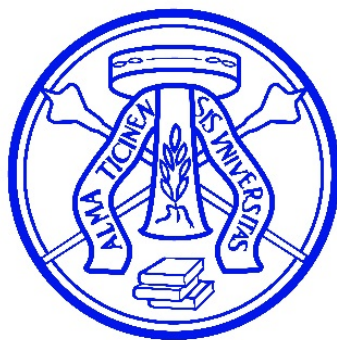




UNIVERSITÀ DEGLI STUDI DI PAVIA

**DOTTORATO IN SCIENZE CHIMICHE E
FARMACEUTICHE E INNOVAZIONE INDUSTRIALE
(XXXVIII Ciclo)**

Coordinatore: Chiar.mo Prof. Giorgio Colombo

**Synthesis of cRGD-based derivatives for
tumor site-specific drug delivery**

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1. INTRODUCTION

1.1 Targeted therapy for anticancer treatment

Cancer remains one of the leading causes of death worldwide and represents a major global health challenge. According to recent epidemiological estimates, cancer incidence and mortality continue to rise, driven by demographic changes such as population aging, lifestyle-associated risk factors and socioeconomic development¹. This increasing burden underscores the need for safer and more effective therapeutic strategies².

Current cancer treatment relies on a combination of surgery, radiotherapy and systemic anticancer therapies, selected according to tumor type, stage, molecular characteristics and patient condition³. Among systemic treatments, cytotoxic chemotherapy has historically played a central role by targeting rapidly proliferating cells through interference with DNA replication, mitosis or cellular metabolism⁴. Despite its clinical efficacy, chemotherapy is associated with significant limitations, including severe systemic toxicity and poor selectivity toward malignant cells, which often results in dose-limiting side effects⁵.

To overcome these drawbacks, the concept of **targeted cancer therapy** has emerged, aiming to selectively interfere with molecular pathways that are critical for tumor growth, survival and progression⁶. This paradigm shift originates from the “*magic bullet*” concept proposed by Paul Ehrlich in 1907, which envisioned therapeutics capable of selectively targeting disease-causing cells while sparing healthy tissues⁷. Advances in cancer biology and molecular oncology have since enabled the translation of this concept into clinical practice.

Molecular targeted therapies, including small-molecule inhibitors and monoclonal antibodies, work by selectively modulating signal transduction pathways frequently dysregulated in cancer, such as those involving receptor tyrosine kinases and downstream signaling proteins⁸. The clinical approval of landmark agents such as trastuzumab and imatinib marked a turning point in oncology, demonstrating that selective targeting of oncogenic drivers could result in significant therapeutic benefit and improved patient outcomes. These successes have established targeted therapy as a cornerstone of precision medicine and first-line treatment for several malignancies^{9–11}.

However, despite their advantages over conventional chemotherapy, targeted therapies face **important challenges**: their clinical efficacy is often restricted to patient subpopulations harboring specific molecular alterations and the emergence of intrinsic or acquired drug resistance frequently limits long-term effectiveness. Moreover, off-target effects and toxicity due to unintended interactions with normal tissues remain significant concerns. These limitations have stimulated the development of advanced therapeutic strategies aimed at improving selectivity, efficacy and safety¹².

In this context, targeted drug delivery systems have gained increasing attention as a means to enhance tumor-specific accumulation of therapeutic and diagnostic agents while minimizing systemic exposure. By integrating molecular targeting with nanotechnology and imaging capabilities, theranostic platforms offer a promising approach to address unmet clinical needs in cancer treatment. Although, the clinical success of targeted therapies critically depends on the identification of robust molecular targets that are both selectively expressed in tumors and capable of mediating efficient cellular internalization¹³. Among the cell-surface receptor classes investigated to date, integrins have emerged as particularly attractive targets¹⁴.

1.2 INTEGRINS: structure and functions

The *extracellular matrix* (ECM) can be defined as a vast three-dimensional network composed of proteins and other molecules that surrounds cells and tissues, providing both structural and biochemical support. Cell adhesion to the extracellular matrix and/or to other cells forms the basis for the development, maintenance and function of various cell and tissue types in multicellular organisms. This adhesion process involves specific transmembrane proteins located on the cell surface, known as *cell-adhesion molecules* (CAMs)¹⁵. CAMs can be grouped into **four major families**: the immunoglobulin superfamily (IgSF), the cadherin family, the selectin family and the integrin family¹⁶. Each of these families includes multiple receptor subtypes, distinguished by the specific functions they perform. Among these families, **integrins** are the most extensively studied due to their central role in cell adhesion and bidirectional signalling.

Integrins are type I transmembrane heterodimeric glycoproteins composed of two distinct chains, referred to as the alpha (α) and beta (β) subunits. A total of 18 α subunits and 8 β subunits have been identified; their various pairings give rise to 24 different receptors. Among these receptors, 16 bind extracellular matrix proteins, while the others are involved in cell-cell interactions¹⁷.

Structurally, integrins share a conserved architecture (*figure 1*), consisting of a large extracellular domain responsible for binding ECM proteins, a transmembrane region composed of 25-29 amino acid residues and a short cytoplasmic tail of 10-70 residues. The extracellular domain of both the α and β subunits comprises an N-terminal globular head for ligand binding and two long C-terminal legs that connect to the transmembrane segment. While α and β subunits differ in domain organization, their coordinated conformational changes ultimately regulate ligand affinity and signal transduction.

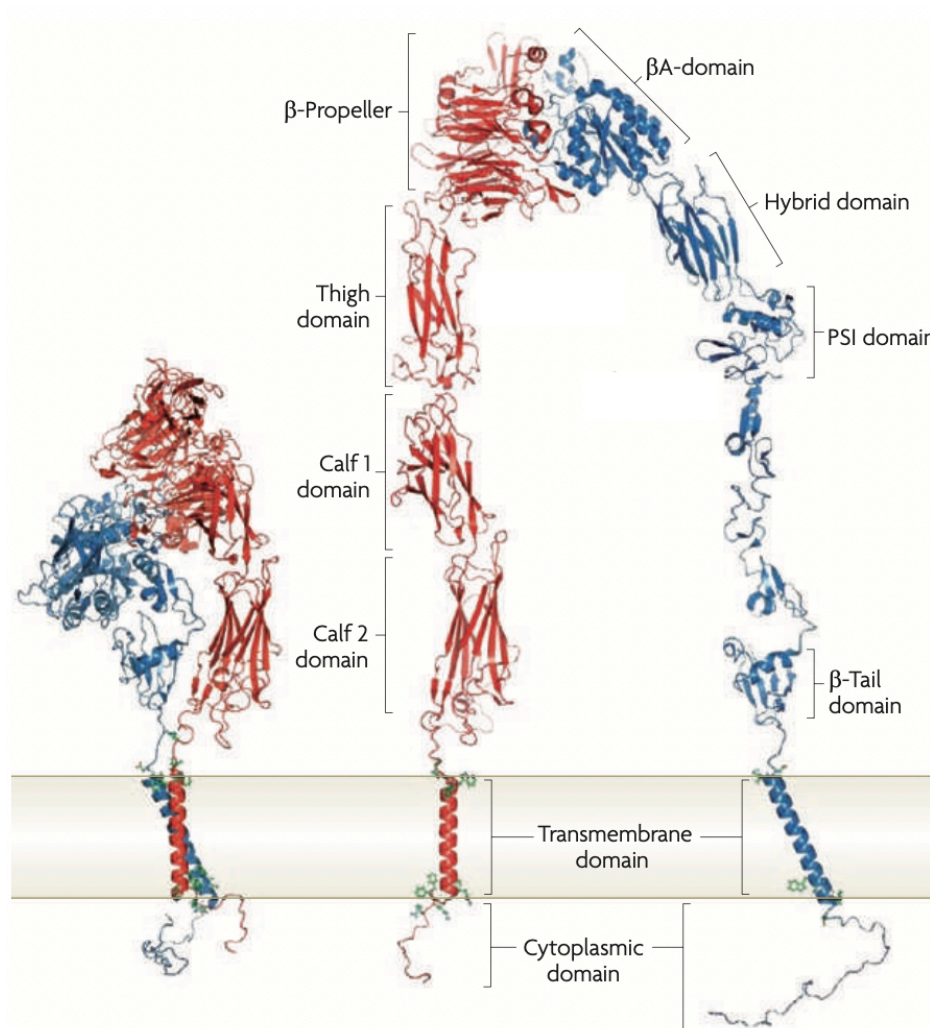


Figure 1 – Schematic representation of the integrin α - and β -subunit structures (shown in red and blue, respectively) and their conformational organization, including the bent inactive/low-affinity state (**left**) and the extended active conformation (**right**). Adapted from Ref. [24]

Once assembled, the two subunits form a binding pocket where the specific ligands for each integrin are recognized. Many α subunits can associate with only one β partner, which allows integrins to be grouped into subfamilies based on the shared β subunit. In contrast, some α subunits, including α_4 , α_6 and especially α_v , can pair with multiple β subunits.^{18,19}

Integrins participate in a wide range of biological processes, but their roles can be broadly summarized into **two major functions**: they enable cell binding to ECM proteins, and they mediate bidirectional signal transduction between the ECM and the cell. Examples of processes in which they play a role include cell migration, proliferation, communication and survival. Among their various functions, they also contribute to blood coagulation, support

For integrin-ligand interactions to be functionally meaningful, the binding event must be capable of regulating signal transduction. However, cell adhesion is a highly dynamic process: cells must rapidly adapt to changes in their pericellular environment, position, and differentiation state. As a result, highly responsive mechanisms are required to mediate signaling initiated either within the cell (*inside-out signaling*) or from the extracellular environment (*outside-in signaling*) (figure 3)^{14,23,24}.

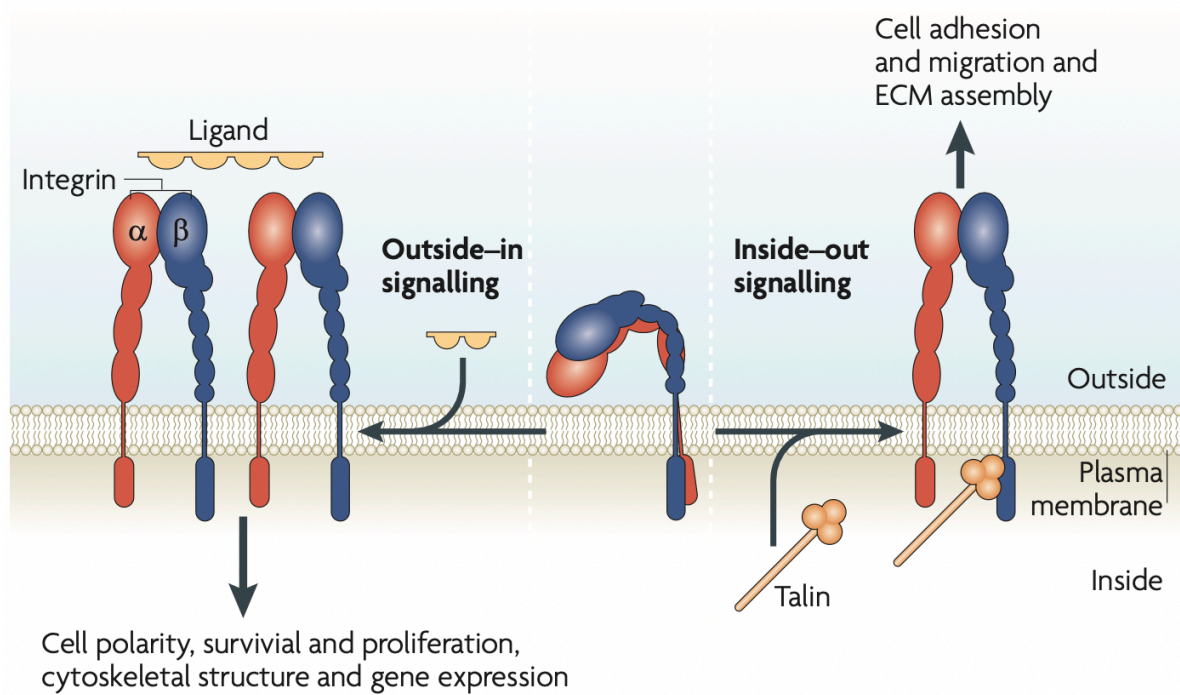


Figure 3 – Bidirectional integrin signaling. Reproduced from Ref. [24]

Because integrins lack intrinsic enzymatic activity, signal transduction is mediated by the assembly of protein complexes on the cytoplasmic face of the cell membrane. Binding to ECM ligands induces conformational changes in integrins, shifting them from an inactive (*unliganded*) to an active (*liganded*) state. According to the “switchblade” model (figure 4), three conformational states have been proposed: low affinity, intermediate affinity and high affinity. In the low-affinity conformation, integrins cannot bind ECM ligands; in this state, the transmembrane and cytoplasmic domains of the α and β subunits remain closely associated. Ligand engagement and intracellular adaptor binding promote conformational rearrangements and receptor extension, shifting integrins toward higher-affinity states and favouring productive signaling²⁵.

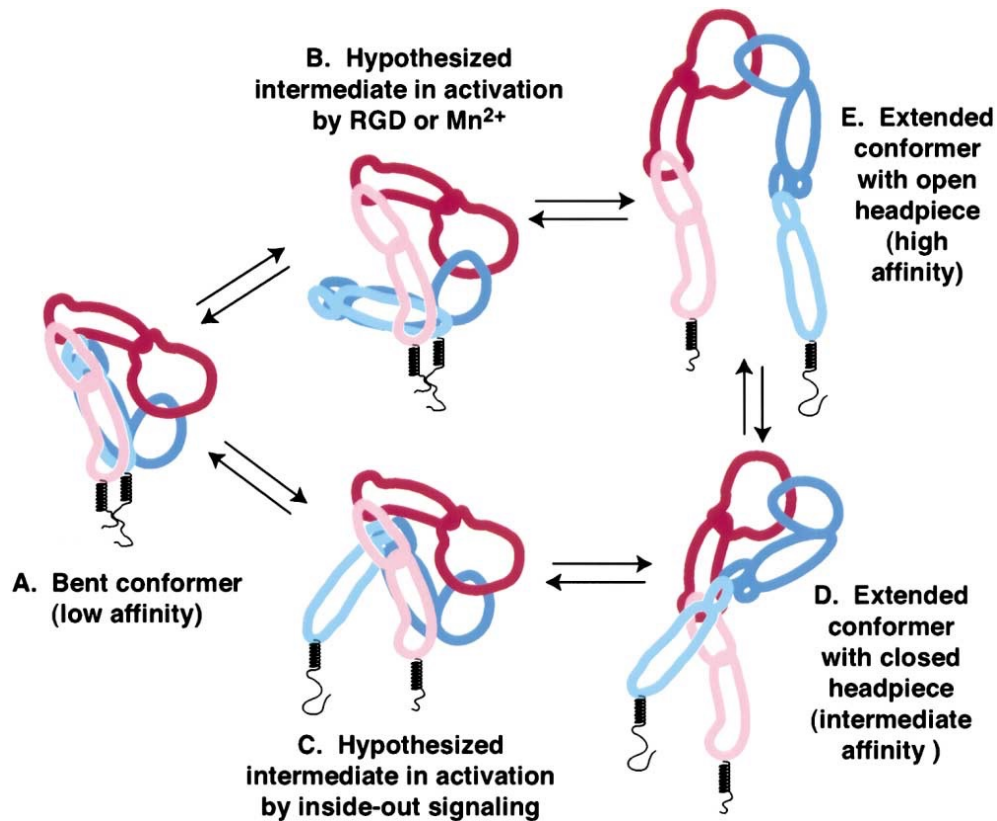


Figure 4 – The 3 conformational states of integrin in the "switchblade" model. Reproduced from Ref. [25]

Integrin activation following ECM engagement promotes the activity of several kinases, enzymes that transfer phosphate groups including the Src tyrosine kinase, which is constitutively associated with the β -subunit cytoplasmic tail. Src-mediated transphosphorylation recruits additional kinases such as *phosphatidylinositol-4-phosphate 5-kinase type γ* (PIP3K γ) and *focal adhesion kinase* (FAK). These signaling events contribute to the activation and recruitment of *focal adhesion proteins* (such as vinculin and talin) through the phosphorylation of intracellular transducers including p130Cas and caveolin, and ultimately activate the MAP/ERK kinase cascade, which regulates proliferative and survival pathways²⁶.

As noted earlier, integrins also mediate signals originating from within the cell. This inside-out signaling is triggered by intracellular forces that stimulate cytoplasmic proteins to bind the β -subunit cytoplasmic tail. Their association alters the receptor's intracellular conformation: the α and β subunits, previously held together by a salt bridge, become separated, resulting in an increased affinity of integrins for their extracellular ligands²⁵.

