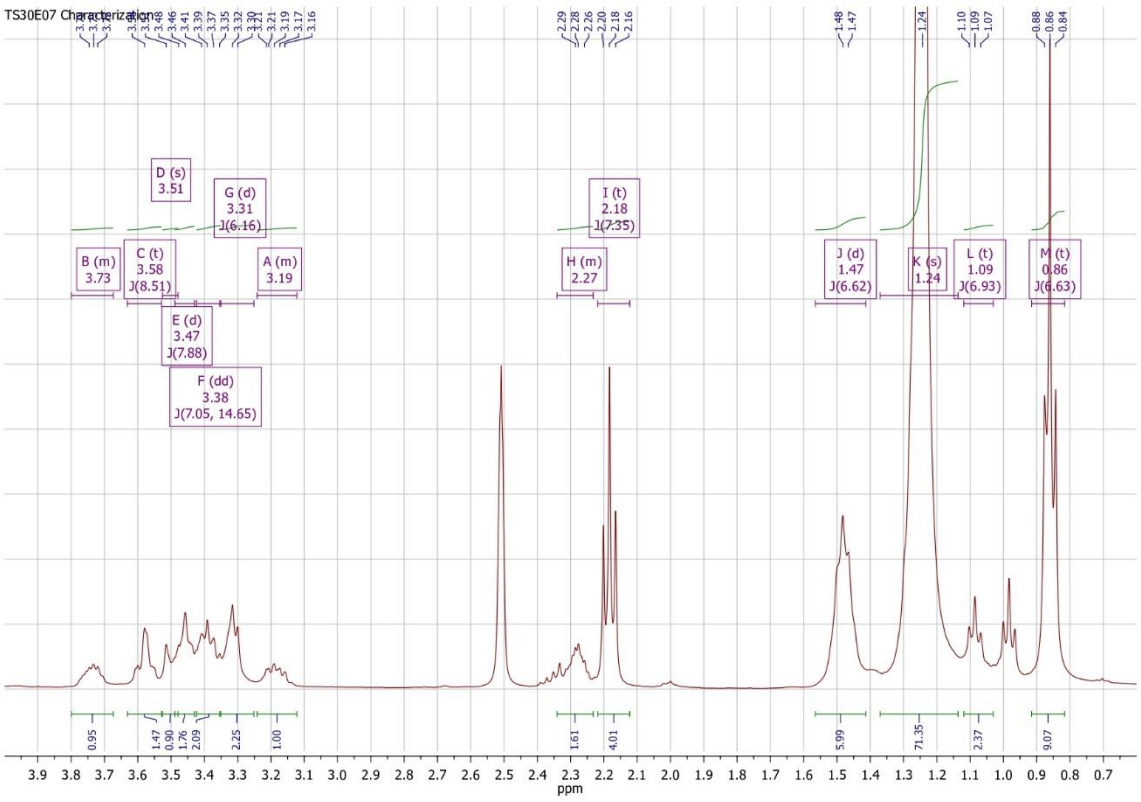


Figure S13: ^{1}H-NMR (DMSO-d_6) of *N*-palmitoyl-*N*-ethyl-glucamide (**6**)

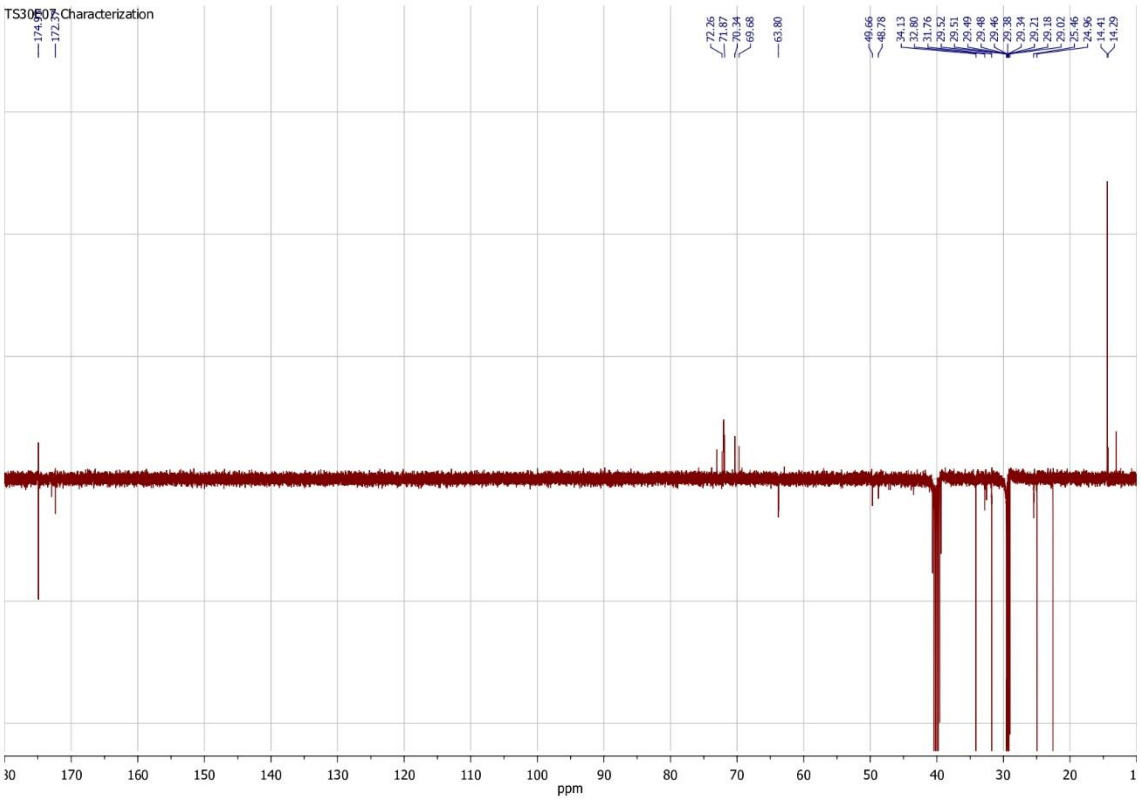


Figure S14: ^{13}C-NMR (DMSO-d_6) of *N*-palmitoyl-*N*-ethyl-glucamide (**6**)

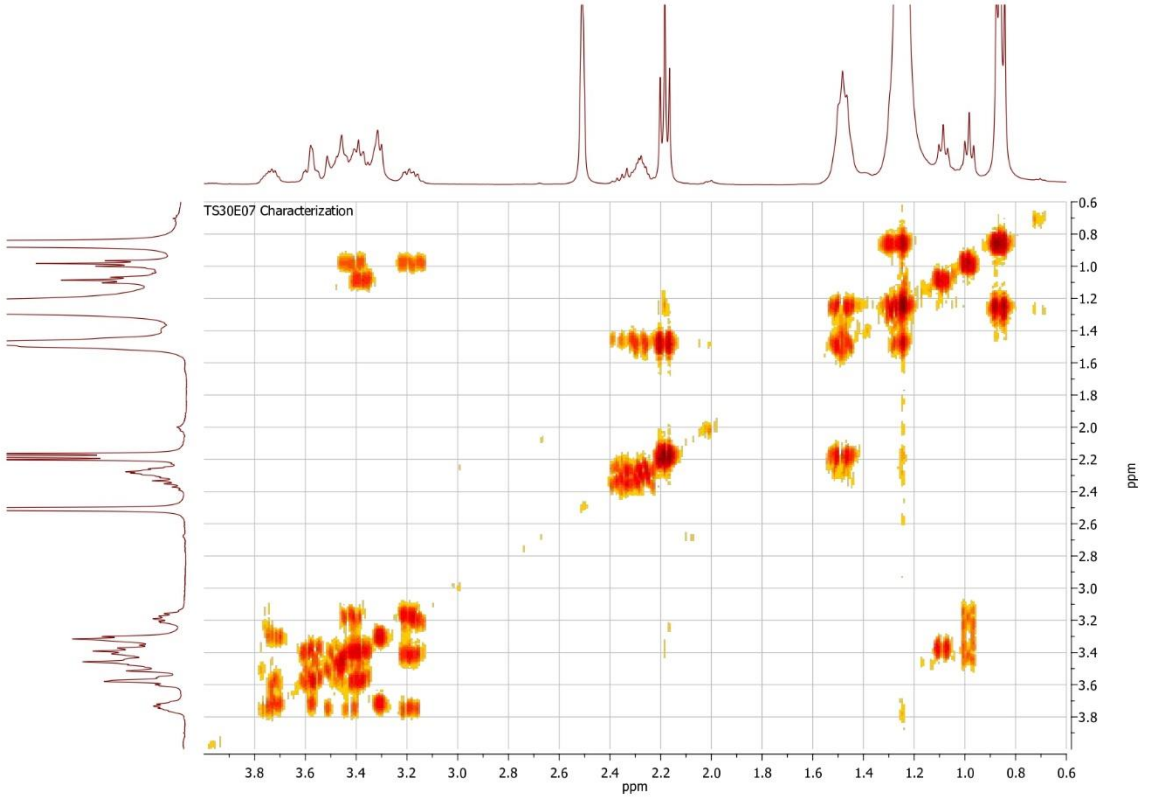


Figure S15: COSY (DMSO-d_6) of *N*-palmitoyl-*N*-ethyl-glucamide (**6**)

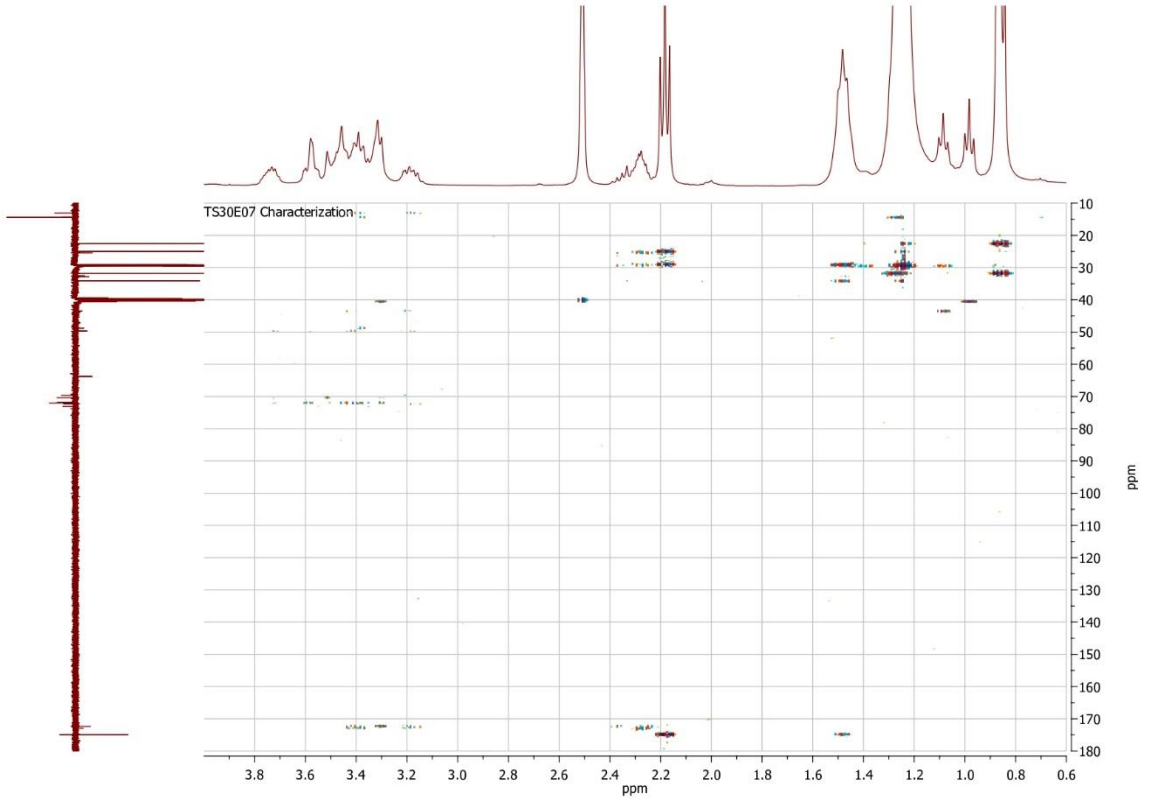


Figure S16: HMBC (DMSO-d_6) of *N*-palmitoyl-*N*-ethyl-glucamide (**6**)

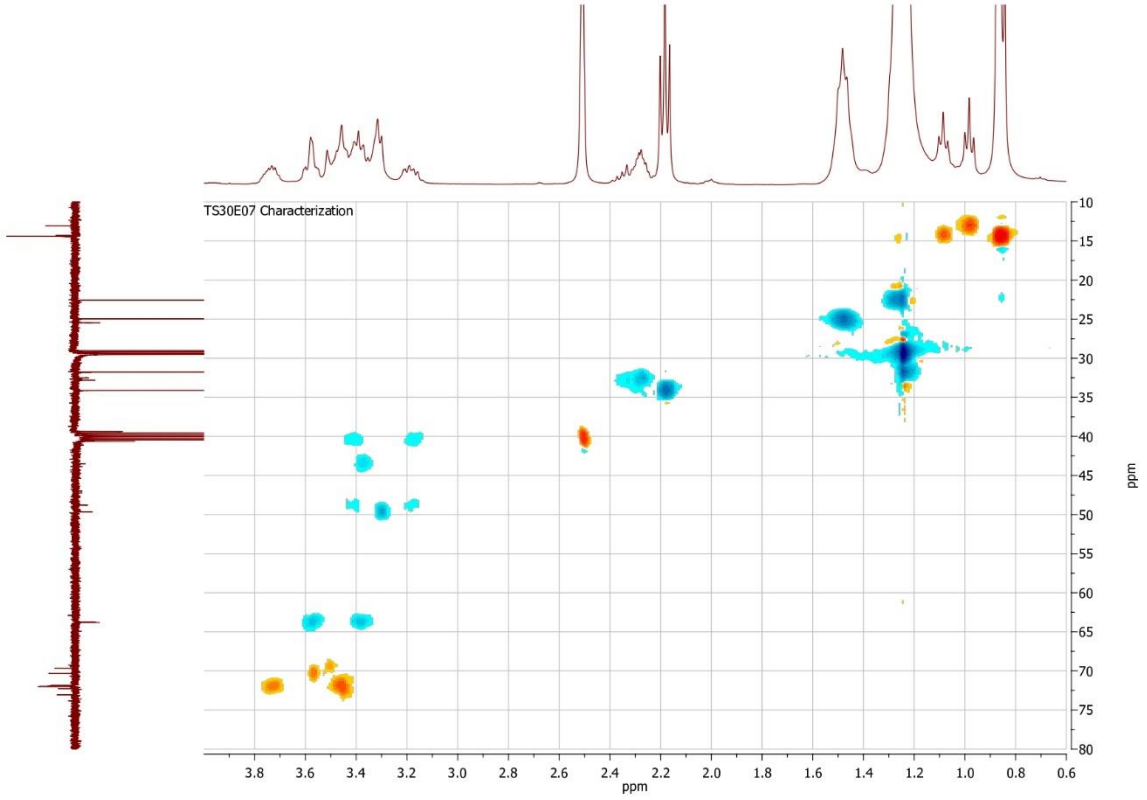


Figure S17: HSQC (DMSO-d_6) of *N*-palmitoyl-*N*-ethyl-glucamide (**6**)

Chapter 4: Conclusions, Collaborations & Acknowledgements

4.1 Conclusions

The core topic of this Thesis was the upgrade of renewable feedstock through biocatalysis. Both the selected starting materials (sunflower lecithin and cheese whey permeate) were successfully transformed into high-value chemicals, even if all the processes developed herein still need to be optimized. As for cheese whey permeate, the enzymatic process applied to commercial lactose (**Chapter 3.2**) as a "proof of concept" has been successfully applied to this biomass and the optimization of the whole bioprocess is currently in progress (reaction and downstream conditions, productivity, screening of new β-galactosidases and lipases as well as immobilization techniques and renewable carriers). To sum up:

Chapter 2 was dedicated to the upgrade of sunflower lecithin, a complex mixture of phospholipids, through its transformation into glycerophosphoinositol (GPI), a natural anti-inflammatory agent, and glycerophosphocholine (GPC), a cognitive enhancer. Although lecithin has an established use as emulsifier in many market sectors, our goal was to get higher-value molecules to enlarge the portfolio of bio-based products derived from this biomass.