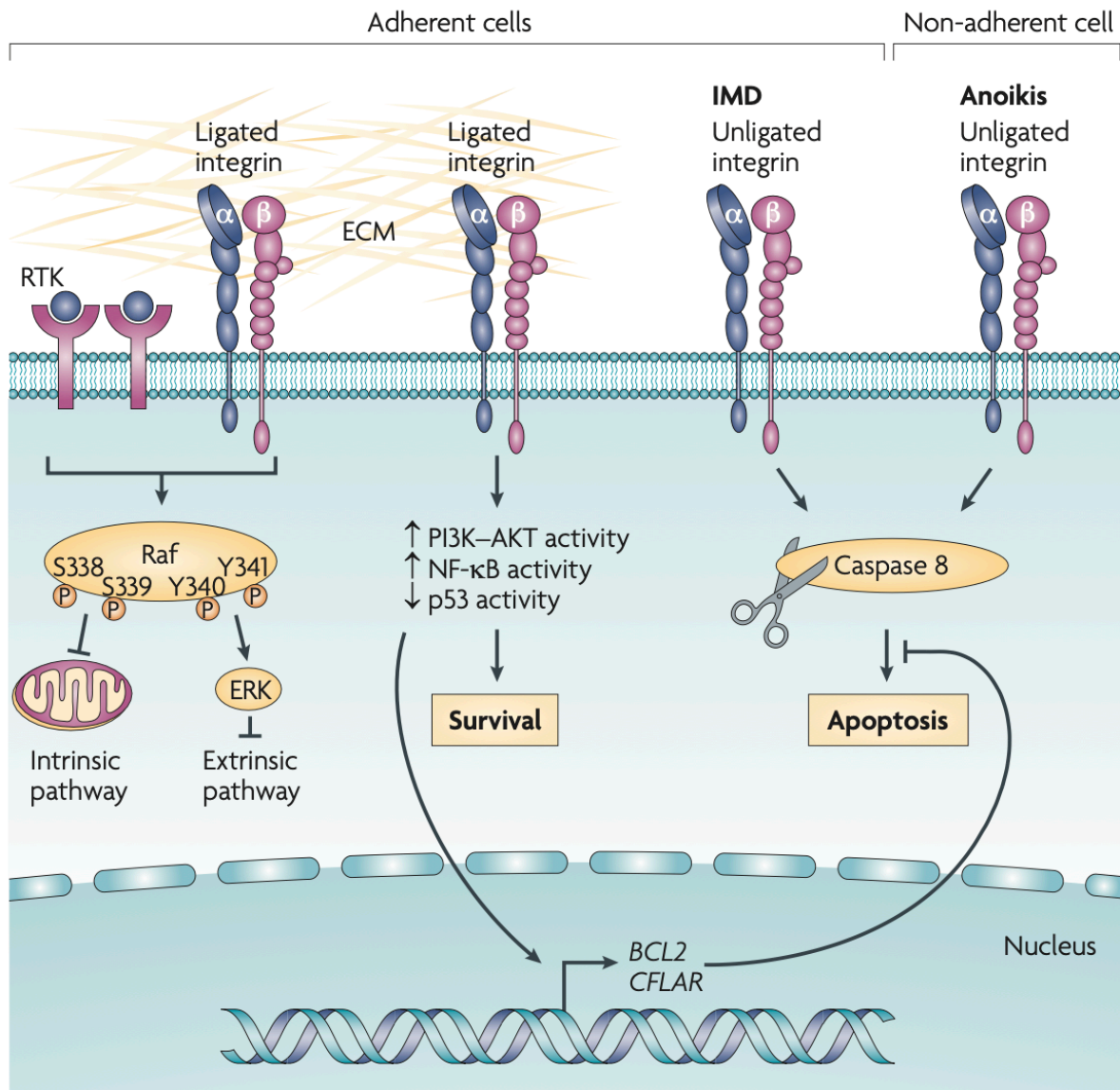


Figure 5 – Integrin-mediated signal activation pathway. Reproduced from Ref. [14]

When interactions between cells and ECM components are impaired, alternative pathways to the MAP/ERK cascade are activated, such as those illustrated in *figure 5*, leading to cell detachment from the ECM. Loss of ECM engagement triggers a specific form of apoptosis known as *anoikis*. Notably, integrins can paradoxically initiate both pro-survival and pro-apoptotic signals: binding to ECM components promotes proliferative signaling, whereas lack of interaction induces signals that lead to cell death¹⁴.

1.3 INTEGRINS: biological role in cancer progression

Although integrins are ubiquitously expressed in human tissues, their expression patterns vary across different cell types: α and β subunits are expressed at distinct levels, giving rise to different receptors with specific ligand-binding profiles. This diversity enables precise control over the ECM components to which cells can adhere. By binding to ECM molecules, integrins activate protein kinases that regulate a wide range of cellular activities, including growth, division, proliferation, differentiation and apoptosis. Based on these functions, it is clear why integrins play a central role in carcinogenesis and tumor progression²³.

Several integrin subtypes are dysregulated in tumors. Among them, integrins such as $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_v\beta_1$, typically not expressed in epithelial or endothelial cells, are instead overexpressed in a variety of cancers including glioma, prostate, pancreatic and ovarian tumors. On tumor cells, $\alpha_v\beta_3$ integrins are markedly upregulated during tumor progression, promoting lamellipodia formation and regulating migration and invasiveness. Through their interactions with matrix metalloproteinase-9 (MMP-9) and the urokinase plasminogen activator receptor (uPAR), enzymes responsible for ECM degradation, they facilitate tumor dissemination. Moreover, by binding various ECM proteins, $\alpha_v\beta_3$ integrins associate with vascular endothelial growth factor (VEGF) receptors and fibroblast growth factor-2 (FGF-2), thereby enhancing tumor cell invasiveness and promoting angiogenesis, the formation of new blood vessels that supply nutrients and oxygen to tumor tissue^{14,27}.

Unlike $\alpha_v\beta_3$, the $\alpha_v\beta_5$ integrin primarily drives tumor invasion and metastasis rather than primary tumor growth. Similar to $\alpha_v\beta_3$, however, $\alpha_v\beta_5$ activates multiple angiogenic pathways. While $\alpha_v\beta_3$ is required for angiogenesis mediated by FGF or tumor necrosis factor- α (TNF- α), VEGF or transforming growth factor- α (TGF- α)-driven angiogenesis depends on $\alpha_v\beta_5$. Downstream $\alpha_v\beta_5$ signaling activates FAK and Raf, protecting endothelial cells from apoptosis induced by loss of ECM attachment, cellular stress, or DNA-damaging agents. Another important endothelial marker is the $\alpha_5\beta_1$ integrin, which correlates with tumor malignancy and invasiveness. During invasion, $\alpha_5\beta_1$ promotes adhesion force generation, focal adhesion assembly and the formation of contractile fibers. Additionally, $\alpha_5\beta_1$ activates pro-survival pathways, regulating cell proliferation both through sustained activation of the

Akt kinase, an apoptosis suppressor, and through the epidermal growth factor receptor (EGFR)²². Furthermore, α_v integrins have been shown to play a key role in activating transforming growth factor- β 1 (TGF- β 1) (figure 6), in maintaining cancer stem cells, and in driving the epithelial-mesenchymal transition (EMT)²⁸.

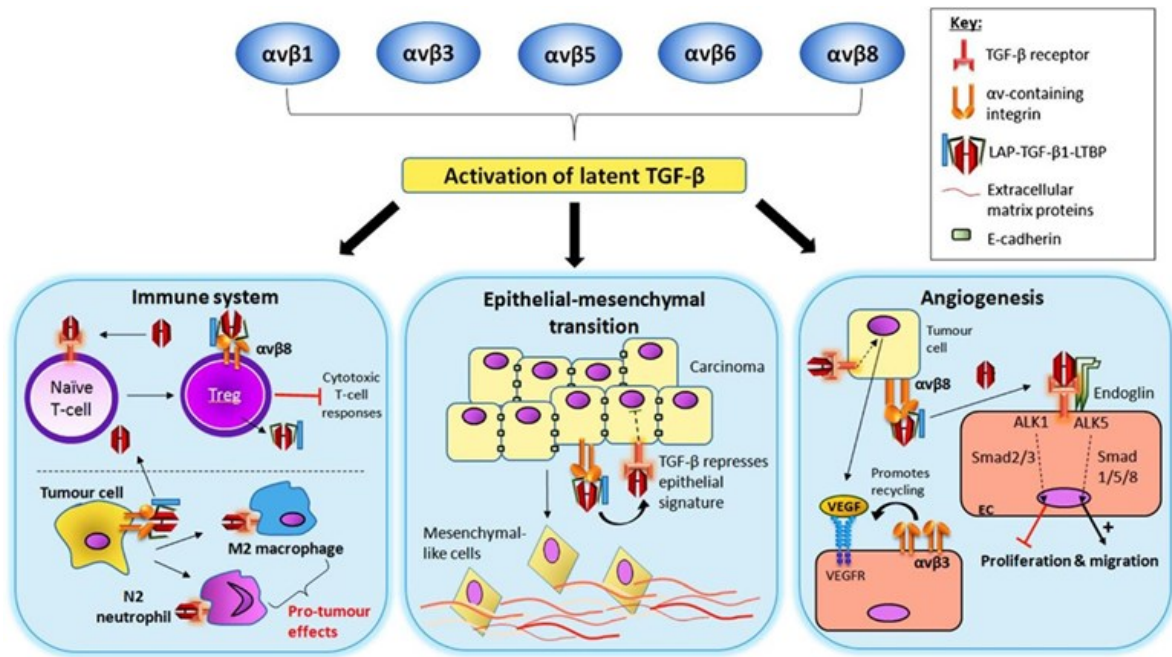


Figure 6 – Integrins involved in TGF- β 1 activation and role in promoting tumor cell survival. Reproduced from Ref. [28]

Collectively, these findings identify $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_1$ and $\alpha_5\beta_1$ integrins as central regulators of tumor invasion, angiogenesis and survival. Their selective overexpression in tumor cells and neovasculature, combined with their ability to undergo ligand-induced internalization, makes them particularly attractive targets for receptor-mediated drug delivery strategies.

1.4 The RGD motif for targeting integrins

Integrin receptors involved in tumor-related processes, such as $\alpha_v\beta_3$ and $\alpha_v\beta_5$, recognize the tripeptide sequence **Arg-Gly-Asp** (RGD)^{29,30}. This amino acid motif is present in many ECM proteins, including fibronectin, vitronectin and osteopontin, and is essential for mediating cell-ECM interactions. However, these proteins are not recognized uniformly by all integrin receptors; their binding affinities differ depending on how the RGD sequence is spatially presented within each protein. Moreover, integrin recognition involves not only the core binding site but also adjacent molecular regions³⁰.

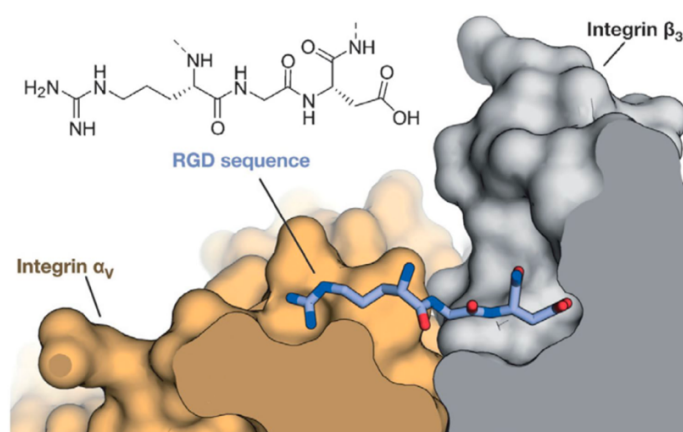


Figure 7 – Crystal structure of the fibronectin's RGD sequence bound to the α_v (in orange) and β_3 (in grey) of the integrin receptor (PDB 4MMX). Reproduced from Mabonga, L. et Al. (*Cancer. Int. J. Pept. Res. Ther.* **2020**, 26 (1), 225–241).

Based on these findings, synthetic peptides and peptidomimetics incorporating the RGD structural motif have been developed as potent integrin ligands and antagonists, with the aim of inhibiting adhesion of anchorage-dependent tumor cells to ECM proteins and thereby modulating integrin-mediated biological functions in cancer cells. The first compound identified as an antagonist of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins was discovered by Kessler and colleagues in 1991³¹. Through spatial screening involving the synthesis of cyclopentapeptides and cyclohexapeptides, they determined the optimal three-dimensional arrangement of the RGD pharmacophoric groups, leading to the development of two cyclic pentapeptides: c[RGDfVG], an inhibitor of fibronectin binding to the $\alpha_{IIb}\beta_3$ integrin, and c[RGDfV], which blocks vitronectin binding to $\alpha_v\beta_3$ ³². Both peptides contain a cyclic

backbone, limiting conformational flexibility, and incorporate a D-amino acid required to maintain the correct spatial orientation of the pharmacophoric groups.

Subsequent *structure-activity relationship* (SAR) studies revealed that the positively and negatively charged side chains of arginine and aspartic acid, the steric constraints imposed by glycine, the hydrogen bond formed with the nitrogen of D-phenylalanine and the hydrophobic interactions mediated by its side chain are all critical for receptor binding and maintenance of the bioactive conformation. In contrast, the valine residue was shown to be non-essential for activity, allowing its removal or substitution (*figure 8*)³³.

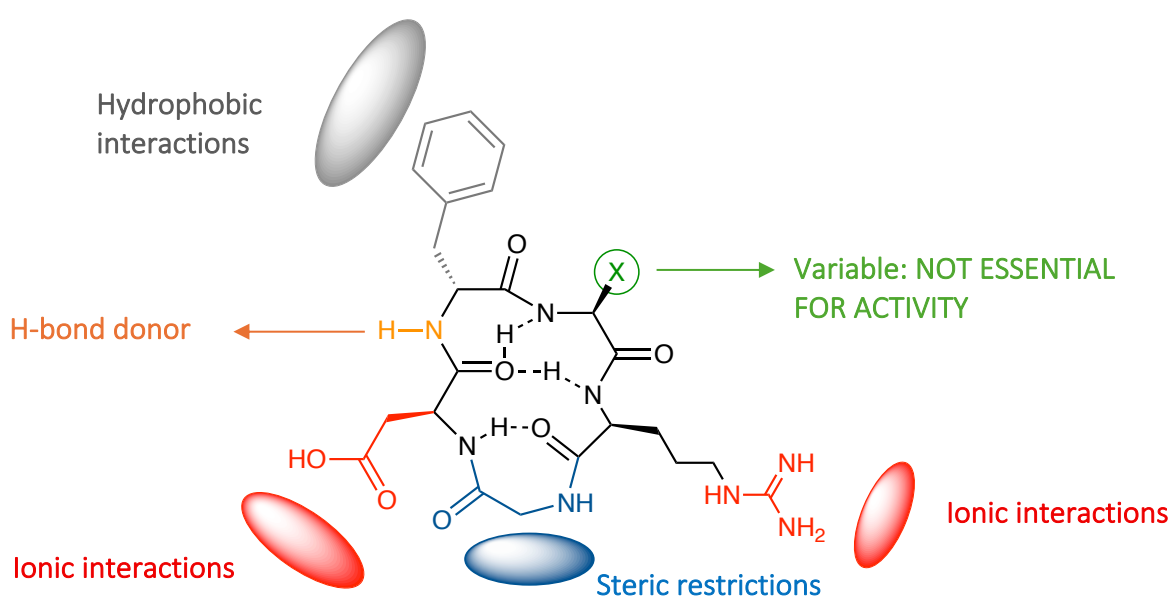


Figure 8 – c(RGDfV) and pharmacophoric model

Starting from the lead compound c[RGDfV], systematic backbone modifications were introduced to improve its pharmacological profile. Specifically, each amide bond was individually N-methylated, generating a series of five monomethylated cyclopentapeptides³⁴. The biological activity of these compounds was evaluated to assess the impact of N-methylation. Among them, c[RGDf-N(Me)V], commonly known as **Cilengitide** (*figure 9*), emerged as the most potent and selective $\alpha_v\beta_3$ integrin antagonist³⁵.

Structural insight into the molecular recognition of RGD ligands by integrins was provided by the crystal structure of the extracellular domain of $\alpha_v\beta_3$ (PDB ID: 1L5G) in complex with cilengitide (*figure 10*). The structure revealed that the ligand binds within a cleft located at the interface between the $\alpha_v\beta$ -propeller and the β_3 β A domains, establishing extensive

contacts with both subunits. Unlike many receptor-ligand interactions that rely predominantly on hydrophobic complementarity, recognition of the RGD motif is governed primarily by electrostatic and metal-coordination interactions.