- **Chapter 2.1**: The "state of the art" on the chemical and enzymatic syntheses of glycerophosphoric acid esters (GPAE) as well as their biological properties and applications were reviewed to give a general overview on the relevance of these molecules and the challenge of their synthesis, especially in the case of GPI.
- **Chapter 2.2**: New chemoenzymatic processes were developed for the preparation of GPI and GPC from sunflower lecithin. This biomass was pre-treated by solvent fractionation and chemical modification to obtain fractions enriched in phosphatidylcholine and phosphatidylinositol, which are precursors of GPC and GPI, respectively, before the biocatalytic step. GPC was obtained in 30% isolated yield (not optimized) by enzymatic hydrolysis of the ethanol soluble fraction of lecithin (enriched in phosphatidylcholine) by using a commercial immobilized lipase B from *Candida antarctica* (Novozym® 435) in *tert*-butanol, which is recognized as a "preferred" solvent. GPI was obtained in 20% isolated

yield (not optimized) by enzymatic hydrolysis in a fully aqueous medium by using a commercial phospholipase A1. To the best of our knowledge, this latter process represents the first synthesis of GPI by enzymatic hydrolysis of lecithin.

- **Chapter 2.3**: A key-intermediate (namely, 2-*O*-acetyl-3,4,5,6-tetra-*O*-benzyl-D-*myo*-inosityl diphenylphosphate) for the synthesis of inositol phosphate derivatives, including GPI, was synthesized and characterized. This research, which further highlighted the complexity of this class of molecules in terms of their preparation, analytical characterization, and isolation, was performed in parallel with the enzymatic hydrolysis of lecithin with the aim to obtain GPI both through a new enzymatic route and a new chemical route as well. Biocatalysis and "conventional" synthetic approaches often walk "hand-in-hand" (and it should be always like that) because they should be envisioned as complementary rather than alternative tools. Moreover, the (re)design of a synthetic scheme by biocatalysis should not be separated from the knowledge of the "conventional" routes to the desired product. Generally speaking, such an assessment allows to weigh up *pros* and *cons* towards the "best" solution (biocatalysis, "conventional" synthesis, or a combination of both), depending on the specific goal. On the way of this research, the biocatalytic route resulted to be far more promising (in spite of the tricky process for product downstream) because of lesser steps, more environmentally friendly conditions, the exploitation of a cheap and abundant biomass; therefore, most of the efforts were eventually concentrated towards it.

Chapter 3 was focused on the synthesis of sugar-based surfactants for food and personal care/cosmetic applications starting from lactose and its hydrolysis products (glucose and galactose) in view of using cheese whey permeate, a crude lactose solution derived from the dairy industry, as a source of the above sugar building blocks, according to the circular economy concept. Cheese whey is a source of proteins and lactose therefore it can be considered a "by-product" rather than a "waste". However, cheese whey production exceeds by far the request of protein and lactose market, thus turning into a true waste with a high environmental impact.

- **Chapter 3.1**: Glucose-based surfactants were synthesized by a two-step chemoenzymatic process. Glucose modification into *n*-butyl glucoside (obtained as an isomeric mixture α/β furanosides + α/β pyranosides) by Fischer glycosidation (90% yield) allowed to achieve a less polar derivative of glucose that could be enzymatically reacted with fatty acids (C12-C16-C18) in a solvent-free system to give glucose-based SFAE (39-65% yields) having emulsifying properties.
- **Chapter 3.2**: A library of alkyl-β-D-galactosides was synthesized by reacting lactose (as well as cheese whey permeate) with naturally occurring alcohols through a transglycosylation reaction catalyzed by the covalently immobilized β-galactosidase from *Aspergillus oryzae* in a ternary system (20-50% yield). In contrast with the Fischer glycosidation of galactose (and glucose, see **Chapter 3.1**), the enzymatic route afforded alkyl galactosides as pure β-pyranosides. *n*-Butyl galactosides were esterified with palmitic acid under the same conditions reported for *n*-butyl glucosides (**Chapter 3.1**) in modest yield (15%). *n*-Butyl galactoside fatty acid esters were shown to generally possess promising interfacial features, although to a different extent; it is worth mentioning that *n*-butyl 6-*O*-palmitoyl-β-D-galactopyranoside, which was prepared by a sequential two-step enzymatic reaction from lactose, led to the most stable emulsion. Having different interfacial properties, anomerically pure and isomeric mixtures of galactoside-based SFAE provide a palette of surfactants that could be used as single ingredients or in combination to tune the physicochemical properties of the final product.
- **Chapter 3.3**: With glucose- and galactose-based SFAE in our hands, the next step was to face what we realized soon to be a true challenge, that is the enzymatic preparation of lactose-based SFAE. Unlike glucose and sucrose esters, lactose-based SFAE have been poorly investigated to date. One of the reasons is the burden associated to their preparation owing to the striking different solubility of lactose and the fatty acid "tail". Therefore, the knowledge about surfactant properties of lactose-based SFAE is lacking as

well. In the attempt to fill this gap, the application of deep eutectic solvents (DES) was explored.

The lipase-catalyzed (trans)esterification of lactose with a number of acyl donors (fatty acids or vinyl esters) was investigated in DES-based systems (e.g. ChCl:U) that were previously reported for the synthesis of glucose-based SFAE. Moreover, the hydrophilic ChCl:lactose "2-in-1" DES system and the hydrophobic Lid:PA combination were assayed, too. The formation of lactose esters was not detected in none of the DES used, but only in a "conventional" system composed of pyridine/THF (benchmark reaction). Computational solubility studies and molecular dynamics (MD) simulations of both reaction media showed that the interaction of DES components with lactose affects its conformation in such a way that the enzymatic reaction may result to be impaired, regardless of the acyl donor used. The unexpected formation of the fatty acid amide (from urea) was observed as a side reaction. Moreover, large acyl donors (fatty acids or vinyl esters higher than C10) displayed a poor solubility in DES and thus did not react even with glucose.

One of the "take home" messages of this work was that the rationalization of "negative" (if referred to the main goal of the study) results can assist in gaining a knowledge which in turn feeds new advancements. In this case, the expectation/perspective is starting from these results to explore and/or design new deep eutectic solvents for the synthesis of glycolipids. The combination of experimental and computational methods can drive the research towards a more rational use of non-conventional media in biocatalysis.

- **Chapter 3.4**: As an alternative to the use of biocatalysis in non-conventional media, the (trans)esterification of lactose by organocatalysis was also explored. Three different α-hydroxycarboxylic acids (DL-lactic acid, L-tartaric acid and citric acid monohydrate) were screened as organocatalysts in neat acyl donors (VP) and in DES (ChCl:U). In none of the tested conditions the target molecule was obtained. This investigation, however, barely scratched the surface of this topic and deserves being further explored.

- **Chapter 3.5:** Reductive amination was evaluated as a further option to transform sugars into less polar derivatives to be used in the preparation of sugar-based surfactants. Also in this case, the first strategy was to set-up a biocatalytic process. To this aim, a small library of (*R*)- and (*S*)-specific ω-transaminases were screened towards arabinose as a benchmark substrate according to literature data but, in this case, none of the assayed enzymes worked out. The chance of using ω-transaminases for the biotransformation of sugars was driven not only by literature, but also by the experience gained during my MSc internship that was focused on the study and development of an immobilized (*S*)-selective ω-transaminase (from *Vibrio fluvialis*) for the (batch and flow) synthesis of an API intermediate (see Appendix 2).

  Lactose and its hydrolysis products (glucose and galactose) were converted into the corresponding *N*-methyl and *N*-ethyl glycamines in moderate to high isolated yields (10-94%) by using the safer 2-picolin borane instead of $NaCNBH_3$. The bottleneck of this work-plan was the following enzymatic acylation with fatty acids (performed, to date, by using *N*-ethyl glucamine as precursor): the reaction in 2M2B occurred very slowly and in a poor yield (80 h, 17% yield), even under flow conditions. The synthetic plan, still in progress, is being directed to the reductive amination of sugars (both in batch and in flow) with longer amines that might modulate the HLB of the resulting glycamines without the need for the acylation step, thus affording a new class of potential surfactants.

4.2 Collaborations & Acknowledgements

The research reported in this Thesis would have not been feasible without the contribution of people from groups with complementary background, expertise, and facilities: **Laboratory of Biocatalysis**, Department of Drug Sciences, University of Pavia, Italy, (*Prof. Daniela Ubiali, Prof. Teodora Bavaro, Dr. Marina S. Robescu, Klodiana Mema*); **Laboratory of Organic Chemistry** and **Laboratory of Physical Chemistry**, Department of Chemistry, University of Milano, Italy, (*Prof. Giovanna Speranza, Prof. Giuseppe Cappelletti, Dr. Sara Sangiorgio, Dr. Eleonora Pargoletti, Dr. Marco Rabuffetti, Mattia Cambò*); **Biocatalysis & Bioprocessing group**, Department of Biological and

Chemical Engineering, Aarhus University, Denmark and Institute of Technical Chemistry, Leibniz University Hannover, Germany (*Prof. Selin Kara, Dr. Santiago Nahuel Chanquia*); **Sustainable Momentum S. L**, Las Palmas de Gran Canaria, Spain (*Prof. Pablo Domínguez de María*); **Institute of Technical Chemistry**, Leibniz University Hannover, Germany (*Dr. Jan Philipp Bittner*); **Hamburg University of Technology**, Institute of Thermal Separation Processes, Hamburg, Germany (*Dr. Simon Müller*).

A 6-month internship (Oct. 2021-March. 2022) was spent in the Biocatalysis & Bioprocessing group, headed by Prof. Selin Kara (Department of Biological and Chemical Engineering, Aarhus University, Denmark); most of the research described in **Chapters 3.3-3.4** was performed in this frametime.

Financial support from Cariplo Foundation (Italy) is gratefully acknowledged. Specifically, **Chapter 2** reports some of the results of the BIOCOSM project (*Biocatalysis for oils and fats in cosmetics*), "Integrated research on industrial biotechnologies and bioeconomy-joint Call 2017 – ID 2017-0978". **Chapter 3** reports some of the results of the BioSurf project (*Integrated platform for the sustainable production of bio-based surfactants from renewable resources*), "Bando Economia Circolare: ricerca per un futuro sostenibile – ID 2020-1094" – https://www.biosurfproject.it/).

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Dr. Jan Philipp Bittner (Institute of Technical Chemistry, Leibniz University Hannover, Germany) and Dr. Simon Müller (Hamburg University of Technology, Institute of Thermal Separation Processes, Hamburg, Germany) are gratefully acknowledged for computational simulations reported in **Chapter 3.3**.

Appendix 1: Permissions

Chemical and Enzymatic Approaches to Esters of sn-Glycero-3-Phosphoric Acid

Author: Giovanna Speranza, Daniela Ubiali, Marco Rabuffetti, et al

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Emulsifying properties of sugar-based surfactants prepared by chemoenzymatic synthesis

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Appendix 2: Papers

During the 3-year PhD program, two more papers were published besides those reported and discussed in this Thesis. Both these papers are partly connected to the core topic of this Thesis, being related to biocatalysis, but the developed biotransformations were not applied to renewable substrates in this case. A brief discussion about these papers is reported below.

The first paper (*ChemCatChem* 2020, 12, 1359–1367) reports the covalent immobilization of the (*S*)-selective transaminase from *Vibrio fluvialis* (*Vf*-ATA) on glyoxyl agarose and the application of this immobilized biocatalyst to the synthesis, under flow conditions, of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine, a chiral building block of the JAK2 kinase inhibitor AZD1480. The immobilized *Vf*-ATA (activity recovery=30%) was used in a packed-bed reactor to set-up a continuous flow biotransformation coupled with a straightforward in-line purification to circumvent the two-step process previously described in literature for the batch reaction.[1] The newly developed biotransformation was run in a homogeneous system including dimethyl carbonate as a green co-solvent. The optically pure amine (ee >99%) was isolated in 35% yield by a in line "catch and release" system based on an ion exchange resin.

This research was carried out within the BIOFLOW project (*BIOFLOW: an innovative platform for the in-flow biocatalytic preparation of high value chemicals*), "Integrated research on industrial biotechnologies 2016– ID 2016-0731", funded by Cariplo Foundation (Italy), during my MSc internship in the Laboratory of Biocatalysis (University of Pavia, Italy). I performed the whole immobilization study and the reactions in batch. Moreover, I collaborated to the production of the enzyme at SCITEC-CNR (Milano, Italy) under the supervision of Dr. Ferrandi and Dr. Monti, and I participated in the preliminary set-up of the flow reactor under the supervision of Prof. Tamborini (University of Milano, Italy).

The second paper (*Catalysts* 2020, 10, 260) reports the immobilization of OYE3, an ene-reductase. Two different immobilization techniques were tested: the covalent immobilization on glyoxyl agarose (GA), and the affinity-based adsorption on EziG^{TM} particles. The activity recovery was comparable (≈50%) for both GA and EziG^{TM}, but the

covalently immobilized enzyme resulted more stable under the activity assay conditions (50 mM phosphate buffer pH 7, 28 °C) and was successfully used for the bioreduction of α-methyl-*trans*-cinnamaldehyde (analytical scale); the immobilized OYE was recycled and re-used for 12 reaction runs. Also in this project, my contribution was about the study of enzyme immobilization.

Both transaminases and ene-reductases are recognized as powerful tools in organic chemistry.[2,3] As previously discussed (see **Chapter 3.5**), transaminases are nowadays entered the established toolbox for the synthesis of enantiopure amines, also at industrial scale. The chemoenzymatic process developed by Merck and Codexis a decade ago for the synthesis of sitagliptin placed a milestone in this frame.[4]

On the other hand, ene-reductases catalyze the stereospecific reduction of activated prochiral C=C double bonds, thus allowing the generation of up to two stereogenic centers within a product. This feature makes ene-reductases a very attractive alternative to conventional asymmetric synthetic schemes that are frequently characterized by many steps, low yields, and a high environmental impact.