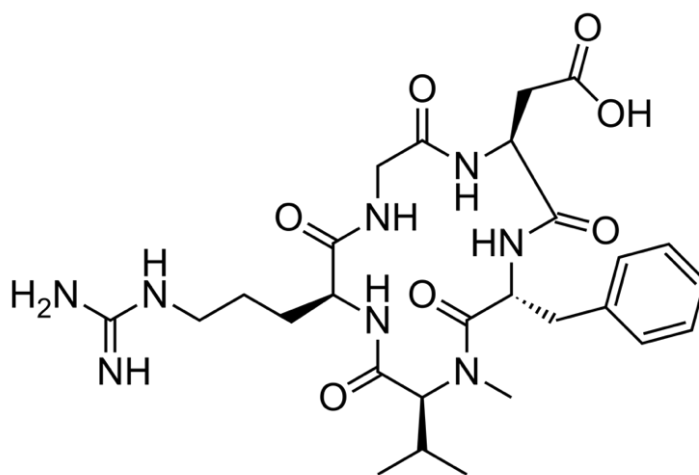


Figure 9 - Chemical structure of Cilengitide®

The Arg and Asp residues of the RGD sequence form a characteristic “*charged clamp*” that anchors the ligand to oppositely charged regions of the receptor. The guanidinium group of Arg inserts into a negatively charged pocket of the α_v subunit, where it is stabilized by a bidentate salt bridge with Asp218 and an additional interaction with Asp150. On the opposite side of the binding interface, the Asp carboxylate coordinates a divalent metal ion located at the *MIDAS* (Metal-Ion-Dependent Adhesion Site) of the β_3 βA domain. The second oxygen atom of the Asp side chain further participates in an extensive hydrogen-bonding network involving Tyr122, Asn215 and Arg214 of β_3 , resulting in complete burial of the Asp residue within the binding pocket.

The central Gly residue occupies the α/β interface and contributes to ligand recognition through close packing interactions. This structural arrangement explains the strict requirement for glycine in integrin-binding RGD motifs, as even minor substitutions disrupt receptor recognition. Notably, only limited hydrophobic contacts are observed, mainly involving the D-Phe residue of cilengitide, whereas the N-methylvaline residue projects toward the solvent and contributes little to direct receptor binding.

Ligand engagement also induces local conformational rearrangements within the β_3 β_A domain. Binding promotes occupancy of the MIDAS site by a divalent cation and the formation of an adjacent ligand-associated metal-binding site (LIMBS), which stabilizes the ligand-binding surface. These observations established the structural basis for RGD-dependent integrin recognition and provided a molecular framework for the rational design of high-affinity integrin antagonists such as cilengitide. Importantly, the crystal structure demonstrated that ligand binding is accompanied by subtle tertiary and quaternary conformational changes within $\alpha_v\beta_3$, supporting the concept that ligand recognition and integrin activation are structurally coupled processes.^{36,37}

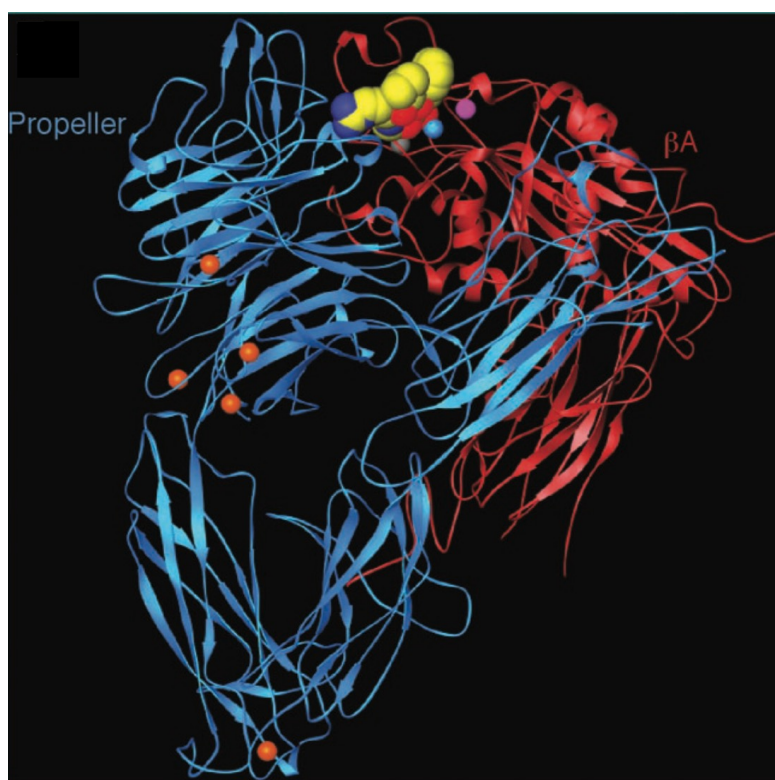


Figure 10 – Ribbon drawing of $\alpha_v\beta_3$ -Cilengitide complex structure (α_v and β_3 subunits are shown in blue and red respectively). The peptide is bound at the propeller- β_A domain interface with the ADMIDAS, MIDAS, and LIMBS metal ions shown. The carbon, nitrogen, and oxygen atoms of cilengitide are shown in yellow, blue, and red, respectively. Adapted from Ref. [36]

Cilengitide demonstrated strong potential as an anticancer agent against multiple tumor types, including glioblastoma, an aggressive brain cancer that remains difficult to treat. It was the first integrin-antagonist peptidomimetic to reach phase III clinical trials. In preclinical and early clinical (phase I/II) studies, Cilengitide exhibited anti-angiogenic and anti-invasive properties, enhanced the radiosensitivity of glioma cells when combined with radiotherapy,

and showed good tolerability both alone and in combination with temozolomide, with minimal toxicity and promising antitumor activity. The phase III trial aimed to evaluate overall survival in glioblastoma patients treated with Cilengitide plus temozolomide and radiotherapy versus the standard temozolomide-radiotherapy regimen.

However, the results did not demonstrate any significant improvement in survival, effectively halting further development of Cilengitide as a therapeutic agent³⁸. Despite its clinical failure as a standalone therapeutic, Cilengitide remains a landmark compound that validated RGD-based targeting of α_v integrins in humans. Importantly, its limitations highlighted the need for alternative strategies in which RGD ligands are employed not as cytotoxic agents themselves, but as targeting moieties for selective drug delivery.

1.5 Integrin-targeting for drug delivery

Although numerous therapeutic strategies against cancer have been developed and refined over the years, including chemotherapy, radiation therapy, surgery and immunotherapy, these conventional approaches still present significant limitations³⁹. Such drawbacks are associated either with the disease itself, such as the rapid proliferation of cancer cells or the development of diffuse metastases or with severe treatment-related side effects. In particular, the major limitations of chemotherapeutic agents arise from their lack of selectivity, which results in damage to healthy tissues, and from the development of multidrug resistance (MDR) in tumor cells⁴⁰.

In this context, the development of targeted drug delivery systems represents a promising strategy to overcome the limitations of conventional chemotherapy and to enhance therapeutic efficacy. These systems are designed to selectively deliver drugs to the site of action, thereby increasing local drug concentration while minimizing systemic exposure and adverse effects, ultimately allowing a reduction in the total administered dose^{41,42}.

Many targeted delivery systems can be conceptually described as comprising **three key elements**: (1) a targeting moiety with high selectivity for the biological target, such as a small molecule or an antibody with nanomolar or sub-nanomolar affinity; (2) a cleavable or non-cleavable linker; and (3) a bioactive compound or a nanostructure capable of carrying therapeutic or imaging agents either internally or on its surface⁴¹.

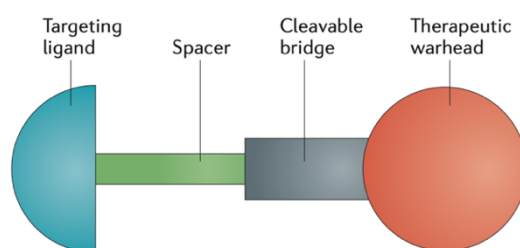


Figure 11 – Schematic representation of a typical ligand-drug conjugate. Reproduced from Ref. [41]

For cancer therapy applications, suitable biological targets are typically overexpressed on tumour cells or on neoangiogenic vessels, while being minimally present in healthy tissues. Additionally, the selected target should undergo internalization upon ligand binding,

enabling intracellular release of the therapeutic payload. In this regard, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are particularly attractive targets as they are highly overexpressed on the surface of various cancer cell types and neoangiogenic vasculature and are known to internalize following ligand interaction¹⁴.

1.5.1 Monoclonal Antibodies (mAbs) and Antibodies Drug Conjugates

Monoclonal antibodies (mAbs) represent one of the most successful classes of targeted therapeutics developed in modern medicine. Produced by B cells and designed to selectively recognize specific antigens, mAbs exert their therapeutic effects through highly specific molecular interactions^{43,44}. Among the five immunoglobulin isotypes (IgG, IgA, IgM, IgD and IgE), IgG, particularly IgG1 and IgG4, is most frequently employed for therapeutic applications due to its prolonged plasma half-life, high affinity and favorable effector functions^{45,46}. Since the approval of rituximab in 1997, monoclonal antibody therapy has expanded rapidly, with over 120 mAbs approved by FDA for oncological and inflammatory indications, highlighting their clinical relevance as precision medicines⁴⁷. Antibody-drug conjugates (ADCs) are an evolution of monoclonal antibody therapy, combining the targeting capability of antibodies with the cytotoxic potency of small-molecule drugs. ADCs are often described as “biological missiles”, in which the antibody functions as a homing device and the cytotoxic payload acts as the warhead^{48,49}.

Upon systemic administration, the antibody selectively binds a cell-surface antigen that is overexpressed on diseased cells. The ADC-antigen complex is subsequently internalized via receptor-mediated endocytosis, followed by lysosomal processing and intracellular release of the drug, ultimately inducing apoptosis of the target cell⁵⁰. The therapeutic performance of ADCs depends on **five key elements**: the target antigen, the antibody scaffold, the linker, the cytotoxic payload and the conjugation strategy. The antigen must be highly and selectively expressed on target cells, exhibit minimal shedding and efficiently undergo internalization. The antibody should display high affinity, long circulation half-life, low immunogenicity and contribute to antitumor activity through antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).

The linker is a critical structural component that must remain stable in circulation while enabling controlled drug release at the target site. Both cleavable and non-cleavable linkers have been developed, with cleavable linkers dominating current ADC designs due to their ability to generate a bystander effect⁵¹. Increasing linker hydrophilicity has emerged as an effective strategy to reduce aggregation, improve pharmacokinetics, and allow higher drug loading^{52,53}. The payload must be extremely potent (typically active in the picomolar range), chemically stable and capable of inducing cell death at low intracellular concentrations. Common payload classes include microtubule inhibitors and DNA-damaging agents^{54,55}.

To date, the U.S. Food and Drug Administration has approved multiple ADCs for clinical use, with HER2-targeting ADCs representing the most prominent example. The continuous increase in clinical trials reflects the strong therapeutic potential of ADCs. Nevertheless, several limitations still restrict their broader application. ADCs require parenteral administration, exhibit limited penetration into solid tumors due to their large molecular size and are affected by the so-called “binding site barrier”, whereby high-affinity antigen binding near blood vessels prevents deeper tumor diffusion. Consequently, ADCs often show reduced efficacy in poorly vascularized or hypoxic tumor regions⁵⁶. Another critical challenge lies in conjugation chemistry. The *drug-to-antibody ratio* (DAR) is a key determinant of efficacy, stability and toxicity. Random conjugation methods, such as lysine coupling, generate heterogeneous ADC populations and may compromise antigen recognition⁵⁷. Site-selective conjugation strategies, including cysteine engineering, incorporation of unnatural amino acids, enzymatic ligation and biorthogonal click chemistry, have been developed to improve DAR control and reproducibility^{57,58}. Despite these advances, only a small proportion of an administered ADC dose, often less than 1%, successfully reaches its intracellular target. This limited delivery contributes to dose-limiting toxicities and increases overall production costs⁵⁹. Several integrin-targeting monoclonal antibodies (mAbs) have successfully reached clinical application, including abciximab ($\alpha_{IIb}\beta_3$), natalizumab (α_4), vedolizumab ($\alpha_4\beta_7$) and etrolizumab (β_7), highlighting the translational potential of integrin-directed therapeutic strategies. Despite the availability of integrin-targeting antibodies, integrin-based ADCs remain relatively underexplored. To date, IMGN-388, an ADC consisting of the pan- α_v integrin antibody intetumumab conjugated to a maytansinoid payload DM4,

represents one of the few examples evaluated in clinical trials^{14,60}. More recently, clinical studies have been initiated for ADCs and peptide-drug conjugates (PDCs) targeting integrins such as $\alpha_v\beta_3$ and $\alpha_v\beta_5$, reflecting renewed interest in leveraging integrins for targeted drug delivery.

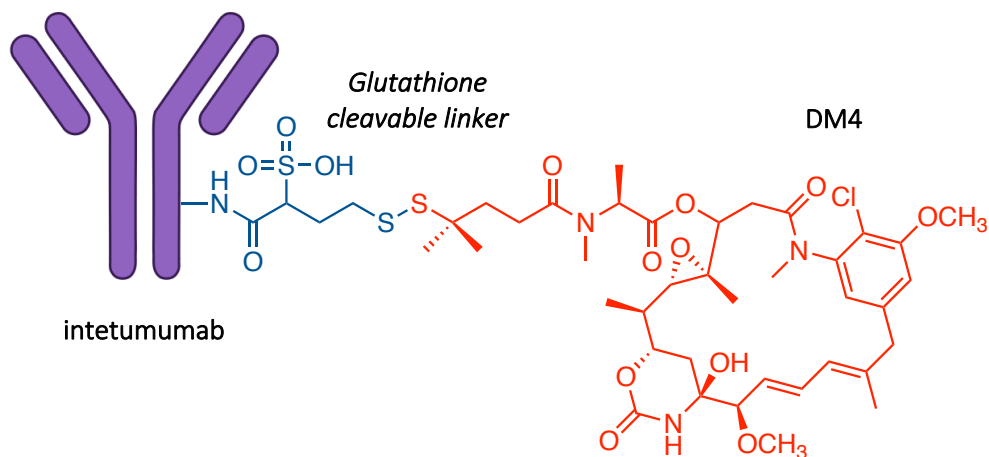


Figure 12 – Structure of Integrin-targeting Antibody drug conjugate IMGN-388

1.5.2 Small molecules Drug Conjugates

Small molecule drug conjugates (SMDCs) represent a promising alternative to antibody-based delivery systems. In these constructs, a cytotoxic payload is covalently linked to a low-molecular-weight ligand that serves as the targeting moiety. Owing to their reduced size, compared with antibodies, SMDCs display favourable pharmacokinetic properties, including efficient extravasation, deep tumour penetration and rapid tissue distribution. Moreover, their pharmacokinetic profile can be readily fine-tuned through relatively simple structural modifications^{61,62}. Despite these advantages, SMDCs may suffer from a limited targeting index (T.I.), defined as the ratio between the selectivity of the conjugate toward cells expressing different levels of the molecular target (e.g., cell lines A and B) and the intrinsic selectivity of the unconjugated cytotoxic agent. A low T.I. reflects insufficient discrimination between target-positive and target-negative cells, thereby limiting therapeutic efficacy and safety^{61,62}.

Within SMDCs, the linker represents a critical structural element governing the biological performance of the entire bioconjugate. Rational linker design is therefore essential to improve the targeting index and overall therapeutic outcome. Ideally, the linker must exhibit

sufficient chemical and metabolic stability to allow the intact conjugate to circulate in the bloodstream and reach the tumour site, while enabling efficient drug release once the SMDC has been internalized by target cells or exposed to the tumour microenvironment. In addition, linker composition can significantly influence pharmacokinetic parameters such as solubility, circulation time and aggregation behaviour. For example, the incorporation of hydrophilic polyethylene glycol (PEG) spacers is frequently employed to enhance aqueous solubility and reduce non-specific aggregation phenomena⁵³.

Linkers used in SMDCs are generally classified into two main categories: *non-cleavable* and *cleavable linkers*. Non-cleavable linkers are suitable when the conjugated drug retains biological activity even when covalently bound to the targeting moiety. These linkers are characterized by high chemical and metabolic stability; however, such stability can limit the efficient intracellular release of the active payload. A representative example is ^{99m}Tc-Maraciclatiside, a single-photon emission computed tomography (SPECT) imaging agent in which the diagnostic radionuclide is linked to an RGD-based $\alpha_v\beta_3$ integrin antagonist through a *non-cleavable* spacer (*figure 13*). In clinical studies, this conjugate demonstrated the ability to identify both malignant and benign breast lesions overexpressing $\alpha_v\beta_3$ integrin, achieving the desired diagnostic effect without requiring linker degradation^{63,64}.