(1) Meadows, R. E.; Mulholland, K. R.; Schürmann, M.; Golden, M.; Kierkels, H.; Meulenbroeks, E.; Mink, D.; May, O.; Squire, C.; Straatman, H.; Wells, A. S. Efficient Synthesis of (S)-1-(5-Fluoropyrimidin-2-Yl)Ethylamine Using an ω-Transaminase Biocatalyst in a Two-Phase System. *Org. Process Res. Dev.* **2013**, *17* (9), 1117–1122. https://doi.org/10.1021/op400131h.

(2) Toogood, H. S.; Scrutton, N. S. Discovery, Characterization, Engineering, and Applications of Ene-Reductases for Industrial Biocatalysis. *ACS Catal.* **2018**, *8* (4), 3532–3549. https://doi.org/10.1021/acscatal.8b00624.

(3) Kelly, S. A.; Pohle, S.; Wharry, S.; Mix, S.; Allen, C. C. R.; Moody, T. S.; Gilmore, B. F. Application of ω-Transaminases in the Pharmaceutical Industry. *Chem. Rev.* **2018**, *118* (1), 349–367. https://doi.org/10.1021/acs.chemrev.7b00437.

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Use of Immobilized Amine Transaminase from *Vibrio fluvialis* under Flow Conditions for the Synthesis of (*S*)-1-(5-Fluoropyrimidin-2-yl)-ethanamine

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We report on the covalent immobilization of the (*S*)-selective amine transaminase from *Vibrio fluvialis* (*Vf*-ATA) and its use in the synthesis of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine, a key intermediate of the JAK2 kinase inhibitor AZD1480. Immobilized *Vf*-ATA on glyoxyl-agarose (activity recovery: 30%) was used in a packed-bed reactor to set-up a continuous flow biotransformation coupled with a straightforward in-line purification to circumvent the 2-step process described in literature for the batch reaction. The newly developed biotransformation was run in a homogeneous system including dimethyl carbonate as a green co-solvent. Optically pure (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (ee >99%) was isolated in 35% yield.

Introduction

Amine transaminases (ATAs, EC 2.6.1.x) catalyze the enantioselective amination of ketones by utilizing simple amines as amino donors and the vitamin B_6-based pyridoxal 5-phosphate (PLP) cofactor as a molecular shuttle.[1–4] Since the first characterization of these enzymatic activities in the late 90s,[5] ATAs have been intensively investigated for the synthesis of chiral amines. These molecules are valuable building blocks for the preparation of APIs including antiarrhythmics (e.g. vernakalant), antibiotics (e.g. moxifloxacin), anti-epilepsy agents (e.g. levetiracetam and pregabalin), and antidiabetics (e.g. sitagliptin).[6] Among the examples of ATAs application on an industrial scale,[7–12] a milestone was placed by Merck and Codexis 10 years ago with the synthesis by transamination of sitagliptin (Januvia®), the first marketed oral antihyperglycemic drug belonging to the gliptin family. This bioprocess allowed the replacement of a high-pressure rhodium-catalyzed asymmetric enamine hydrogenation step by applying a highly engineered ATA variant.[7] Later on, a number of reports about ATA immobilization for the synthesis of this API were released.[13–16] These outstanding achievements have demonstrated that, if optimized, ATA-based enzymatic platforms can be competitive with the established manufacture processes, since align selectivity, efficiency and sustainability.[17]

Another example of a pre-industrial ATA-catalyzed process was developed by AstraZeneca for the production of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (**3**, Scheme 1A), a key intermediate in the synthesis of the JAK2 kinase inhibitor AZD1480, a molecule targeting idiopathic myelofibrosis and polycythaemia rubra vera.[9,18] The well-known ATA from *Vibrio fluvialis* (*Vf*-ATA)[19–22] was selected among 60 transaminases; process conditions were optimized on a gram scale starting from the fluorinated ketone (**1**) and using (*S*)-α-methylbenzylamine (**2**, (*S*)-MBA) as the amino donor.

The reaction was performed in a biphasic system by using toluene to pull out the co-product acetophenone (**4**). The continuous extraction was necessary to shift reaction equilibrium and to avoid product inhibition effects.[18] Since the synthesized amine is water soluble, the purification protocol relies on the conversion of the amine to the corresponding Boc-derivative followed by extraction and precipitation of the amine hydrochloride with HCl in *iso*-propanol. Besides co-product removal through the use of a biphasic system as described above, unfavorable reaction equilibrium of ATA-catalyzed amine synthesis as well as product inhibition suffered by these enzymes have been managed in different ways. The use of amino donors in large excess combined with the *in situ* removal of amine-derived by-products have been pursued, either

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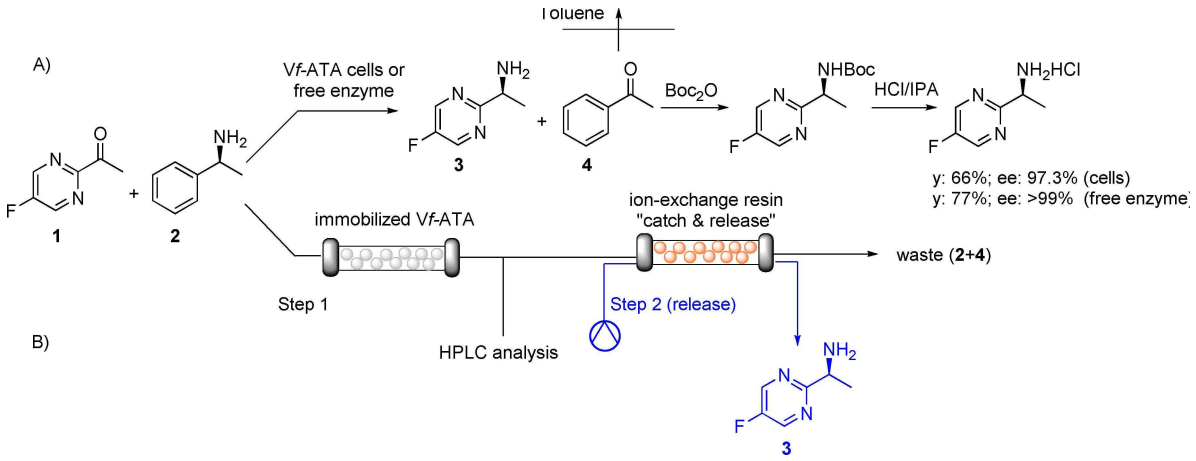


Scheme 1. Synthesis of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (3) catalyzed by *Vf*-ATA. A) Synthetic route as reported in Ref. [18]. B) Synthetic route investigated in the present work.

through an ancillary enzymatic reaction, or by exploiting specific diamines generating by-products that spontaneously undergo cyclization/tautomerization.[23–24]

Given the increasing evidences supporting the use of biocatalysis for industrial scale synthesis of APIs, a number of innovative technologies have been considered as complementary tools for the development of intensified and industrially relevant biocatalytic processes. In particular, there is a growing interest in performing biocatalyzed transformations in continuous flow reactors.[25–28] Both biocatalysis and continuous processing do have the features that make these technologies as the foremost key green research areas for sustainable manufacturing of APIs and fine chemicals. Biocatalysts, either enzymes or whole cells, are typically immobilized onto the reactor wall or on particles of a carrier material, which is then packed into a column to form a packed-bed reactor with a plug-flow behavior.[29] Among the various methods for enzyme immobilization, covalent immobilization is probably the most successful approach, allowing a robust attachment of the enzyme. Powdery materials can be used as carriers and packed in a fixed bed reactor as well as porous monoliths, which have been successfully used for ATA immobilization.[30–31] Flow chemistry, along with enzyme immobilization, can circumvent some critical issues of biocatalytic reactions, thus positively influencing the outcome of the biotransformation.[32] In particular, whereas enzyme immobilization can improve enzyme stability, operating under flow-conditions can significantly reduce substrate/product inhibition effects and push forward unfavorable reaction equilibria through the continuous removal of the product from the reaction site. Moreover, a better process control makes the reaction more efficient and minimizes waste generation.

Based on these premises, we developed a new protocol integrating flow biocatalysis with downstream processing for the end-to-end synthesis of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (**3**), using immobilized *Vf*-ATA as the biocatalyst.

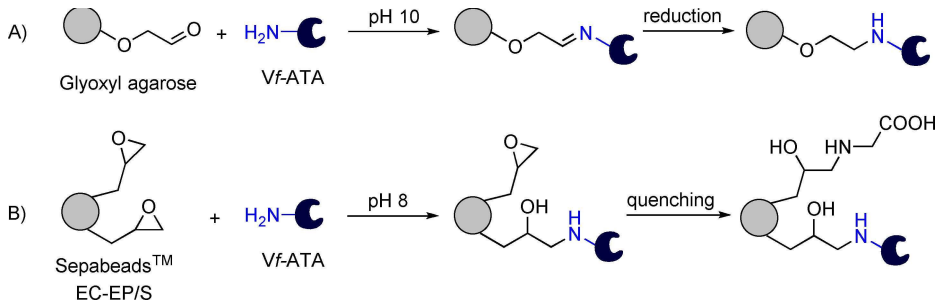
Results and Discussion

The amine transaminase from *Vibrio fluvialis* (*Vf*-ATA) was successfully produced by recombinant expression in *E. coli* BL21 (DE3) cells from the corresponding codon-optimized synthetic gene (GenBank AEA39183.1). Subsequent purification by nickel-nitrilotriacetic acid (Ni-NTA) chromatography afforded 80 mg of pure protein from 1 L culture (Supporting Information, Figure S1). The specific activity of the purified enzyme was determined spectrophotometrically in the transamination reaction between the benchmark substrates (*S*)-MBA (**2**) and pyruvate (Supporting Information, Scheme S1). Two different carriers were chosen for the immobilization: glyoxyl-agarose and Sepabeads™ EC-EP/S. Both carriers rely on the covalent interaction enzyme-support and have been demonstrated to be versatile for the immobilization of many enzymes.[33–34] Moreover, both glyoxyl-agarose and Sepabeads™ EC-EP/S have been successfully applied to biotransformations under flow conditions.[35–36]

Vf-ATA immobilization on glyoxyl-agarose

The immobilization of *Vf*-ATA on glyoxyl-agarose was performed at pH 10 to obtain a "multi-point" interaction through the lysine residues of the enzyme and the aldehyde groups of the carrier. The resulting enzyme-carrier bonds are weak Schiff bases and are thus reversible. Chemical reduction of the immobilized enzyme (usually by $NaBH_4$ or $NaCNBH_3$) is frequently applied[37–38] to convert enzyme-carrier bonds into stable secondary amino groups (Scheme 2A).

This immobilization protocol gave quantitative yields in terms of immobilized protein and immobilized activity: at the endpoint (3 h) neither residual protein nor activity were found in the supernatant, but the activity recovery was very poor (3%).



Scheme 2. *Vf*-ATA immobilization. A) Immobilization on glyoxyl-agarose was performed in sodium bicarbonate (pH 10, 50 mM) under mechanical stirring (protein loading: 2 mg g^{-1}; carrier/total volume: 1 g/10 mL). Experiments were performed at 4 °C or r.t. for 3 h, with or without glycerol (20% v/v). $NaBH_4$ or $NaCNBH_3$ (1 mg mL^{-1}) was used as reducing agent. B) Immobilization on Sepabeads™ EC-EP/S was performed in phosphate buffer (pH 8.0, 50 mM) under mechanical stirring (protein loading: 1 mg g^{-1}; carrier/total volume:1 g/10 mL). Experiments were performed at r.t for 24 h. Quenching was performed with 3 M glycine in phosphate buffer (pH 8.5, 50 mM, 4 mL) under mechanical stirring at r.t. for 20 h.

However, the activity assay performed on the immobilization suspension when the supernatant was already non-active, but before adding the reducing agent, showed activity (data not shown), thus suggesting that the reduction step might be the cause of enzyme inactivation. To confirm whether the reduction was the critical step, three immobilization trials were performed in parallel by following the protocol described in the Experimental Section. When both protein content and activity were negligible in the supernatant (3 h), two aliquots of the same immobilized biocatalyst were reduced with either $NaBH_4$ or $NaCNBH_3$, respectively, whereas the third aliquot was not reduced (Figure 1A). The presence of the cyano group in $NaCNBH_3$ makes this reducing agent milder than $NaBH_4$; in fact, $NaCNBH_3$ is generally used to selectively reduce imines even in presence of aldehydes and ketones under neutral or weakly acid conditions. Although in all cases the activity recovery was $<10\%$, the highest activity recovery was obtained for the non-reduced biocatalyst and when the reduction was performed by using $NaCNBH_3$. Since the reversibility of the imine groups could be a limitation in the use of the immobilized enzyme, $NaCNBH_3$ was selected as the reducing agent for the following immobilization trials. To further increase the activity recovery of the immobilized *Vf*-ATA, the effect of glycerol and temperature were evaluated (Figure 1B).

In fact, glycerol is routinely used to preserve proteins under storage.[39] For this reason, glycerol has been used as an additive in some immobilization procedures; typically, the amount of glycerol does not exceed 20% v/v.[40] Furthermore, a recent report on *Vf*-ATA thermostability[41] showed that this enzyme retains 100% activity at 4 °C for more than 50 h, and 80% activity when it is incubated at 25–37 °C. In the presence of glycerol (20% v/v) and at low temperature (4 °C), the yields in terms of immobilized protein and activity remained quantitative and both the immobilization experiments showed a remarkable increase of the activity recovery (around 30%, Table S1). From these results, 4 °C and $NaCNBH_3$ as the reducing agent were selected for the following immobilization experiments. The activity recovery (30%) was considered suitable to switch from batch to flow applications, in agreement with previous reports.[36] Immobilization results for glyoxyl-agarose are summarized in Figure 1.

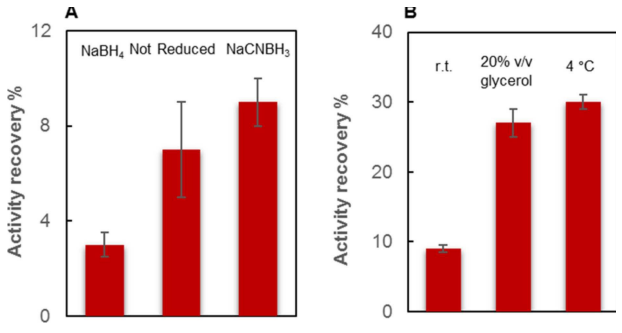


Figure 1. A) Effect of the reducing agent on the activity recovery (observed activity/starting activity)x100. B) Effect of glycerol and temperature on the activity recovery ($NaCNBH_3$). These immobilization experiments were performed with 0.5 g of glyoxyl-agarose in a final volume of 5 mL by using the same protein loading (2 mg g^{-1}), activity loading (4.58 U g^{-1}) and run time (3 h). For the reduction step, 5 mg of the reducing agent were added and the mixture was stirred for further 30 min.