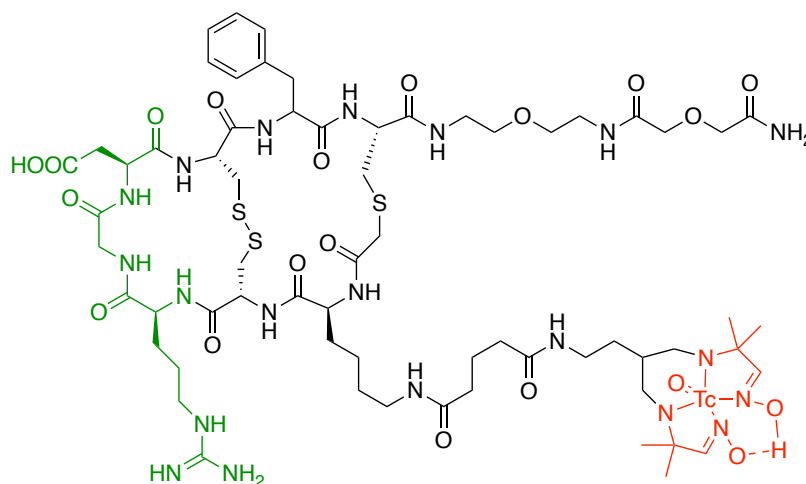
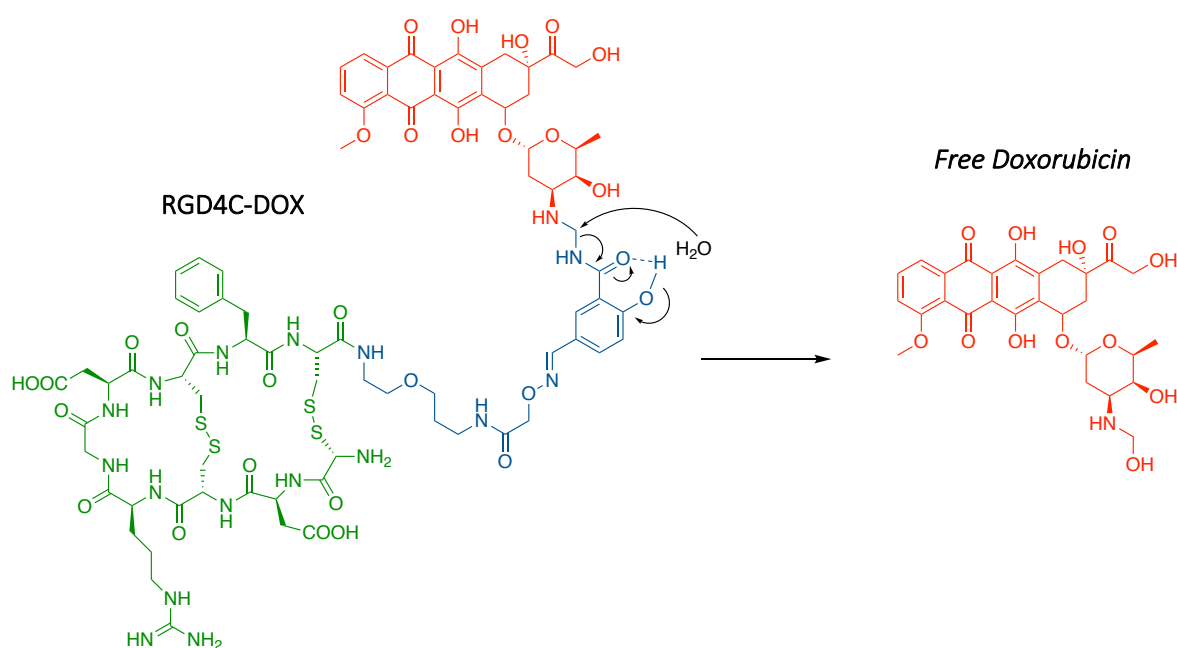


Figure 13 – ^{99m}Tc-Maraciclatiside's chemical structure; RGD binding sequence is highlighted in green

In contrast, cleavable linkers are specifically engineered to release the cytotoxic drug in response to defined stimuli, enabling precise spatial and temporal control over drug activation. Among these, pH-responsive linkers exploit the acidic conditions characteristic of the intracellular compartments of tumour cells, such as endosomes and lysosomes, which

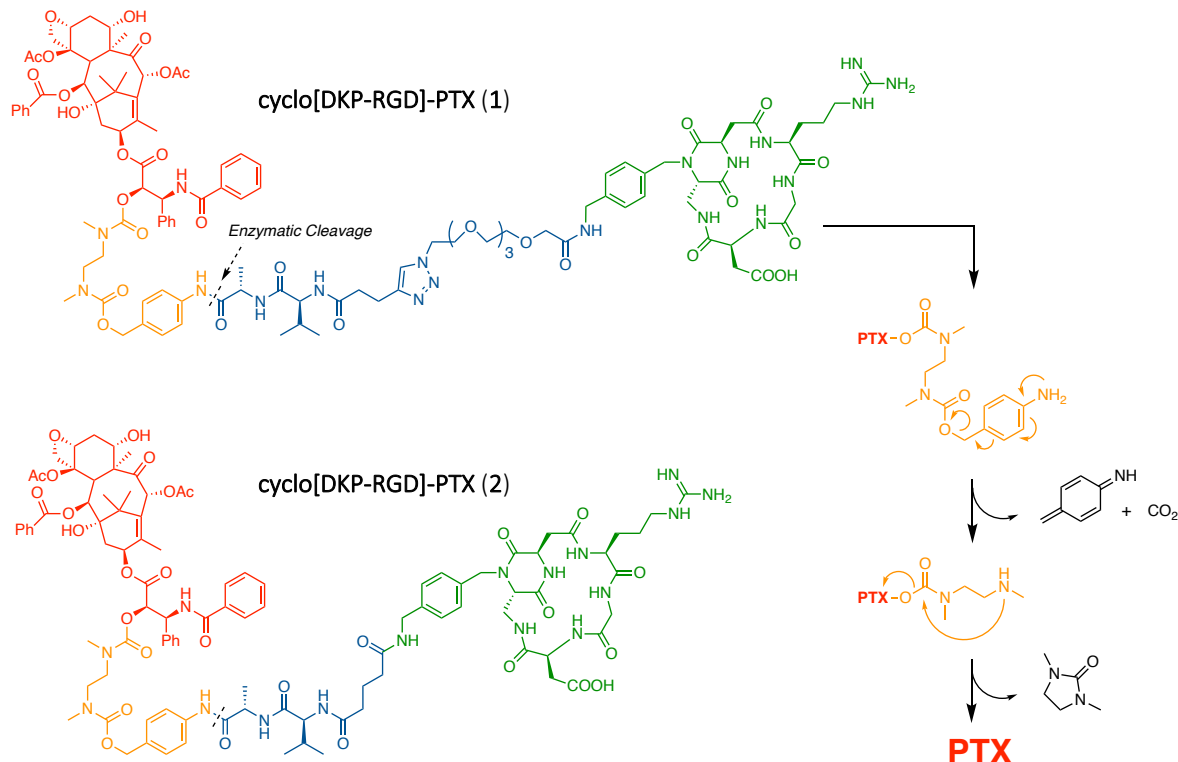
arise from the high metabolic activity of cancer cells⁶⁵. This strategy relies on the incorporation of acid-labile functional groups, such as hydrazones, imines, *cis*-aconityl moieties, trityl groups, N-Mannich bases, esters, acetals and β -thiopropionates, that remain stable at physiological pH but undergo rapid hydrolysis following cellular uptake^{65–67}. An illustrative example is the RGD4C-DOX conjugate, in which doxorubicin (DOX) is linked to an $\alpha_v\beta_3$ integrin-targeting ligand via an N-Mannich base. This conjugate is rapidly cleaved either in the acidic tumour microenvironment or after internalization, resulting in the preferential release of an active doxorubicin metabolite at the intended site of action⁶⁸ (scheme 1).



Scheme 1 – Hydrolysis Mechanism of the N-Mannich base linker in the RGD4C–DOX conjugate and release of the active doxorubicin metabolite

Another highly promising approach involves the use of enzyme-cleavable linkers incorporating **short peptide sequences** or other functional motifs recognized by intracellular proteases. These enzymes, which display low activity in the bloodstream, become highly active within endosomal and lysosomal compartments following internalization of the SMDC⁶⁵. A notable example is provided by a cyclo[DKP-RGD]-paclitaxel (PTX) conjugates, in which the linker contains a Val-Ala dipeptide sequence specifically cleaved by lysosomal enzymes, particularly *cathepsin B*. This SMDC exhibited a markedly improved targeting index (T.I. = 9.0), highlighting the crucial role of the linker in governing selective drug release. In this system, cleavage of the peptide segment triggers a *self-immolative* degradation process,

ultimately liberating the active cytotoxic agent^{69,70} (*scheme 2*). Collectively, these examples underscore the central importance of linker design in optimizing SMDC efficacy, selectivity and therapeutic potential.



Scheme 2 – Cleavage mechanism of cyclo[DKP-RGD]-PTX; After cyclo[DKP-RGD]-mediated internalization (green), the Val-Ala recognition sequence (blue) is cleaved by lysosomal enzymes. This triggers self-immolative degradation of the spacer-PABC unit (orange), leading to breakdown of the PTX-spacer prodrug (red). The terminal amino group of the spacer then promotes an intramolecular nucleophilic substitution, resulting in release of free PTX.

1.5.3 Integrin Targeting Nanoparticles Delivery Systems

Nanocarrier-based drug delivery systems offer several advantages over conventional chemotherapy, including improved pharmacokinetics, enhanced tumor accumulation, protection of the payload from premature degradation and reduced off-target effects⁷¹. When combined with integrin-targeting ligands, nanotherapeutics enable preferential delivery of bioactive agents to diseased tissues. The multivalent presentation of targeting moieties on the nanoparticle surface increases ligand-receptor interactions, thereby enhancing binding avidity and cellular uptake through receptor-mediated endocytosis^{72,73}. RGD-based peptides are among the most widely used ligands for integrin targeting due to their high affinity, small size, synthetic accessibility and low immunogenicity. RGD-

functionalized nanoparticles have been extensively investigated in preclinical studies for the delivery of chemotherapeutics, nucleic acids and imaging agents. For example, $\alpha_v\beta_3$ -targeting nanoparticles obtained by conjugating RGD peptides to PEGylated chitosan-poly(ethyleneimine) hybrids have demonstrated efficient siRNA delivery and high gene silencing activity. Similarly, RGD-modified polymeric nanoparticles based on PEG-PLA have been employed to deliver poorly soluble chemotherapeutic agents such as paclitaxel and docetaxel, improving their solubility and reducing dose-limiting toxicity^{74–76}.

Nanoparticles can exploit both passive and active targeting mechanisms to accumulate at tumor sites. Passive targeting relies on the Enhanced Permeability and Retention (EPR) effect, a phenomenon associated with the abnormal architecture of tumor vasculature. Rapid and disorganized neoangiogenesis leads to increased vascular permeability, allowing nanoparticles, typically below 400 nm, to extravasate into the tumor interstitial space. Impaired lymphatic drainage further promotes nanoparticle retention within the tumor microenvironment^{77,78}. Active targeting is achieved by nanoparticle surfaces functionalization with ligands such as RGD peptides or integrin antagonists. This strategy aims to enhance cellular uptake via integrin-mediated endocytosis, improve intracellular drug release, and ultimately increase therapeutic efficacy while reducing required doses and systemic side effects. Although active targeting does not always significantly increase overall tumor accumulation compared to passive delivery, it has been shown to improve cellular internalization and intracellular drug localization, which are critical parameters for therapeutic success^{79–81}.

Integrin-targeted nanocarriers can be classified according to their chemical nature, including polymeric, protein-based, lipid-based and inorganic systems. Polymeric nanoparticles are the most extensively studied due to their biodegradability, relatively low toxicity and high versatility. They can be engineered into various architectures, such as micelles, nanocapsules and solid nanoparticles, and readily modified through surface functionalization, for instance by PEGylation, to improve solubility and reduce nonspecific protein adsorption^{82,83}. Protein-based nanocarriers offer improved biocompatibility and solubility compared to synthetic polymers. Albumin, in particular, has been widely used owing to its favourable pharmacokinetic profile and natural role in drug transport. An

example of an integrin-targeted protein-based system involves albumin nanoparticles functionalized with cyclo[RGDyK] and loaded with gemcitabine, which demonstrated enhanced intracellular uptake and improved therapeutic index *in vitro*^{84,85} (figure 14).

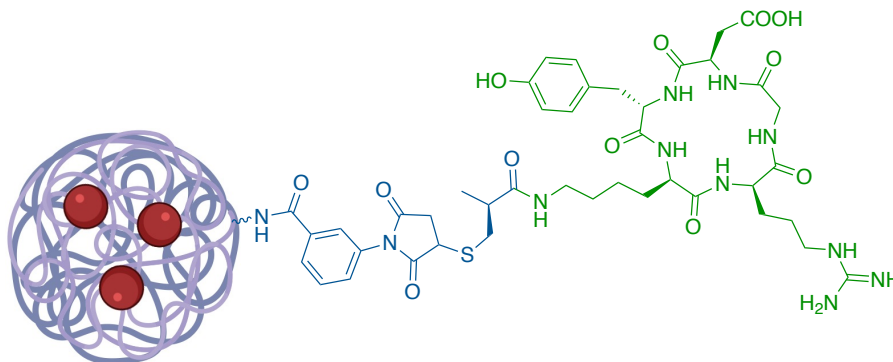


Figure 14 – Albumin nanoparticle, loaded with gemcitabine (in red), functionalized with cyclo[RGDyK]C (in green)

Among lipid-based systems, liposomes represent a clinically established platform capable of encapsulating both hydrophilic and hydrophobic agents. RGD-functionalized liposomes have been developed as theranostic systems combining chemotherapeutic drugs and imaging agents, enabling simultaneous tumor targeting, therapy and diagnostics^{86–88}. Finally, Inorganic nanoparticles, such as gold nanorods and graphene oxide, have also been investigated for integrin-targeted delivery, particularly in theranostic and photothermal applications.

Although concerns regarding long-term toxicity remain, these systems benefit from unique optical and physical properties^{89–91}. Despite their promise, integrin-targeted nanotherapeutic systems face several challenges. The EPR effect is highly variable among tumor types and patients, often leading to inconsistent nanoparticle accumulation. Furthermore, high interstitial pressure and dense cellular architecture within tumors limit deep tissue penetration. Active targeting may also be hindered by the binding-site barrier effect, where strong ligand-receptor interactions near blood vessels prevent diffusion into deeper tumor regions⁷⁹. Nevertheless, strategies such as stimuli-responsive nanoparticles, lymph node-targeted delivery and immunomodulatory nanocarriers are being explored to overcome these limitations⁸².

Within the context of this PhD work, integrin-targeted nanocarriers, particularly those based on RGD peptides, represent a valuable platform for improving selective drug delivery. Their ability to combine multivalent targeting, enhanced cellular uptake and modular design makes them an attractive alternative or complement to antibody-based delivery systems. Continued optimization of ligand density, carrier architecture and release mechanisms is expected to further enhance the therapeutic potential of RGD-based drug delivery systems in cancer treatment.

2. RESULTS AND DISCUSSION

2.1 Introduction to the work

This PhD project aims to design, synthesize and evaluate novel cRGD-based conjugates for targeted anticancer applications, combining nanoparticle-mediated drug delivery, small-molecule drug conjugation and advanced peptidomimetic scaffold development. The central molecular element of this work is the **3a-RGD** peptidomimetic (*figure 15*), a conformationally constrained cyclic RGD ligand derived from azabicyclolactam-based reverse-turn mimetics. Owing to its structural rigidity and the possibility of introducing functional groups on the bicyclic framework, this scaffold represents a versatile platform for the development of targeted bioconjugates and delivery systems.

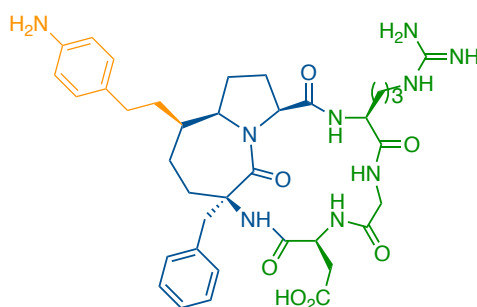


Figure 15 – Chemical structure of 3a-RGD

The first objective concerns the optimization of the synthetic strategy for the preparation of the 3a-RGD scaffold, with particular focus on improving the efficiency and scalability of key transformations involved in the construction of the bicyclic azabicyclolactam core. Special attention was devoted to the Ru-catalyzed cross-enyne metathesis/ring-closing metathesis sequence and to the late-stage functionalization of the peptide through installation of an azide linker suitable for “click-chemistry” reactions such as Huisgen 1,3-dipolar cycloaddition^{92,93}.

The second part of the work explores the development of integrin-targeted nanocarrier systems, in which the 3a-RGD ligand is employed as a targeting moiety for nanoparticle surface functionalization. In particular, three different nanosystems were investigated: doxorubicin-loaded liposomes designed as theranostic platforms for targeted delivery to triple-negative breast cancer cells, chitosan-based nanoparticles developed as a potential mucoadhesive system for intranasal delivery and epithelial transport and a dual-

functionalized silk fibroin nanoparticle platform designed to achieve a multistep targeting strategy, combining blood-brain barrier (BBB) crossing and tumor-specific delivery.

The third research area focuses on the development of small-molecule drug conjugates based on cyclic RGD targeting vectors. In this part of the work, the 3a-RGD ligand was conjugated to naphthalene diimide (NDI) derivatives, a class of molecules known for their ability to bind and stabilize G-quadruplex DNA structures. The design of these conjugates included the incorporation of cleavable linkers capable of releasing the cytotoxic payload following receptor-mediated internalization, thereby combining targeted delivery with controlled intracellular drug activation.

In parallel with these applications, the thesis also investigates the development of new synthetic methodologies for the preparation of functionalized azabicyclic lactam scaffolds, aimed at expanding the structural diversity of conformationally constrained dipeptide mimics that can be employed in RGD-based peptidomimetics.

Although these research directions involve different chemical systems and experimental approaches, they are united by a common conceptual framework: the development of integrin-targeted molecular platforms capable of improving the selective delivery of therapeutic agents to tumor cells. Through the combination of synthetic organic chemistry, peptide design and nanomedicine strategies, this work contributes to the development of new tools for integrin-mediated targeted drug delivery in cancer therapy.

2.2 Previous studies on RGD integrin antagonists

The development of **cilengitide** (EMD 121974) represents one of the most illustrative examples of how conformationally constrained RGD ligands can be translated from structure-based design into advanced preclinical and clinical evaluation (see [Chapter 1.4](#)). As a cyclic pentapeptide targeting α_v integrins, cilengitide showed high affinity for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ subtypes, and it became a reference compound for integrin antagonism and anti-angiogenic strategies.

Early NMR and molecular modeling studies performed by *Kessler and co-workers* demonstrated that the cyclic pentapeptide c(RGDfV), a precursor of cilengitide, preferentially adopts a $\beta_{II'}/\gamma$ -turn conformation in solution, with the D-Phe residue occupying the i+1 position of the $\beta_{II'}$ turn. This structural motif was subsequently identified as a key conformational feature associated with high integrin-binding activity and therefore served as the basis for the design of numerous conformationally constrained analogues in which the D-Phe-Val segment was replaced by β -turn mimetics. These modifications were intended to reduce the conformational freedom of the peptide while preserving the spatial arrangement of the RGD pharmacophore required for integrin recognition³².

Definitive structural insight into the bioactive conformation of cRGD ligands was later provided by the X-ray crystal structure of the $\alpha_v\beta_3$ integrin extracellular domain in complex with cilengitide (PDB ID: 1L5G). The bound ligand adopts an almost extended RGD arrangement characterized by an *inverse γ -turn* with Asp in the i+1 position and a distorted *$\beta_{II'}$ -turn* involving Gly and Asp at the i+1 and i+2 positions, respectively. In this receptor-bound conformation, the distance between the C β atoms of Arg and Asp is approximately 8.9 Å, a geometrical parameter considered important for integrin recognition. The crystal structure further revealed the molecular basis of ligand binding. The Arg guanidinium group forms a network of electrostatic interactions with Asp150 and Asp218 of the α_v subunit, while one oxygen atom of the Asp side-chain carboxylate coordinates the divalent metal ion located at the MIDAS of the β_3 subunit. Additional stabilization is provided by hydrogen bonds involving the Asp backbone -NH and the carbonyl oxygen of Arg216, as well as

interactions between the second Asp carboxylate oxygen and the backbone amides of Asn215 and Tyr122. The central Gly residue is positioned in close contact with the integrin surface, explaining the strict requirement for glycine within the RGD sequence. Together, these interactions generate the characteristic “*charged clamp*” responsible for high-affinity recognition of RGD-containing ligands by $\alpha_v\beta_3$ integrin. Comparison of the solution and receptor-bound structures suggests that although cyclic RGD peptides exhibit a preferred $\beta II'/\gamma$ -turn organization in solution, some degree of conformational adaptation occurs upon receptor binding⁹⁴. These observations explain why successful integrin antagonists generally combine conformational preorganization with sufficient flexibility to adopt the optimal geometry required for productive interactions within the integrin binding pocket.

2.2.1 *Structural turn mimetics*

Replacing the peptide backbone with a small-molecule unit able to induce a defined turn conformation represents an attractive strategy to improve drug-like properties including metabolic stability, oral bioavailability and pharmacokinetic behaviour. In this framework, *turn mimetics* behave as molecular scaffolds that orient key substituents in a defined three-dimensional arrangement, reproducing the bioactive geometry while reducing conformational entropy penalties upon binding. The biological activity of peptides is strongly dependent on their three-dimensional conformation. In particular, the spatial disposition of atoms along the peptide backbone defines the secondary structure. Due to the electronic and geometric constraints of the amide bond, and the limited rotational freedom around adjacent bonds, peptide chains populate discrete conformational regions. The torsion angles around the N-C α and C α -C bonds-known as ϕ (phi) and ψ (psi), respectively, largely determine the accessible secondary-structure motifs⁹⁵. The most common secondary structures found in proteins include α -helices, β -sheets and *reverse turns*. Reverse turns comprise γ -, β - and α -turns correspond to motifs involving 3, 4 and 5 amino acid residues, respectively. In γ -turns, the carbonyl oxygen of residue i can form an intramolecular hydrogen bond with the amide hydrogen of residue $i+2$, whereas in β - and α -turns the equivalent interaction involves residues $i+3$ and $i+4$, respectively. Turn classification is based on backbone geometry and is described by the torsional angles of residues $i+1$ and $i+2$ (for β -turns) or $i+1$ (for γ -turns)^{96,97}.

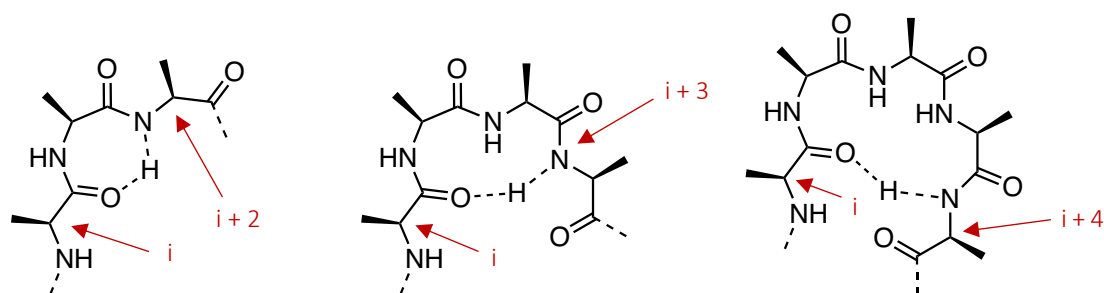


Figure 16 – representation of the structure of γ -, β - and α -turn (from left to right). Adapted from Ref. [96]

Among all turn motifs, β -turns are the most frequently observed and have been extensively investigated. Consequently, significant efforts have been devoted to the development of structural motifs capable of mimicking their geometry. Several classes of reverse-turn mimetics have been explored, including *bicyclic peptides*, *benzodiazepines*, *carbohydrate-based scaffolds*, *spirocycles* and *trans-pyrrolidine-3,4-dicarboxamides*⁹⁸.

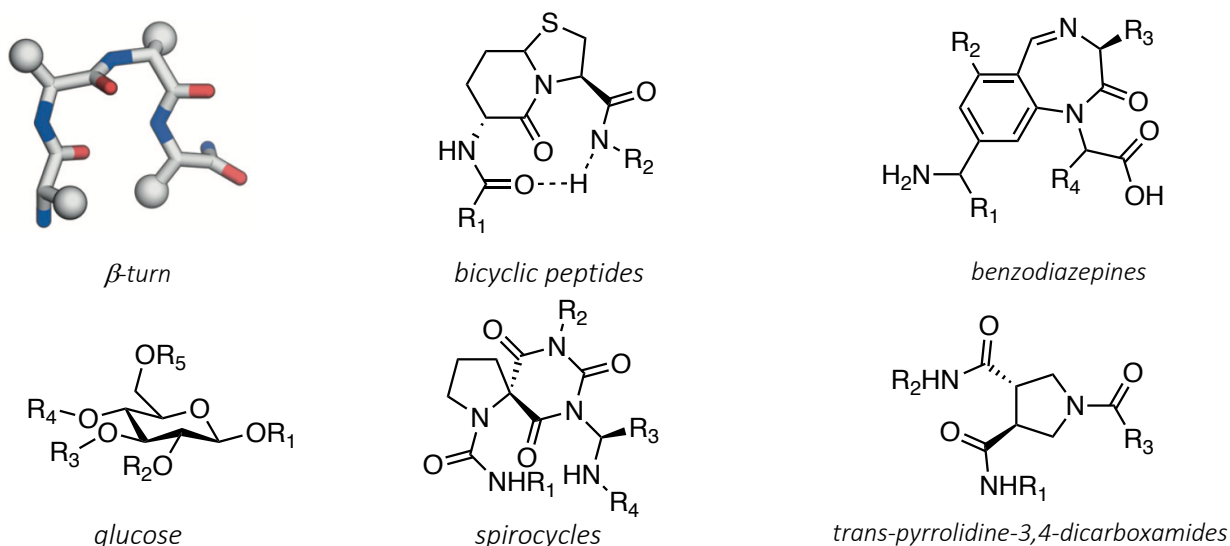


Figure 17 – Scaffolds that mimic β -turn conformations. Adapted from Ref. [98]

2.2.2 Bicyclic peptides as reverse-turn inducers

Early evidence that bicyclic systems could effectively mimic peptide turns was provided by Nagai and Sato, who demonstrated that specific bicyclic compounds could reproduce the conformation adopted by the D-Phe-L-Pro sequence in **gramicidin S**, a peptide antibiotic. In particular, the first bicyclic structures reported were based on a 1-aza-2-oxabicyclo[4.3.0]alkane framework containing a sulfur atom in the five-membered ring and were shown to preferentially adopt a $\beta II'$ -turn⁹⁹.

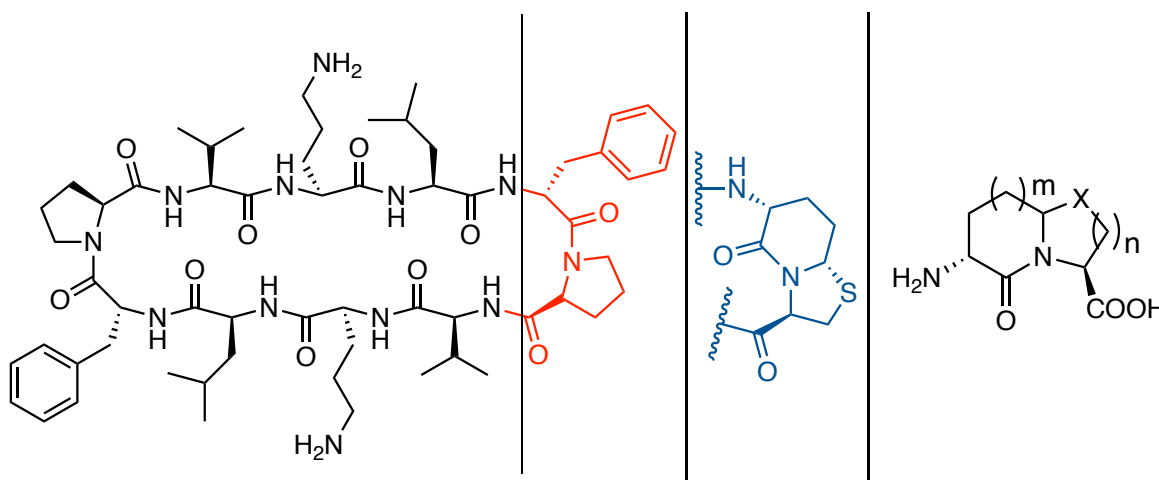


Figure 18 – Structure of **gramicidin S**: comparison of the D-Phe-L-Pro sequence (in red) and the dipeptide synthesized by Nagai and Sato (in blue); Generic structure of an amino acid with azabicyclo[XY0]alkane structure

This discovery paved the way for the design of bicyclic scaffolds as structural elements capable of enforcing β -turn geometries. Subsequent studies led to the synthesis of several constrained amino acids featuring 1-aza-2-oxabicyclo[X.Y.0]alkane motifs. These bicyclic systems can be considered conformationally locked substitutes of dipeptide fragments such as Ala-Pro and Phe-Pro. A common feature among these scaffolds is the presence of a proline-derived structural element, while both lactam ring size and stereochemical configuration may vary. Notably, [5.3.0] and [4.3.0] fused bicyclic systems have emerged as valuable intermediates for the preparation of conformationally restricted compounds showing activity as $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_v\beta_1$ integrin antagonists, as well as antagonists of the bradykinin B2 receptor¹⁰⁰. Moreover, related scaffolds have also been exploited in the development of inhibitors of thrombin/factor VII, inhibitors of apoptosis proteins (IAPs), and modulators of prostaglandin F2 α receptors¹⁰¹.

A notable example of this approach is provided by *Belvisi et al.*, who exploited a small library of constrained cyclic pentapeptides to identify potent antagonists of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins¹⁰². These ligands are characterized by the presence of a conformationally restricted azabicyclolactam scaffold, which has been shown by conformational and spectroscopic investigations to act as an efficient *reverse-turn inducer* when incorporated into peptide sequences. The synthesized scaffolds were subsequently employed as building blocks for the preparation of RGD cyclopentapeptides, whose integrin antagonistic activity was evaluated against purified $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors. Among the investigated compounds, the derivative **1a-RGD** emerged as the most active analogue, exhibiting high affinity for both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, with IC_{50} values of 6.4 and 7.7 nM, respectively (*Table 1*). Unlike cilengitide (EMD121974), which displayed a marked preference for $\alpha_v\beta_5$ in the same assay, 1a-RGD showed a balanced dual-target profile with comparable affinity toward both receptors. Computational docking studies suggested that the enhanced activity of 1a-RGD arises from the preservation of the key interaction's characteristic of RGD ligands within the $\alpha_v\beta_3$ binding pocket, together with an additional T-shaped aromatic interaction between the benzyl substituent of the azabicyclic scaffold and Tyr122 of the β_3 subunit. Furthermore, cell-based assays demonstrated that 1a-RGD effectively inhibited human umbilical vein vascular endothelial cells (HUVEC) adhesion to vitronectin (IC_{50} = 9.8 μ M), confirming its functional integrin antagonism^{94,102}. This strategy ultimately led to the identification of a potent dual $\alpha_v\beta_3/\alpha_v\beta_5$ antagonist with inhibitory potency comparable to, or slightly higher than, that of the clinical candidate cilengitide toward $\alpha_v\beta_3$ integrin. *In vitro* studies confirmed the ability of this compound to inhibit cell migration and induce *anoikis* in glioblastoma cell lines¹⁰³.