Stability assay of *Vf*-ATA immobilized on glyoxyl-agarose

The immobilized enzyme must be active at least for the time required by the reaction to be completed. However, it is desirable that the biocatalyst maintains its activity for a longer period, thus allowing its reuse for more reaction cycles. *Vf*-ATA immobilized on glyoxyl-agarose (at 4 °C and reduced with $NaCNBH_3$) was incubated with the reaction mixture containing all the reactants with the exception of the prochiral ketone (**1**, amino acceptor) due to its high cost. The immobilized biocatalyst maintained about 50% of the starting activity after 72 h (Figure 2).

Although a striking difference of stability between native and immobilized enzyme did not emerge, the clear advantage of the immobilized biocatalyst is its easier separation from the reaction medium and application for in continuum biotransformations.

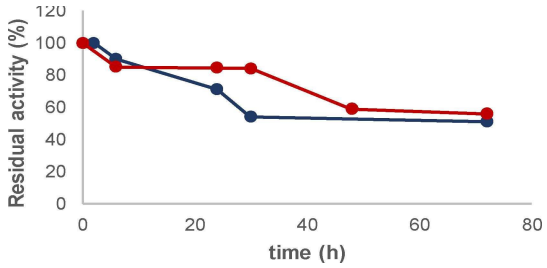


Figure 2. Stability of *Vf*-ATA immobilized on glyoxyl-agarose (blue) compared to free *Vf*-ATA (red). Experimental conditions: phosphate buffer (pH 8.0, 100 mM) containing DMSO (2% v/v) in the presence of 10 mM (*S*)-MBA and 1 mM PLP. Immobilized *Vf*-ATA: 50 mg, final volume: 1 mL; free *Vf*-ATA: 2.6 mg, final volume: 3 mL. The residual activity was determined by the spectrophotometric assay (see Experimental Section).

Vf-ATA immobilization on Sepabeads™ EC-EP/S

Besides glyoxyl-agarose, epoxy carriers are probably the most used supports to immobilize enzymes. These resins do not need to be pre-activated since epoxides act as the reactive groups.[34] Thus, immobilization of *Vf*-ATA was also performed on Sepabeads™ EC-EP/S. Enzymes bind covalently to this support by exploiting the nucleophilic features of lysine ε-amino groups, thus generating a multi-point interaction (as for glyoxyl-agarose). In this case, a stable secondary amine bond is formed without the need for any further step (Scheme 2B). However, epoxides are much more reactive than aldehydes and may still react either with the protein, even after the immobilization, or with substrates and/or products of the target reaction. For this reason, unreacted epoxy groups of Sepabeads™ EC-EP/S must be quenched in order to avoid side-reactions.[42–43] Glycine was chosen for the post-immobilization quenching according to a literature protocol in which a derivatized Sepabeads™ EC-EP/S was used to immobilize the transaminase from *Halomonas elongata* (HEWT).[36] The efficient immobilization of an engineered ATA mutant from *Chromobacterium violaceum* on a properly designed epoxy-functionalized carrier has been also recently described for continuous-flow applications.[44]

Vf-ATA immobilization on Sepabeads™ EC-EP/S gave quantitative yields in terms of immobilized protein and immobilized activity after 24 h incubation. The activity of this biocatalyst was determined by a discontinuous assay because, unlike glyoxyl-agarose, epoxy-acrylic carriers are bead-like materials that interfere with the spectrophotometric analysis. Although the activity of *Vf*-ATA immobilized on Sepabeads™ EC-EP/S was remarkable (activity recovery: ≈50%, Table S2), it was observed that acetophenone (**4**), i.e. the product of the investigated biotransformation, was adsorbed by the carrier, thus affecting its accurate quantification. The quenching with glycine increased the hydrophilicity of the carrier, thus decreasing (but not eliminating) the acetophenone adsorption. This adsorption phenomenon was confirmed by incubating Sepabeads™ EC-EP/S (without the enzyme) with a solution of acetophenone at a known concentration and monitoring the supernatant absorbance at 245 nm over time (data not shown).

Taking in consideration this phenomenon, glyoxyl-agarose appeared a more convenient carrier due to the acceptable activity recovery and stability. Moreover, this carrier was already shown to be suitable for flow applications.[35]

Batch synthesis of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (3)

The enzymatic synthesis of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (**3**) was performed by using *Vf*-ATA immobilized on glyoxyl-agarose in view of transferring the bioconversion from batch to a flow system. As a preliminary approach, the amination of the non-fluorinated ketone was carried out with the aim of exploring the reaction conditions, testing the feasibility of the bioconversion with the newly immobilized enzyme, and setting-up a downstream process that might be more straightforward than that described in literature.[18] (*S*)-MBA (**2**) was selected as the amino donor because it was also used in the activity assay and it is commonly used as benchmark substrate with (*S*)-selective transaminases (both in batch and in flow systems).[20,36,45] Moreover, (*S*)-MBA is the amino donor used in the synthesis of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine reported by Meadows *et al.*[18] The reaction was performed at pH 8, according to the enzymatic assay conditions (see also Figure S2). In contrast to literature,[18] in this work a monophasic system containing DMSO (2%) as the co-solvent was used. As reported in Table 1, a 30% conversion was observed which was comparable with the results obtained with the non-fluorinated ketone (data not shown). Conversions (%) were calculated by HPLC, using the acetophenone calibration curve.

After the reaction, the biocatalyst was easily separated from the reaction mixture by filtration and showed a residual activity of 50%. This result was consistent with the stability assay of the immobilized *Vf*-ATA. Furthermore, no protein release was detected in the reaction (Bradford assay),[46] as a consequence of the covalent immobilization.

The data obtained in the batch reaction were considered suitable to switch the bioconversion to a flow system and thus to run the study both of the reaction and the downstream in such a set-up.

Table 1. Batch synthesis of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (**3**) catalyzed by *Vf*-ATA immobilized on glyoxyl-agarose.

Substrate [mM]	*Vf*-ATA [U][a]	Ratio [acceptor: donor]	Conversion [%][b]	Time [h]
20	1.80	1:1	30	31

[a] The biocatalyst was immobilized at 4 °C and $NaCNBH_3$ was used as the reducing agent. Protein loading: 2 mg g^{-1}; activity recovery: 30%. The specific activity, determined by spectrophotometric assay, was 2.76 U g^{-1}, [b] Conversion was calculated by HPLC. The reaction was performed in duplicate.

Flow synthesis of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (3)

A packed-bed reactor with immobilized *Vf*-ATA was prepared for the continuous flow application. First, the effect of residence time and temperature on the reaction outcome was evaluated using a 0.65 mL volume bioreactor (immobilized *Vf*-ATA = 0.65 g) and flowing through it a solution of 1-(5-fluoropyrimidin-2-yl)-ethan-1-one (**1**, 20 mM), (*S*)-MBA (**2**, 20 mM) and PLP (1 mM) in phosphate buffer (100 mM, pH 8) containing DMSO (2% v/v). Conversions (%) were calculated by HPLC, using the acetophenone calibration curve. As reported in Table 2, a 40% conversion was achieved in 10 minutes of residence time (entry 2); a further increase of the residence time was not beneficial (entries 3 and 4). Keeping constant the residence time (10 min), concentration of the substrates in the feeding flow was varied, but the conversion was dramatically reduced (entry 6). Also, an increase of the temperature up to 38 °C did not speed up the reaction (entry 5).

Under a similar degree of conversion (i.e. c = 30% after 31 h for the batch biotransformation and c = 26% in 5 minutes for the flow biotransformation), the specific reaction rate (*r*) of the flow reaction resulted to be about 22-fold higher than the batch reaction ($r_{flow} = 0.52\ \mu mol\ min^{-1}\ mg^{-1}$; $r_{batch} = 0.024\ \mu mol\ min^{-1}\ mg^{-1}$), probably due to the high local concentration of the biocatalyst in the packed-bed reactor.[47]

To increase the sustainability of the proposed protocol, we tested dimethyl carbonate as the co-solvent (5% v/v) for the flow reaction and evaluated the reaction outcome under continuous work.

To this aim, a 2 mL bioreactor was prepared and 100 mL of the substrate solution was flowed through it under the following conditions: residence time = 10 min, T = 28 °C, P = atm. Overall, a 38% conversion was obtained. Finally, an in-line purification procedure for product isolation was developed (Scheme 1B).

A first attempt to extract the produced amine using an inlet of an organic solvent (*i.e.* EtOAc) and a membrane phase separator was performed. However, only a low amount of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (**3**) was recovered in the organic phase (< 5%). Therefore, we exploited a catch-and-release strategy, using a column packed with a strongly acidic resin (Dowex Marathon C) to trap any unreacted (*S*)-MBA and the desired amine. Then, after a washing step with distilled water, an inlet of aqueous ammonia (1 M) was used to release the amine product that, due to the different pka compared to (*S*)-MBA, was collected separately and isolated in 35% yield.

Table 2. Flow synthesis of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (**3**) catalyzed by immobilized *Vf*-ATA.[a]

Entry	Substrate [mM]	Residence time [min]	Temperature [°C]	Conversion [%][b]
1	20	5	28	26
2	20	10	28	40
3	20	15	28	39
4	20	30	28	39
5	20	10	38	38
6	40	10	28	17

[a] Immobilized *Vf*-ATA: 0.65 g; reactor volume: 0.65 mL; ratio acceptor/donor 1:1; PLP 1 mM; flow stream: phosphate buffer (100 mM, pH 8.0) containing DMSO (2% v/v); atmospheric pressure, [b] Conversion was calculated by HPLC.

Determination of the enantiomeric excess of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (3)

Upon in-line purification, the target amine was submitted to a HPLC chiral analysis to assess the enantiomeric excess. The absolute configuration of a chiral amino donor has to match the stereospecificity of the ATAs in order to be accepted: this feature explains why transaminases can be employed in the kinetic resolution of racemic amines.[48] For this reason, *Vf*-ATA, which is a (*S*)-selective ATA, should not be able to produce amines with (*R*)-configuration. Surprisingly, the chiral HPLC analysis of the amine synthesized enzymatically did result in a 90% enantiomeric excess for the *S*-enantiomer which was totally unexpected. To shed light on this result, new experiments were performed. Taking into account that both immobilization and the presence of organic co-solvents can alter enzyme selectivity,[49–50] the reaction was repeated both by using the non-immobilized enzyme and either DMSO or dimethyl carbonate as co-solvent. However, it is worth to underline that, to the best of our knowledge, decrease and/or inversion of enantioselectivity have never been reported for transaminases.

The biotransformation performed in batch by using the soluble *Vf*-ATA under the same conditions of the flow reaction (donor and acceptor: 200 μmol; PLP: 20 μmol; medium: 100 mM phosphate buffer, pH 8.0, 5% v/v dimethyl carbonate) gave the same ee% (90%): this result thus ruled out the influence of immobilization on the incomplete (*S*)-enantioselectivity of *Vf*-ATA.

The same result was obtained upon analyzing the reaction mixtures containing two different co-solvents (DMSO and dimethyl carbonate, respectively), thus excluding a role of these solvents, too.

Our attention was then focused on PLP, the co-factor of ATAs. PLP is an activated aldehyde and the formation of imines is its natural role. It has been reported that PLP, upon the formation of a Schiff base with α-amino acid esters or amines, enhances racemization through a reversible proton migration.[51] This phenomenon has been exploited for the dynamic resolution of a number of racemic substrates.[52–54] The hypothesized mechanism of PLP toward (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (**3**) is reported in Scheme S2 (Supporting Information).

The chiral analysis of a batch biotransformation which was performed without adding PLP showed a complete ee%, thus supporting our hypothesis concerning the role of PLP in the amine racemization. In this case, the (*S*)-amine formation catalyzed by *Vf*-ATA relies exclusively on the PLP intrinsically bound to *Vf*-ATA. We concluded, indeed, that the driving force of the side-reaction was the concentration of PLP used in the

reactions (1-2 mM) which is probably uselessly high for the biotransformation, although consistent with other reports on transaminases.[55–58] Furthermore, the hypothesized mechanism might be enhanced by the alkaline pH of the reaction mixture: at this pH value (around 8–9), ATAs show the highest activity, but amines are predominant in their neutral form. Thus, the nucleophilic reaction of amines with the PLP aldehyde group is plausible to occur. On the other hand, although the absence of PLP in the reaction mixture allowed to restore the complete enantioselectivity of *Vf*-ATA and to achieve a ee > 99%, this condition was detrimental for the biocatalyst. After this reaction, immobilized *Vf*-ATA lost almost completely its activity. The concentration of PLP is therefore a crucial parameter which deserves to be further investigated.

In this context, we tested the reaction without PLP under continuous flow using the conditions reported above. However, a very low conversion (< 5%) was obtained. Therefore, increasing amounts of PLP (i.e., 0.1 mM and 0.25 mM) were added to the substrate solution that was flowed through the bioreactor and the performance of the biotransformation was evaluated. Using 0.1 mM PLP, again, a very low conversion in 10 minutes of residence time was observed, whereas, using 0.25 mM PLP, a conversion comparable to the previous experiments (38%) was achieved. The amine was isolated as reported above and the enantiomeric excess was assessed by chiral HPLC, resulting to be > 99%.

It is worth reporting that the use of tailor-made carriers which can enable the co-immobilization of transaminase and PLP has been recently described.[38] Through this strategy, the exogenous addition of cofactor is avoided, since a self-sufficient source of PLP is ensured by the co-immobilized preparation for an efficient catalysis.

Conclusions

A new protocol for the synthesis of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (**3**), which integrates flow biocatalysis with an in-line downstream processing, was successfully developed.

The main benefits of the set-up herein described include the increased specific reaction rate in comparison with the batch protocol and a significantly reduced time of the overall process (reaction and product recovery). Interestingly, a possible role played by the cofactor PLP in transaminase-catalyzed amination of ketones was highlighted; albeit essential for the catalysis, PLP seemed to affect the enantioselectivity of the transamination reaction and, indeed, its concentration needs to be finely tuned. These results demonstrated that the combination of biocatalysis and flow chemistry should be regarded as a truly competitive approach with established fully chemical protocols.

Experimental Section

General

All reagents and solvents were purchased from Sigma-Aldrich® Srl (Milan, Italy) unless otherwise stated. 1-(5-Fluoropyrimidin-2-yl)-ethan-1-one (**1**) was purchased from Fluorochem (Hadfield, UK). For the production, purification and characterization of the enzyme, the following instruments were used: Thermoshaker INNOVA 42 (New Brunswick); Sorvall RC6 Plus (Thermo) centrifuge, A.L.C. 4226 centrifuge, Omni-Ruptor 250 sonicator, UV-VIS V-530 (Jasco) spectrophotometer or a UV 1601 spectrophotometer (Shimadzu).

Agarose gel 6B-CL was purchased from Amersham Biosciences (Uppsala, Sweden) and activated to glyoxyl-agarose as previously reported.[59] Sepabeads™ EC-EP/S was kindly supplied by Resindion srl (Binasco, Italy).

The flow experiments were performed by using a R2 +/R4 flow reactor (Vapourtec).

Immobilization yields

The immobilization yields (%) were determined as: immobilized protein, immobilized activity and activity recovery as reported by Sheldon *et al.*[60] according to the following equations:

Immobilized protein (%): (immobilized protein/loaded protein) x 100; immobilized activity (%): (immobilized activity/loaded activity) x 100; activity recovery (%): (observed activity of the immobilized enzyme/starting activity) x 100.

Specific reaction rate

The specific reaction rate for batch (r_{batch}) was calculated from the amount of the product (P, μmol), the reaction time (t, min), and the mass of biocatalyst employed (m_E, mg): $r_{batch} = nP/t\ m_E$ (μmol min^{-1} mg^{-1}). The specific reaction rate for flow biotransformation (r_{flow}) was calculated from the concentration of the product ([P], μmol mL^{-1}), the flow rate (f, mL min^{-1}), and the mass of protein employed (m_E, mg): $r_{flow} = [P] \times f/m_E$ (μmol min^{-1} mg^{-1}).

Vf-ATA expression and purification

The amine transaminase from *Vibrio fluvialis* (*Vf*-ATA) was produced as previously reported.[57] Briefly, *E. coli* BL21(DE3) cells carrying the expression plasmid for *Vf*-ATA were streaked on LB-Agar plates containing kanamycin 30 μg mL^{-1} (LB_{KAN30}) and incubated overnight at 37 °C. Colonies were aseptically removed, inoculated in 50 mL of LB_{KAN30} medium and incubated overnight at 37 °C and 220 rpm. The pre-cultures were used for the inoculation of 500 mL of LB_{KAN30} medium, then incubated at 37 °C and 220 rpm. When the OD_{600} value was between 0.5 and 1, IPTG (0.5 M stock solution) was added till a final concentration of 1 mM to induce the expression. The *Vf*-ATA cultures were incubated at 30 °C and 220 rpm for 24 h. At the endpoint of the expression, the cells were recovered by centrifugation at 5000 rpm and 4 °C for 30 min and resuspended in 20 mL of washing buffer (500 mM NaCl, 20 mM imidazole, 20 mM potassium phosphate buffer, pH 7.0). Cells were lysed by three sonication cycles at 0 °C and the cell lysate was centrifuged at 10000 rpm and 4 °C for 30 min. The transaminase was purified by IMAC using Ni-NTA Sepharose 6 Fast Flow resin (GE Healthcare, Italy) as the stationary phase according to the manufacturer's instruction. The eluates containing the target protein were collected and dialyzed for 16 h at 4 °C in 50 mM phosphate buffer pH 9. After the dialysis, samples were stored at −80 °C. Protein content was measured

using the Bio-Rad Protein Assay according to the method of Bradford; bovine serum albumin (BSA) was used as standard protein. Protein purity was verified by SDS-PAGE analysis (10% T, 2.6% C). The molecular weight protein standard mixture from BioRad (Karlsruhe, Germany) was used as reference. Gels were stained for protein detection with Coomassie Brilliant Blue.

Glyoxyl-agarose preparation

Glyoxyl-agarose was prepared as reported by Guisan.[59] Briefly, agarose (10 g) was suspended in deionized water (2.8 mL) at 4 °C under mechanical stirring. A 1.7 M solution of NaOH (4.8 mL) containing $NaBH_4$ (14.3 mg mL^{-1}) was added. Etherification was carried out in an ice bath by adding dropwise glycidol (3.4 mL). The reaction was carried out for 18 h. After the incubation period, the suspension was filtered and the carrier was washed with deionized water. Oxidation was initiated by adding 100 mM $NaIO_4$ (68 mL). The reaction was carried out for 2 h at room temperature, then the carrier was filtered under reduced pressure and washed thoroughly with deionized water and stored at 4 °C.

Vf-ATA immobilization on glyoxyl-agarose

Vf-ATA (2 mg; 9.3 U) was incubated with glyoxyl-agarose (1 g) in $NaHCO_3$ buffer (pH 10, 50 mM, 10 mL) at 4 °C for 3 h under mechanical stirring. Chemical reduction of imines was carried out over 30 min by adding $NaCNBH_3$ (10 mg) to the mixture. The immobilized enzyme was then filtered under vacuum and washed thoroughly with deionized water and K_2HPO_4 buffer (pH 8, 50 mM) and stored at 4 °C. The immobilization procedure was monitored by evaluating the residual protein and the residual activity of the supernatant by Bradford[46] and activity assay, respectively. For the flow reactions, a protein loading of 5 mg/g was used.

Vf-ATA immobilization on Sepabeads™ EC-EP/S

Vf-ATA (1 mg; 1.23 U) was incubated with Sepabeads™ EC-EP/S (1 g), previously hydrated with deionized water (10 mL) under gentle shaking for 1 h in phosphate buffer (pH 8, 50 mM, 5 mL), at room temperature for 24 h under mechanical stirring. The immobilization procedure was monitored by evaluating the residual protein and the residual activity of the supernatant by Bradford assay[46] and activity assay, respectively. The immobilized enzyme was then filtered, washed with deionized water and re-suspended in K_2HPO_4 buffer (pH 8.5, 50 mM, 4 mL) containing glycine (3 M) and stirred for further 20 h to quench the unreacted epoxides.[36,43] The biocatalyst was eventually filtered under vacuum, washed thoroughly with deionized water and K_2HPO_4 buffer (pH 8, 50 mM) and stored at 4 °C.

Vf-ATA activity assay

Enzyme specific activity was determined by a spectrophotometric assay (Scheme S1) as previously reported.[45] Briefly, phosphate buffer (pH 8, 50 mM, 3 mL) containing sodium pyruvate (2.5 mM), (*S*)-α-methyl benzylamine ((*S*)-MBA, 2.5 mM) and DMSO (0.25% v/v) was poured in a quartz cuvette. Soluble *Vf*-ATA (6 μL) was added and the absorbance of acetophenone (ε = 12 mM^{-1} cm^{-1}) at 245 nm was detected for 60 s under the kinetic mode. One unit (U) is defined as the amount of enzyme which produces one μmol of acetophenone per minute under the above conditions. For the immobilized *Vf*-ATA, the same assay was performed by using 10–15 mg of the immobilized biocatalyst under magnetic stirring for 180 s. Enzymatic activity was determined according to Equation (1). The measurements were performed at least in duplicate.

$$\frac{U}{mL \text{ or } g} = \frac{\Delta A/min \times Vs}{\varepsilon \times E} \quad (1)$$

Equation 1. △A/min = slope of Abs *vs* time curve (mAU min^{-1}), Vs = total assay volume (mL), ε = acetophenone molar absorbivity (12 mM^{-1}cm^{-1}), E = amount of soluble enzyme (mL) or immobilized enzyme (g)

The activity of *Vf*-ATA immobilized on Sepabeds™ EC-EP/S was determined by a discontinuous assay. Three aliquots of immobilized *Vf*-ATA (15 mg) were placed in plastic tubes and the assay solution (2 mL) was added. The samples were kept under gentle stirring (rotary shaking); every two minutes, a sample was quenched with HCl (2 M, 2 mL) and the absorbance at 245 nm was registered, as a single reading. Acetophenone (μmol) was quantified by a calibration curve and used to determine the enzyme activity.

Residual activity of *Vf*-ATA immobilized on glyoxyl-agarose

A stock solution containing (*S*)-MBA (64.5 μL, 500 μmol), DMSO (1 mL) in phosphate buffer (pH 8, 100 mM, 49 mL) and PLP (12.4 mg, 50 μmol) was prepared. Aliquots (50 mg) of immobilized *Vf*-ATA were placed in Eppendorf micro tubes and 1 mL of the stock solution was added to each Eppendorf. The samples were kept under stirring (rotary shaker) at 25 °C. At fixed times, each sample was filtered under vacuum, rinsed thoroughly with distilled water, conditioned with phosphate buffer (pH 8, 50 mM) and dried for 5 min. Residual activity (U g^{-1}) was measured by submitting the filtered immobilized enzyme (10–15 mg) to the spectrophotometric assay as reported above and compared with the activity (U g^{-1}) of the enzyme before incubation, which was considered as 100%.

Batch synthesis of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (3)

1-(5-Fluoropyrimidin-2-yl)-ethanone **1** (28.0 mg, 200 μmol) was dissolved in DMSO (200 μL) and added to phosphate buffer (pH 8, 100 mM, 9.8 mL) containing PLP (4.9 mg, 20 μmol) and (*S*)-MBA **2** (25.8 μL, 200 μmol) in a plastic flask. Immobilized *Vf*-ATA (652 mg, 1.80 U) was added and the mixture was kept under mechanical stirring. Samples were taken, diluted with bidistilled water (1:10) and analyzed by HPLC. Once the highest conversion was achieved, the biocatalyst was recovered by filtration under vacuum.

Flow synthesis of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (3)

Immobilized *Vf*-ATA (2.0 g) was packed into a glass column (Omnifit, 6.6 mm i.d. x 100 mm length). The packed-bed reactor was washed with a 100 mM phosphate buffer pH 8 at 100 μL min^{-1} for 10 min. The obtained reactor volume was 2.0 mL. A solution (100 mL) of 1-(5-fluoropyrimidin-2-yl)-ethan-1-one (**1**, 20 mM), (*S*)-MBA (**2**, 20 mM) and PLP (1 mM) in phosphate buffer (pH 8, 100 mM) with 5% v/v dimethyl carbonate was prepared. The solution was pumped at 0.20 mL min^{-1} (residence time = 10 min) through the bioreactor maintained at 28 °C. The exiting flow stream was directed into a column packed with Dowex Marathon C resin (reactor volume: 5 mL) that was previously activated with 2 M HCl and washed with H_2O. The desired amine was trapped by the resin. At the end of the reaction, the column packed with Dowex Marathon C resin was washed by flowing H_2O at 0.25 mL min^{-1} for

20 min. (*S*)-1-(5-Fluoropyrimidin-2-yl)-ethanamine **3** was then released using a 1 M solution of NH_4OH at 0.25 mL min^{-1} for 30 min. The flow stream was collected in fractions of 2.0 mL; TLC assays were performed to select the samples containing the pure product. After evaporation of the solvent, the pure amine was recovered in 35% isolated yield (99 mg). ^1H NMR ($CDCl_3$) δ 8.60 (s, 2H), 4.65 (br s, 2H), 4.07-4.15 (m, 1H), 1.20 (d, 3H).

Chemical synthesis of racemic 1-(5-fluoropyrimidin-2-yl)-ethanamine

The chemical synthesis of racemic 1-(5-fluoropyrimidin-2-yl) ethanamine was performed slightly modifying a previously reported procedure.[61] i) 1-(5-Fluoropyrimidin-2-yl)-ethan-1-one **1** (200 mg, 1.43 mmol) was dissolved in MeOH (5 mL) at room temperature. The solution was cooled at 0 °C and $NaBH_4$ (54 mg) was added. The resulting mixture was stirred at room temperature for 1 h. Water (2 mL) was added and the organic solvent was removed under reduced pressure; the aqueous phase was extracted with EtOAc (3 x 3 mL). The organic extracts were dried over Na_2SO_4 and, after evaporation of the volatiles under reduced pressure, 1-(5-fluoropyrimidin-2-yl)ethan-1-ol was obtained as a red oil (75% yield). ii) The alcohol obtained from the previous step (152 mg, 1.07 mmol) was dissolved in CH_2Cl_2 (2 mL), and triethylamine (297 μL, 2.14 mmol) was added at room temperature. The solution was cooled to 0 °C, and methanesulfonyl chloride (100 μL, 1.28 mmol) was added dropwise. The resulting mixture was allowed to stir at r.t. for 2 h. The mixture was washed with 1 M HCl (2×2 mL), the organic phase was dried over Na_2SO_4 and evaporated under reduced pressure to give 1-(5-fluoropyrimidin-2-yl)ethyl methanesulfonate (90% yield). iii) The residue (212 mg, 0.96 mmol) was dissolved in DMF (5 mL) and treated with sodium azide (62 mg, 0.96 mmol). The resulting mixture was stirred at r.t. for 72 h and was then partitioned between EtOAc (5 mL) and brine (10 mL). The organic layer was collected, dried over Na_2SO_4, and evaporated under reduced pressure. The crude material was purified by flash column chromatography as a colorless oil (55% yield). iv) 2-(1-Azidoethyl)-5-fluoropyrimidine (88 mg, 0.53 mmol) was dissolved in MeOH (5 mL) and submitted to a hydrogenation reaction in a Thales-Nano H-Cube Mini (cartridge: Pd/C 10%, flow rate: 1 mL min^{-1}). The exiting solution was evaporated under reduced pressure to afford racemic 1-(5-fluoropyrimidin-2-yl) ethanamine as a pale yellow oil in quantitative yield. HPLC chiral analysis retention times (t_R): (*S*)-MBA (**2**) = 3.8 min, (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (**3**) = 5.9 min; (*R*)-1-(5-fluoropyrimidin-2-yl)-ethanamine = 6.1 min; 1-(5-fluoropyrimidin-2-yl)-ethan-1-one (**1**) = 8.0 min.

Analytical methods

HPLC analyses of batch reactions were performed on a HPLC Chromaster 600 bar System, Merck Hitachi VWR, equipped with a Purospher® STAR RP-18 endcapped (3 μm), Hibar HR 100–2.1, UHPLC column, Sorbent Lot No. TA 1822868 (Merck KGaA, Darmstadt, Germany). Injection volume: 2 μL; mobile phase: H_2O + TFA 0.05% (A)/ACN + TFA 0.05% (B); gradient conditions: 0–5 min 100% A; 5→20 min 50% (A)/50% (B); flow rate: 0.3 mL min^{-1}; λ: 245–254 nm. Retention times (t_R): (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (**3**) = 3.60 min, 1-(5-fluoropyrimidin-2-yl)-ethan-1-one (**1**) = 13.40 min, (*S*)-MBA (**2**) = 14.37 min, acetophenone (**4**) = 20.90 min.

HPLC analyses of the flow reactions were performed on a Breeze 2 HPLC System, Waters, equipped with a Waters 2489 UV–vis detector (Waters, Milford, MA) and a Phenomenex Synergi column (150 mm ×4.6 mm, 4 μm). Injection volume: 10 μL; mobile phase: H_2O + 0.05% TFA (A)/ACN + 0.05% TFA (B); gradient conditions: 0–9 min 90% (A)/10% (B), 9→14 min 50% (A)/50% (B); flow rate: 1.0 mL min^{-1}; λ: 254 nm. Reaction samples were diluted with a solution 1:10 H_2O/ACN + 0.05% TFA. Retention times (t_R): (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine (**3**) = 2.7 min, (*S*)-MBA (**2**) = 5.6 min, 1-(5-fluoropyrimidin-2-yl)-ethan-1-one (**1**) = 7.0 min, acetophenone (**4**) = 16.4 min.

HPLC chiral analyses were performed using a Jasco PU-980 pump (PerkinElmer, Inc., Waltham, MA) equipped with a UV–vis detector Jasco UV-975 and a Phenomenex Lux Amylose 2 column (150 mm × 4.6 mm, 3 μm). Injection volume: 10 μL; mobile phase: hexane + 0.5% diethylamine/iso-propanol + 0.5% diethylamine, isocratic elution 80:20; flow rate: 1.0 mL min^{-1}.