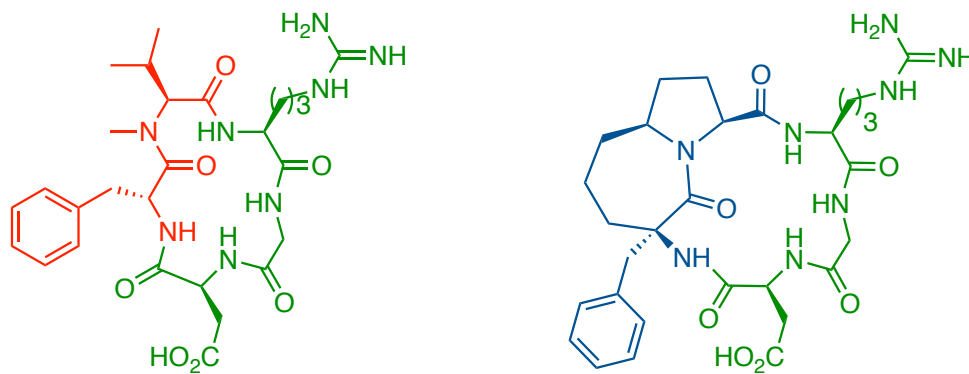


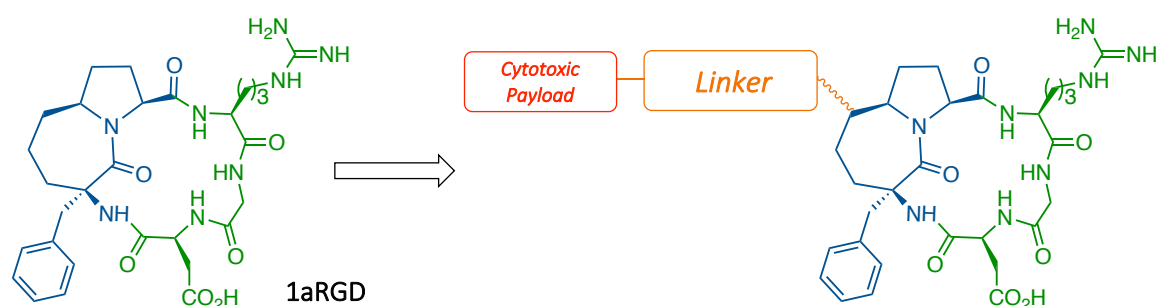
Figure 19 – A comparison between Cilengitide® (left) and 1a-RGD (right) structures

Table 1 - Inhibition of [¹²⁵I] echistatin binding to purified $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins by selected cyclic RGD ligands

Compound	IC ₅₀ ($\alpha_v\beta_3$) [nM]	IC ₅₀ ($\alpha_v\beta_5$) [nM]	$\alpha_v\beta_5/\alpha_v\beta_3$ Selectivity*
Echistatin	0.28 ± 0.08	0.29 ± 0.02	1.0
c(RGDfV)	195.9 ± 16.8	0.11 ± 0.03	1781
EMD121974 (Cilengitide)	18.9 ± 3.1	0.13 ± 0.01	145
ST1646	3.7 ± 0.6	1.39 ± 0.2	2.7
1a-RGD	6.4 ± 0.1	7.7 ± 0.04	0.83

IC₅₀ values represent the concentration required to inhibit 50% of echistatin binding. Data adapted from Ref. [94]. Data are reported as mean ± SD. *Calculated as IC₅₀($\alpha_v\beta_3$)/IC₅₀($\alpha_v\beta_5$); values >>1 indicate preference for $\alpha_v\beta_5$, whereas values close to 1 indicate similar affinity for both receptors

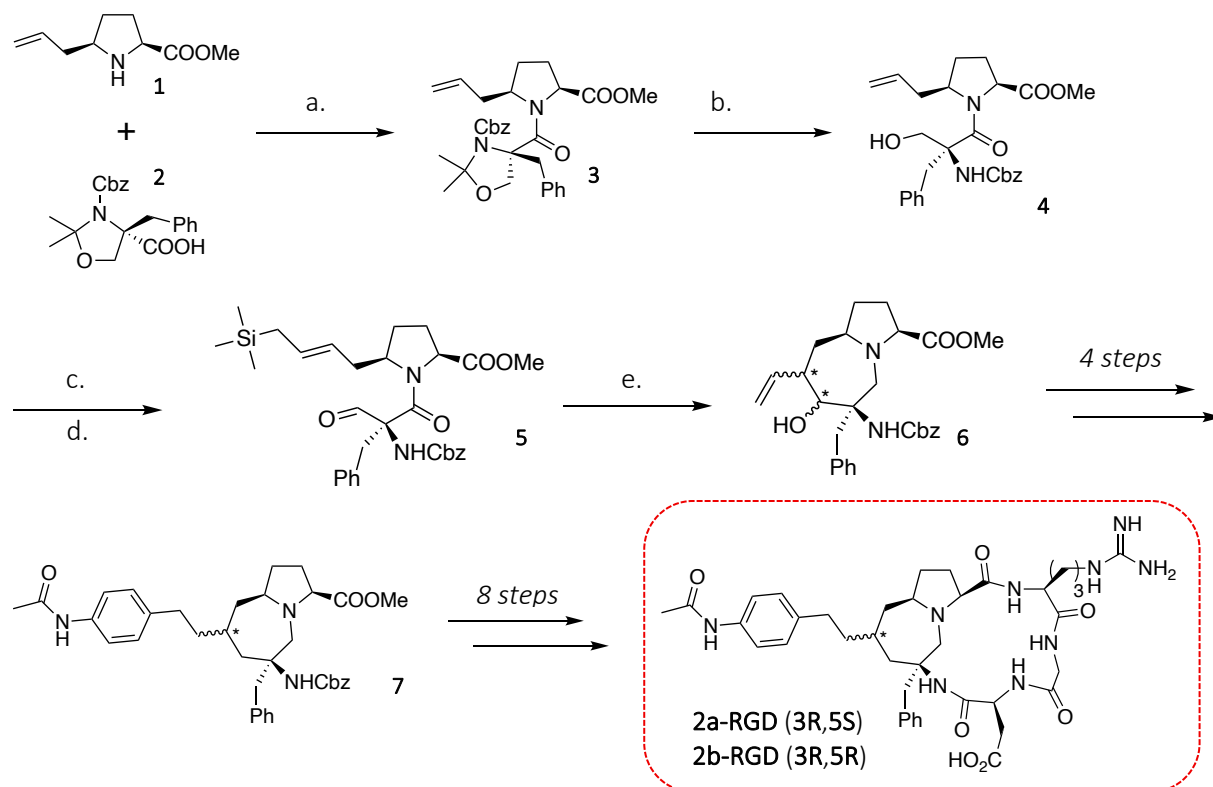
Building on these results, a new synthetic strategy was developed to introduce additional functionalizable handles on the lactam ring of **1a-RGD**, enabling the preparation of novel RGD-based bioconjugates. One of the most attractive features of these scaffolds is the possibility to design synthetic routes allowing the installation of different functional groups on the lactam ring. Such groups can be exploited either to introduce structural modifications aimed at improving receptor affinity, or as conjugation points for linkers used to attach bioactive payloads (e.g., cytotoxins), nanoparticles, liposomes or molecular imaging agents (*scheme 3*). This goal was achieved through the synthesis of **2a-RGD** and **2b-RGD**, in which the synthetic key step consisted of an intramolecular **Sakurai allylation**¹⁰⁴ (*scheme 4*).



Scheme 3 – Generic structure of a SMDC 1a-RGD based

This transformation enabled the formation of the seven-membered lactam ring in a single step while simultaneously introducing a hydroxyl group and a vinyl substituent at C5. The choice of functionalization site was guided by the need to minimize interference with integrin recognition. In line with these considerations, preliminary computational studies suggested that the side chain moiety does not affect the active RGD conformation, and

subsequent *in vitro* studies confirmed these findings by showing retention of affinity towards $\alpha_v\beta_3$ integrin in biotinylated vitronectin displacement assays compared to **1a-RGD**¹⁰⁵ (Table 2).



Scheme 4 – a) PyBroP, DIPEA, DMAP, DCM, rt, 24 h, y: 85%; b) Bi(OTf)₃, CH₃CN, rt, 10 min, y: 86%; c) (COCl)₂, DMSO, TEA, DCM, -60°C → 0°C, 2 h, y: 88%; d) Allyltrimethylsilane, HG2, DBBQ, DCM, reflux, 8 h, y: 84%; e) Sc(OTf)₃, DCM, -10°C then rt, 5 h, y: 86%

Table 2 - Binding affinity of selected cyclic RGD ligands toward $\alpha_v\beta_3$ integrin determined by biotinylated vitronectin displacement assays

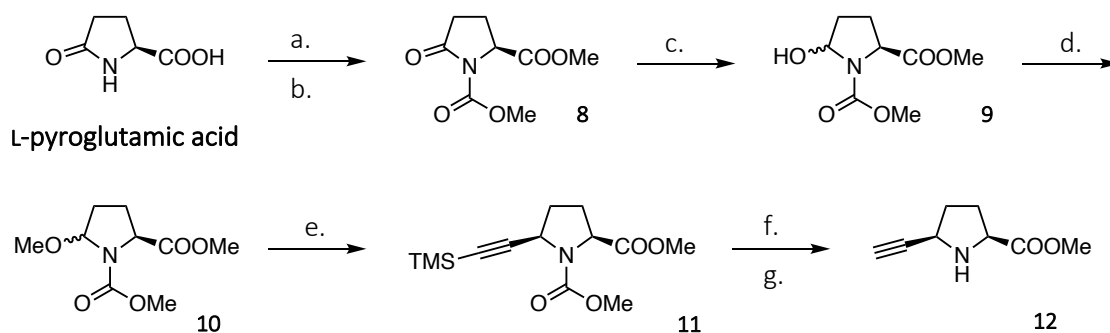
Molecule	$\alpha_v\beta_3$ IC ₅₀ (nM)
2a-RGD	16.3 ± 2.7
2b-RGD	9.3 ± 5.6
1a-RGD	3.3 ± 0.1
c(RGDfv)	3.2 ± 1.3

Lower IC₅₀ values indicate higher affinity for the receptor. Data are reported as mean ± SD. Data adapted from Ref. [105]. Data are reported as mean ± SD.

2.3 Synthesis of new generation RGD-based peptidomimics

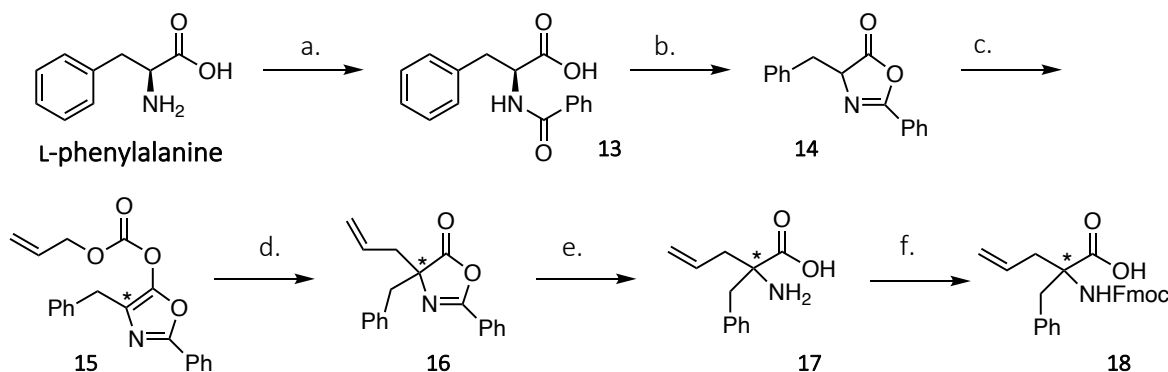
Despite the success of bicyclic scaffolds as reverse-turn inducers, the preparation of these conformationally constrained building blocks often requires multistep synthetic routes and provides limited opportunities for late-stage functionalization.

Starting from this evidence, Serra and coworkers investigated new synthetic protocols aimed at obtaining readily functionalizable bicyclic lactam scaffolds in a reduced number of synthetic steps. To access azabicycloalkane scaffolds bearing an exocyclic olefin on the seven-membered lactam ring, an alternative strategy based on *ring-closing enyne metathesis* (RCEYM) reaction was developed. In this approach, the key step was the conversion of a suitably assembled enyne dipeptide precursor into a cyclic diene scaffold carrying an exocyclic double bond at C6¹⁰⁶. The **two key building blocks** were first prepared: a 5-ethynylproline derivative and an α -allyl quaternary α -amino acid. The 5-ethynylproline fragment was obtained from L-pyroglutamic acid through sequential protection of the amino and carboxyl groups (as methyl carbamate and methyl ester), followed by reduction and O-methylation steps, and the final introduction of the ethynyl moiety via Lewis acid-catalyzed addition of bis(trimethylsilyl)acetylene, yielding compound **11** as a single diastereoisomeric compound (30% yield over 5 steps). After chromatographic purification, deprotection of both the trimethylsilyl and the carbamate groups provided the target compound **12** with a overall 21% yield (scheme 5).



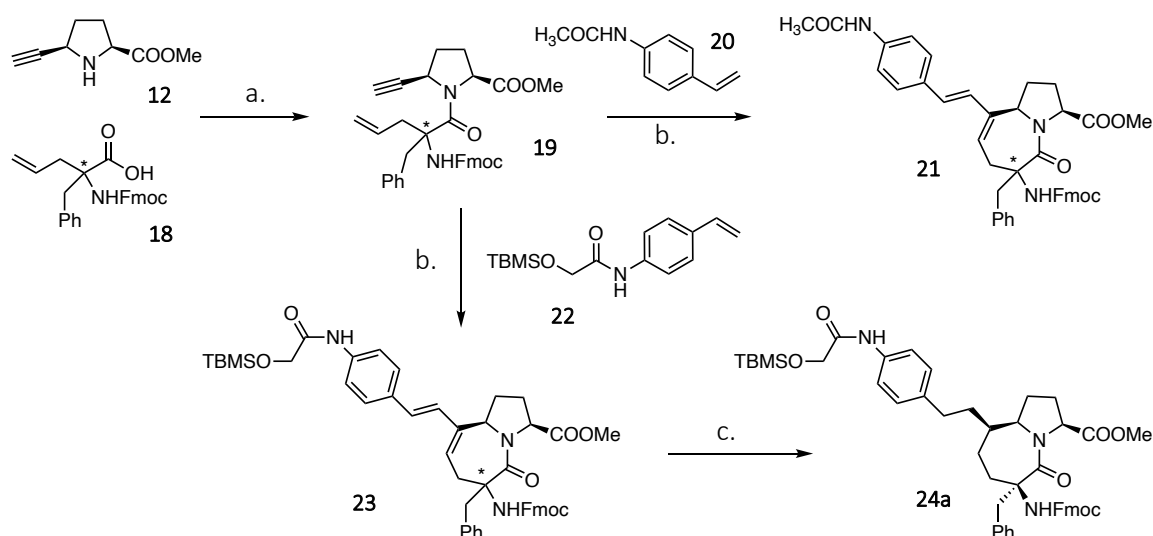
Scheme 5 – a) SOCl_2 , MeOH, 0 °C, y: 99% b) LiHMDS , ClCO_2CH_3 , THF, -78 °C, y: 83% c) LiEt_3BH , THF, -78 °C; d) $\text{CH}(\text{OCH}_3)_3$, PPTS, MeOH, rt, y: 90%; e) BTMSA, SnCl_4 , AlCl_3 , DCM, -45 °C, y: 40%; f) TBAF, THF, -20 °C, y: 90%; g) TMSI, DCM, reflux, y: 78%

In parallel, the α -allyl quaternary amino acid building block was synthesized from N-benzoyl-L-phenylalanine via azlactone formation (DCC-mediated cyclodehydration), followed by enolization and allylation to the corresponding allyl enol carbonate. A key Pd-catalyzed Tsuji decarboxylative allylation installed the quaternary allyl substituent, and subsequent azlactone ring opening with TFA, followed by Fmoc protection, afforded the final N-Fmoc protected α -allyl quaternary amino acid as a racemic mixture (*scheme 6*).



Scheme 6 – a) BnCl , NaOH 6N, 0°C 2 h $\rightarrow$ HCl 6N; b) DCC, DCM, 0°C , y : 80%; c) Allyl chloroformate, Et_3N , THF, 0°C , y : 99%; d) $\text{Pd}_2(\text{dba})_3 \cdot \text{CHCl}_3$, dppe , THF, y : 99%; e) TFA, 100°C ; f) Fmoc-Cl, NaHCO_3 , THF/ H_2O , y : 45%

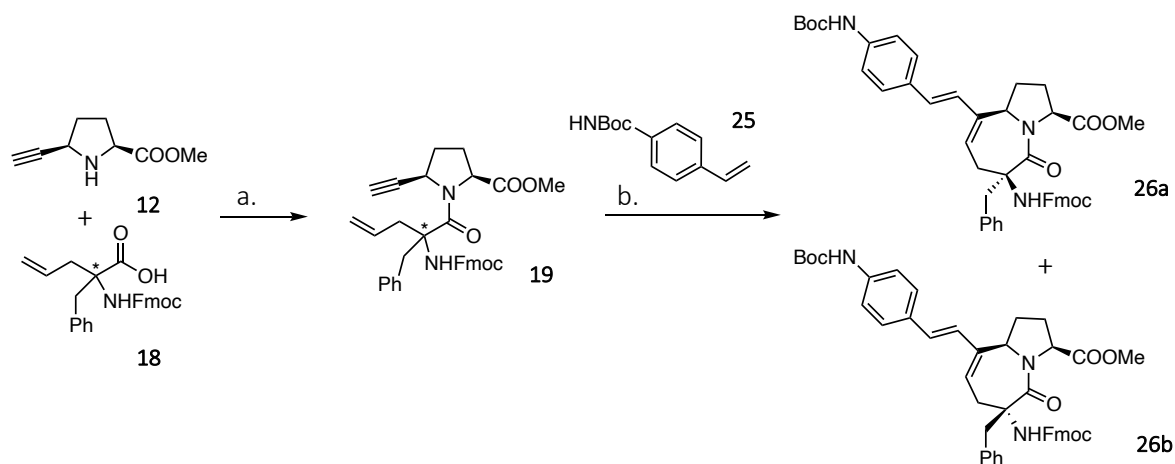
The compound **18** was subsequently condensed with the ethynylproline **12** derivative using PyBrop to furnish the enyne dipeptide **19**. Notably, compound **19** proved unreactive under direct RCEYM conditions, but it underwent a highly productive Ru-catalyzed domino cross-enyne metathesis/ring-closing metathesis (CEYM/RCM) in the presence of a styrene partner (*scheme 7*), through an *exo* “yne-then-ene” pathway¹⁰⁶.