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Immobilization of Old Yellow Enzymes via Covalent or Coordination Bonds

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Abstract: Ene-reductases (ERs) belonging to the old yellow enzyme (OYE) family have been thoroughly investigated for the stereospecific reduction of activated prochiral C=C double bonds. In this work, OYE3 was immobilized both by covalent binding on glyoxyl-agarose (OYE3-GA), and by affinity-based adsorption on EziG^{TM} particles (OYE3-EziG). The immobilized OYE3-GA was demonstrated to be active (activity recovery = 52%) and to retain almost 100% of its activity under the enzymatic assay conditions (50 mM phosphate buffer pH 7, 28 °C) for six days, whereas the activity of the non-immobilized enzyme dropped to 50% after two days. In the case of EziG^{TM}, the highest activity recovery (54%) was achieved by using the most hydrophilic carrier (EziG^{TM} Opal) that was selected for the full characterization of this type of enzyme preparation (stability, recycling, re-use, enzyme leakage). OYE3-EziG was slightly less stable than OYE3-GA under the same experimental conditions. OYE3-GA could be recycled and re-used for up to 12 reaction cycles in the bioreduction of α-methyl-*trans*-cinnamaldehyde; after 12 runs, the highest conversion achieved was 40%. In the case of the co-immobilized OYE3/GDH-EziG, the conversion dropped to 56% after two reaction cycles. No enzyme leakage was detected over 48 h for both OYE3-GA and OYE3/GDH-EziG (50 mM phosphate buffer pH 7, 28 °C). These seed results pave the way for a true optimization of the immobilization of OYE3, as well as for the use of immobilized OYE3 for preparative applications both in batch and continuous flow conditions.

Keywords: biocatalysis; enzyme immobilization; old yellow enzymes; glyoxyl-agarose; affinity-based immobilization

1. Introduction

Ene-reductases (ERs) belonging to the old yellow enzyme (OYE) family (EC 1.6.99.1) are flavin mononucleotide (FMN)-containing oxidoreductases, which are able to catalyze the stereoselective reduction of C=C double bonds activated by the presence of a suitable electron-withdrawing group (EWG) as a substituent of the alkene moiety [1,2]. The reaction occurs through a stereospecific anti hydrogen addition to the C=C double bond, according to the following steps [3]. The enzyme-bound flavin (reduced by NAD(P)H cofactor to $FMNH_2$) transfers a hydride to the olefin carbon atom in β position with respect to the EWG. A proton is delivered by a tyrosine side-chain (Tyr196 for OYE1-3) of the enzyme active site to the α-carbon atom (with respect to EWG) on the opposite face of the alkene. For the application of OYE-mediated reductions in synthetic sequences of practical interest, it

is advantageous to promote the in situ regeneration of the reduced nicotinamide cofactor by using a NAD(P)H-dependent glucose dehydrogenase (GDH) with glucose as a sacrificial co-substrate.

The OYE-mediated bioreduction has been widely investigated in the last decade to establish substrate scope and stereoselectivity of these enzymes [4–6], and to study the combination of this reaction with other chemo- and/or biocatalyzed transformations, both in concomitant and subsequent cascade procedures [7,8]. Several works showed the advantages of using OYE-catalyzed hydrogenation to prepare chiral building blocks for the synthesis of APIs (Active Pharmaceutical Ingredients) [9–12], flavors and fragrances [13–15].

The synthetic potential of the OYE-mediated bioreduction can be further expanded by OYE immobilization on solid carriers. If OYEs are converted into stable, readily-recoverable and recyclable heterogeneous catalysts, then their suitability for large-scale applications can be enhanced, in agreement with one of the key green chemistry research areas established by the ACS Green Chemistry Institute® Pharmaceutical Roundtable (catalyst immobilization without significant loss in kinetics) [16].

Studies on the immobilization of oxidoreductases are gradually increasing, but still only a few examples of ER immobilization have been described to date. Recently, a recombinant ER from the OYE family and its cofactor-recycling partner enzyme GDH (commercially available from Amano Enzyme Inc.) were co-immobilized by using two immobilization methods (i.e., cross-linked enzyme aggregates (CLEAs) and a so-called "biomimetic" immobilization (BI)) by entrapment within a network of fused silica particles. Activity recovery of both enzyme preparations were about 45%. Immobilization enhanced the thermal stability (50 °C) of both enzymes and markedly increased their resistance to acidic pH (5-6) in comparison with the non-immobilized enzyme. Moreover, both immobilized enzymes were successfully recycled and re-used for up to 14 reaction cycles. The catalytic "performance" of the CLEA preparation in the reduction of 4-(4-methoxyphenyl)-3-buten-2-one resulted to be superior, both to the non-immobilized enzymes and the entrapped enzymes by BI [17].

In a very recent study, an ER from *Thermus scotoductus* SA-01 (*Ts*OYE) was encapsulated with a light-harvesting dye in an alginate hydrogel for NADH-free, photobiocatalytic asymmetric hydrogenation of 2-methylcyclohexenone. The *Ts*OYE encapsulated in alginate hydrogel exhibited enhanced stability against external stresses (e.g., heat, organic solvents), also on a repeated use. However, the long-term reusability of *Ts*OYE alginate hydrogel was hampered by photobleaching and leakage of the dye used [18].

OYE immobilization is still under-investigated and, to the best of our knowledge, no attempt of immobilization has ever been reported for OYE3, one of the most performing members of this enzyme class. The impressive results obtained in OYE3-catalyzed biotransformations in the past years and the benefits envisaged for ER immobilization urged us to undergo a "seed-study" in this frame.

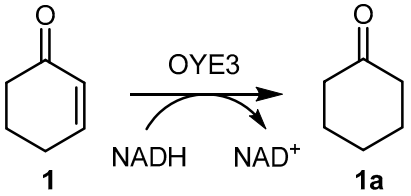
In this work, OYE3 was immobilized both by covalent binding on glyoxyl-agarose (OYE3-GA), and by metal affinity interaction on EziG™ particles (OYE3-EziG). The GA carrier [19,20], widely explored for the irreversible immobilization of enzymes by multipoint attachment, has proven its efficiency and versatility as immobilization support by affording robust biocatalysts, both for batch and flow applications [21,22]. On the other hand, EziG™ is a novel carrier made of controlled porosity glass (CPG) particles containing chelated Fe(III) for His-tag binding. This carrier can specifically immobilize tagged enzymes from a crude mixture by affinity interaction and is, thus, frequently used to combine immobilization and purification steps [23,24]. In this context, EziG™ was studied to explore a milder immobilization technique based on non-covalent interaction.

2. Results and Discussion

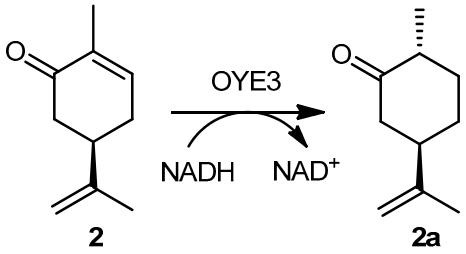
2.1. Enzyme Activity Assays

In order to characterize OYE3, the enzyme activity was assessed spectrophotometrically, as described for other ERs [25–27], by measuring the consumption of NADH at 340 nm in the reduction of cyclohex-2-enone (1) (see Scheme 1 and Materials and Methods, Section 3.3.1). This assay was found to

be suitable for the soluble enzyme, but not for assessing the activity of the immobilized enzyme (and, indeed, to calculate the activity recovery after immobilization) because of the interference of the solid carrier with the spectrophotometric readouts. Therefore, a GC-MS assay (see Scheme 2 and Materials and Methods, Section 3.3.2) based on the reduction of (*R*)-carvone (2) in the presence of stoichiometric NADH was used. Unless otherwise stated, this latter assay was used to determine the activity of both soluble and immobilized OYE3.

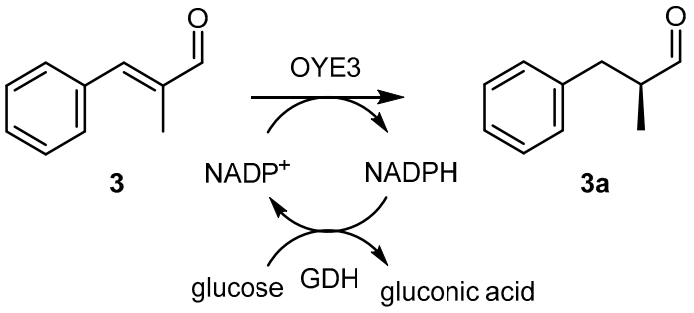


Scheme 1. Reduction of cyclohex-2-enone (1) to cyclohexanone (1a) catalyzed by OYE3 (spectrophotometric activity assay). Experimental conditions: 50 mM phosphate buffer pH 7 (0.5% DMSO), [substrate] = 0.05 mM, [NADH] = 0.2 mM, 28 °C, reaction time = 80 s, volume = 1 mL.



Scheme 2. Reduction of (*R*)-carvone (2) to (2*S*,3*R*)-dihydrocarvone (2a) catalyzed by OYE3 (GC-MS activity assay). Experimental conditions: 50 mM phosphate buffer pH 7 (1% DMSO), [substrate] = 1 mM, [NADH] = 1 mM, 28 °C, reaction time = 30 min, volume = 1 mL.

A spectrophotometric assay was also used to assess the activity of soluble GDH by measuring the formation of NADH over time at 340 nm as a result of glucose oxidation (experimental conditions: 50 mM phosphate buffer pH 7, [substrate] = 20 mM, [NAD^+] = 0.2 mM, 28 °C, reaction time = 2 min, volume = 1 mL). Glucose dehydrogenase was coupled to OYE3 for cofactor regeneration in the reduction of α-methyl-*trans*-cinnamaldehyde (3) (see Scheme 3 and Materials and Methods, Section 3.4).



Scheme 3. Reduction of α-methyl-*trans*-cinnamaldehyde (3) to (*S*)-α-methyl-β-phenylpropanal (3a) catalyzed by OYE3. The regeneration of $NADP^+$ was ensured by the GDH glucose system. Experimental conditions: 50 mM phosphate buffer pH 7 (1% DMSO), [substrate] = 5 mM, [$NADP^+$] = 0.1 mM, [glucose] = 20 mM, GDH = 10 μL; 5 mg mL^{-1}, 28 °C, reaction time = 1 h, volume = 1 mL.

2.2. Preliminary Study of OYE3 Stability at Alkaline pH

Covalent immobilization of enzymes on glyoxyl-agarose (GA) relies on the formation of imino bonds between the non-protonated ε-amino groups of enzyme Lys residues and the aldehyde groups

of the carrier [19,20]. This immobilization is required to be carried out at alkaline pH (i.e., pH ≥ 10) and a final chemical reduction step is necessary for the generation of irreversible C-N bonds. Whenever compatible with the enzyme stability, sodium borohydride is used to this aim, since it transforms Schiff's bases into secondary amino bonds and the unreacted aldehyde groups of the carrier into inert hydroxyl moieties. Alternatively, the milder sodium cyanoborohydride or 2-picoline borane can be used for those enzymes which are sensitive to sodium borohydride reduction (but unreacted aldehyde groups of the carrier remain unchanged in this case) [28]. Thus, the first step before immobilization on GA was to assay the stability of OYE3 at alkaline pH. A pH "window" of 8–10 was considered and 3 h was selected as the endpoint (according to the general protocol for this type of immobilization) [19]. Enzyme activity before and after incubation at pH 8–10 (see Section 3.5, Materials and Methods) was assessed spectrophotometrically using the cyclohex-2-enone assay, Scheme 1). Data of the stability assay at pH 8–10 are reported in Figure 1. The residual activity of OYE3 after 3 h at pH 10 was about 70%. This result was considered acceptable for the immobilization on GA.

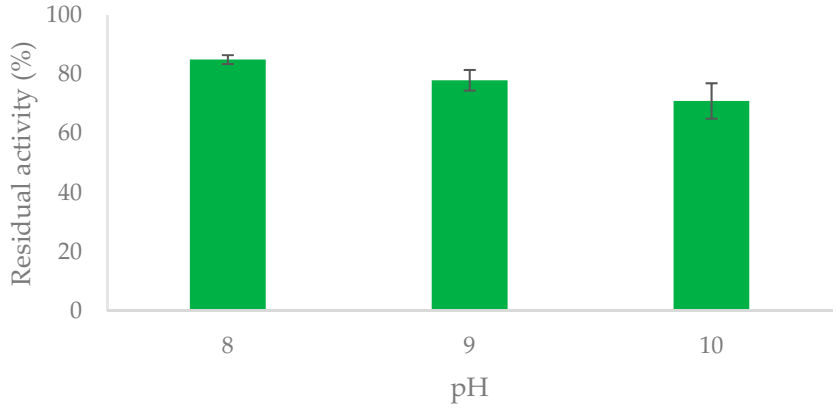


Figure 1. Residual activity (%) of soluble OYE3 upon incubation at pH 8–10 (endpoint—3 h). Experimental conditions: 50 mM phosphate buffer, pH 8; 50 mM Tris-HCl buffer, pH 9; 50 mM NaOH-glycine buffer, pH 10; 20 °C. Enzyme activity was determined as reported in Scheme 1. Residual activity (%) was calculated as reported in Section 3.5 [29].

2.3. Immobilization of OYE3

Immobilization of OYE3 on GA (OYE3-GA) was performed according to a standard protocol by using a 2 mg g^{-1} protein loading [19], as described in Section 3.6 (Materials and Methods). In order to extend the enzyme stability during the immobilization, the reaction was performed at 4 °C. After 3 h, almost all the protein was immobilized (immobilization yield = 95%), as revealed by protein measurements of the supernatant from the immobilization reaction. The activity recovery, after reduction of the immobilized enzyme with $NaBH_4$, was 52% (Table 1).

Table 1. Results of OYE3 immobilization.

Carrier	Enzyme Loading (mg g^{-1})	Immobilization Yield [1] (%)	Activity [2] (U g^{-1})	Activity Recovery [3] (%)
Glyoxyl-agarose	2.0	95	0.023	52
EziG™ Opal	4.2	100	0.037	54
EziG™ Coral	4.2	100	0.015	23
EziG™ Amber	4.2	100	0.014	19

[1] Calculated as reported in Section 3.6 [29]. [2] Determined by the (*R*)-carvone (2) assay (see Scheme 2). [3] Calculated as reported in Section 3.6 [29]. Activity measurements were performed at least in duplicate. Deviation from mean values was below 5%.

For the immobilization by metal-coordination, three EziG^{TM} resins were tested (namely Opal or EziG 1, Coral or EziG 2, and Amber or EziG 3). According to the indications of the supplier, Opal is a hydrophilic carrier (glass), whereas Coral and Amber are hydrophobic and semi-hydrophilic, respectively. The lower hydrophilicity of Coral and Amber in comparison with Opal is due to the presence of different polymeric coatings on the particle silica surface [30]. Specifically, EziG^{TM} Coral is made from hybrid CPG with polyvinyl benzyl chloride, whereas EziG^{TM} Amber is produced from hybrid CPG with a blended co-polymer. Pore size distribution of the carriers ranges from ~500 Å as standard (Opal) to ~300 Å (Coral and Amber, due to the polymer coating). According to the indications of the supplier, a higher protein loading (4.2 mg g^{-1}) was applied to EziG^{TM} carriers. In all cases, the immobilization was complete. The activity recovery for EziG^{TM} Opal was 54%, thus resulting comparable to that previously obtained with GA. In the case of the hybrid CPG Coral and Amber, the activity recovery was less than half with respect to EziG^{TM} Opal. Under the same binding chemistry, it is plausible that this result may be ascribed to the effect of the polymer coating in tuning the hydrophilicity–hydrophobicity balance of the carrier. However, a systematic study on these carriers was not performed at this stage. Due to the positive result achieved with EziG^{TM} Opal, this carrier was selected for the full characterization of this type of enzyme preparation (stability, recycling, re-use, enzyme leakage).

2.4. Stability of Immobilized OYE3

Increased stability is recognized as one of the most important advantages of immobilized enzymes [31,32]. To verify whether this "general rule" might be applied also to immobilized OYE3 preparations, the stability of both OYE3-GA and OYE3-EziG (Opal) was tested under the experimental conditions used for the reduction of (*R*)-carvone (2) (Scheme 2). The stability assay was carried on for six days (Figure 2). From the stability time course, it clearly emerged that immobilization exerted a stabilizing effect on the soluble enzyme. The OYE3-GA retained almost 100% of its activity, whereas the activity of the non-immobilized enzyme dropped to 50% after two days. On the other hand, OYE3-EziG was slightly less stable than OYE3-GA, but it still retained about 90% of its starting activity after 144 h.

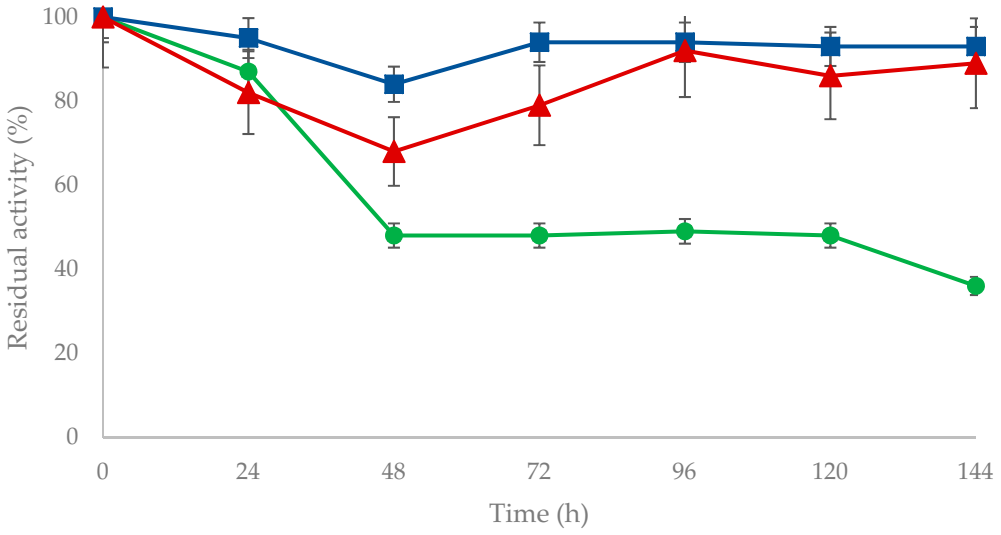


Figure 2. Stability of soluble OYE3 (green circles), OYE3-GA (blue squares) and OYE3-EziG (red triangles). Experimental conditions: 50 mM phosphate buffer pH 7 (1% DMSO), (*R*)-carvone and NADH = 1 mM, 28 °C, volume = 1 mL.

2.5. Recycling of Immobilized OYE3

Biocatalyst recycling and re-use is a further strength point of immobilized enzymes [31–33]. Thus, both OYE3-GA and OYE3-EziG were used in the bioreduction of α-methyl-*trans*-cinnamaldehyde (3, Scheme 3), then recovered and added to a newly-prepared reaction mixture.

A catalytic amount of the cofactor ($NADP^+$) was used since a GDH glucose regeneration system was coupled to the main biotransformation. In the case of OYE3-GA, GDH was added as a soluble enzyme to each reaction cycle. In the case of OYE3-EziG, a co-immobilized preparation OYE3/GDH was used, taking advantage of the His-tag displayed by both enzymes. The enzymes (OYE3 and GDH) were incubated with EziG™. Activity recovery for OYE3 was 55%. OYE3-GA could be recycled and re-used for up to 12 reaction cycles. The highest conversion achieved after 12 runs was 40% (Figure 3). Moreover, it is worth noting that OYE3-GA retained completely its activity up to the fifth reaction cycle. In the case of OYE3/GDH-EziG, the initial conversion dropped to 56% after two reaction cycles. Interestingly, when the conversion % was almost negligible (reaction 11) and the biotransformation was supplemented with a fresh aliquot of soluble GDH, the reaction afforded a 30% conversion, thus suggesting that the gradual drop of the conversions registered for OYE3/GDH-EziG might depend either on the loss of activity of immobilized GDH or on its leakage from the carrier and/or deactivation, thereof.

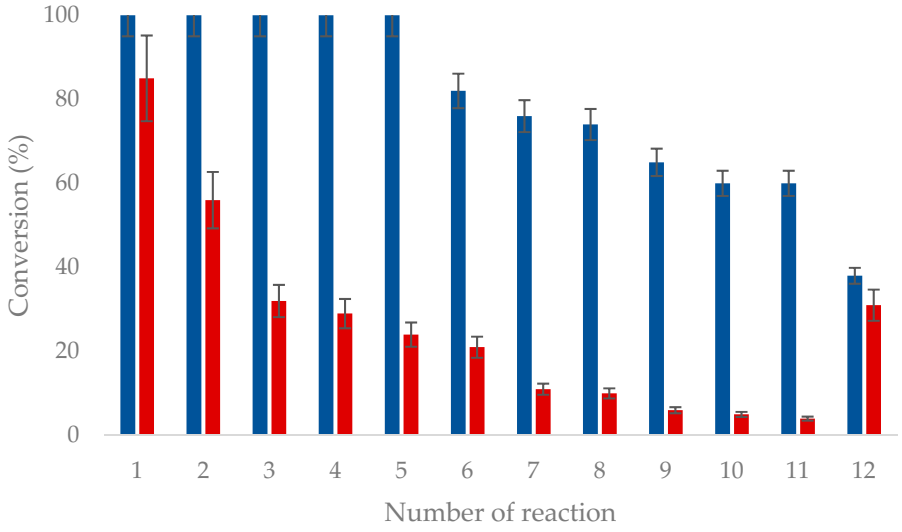


Figure 3. Recycling of immobilized OYE3-GA (blue) and OYE3/GDH-EziG (red). Experimental conditions as reported in Scheme 3.

2.6. Protein Leakage Assay of Immobilized OYE3

Protein leakage from the immobilization carrier is undesirable for several reasons: Loss of activity of the biocatalyst, lack of data reproducibility in the investigated biotransformation, costs and product contamination, to name a few [29]. Covalent immobilization of the enzyme to the carrier generally prevents the protein from leaching from the support surface. In this context, glyoxyl-agarose and other surface-functionalized carriers (i.e., epoxy carriers) are frequently preferred because of the "irreversible" nature of the bonds generated between the enzyme and the carrier. On the other hand, the high affinity and the specificity of the binding interaction based on the His-tag as in EziG™-type carriers is plausible to generate a strong enzyme-support interaction which might prevent enzyme leakage. However, predictions about how an enzyme will respond to an immobilization process are often hard to make. Enzyme leakage is routinely verified by protein measurements [34] of the supernatant in which the immobilized preparation is incubated under specific conditions dictated by the type of information needed (e.g., reaction or storage conditions). Both OYE3-GA and OYE3/GDH-EziG were, thus, incubated in the reaction buffer (50 mM phosphate buffer pH 7) at 28 °C under stirring for 48 h.

At scheduled times, samples of the incubation buffer were collected and submitted to SDS-PAGE analysis; silver staining, due to its high sensitivity in the low nanogram range, was used to detect protein traces after electrophoretic separation [35]. From the inspection of the SDS-PAGE gel (data not reported), no enzyme traces were detected for either OYE3-GA or OYE3/GDH-EziG. This suggests that the loss of activity of both immobilized enzymes registered during the recycling and re-use study, although to a different extent, cannot be ascribed to protein leakage phenomena.

3. Materials and Methods

3.1. General

Chemicals and solvents were purchased from Merck Sigma-Aldrich® Srl (Milano, Italy) and used without further purification. Agarose gel 6B-CL was purchased from Amersham Biosciences (Uppsala, Sweden) and activated to glyoxyl-agarose (GA), as previously reported [19]. EziG™ carriers were kindly provided by EnginZyme AB (Sweden) as free samples. GC-MS analyses were performed using a HP-5MS column (30 m × 0.25 mm × 0.25 µm, Agilent). The following temperature program was employed: 60 °C (1 min), 6 °C min^{-1}/150 °C (1 min), 12 °C min^{-1}/280 °C (5 min). Bradford assay [33] was performed by using a Shimadzu UV-1601 spectrophotometer and bovine serum albumin (BSA) was used as reference standard protein. All the enzymatic reactions were kept under stirring and controlled temperature by using a thermoshaker New Brunswick™ Excella® E24. Reaction extraction and phase separation were accomplished through a ZX3 Advanced Vortex Mixer from VELP Scientifica® and a Microcentrifuge ScanSpeed Mini (LaboGene™), respectively.

3.2. Enzyme Production

The enzymes OYE3 from *Saccharomyces cerevisiae* and GDH from *Bacillus megaterium* were prepared as His-tagged proteins and purified according to standard methods [36]. Concentration of the purified OYE3 was in the range 2–3 mg mL^{-1}, whereas the concentration of GDH was 3–4 mg mL^{-1}. Activity of OYE3 was measured both by the cyclohex-2-enone assay (see Section 3.3.1 and Scheme 1) and the (*R*)-carvone assay (see Section 3.3.2 and Scheme 2), resulting in 0.09 ± 0.008 U mg^{-1} and 0.13 ± 0.007 U mg^{-1}, respectively. Activity of GDH was measured by a spectrophotometric assay (see Section 3.3.1), resulting in 6.7 ± 0.35 U mg^{-1}.

3.3. Activity Assays

3.3.1. Spectrophotometric Enzymatic Assay

(a) For the OYE activity assay, in a 2 mL cuvette, a solution of cyclohex-2-enone (5 µL; stock solution = 10 mM in DMSO) and a solution of NADH (20 µL; stock solution = 10 mM in water) were added to 50 mM phosphate buffer pH 7 (916 µL). The reaction was carried out at 28 °C and started by adding to the blank solution 59 µL of OYE3 (177 µg, conc. = 3 mg mL^{-1}).

(b) For the GDH activity assay, in a 2 mL cuvette, a solution of glucose (205 µL; stock solution = 1 M in water) and a solution of NAD^+ (20 µL; stock solution = 10 mM in water) were added to 50 mM phosphate buffer pH 7 (960 µL). The reaction was carried out at 28 °C and started by adding to the blank solution 2 µL of GDH (6 µg, conc. = 3 mg mL^{-1}).

The enzymatic activity (U mL^{-1}) was calculated from the consumption formation of NADH, for (a) and (b), respectively, at 340 nm ($\varepsilon_{NAD(P)H}$ = 6200 M^{-1} cm^{-1}) according to the following equation:

$$\frac{U}{mL} = \frac{\Delta Abs/min \cdot V_{tot}}{V_E \cdot \varepsilon}$$

ΔAbs/min: slope of Abs vs. time curve (mAU min^{-1}), V_{tot}: total assay volume (mL), V_E: volume of soluble enzyme used in the assay (mL), ε: NAD(P)H molar absorptivity (6200 M^{-1} cm^{-1}).

One unit (U) is defined as the amount of enzyme that converts 1 μmol of substrate in the product in 1 min under specific temperature and pH conditions.

3.3.2. GC-MS Enzymatic Assay

In a 2 mL test-tube, a solution of (*R*)-carvone (10 μL; stock solution = 100 mM in DMSO) and 15 μL of soluble OYE3 (conc. = 3 mg mL^{-1}) or immobilized OYE3 (ca. 20 mg for OYE3-GA or 45 mg for OYE3-EziG) were added to 50 mM phosphate buffer, pH 7 (final volume = 1 mL). The reaction was started by adding NADH (100 μL; stock solution = 10 mM in water) and the mixture was kept at 28 °C under stirring (thermoshaker) for 30 min, then vortexed for 30 s in the presence of CH_2Cl_2 (200 μL). The organic phase was separated by centrifugation (2 min, 13500 rpm), dried over Na_2SO_4 for 5 min, and analyzed by GC-MS (see Section 3.1 for analytical conditions). (*R*)-Carvone: GC-MS (EI) t_R = 12.99 min: mz (%) = 150 (M^+, 15), 135 (10), 108 (40), 93 (40), 82 (100). Product: GC-MS (EI) t_R = 11.83 min: mz (%) = 152 (M^+, 30), 137 (25), 123 (10), 109 (55), 95 (100). The enzymatic activity (U mL^{-1} for the non-immobilized enzyme, or U g^{-1} for the immobilized enzyme) was calculated from the percentage of substrate conversion after 30 min according to the following equation:

$$\frac{U}{mL \text{ or } g} = \frac{[S] \cdot c}{t_R \cdot E}$$

[S]: substrate concentration (mM), c: conversion (%), t: reaction time (30 min), E: amount of soluble enzyme (mL) or immobilized enzyme (g).

One unit (U) is defined as the amount of enzyme that converts 1 μmol of substrate in the product in 1 min under specific temperature and pH conditions.

3.4. OYE3-Mediated Reduction of α-Methyl-Trans-Cinnamaldehyde (3)

In a 2 mL test tube, a solution of α-methyl-*trans*-cinnamaldehyde (10 μL; stock solution = 500 mM in DMSO) was added to 50 mM phosphate buffer pH 7 (838 μL) followed by a solution of $NADP^+$ (10 μL; stock solution = 10 mM in water), glucose (20 μL; stock solution = 1 M in water), and GDH (10 μL; 5 mg mL^{-1}). The reaction (final volume = 1 mL) was started by adding 160 μg of OYE3 (non-immobilized enzyme = 53 μL; OYE3-GA = 80 mg; OYE3-EziG = 170 mg) and kept under stirring (thermoshaker) at 28 °C for 1 h. At the endpoint, in the case of the immobilized enzyme, the reaction was stopped by removing the biocatalyst by centrifugation (4 min, 13,200 rpm). The reaction mixture was vortexed for 30 s in the presence of CH_2Cl_2 (400 μL). The organic phase was separated by centrifugation (2 min, 13,200 rpm), dried over Na_2SO_4 for 5 min, and analyzed by GC-MS (see Section 3.1 for analytical conditions). α-Methyl-*trans*-cinnamaldehyde = GC-MS (EI) t_R = 14.60 min = mz (%) = 146 (M^+, 70), 145 (100), 117 (80), 115 (70), 91 (50). Product: GC-MS (EI) t_R = 11.68 min = mz (%) = 148 (M^+, 45), 133 (25), 105 (25), 91 (100). The conversion was evaluated from the ratio of the peak areas of product and substrate.