Scheme 7 – a) PyBrop[®], DCM, 50°C , y : 50%; b) 10% HG2, Tol reflux (**21** y : 54%; **23** y : 56%); c) Pd/C, H_2 , MeOH, y : 77%

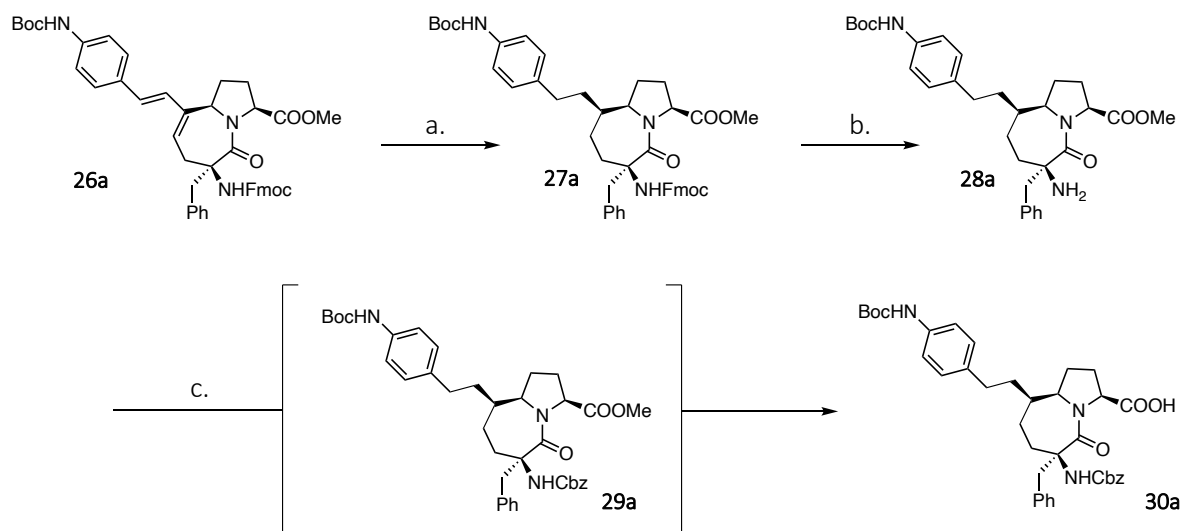
This transformation offered an important advantage: by selecting appropriately functionalized styrene derivatives, it was possible to install a spacer already equipped with a conjugation handle, thereby simplifying the overall route and reducing time and costs. Reaction optimization demonstrated that the most effective protocol consisted of the slow addition of the Hoveyda-Grubbs second generation catalyst solution over several hours by syringe pump, delivering the bicyclic lactam scaffold with excellent stereochemical control. In particular, the formation of the exocyclic double bond was highly stereoselective, with only the *E* isomer observed for both the diastereomeric scaffolds, which differ in the configuration at the quaternary C3 stereocentre. The two diastereomers can be easily separated chromatographically. The downstream sequence toward the RGD derivative was typically carried out on the azabicyclic lactam scaffold bearing the “R” configuration at C3: selective hydrogenation of the diene motif (using a Pd/C-ethylenediamine complex) prevented silyl ether cleavage and provided a single diastereoisomeric saturated product, which was then converted into the corresponding Cbz-protected scaffold suitable for peptide conjugation¹⁰⁶.

In his PhD thesis Dr. Bernardi established robust conditions for both enyne assembly and scaffold elaboration, enabling a practical entry to the RGD-binding pathway¹⁰⁵. In particular, the condensation reaction between compounds **12** and **18**, using BOP-Cl and NaHCO₃ in THF at 50 °C, was optimized to provide the metathesis precursor **19** in 70% yield on gram scale. Subsequent domino CEYM/RCM with the styrene derivative **25** (N-Boc 4-vinylaniline) delivered the functionalized scaffold **26** in 54% yield as a mixture of diastereoisomers.



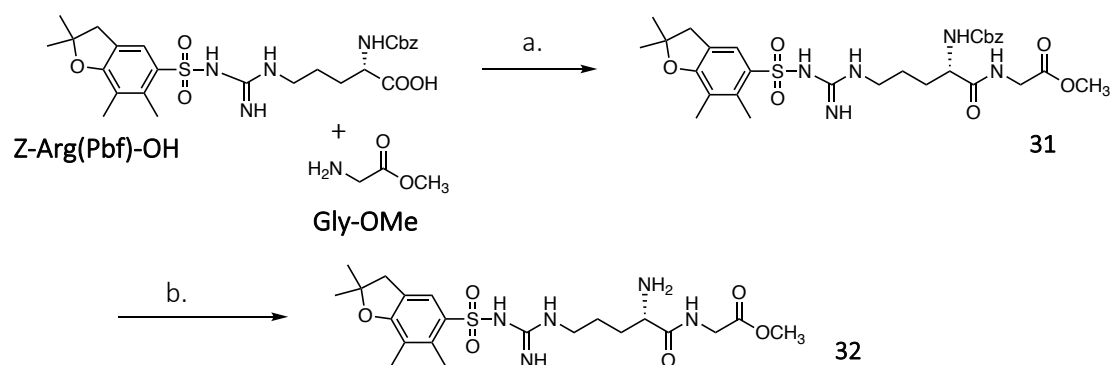
Scheme 8 – a) BOP-Cl, DCM, 50 °C, *y*: 70%; b) 10% HG2, toluene, reflux, *y*: 54% (**26a** *y*: 24%, **26b** *y*: 30%)

The C3-(R)-configured diastereoisomer **26a** was efficiently reduced under hydrogen atmosphere with PtO_2 to give **27a** (scheme 9), and the subsequent deprotection of the Fmoc group with piperidine furnished the corresponding free amine **28a** in 85% yield. A concise one-pot sequence using $(\text{Cbz})_2\text{O}$ to introduce the Cbz protecting group followed by LiOH-mediated ester hydrolysis then afforded the free carboxylic acid scaffold intermediate **30a** in excellent overall yield (96%), establishing a versatile platform for peptide coupling.



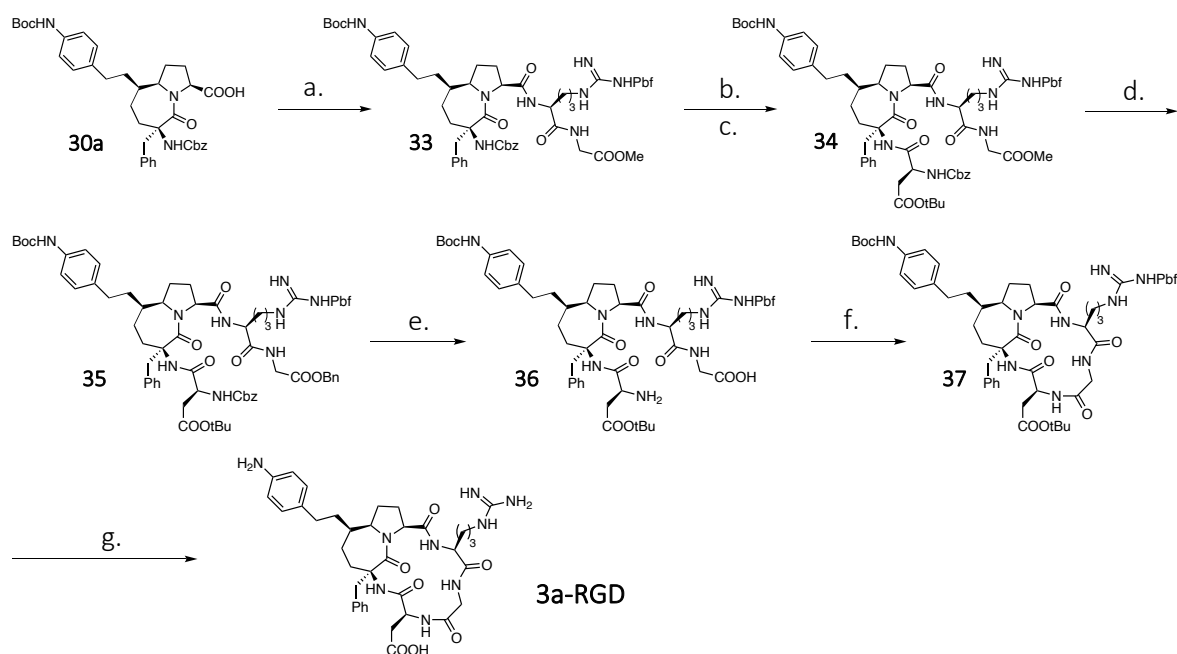
Scheme 9 – a) H_2 , PtO_2 (cat.), MeOH, y : 91%; b) piperidine, THF, y : 85%; c) $(\text{Cbz})_2\text{O}$, THF, 0°C rt. Then, LiOH (1.6 M in H_2O), 60°C , y : 96% (over two steps)

On this basis, the development of the RGD conjugation protocol was achieved by first assembling the Arg-Gly dipeptide through condensation of N-Cbz-(Pbf)-arginine with L-glycine methyl ester under mixed anhydride conditions (isobutyl chloroformate/N-methylmorpholine, scheme 10), followed by hydrogenolysis of the Cbz group and coupling of the resulting dipeptide **32** to the Cbz-protected bicyclic scaffold **30a** using HATU (scheme 11).



Scheme 10 – a) $\text{NH}_2\text{-Gly-OMe}$, NMM, $i\text{BuOCOCl}$, THF, $-30^\circ\text{C} \rightarrow \text{rt}$, y : 80%; b) H_2 , $\text{Pd}(\text{OH})_2/\text{C}$, MeOH, y : 99%

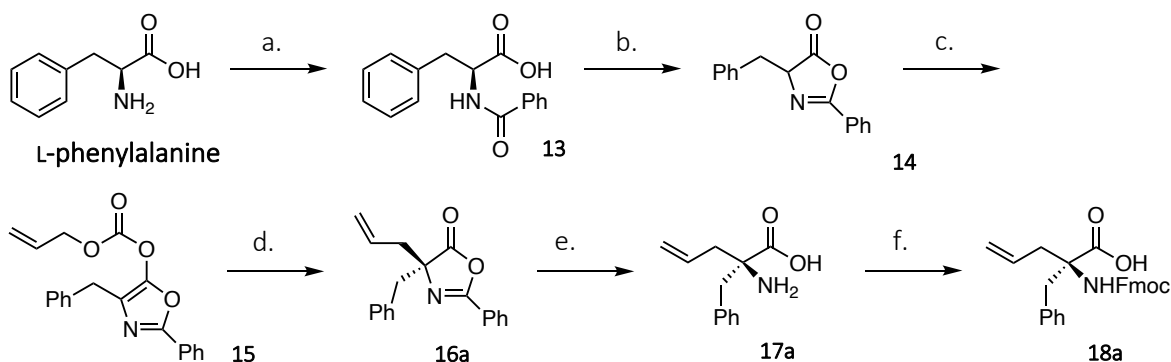
After a second hydrogenolysis step, the Asp residue was introduced to afford the protected linear pentapeptide, and the glycine methyl ester was converted into the benzyl ester via transesterification with $\text{Ti}(\text{O}i\text{Pr})_4$ and benzyl alcohol. Contemporary hydrogenolysis of the Cbz and benzyl ester groups, followed by intramolecular HATU-mediated cyclization between Gly and Asp, enabled the isolation of the protected cyclopentapeptide **37**. Subsequent TFA deprotection in the presence of scavengers, followed by semipreparative RP-HPLC purification, furnished the target **3a-RGD** in 62% yield (*scheme 11*). As previously described in the previous chapter for compound **2a-RGD**, computational and molecular dynamics studies carried out on this new derivative have shown that the introduction of the side chain on the azabicycloalkane scaffold has no effect on the active conformation of the RGD sequence, thus maintaining the affinity for the receptor unchanged¹⁰⁵.



Scheme 11 – a) **32**, HATU, THF, rt, y: quant.; b) H_2 , $\text{Pd}(\text{OH})_2/\text{C}$, MeOH $\rightarrow$ c) Z-Asp(OtBu)-OH, HATU, THF, rt, y: 96%; d) BnOH, $\text{Ti}(\text{O}i\text{Pr})_4$, THF, 90 °C; e) H_2 , $\text{Pd}(\text{OH})_2/\text{C}$, MeOH, rt; f) HATU, DIPEA, THF, rt; g) TFA, scavengers, y: 62%.

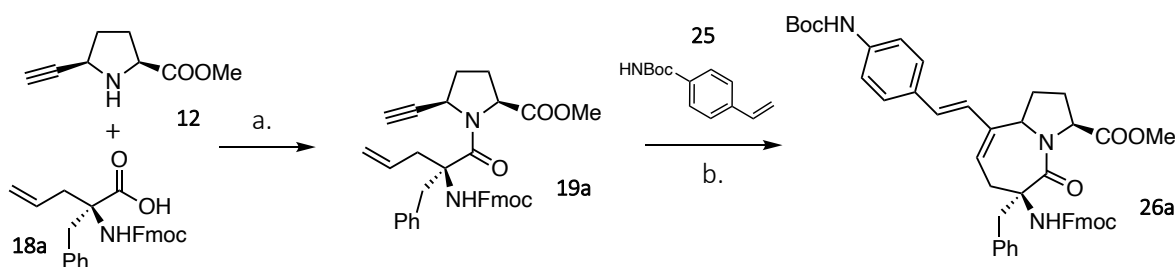
Building on these results, Dr. Bisbano's doctoral thesis work focused on improving the overall efficiency of the **3a-RGD** synthesis, particularly by maximising access to the single diastereoisomer required for the final construct¹⁰⁷. Since the domino CEYM/RCM step provided a mixture of diastereoisomers but only one (**26a**) was advanced, an asymmetric strategy was explored upstream to bias the formation of the productive scaffold. This

optimization relied on the preparation of the α -allyl quaternary amino acid, one of the two key precursors required for the synthesis of the bicycrolactam scaffold, via an enantioselective *Pd*-catalyzed *Tsuji-Trost allylation* using the (R,R)-DACH-phenyl Trost ligand. This reaction provided an allyl-oxazolone intermediate, which was hydrolyzed with TFA to the corresponding α -allyl phenylalanine derivative. Following recrystallization, the enriched (R)-enantiomer was isolated with high enantiomeric excess (84% *e.e.*) and converted to the Fmoc-protected amino acid **18a** (scheme 12).



Scheme 12 – a) $BnCl$, $NaOH$ 6N, $0^\circ C$ 2 h $\rightarrow$ HCl 6N; b) DCC , DCM , $0^\circ C$; c) Allyl chloroformate, Et_3N , THF , $0^\circ C$; d) $[PdCl(allyl)]_2$, (R,R)-DACH-phenyl Trost, CH_3CN , y : 100%; e) TFA , $100^\circ C$, y : 61%, *ee* 84%; f) $Fmoc-Cl$, $NaHCO_3$, THF/H_2O , y : 80%

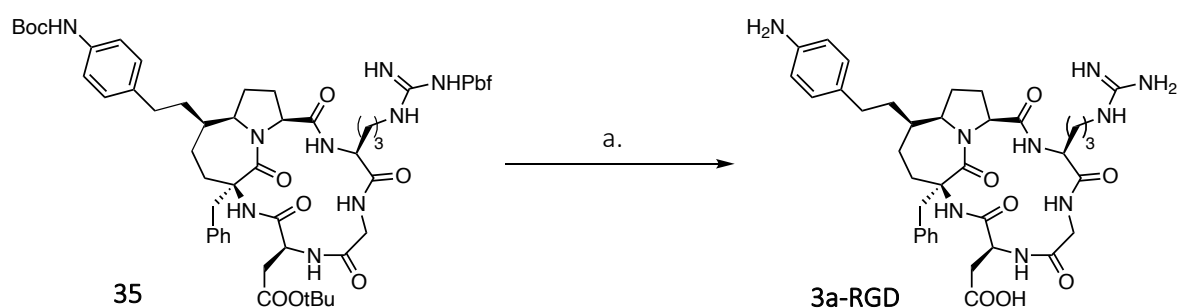
Although condensation with the ethynylproline building block **12** proceeded in lower yield compared to the racemic protocol, the subsequent metathesis gave a significantly improved yield of the desired bicyclic scaffold, so that the two-step yield (condensation and metathesis) was slightly higher overall; importantly, the enantioenriched route reduced the waste of the synthetically demanding ethynylproline fragment, representing a tangible practical benefit.