3.5. Stability of OYE3 in the pH Range 8-10

The stability of non-immobilized OYE3 was assayed in the pH range 8–10 by incubating 100 μL of the enzyme solution (stored in 20 mM phosphate buffer pH 8.5 containing 200 mM imidazole and 500 mM NaCl) in 100 μL of 50 mM phosphate buffer pH 7.5 (final pH = 8), 50 mM Tris-HCl buffer pH 9.5 (final pH = 9), or 50 mM NaOH-glycine buffer pH 11.5 (final pH = 10) at 20 °C. After 3 h, 100 μL were withdrawn from each sample and the enzymatic activity was measured spectrophotometrically in the reduction of cyclohex-2-enone (see Section 3.3.1). The residual activity was calculated according to the following equation:

$$\text{Residual activity } (\%) = \frac{\text{observed activity at the endpoint}}{\text{starting activity}} \times 100$$

3.6. Covalent Immobilization of OYE3 on Glyoxyl-Agarose (OYE3-GA)

Immobilization of OYE3 on glyoxyl-agarose was performed following the protocol previously reported [19]. Briefly, glyoxyl-agarose (1.8 g = 2.52 mL) was suspended in 50 mM Na_2CO_3 buffer (14.3 mL) at pH 10. After the addition of the desired amount of protein (1.2 mL, loading: 2 mg g^{-1}), the suspension was kept under mechanical stirring for 3 h at 4 °C. Chemical reduction of Schiff bases and unreacted aldehyde groups was carried out over 30 min by adding $NaBH_4$ to the mixture (18 mg; 1 mg mL^{-1} of suspension). The immobilized enzyme was then filtered and washed with 10 mM phosphate buffer pH 7 and deionized water.

The immobilization reaction was monitored by measuring the amount of protein in the supernatant before the addition of the carrier and after 1.5 h and 3 h [34]. The immobilization yield (%, endpoint = 3 h) was calculated according to the following equation [29]:

$$\text{Yield } (\%) = \frac{\text{immobilized protein}}{\text{starting protein}} \times 100$$

The activity of the immobilized OYE3 was measured by the GC-MS enzymatic assay reported in Section 3.3.2 (reduction of (*R*)-carvone, 2). The activity recovery (%) was calculated according to the following equation [29]:

$$\text{Activity recovery } (\%) = \frac{\text{observed activity}}{\text{starting activity}} \times 100$$

3.7. Immobilization of OYE3 by Affinity-Based Adsorption (OYE3-EziG)

Immobilization of OYE3 by affinity-based adsorption on EziG™ (Opal, Coral, and Amber) was performed following the protocol of the supplier with minor modifications. EziG™ (100 mg) was added to a 2 mL test tube containing the enzyme (140 µL, loading = 4.2 mg·g^{-1} of dry carrier) diluted in 50 mM phosphate buffer pH 7 (1.5 mL) at r.t. After 30 min, the test tube was centrifugated (5 min, 13,200 rpm) and the supernatant was removed by pipetting. Following supplier's instructions, the wet enzyme preparation was stored as such (when the immobilized enzyme was filtered under vacuum, it showed an almost complete loss of activity. The weight of the wet carrier was estimated to be 4.5-fold higher than the dry carrier. The activity recovery (%) was referred to the wet carrier weight).

The immobilization yield (%, endpoint = 30 min) was calculated as reported above (Section 3.6). The activity of the immobilized OYE3 was measured by the (*R*)-carvone assay (see Section 3.3.2.) and the activity recovery (%) was calculated as described above (Section 3.6).

Co-immobilization of OYE3 and GDH on EziG™ Opal was carried out by following the procedure applied to OYE3 immobilization. Briefly, EziG™ Opal (100 mg) was added to 50 mM phosphate buffer pH 7 (1.5 mL) containing OYE3 (140 µL, U = 0.06) and GDH (45 µL, U = 0.87). Monitoring and work-up of the immobilization mixture was performed as above reported.

3.8. Stability of Non-Immobilized OYE3, OYE3-GA, and OYE3-EziG

For each endpoint, non-immobilized or immobilized OYE3 (42 µg of protein) was incubated in a solution containing 50 mM phosphate buffer pH 7 (890 µL) and (*R*)-carvone (10 µL; stock solution = 100 mM in DMSO) at 28 °C. Each reaction was started by adding NADH (100 µL; stock solution: 10 mM in water). The final volume was 1 mL. The enzyme activity was assessed by the standard activity assay described in the Section 3.3.2 (reduction of (*R*)-carvone). The enzyme activity was compared with the starting activity which was considered as 100%.

3.9. Protein Leakage Assay

For each endpoint (1-3-6-24-48 h), 42 µg of protein (corresponding to 21 mg of OYE3-GA and 45 mg of OYE3/GDH-EziG) were incubated in 50 mM phosphate buffer pH 7 (200 µL). The suspensions

were kept at 28 °C under stirring (thermoshaker). At each endpoint, a sample (15 µL) was withdrawn from the supernatant and analyzed by SDS-PAGE. GDH and OYE3 (450 ng, corresponding to 0.15 µL of each stock solution) were loaded in the gel as reference standards. Precision Plus Protein™ Unstained Protein Standards (10–250 kD) was used as molecular weight proteins size marker. PAGE analysis was performed in a Mini-Protean 3 system (Bio-Rad). SDS-PAGE was carried out on 12% polyacrylamide (running buffer = 50 mM MES, 50 mM Tris base, 0.1 % w/v SDS and 1 mM EDTA). Proteins were stained by means of a silver staining kit (SilverQuest™ kit, Thermofisher) according to supplier's protocol.

3.10. Recycling of Immobilized Enzymes

Recycling of immobilized OYE3-GA and OYE3/GDH-EziG was performed by evaluating the conversion (%) of α-methyl-*trans*-cinnamaldehyde (3) into 3a (see Section 3.4). At the end of the reaction, the immobilized biocatalyst was washed twice with 1 mL of 50 mM phosphate buffer pH 7, separated through centrifugation, and then re-suspended under the conditions above described for the next reaction cycle.

4. Conclusions

Ene-reductases are a powerful tool for the stereoselective reduction of activated C=C double bonds under mild and environmentally friendly conditions. Due to the great interest in these enzymes for preparative applications, the access to stable and reusable heterogeneous biocatalysts is being sought. Immobilization of OYE3 both on the well-known glyoxyl-agarose (GA) by covalent binding and on controlled pore glass EziG^{TM} by affinity-based adsorption was shown to produce active, stable and reusable biocatalysts. Although activity recovery for both enzyme preparations was similar (≈50%), OYE3-GA appeared to be superior about stability and recycling properties. In this latter regard, OYE3-GA could be re-used up to five reaction cycles without any loss of activity. Still, upon a prolonged re-use (up to 12 runs), this immobilized biocatalyst allowed to obtain a 40% conversion in the reduction of α-methyl-*trans*-cinnamaldehyde (3). On the other hand, in the case of the co-immobilized OYE3/GDH-EziG, a drop of conversion was observed after two reaction cycles, presumably due to deactivation of GDH. Interestingly, no evidence of enzyme leaching from both the supports was registered.

Author Contributions: Conceptualization, E.B. and D.U.; methodology, F.T., D.C. and R.S.; investigation, F.T., D.C., R.S. and T.B.; data curation, F.T.; writing (original draft preparation, review and editing) F.T., T.B., E.B., D.M. and D.U.; supervision, E.B., D.M. and D.U.; all authors critically reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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Appendix 3: List of Publications, Poster and Oral Communications

3.1 Publications

6) **Semproli R.**^, Robescu M.S.^, Sangiorgio S., Pargoletti E., Bavaro T., Rabuffetti M., Cappelletti G., Speranza G. *, Ubiali D. *, «From lactose to alkyl galactoside fatty acid esters as non-ionic biosurfactants: a two-step enzymatic approach to cheese whey valorization». ^equal contribution. *ChemPlusChem*, **2022**, in press IF: 3.210 (Research Paper)

5) Sangiorgio S., Pargoletti E., Rabuffetti M., Robescu M.S., **Semproli R.**, Ubiali D., Cappelletti G. *, Speranza G. *, «Emulsifying properties of sugar-based surfactants prepared by chemoenzymatic synthesis». *Colloids Interface Sci. Commun*., **2022**, 48, 100630 IF: 5.633 (Rapid Communication)

4) Sangiorgio S., Cambò M., **Semproli R.**, Ubiali D., Speranza G., Rabuffetti M. *, «2-O-Acetyl-3,4,5,6-tetra-*O*-benzyl-D-*myo*-inosityl diphenylphosphate: a new useful intermediate to inositol phosphate and phospholipids». *Chirality*, **2022**, 34, 1038–1043 IF: 2.437 (Short Communication)

3) **Semproli R.**, Robescu M.S., Cambò M., Mema K., Bavaro T., Rabuffetti M., Ubiali D. *, Speranza G. *, «Chemical and enzymatic approaches to esters of *sn*-glycero-3-phosphoric acid». *Eur. J. Org. Chem.,* **2021,** 4027-4037 IF: 3.261 (Minireview)

2) Tentori F., Bavaro T., Brenna E. *, Colombo D., Monti D., **Semproli R.**, Ubiali D*. «Immobilization of Old Yellow Enzymes via covalent or coordination bonds». *Catalysts,* **2020**, 10, 260 IF: 4.501 (Communication)

1) **Semproli R.**^, Vaccaro G.^, Ferrandi E.E., Vanoni M., Bavaro T., Marrubini G., Annunziata F., Conti P., Speranza G., Monti D*., Tamborini L.*, Ubiali D.* «Use of immobilized amine transaminase from *Vibrio fluvialis* under flow conditions for the synthesis of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine». *ChemCatChem*, **2020**, 12, 1359 –1367 IF: 4.495 ^equal contribution (Research Paper)

3.2 Communications as Presenting Author (P=poster, O=oral)

P4) **Semproli R.**, Robescu M.S., Sangiorgio S., Pargoletti E., Bavaro T., Rabuffetti M., Cappelletti G., Speranza G., Ubiali D., «From lactose to alkyl galactoside fatty acid esters as non-ionic surfactants: a two-step enzymatic approach to cheese whey valorization» (P8-13, pag. 150), *10th International Congress on Biocatalysis (BioCat 2022),* August 28th-September 1st, **2022**, Hamburg, Germany

P3) **Semproli R.**, Ubiali D., Bavaro T., De Rosa M.C., Pirroli D., Righino P., Pappalardo V.M., Zaccheria F., Ravasio N., «Esters of chlorogenic acid as α-glucosidase inhibitors with a potential antiviral activity. Synthesis, molecular docking and in vitro assays» (PC_62, pag.

101), *MedChem 2021 Paul Ehrlich Virtual Meeting (PEVM 2021),* July 26th-28th, **2021**, Catanzaro, Italy (on-line event)

P2) Sangiorgio S.,^ **Semproli R.**,^ Bavaro T., Cappelletti G., Marrubini G., Nasserian S., Rabuffetti M., Robescu M.S., Speranza G., Ubiali D. «Chemoenzymatic synthesis of alkyl-glycoside fatty acid esters as biosurfactants» (PC-21, pag. 68), ^equal contribution *6th International Conference on Biocatalysis in Non-Conventional Media* (BNCM2021), May 6th-8th, **2021**, Milano, Italy (on-line event)

P1 & O1) Semproli R.^, Vaccaro G.^, Ferrandi E.E., Vanoni M., Bavaro T., Marrubini G., Annunziata F., Conti P., Speranza G., Monti D*., Tamborini L.*, Ubiali D.* «A new synthesis of (*S*)-1-(5-fluoropyrimidin-2-yl)-ethanamine catalyzed by immobilized ω-transaminase from *Vibrio fluvialis* under flow and green conditions» (FL-31, pag 135). ^equal contribution *Merck Young Chemists Symposium*, November 25th-27th, **2019**, Rimini, Italy (flash poster communication)

3.3 Communications as co-Author (P=poster, O=oral)

P4) Cappelletti G., Pargoletti E., Rabuffetti M., Robescu M.S., Sangiorgio S., **Semproli R.**, Ubiali D., Speranza G. «The BioSurf project: a sustainable production of bio-based surfactants from renewable resources» (P37, pag. 115), *Solutions in Chemistry*, November 8th-11th, **2022**, Sveti Martin na Muri, Croatia

O3) Robescu M.S., **Semproli R.**, Sangiorgio S., Pargoletti E., Bavaro T., Rabuffetti M., Cappelletti G., Speranza G., Ubiali D. «Cheese whey permeate gets a new look by turning into biosurfactants» (pag. 63), *European Federation of Biotechnology virtual conference* (*efb2022*), October 4th-5th, **2022** (on-line event) (flash poster communication)

O2) Robescu M.S., **Semproli R**., Sangiorgio S., Pargoletti E., Bavaro T., Rabuffetti M., Cappelletti G., Speranza G., Ubiali D. «A two-step enzymatic approach to cheese whey valorization: Synthesis of alkyl galactoside fatty acid esters as non-ionic biosurfactants» (O6, pag. 15), *4th Workshop I Chimici per le Biotecnologie*, July 1st, **2022**, Parma, Italy

O1) Sangiorgio S., Pargoletti E., Rabuffetti M., Nasserian S., Robescu M.S., **Semproli R**., Ubiali D., Cappelletti G., Speranza G. «Chemoenzymatic synthesis of alkyl glycoside fatty acid esters and investigation of their emulsifying properties» (O15, pag. 95), *"Attilio Corbella" International Summer School on Organic Synthesis* (*ISOS 2022, XLVI edition*), June 12th-16th, **2022**, Gargnano, Italy

P3) Donzella S., Fumagalli A., Sangiorgio S., Rabuffetti M., Robescu M.S., **Semproli R.**, Speranza G., Ubiali D., Molinari F., Compagno C. «Sustainable production of bio-based surfactants from renewable resources by using oleaginous yeasts» (P9, pag. 43), *An International Young Investigator Symposium* (2nd *NextGenBioCat 2022*), April 25th-26th, **2022**, Delft, the Netherlands

P2) Sangiorgio S., **Semproli R.,** Bavaro T., Cappelletti G., Marrubini G., Rabuffetti M., Nasserian S., Robescu M.S., Ubiali D., Speranza G. «Sugar-based surfactants:

chemoenzymatic synthesis and interfacial properties evaluation» (pag. 135), *Green Chemistry PostGraduate Summer School*, July 4th-9th, **2021**, Venezia, Italy

P1) Colombo D., Tentori F., Bavaro T., Brenna E., Monti D., **Semproli R.**, Ubiali D. «Covalent or coordination bond - Two different strategies for the immobilization of Old Yellow Enzymes» (PC-8, pag. 55), *6th International Conference on Biocatalysis in Non-Conventional Media* (BNCM2021), May 6th-8th, **2021**, Milano, Italy (on-line event)