Scheme 13 – a) $BOP-Cl$, $NaHCO_3$, THF , $50^\circ C$, y : 51%; b) $HG2$ (10% mol), $toluene$, $80^\circ C$, y : 40%

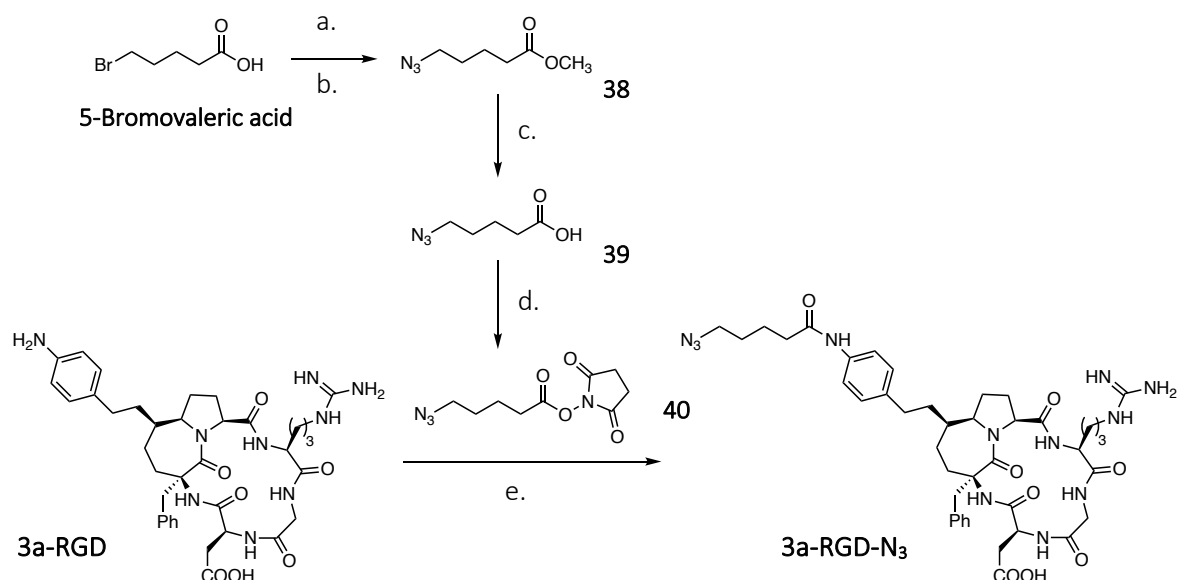
Additional refinements were obtained in the late-stage peptide sequence, including a markedly improved Fmoc deprotection yield and a higher-yielding transesterification to the benzyl ester. Finally, particular attention was devoted to the last deprotection step required

to obtain 3a-RGD from the fully protected cyclopeptide, since classical TFA conditions in the presence of radical scavengers (anisole, ethane-1,2-dithiol (EDT) and thioanisole) often led to complex mixtures and challenging purifications. Two deprotection cocktails incorporating triisopropylsilane (TIS) were compared: a more complex mixture including thioanisole/phenol/H₂O/EDT/TIS and a simplified TFA/TIS/H₂O system. While the former afforded the target product in comparable yield but required prolonged reaction time and gave many side products, the simplified mixture provided cleaner conversion, shorter reaction time and similar isolated yield, amounting to 73% yield, after preparative HPLC purification, thus being selected as the preferred procedure (*scheme 14*).



Scheme 14 - a) 95:2.5:2.5 TFA/TIS/H₂O, γ : 73%

Late-stage installation of a linker bearing an azide functional group suitable for click-chemistry conjugation ultimately afforded the final compound **3a-RGD-N₃** (*scheme 15*).



Scheme 15 – a) SOCl₂, MeOH, 0 °C → rt; b) NaN₃, DMF, 80 °C MW, γ : 97% over two steps; c) LiOH, MeOH, γ : 100%; d) NHS, EDC*HCl, DCM, γ : 97% e) **40**, Et₃N, DMF γ : 62%

2.4 Optimization of 3a-RGD synthesis

Building on the synthetic platform established in the PhD theses of Dr. Bernardi and Dr. Bisbano, my doctoral work focused on the further optimization of the synthetic sequence toward the C6-functionalized azabicycloalkane scaffold and its corresponding RGD cyclopentapeptide conjugate. In particular, attention was focused on two key bottlenecks of the overall route: (1) the Ru-catalysed *domino cross-enyne metathesis/ring-closing metathesis* (CEYM/RCM) step used to generate the bicyclic core and (2) the late-stage attachment of an azide-containing linker to the 3a-RGD peptide.

2.4.1 CEYM/RCM step optimization

Although the metathesis protocol previously reported proved reliable on small scale, a major limitation emerged during scale-up experiments. When the reaction was performed on quantities exceeding 200 mg of the enyne dipeptide precursor, a significant drop in isolated yield was consistently observed, decreasing from approximately 40% to only 20-25%. This reduction severely limited the preparation of the scaffold in moderate amounts, since the long reaction times combined with the limited throughput of the precursor processing prevented rapid access to gram-scale quantities. To overcome this drawback, the possibility of applying microwave irradiation was investigated, with the aim of drastically shortening reaction times while maintaining, or potentially improving, the efficiency of the transformation¹⁰⁸. Starting from the original conditions, 0.01 M dipeptide concentration in toluene and slow addition of Hoveyda-Grubbs second generation catalyst (HG2, 15 mol%) at 80 °C, the methodology was translated to a microwave-assisted protocol. The dipeptide concentration was initially maintained at 0.01 M using 100 mg of dipeptide to reach the maximum capacity allowed by the largest microwave vial (20 mL), while catalyst loading was initially reduced to 5 mol% and 10 mol% in two separate trials. The Styrene partner **25** was maintained at the same molar ratio 2:1 relative to **19a**. The reactions were subjected to repeated 15 min microwave cycles, with TLC monitoring after each cycle in order to monitor the consumption of reagents. The best outcome was achieved using 10 mol% HG2 under microwave irradiation at 120 °C, where complete consumption of the starting

material was observed after three 15 min cycles. After chromatographic purification, the desired bicyclic scaffold was isolated in 45% yield, a result that was subsequently confirmed and reproduced using a single 45 min irradiation cycle. A second set of experiment was conducted to evaluate the impact of concentration for the outcome of the reaction. The optimal result was obtained when the reaction was performed at a slightly lower concentration (0.008 M), while maintaining the previously optimized conditions. Under these conditions, compound **26a** was obtained in an improved isolated yield of 61%.

Table 3 – Optimization of the RCEYM metathesis of the enyne dipeptide precursor under conventional and microwave-assisted conditions

Reaction Method	Concentration	Catalyst (HG2)	Temperature	Reaction Time	Scale	Isolated Yield
<i>Slow infusion</i>	Starting 0.01 M	15 mol%	80 °C	7 h	≤ 200 mg	40%
<i>Slow infusion</i>	Starting 0.01 M	15 mol%	80 °C	7 h	> 200 mg	20-25%
<i>Microwave-assisted</i>	0.01 M	5 mol%	120 °C	3 x 15 min cycles	≤ 200 mg	25%
<i>Microwave-assisted</i>	0.01 M	10 mol%	120 °C	3 x 15 min cycles	≤ 100 mg	45%
<i>Microwave-assisted</i>	0.01 M	10 mol%	120 °C	45 min (single cycle)	≤ 100 mg	45%
<i>Microwave-assisted</i>	0.08 M	10 mol%	120 °C	45 min (single cycle)	≤ 100 mg	61%

Importantly, this microwave-assisted approach, due to its short reaction times, allowed multiple runs in a single day, enabling the conversion of gram-scale amounts of precursor in a much shorter overall time. At the same time, it provided an increased maximum yield and required lower catalyst loading compared to the original syringe-pump protocol. Overall, this optimization represented a substantial improvement in both the practicality and scalability of the metathesis step, which constitutes the central transformation for scaffold construction.

2.4.2 Optimization of the linker attachment step

A second crucial aspect of my work involved optimizing the installation of an azide-functionalized linker on the side chain of the 3a-RGD cyclopentapeptide, a key prerequisite for subsequent bioconjugation via click chemistry. Under the previously developed conditions, linker coupling was performed in DMF using an excess of triethylamine (10 equivalents) to ensure that the amine functionality of 3a-RGD remained unprotonated and reactive. However, a major drawback of this procedure was the formation of a by-product displaying an HPLC retention time very close to that of the desired conjugate, as shown in *figure 20*, making purification difficult and lowering the overall efficiency.

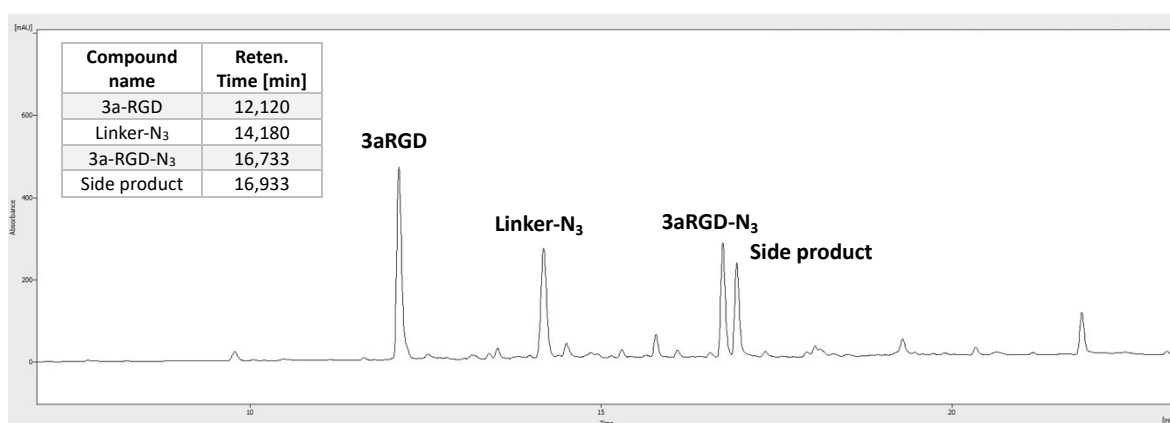


Figure 20 – HPLC monitoring chromatogram of 3a-RGD-N₃ synthesis with Et₃N in DMF

After confirming the stability of the compound in DMF alone, we hypothesized that the formation of the by-product, subsequently identified by mass spectrometric analysis as a derivative of 3a-RGD, was associated with the use of triethylamine as the base. To address this issue, systematic modification of the reaction medium was pursued by introducing an aqueous buffered system, with the aim of suppressing or at least limiting the formation of this undesired by-product while maintaining conditions suitable for efficient linker coupling. To test this hypothesis, an initial set of experiments was designed in which pure DMF was replaced with mixtures of DMF and phosphate-buffered saline (PBS) at pH 8.0 and pH 7.4 (75:25 v/v). HPLC monitoring revealed that the original impurity was no longer formed but a different side-product appeared instead (*figure 21*).

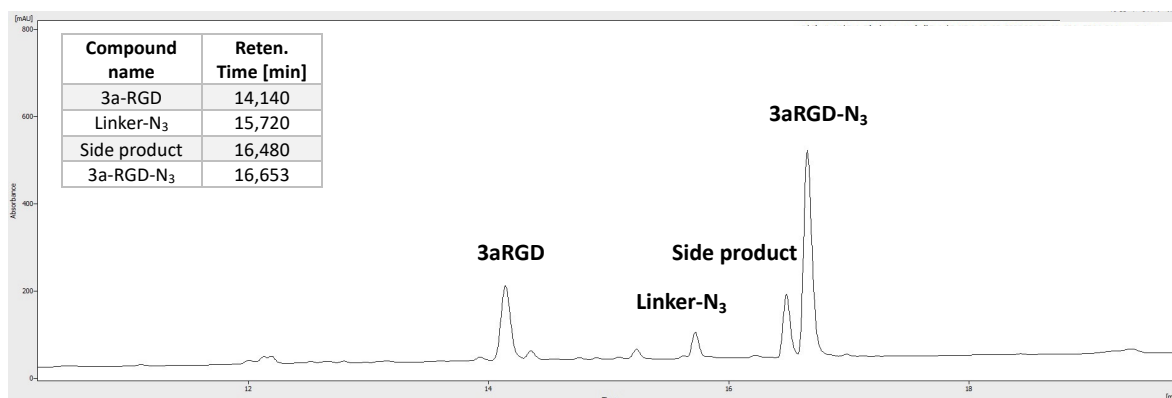


Figure 21 – HPLC monitoring chromatogram of 3a-RGD-N₃ synthesis with PBS buffer pH 7.4

Based on these results, further solvent systems were evaluated by replacing PBS with *Good's buffers*¹⁰⁹, namely **HEPES** (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and **MES** (2-(N-morpholino) ethanesulfonic acid), both at pH 8.0 and in 50:50 (v/v) mixtures with DMF. The choice of these buffering agents was driven by their well-known chemical inertness and minimal involvement in side reactions. Unlike phosphate buffers, *Good's buffers* are zwitterionic, exhibit low metal-binding affinity and lack nucleophilic functionalities that could interfere with linker-coupling processes. In addition, both HEPES and MES possess high buffering capacity in the near-neutral to mildly basic pH range, allowing maintenance of sufficiently basic conditions to keep the amine functionality of 3a-RGD in its free form while avoiding the use of excess organic base. The test conducted under these new experimental conditions, after HPLC monitoring, showed that in both cases the impurities detected under the previous conditions (DMF/Et₃N and DMF/PBS) were completely suppressed, indicating that modulation of both pH control and buffer composition played a key role in improving reaction selectivity (*figure 22*).

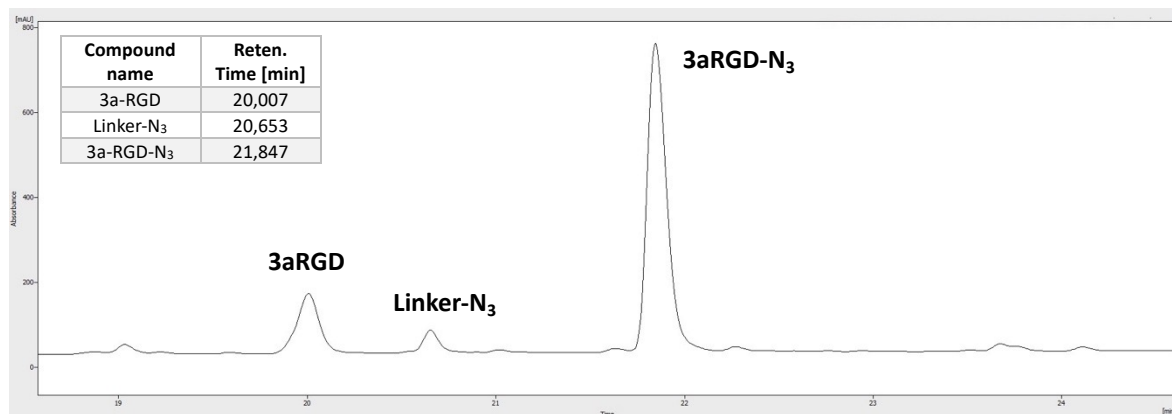


Figure 22 – HPLC monitoring chromatogram of 3a-RGD-N₃ synthesis with HEPES buffer pH 8.0

Among the two buffered systems, the **DMF/HEPES** mixture was selected as the optimal medium, as reaction completion was achieved more rapidly than in the presence of MES buffer. This behaviour is consistent with the higher buffering efficiency of HEPES at pH 8.0 and its superior solubility and compatibility with organic co-solvents, which likely facilitate more favourable reaction kinetics under these conditions.

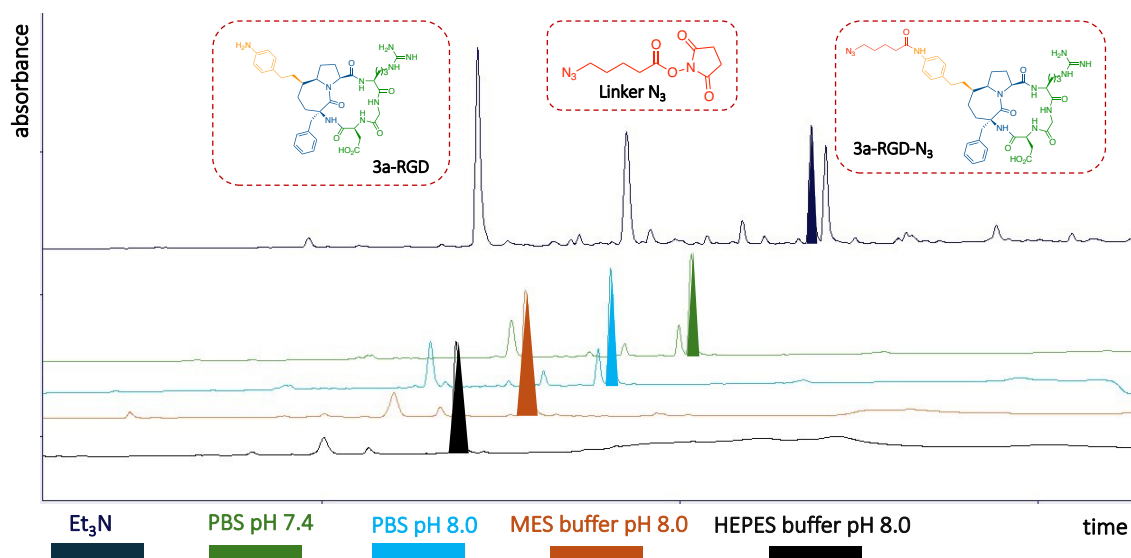


Figure 23 -Visual overlay of HPLC monitoring chromatograms for all different reaction conditions tested

The adoption of this optimised protocol resulted in a dramatic improvement of the isolated yield, which increased from 62% to 90%, while also simplifying purification by significantly reducing the formation of closely eluting by-products. This enhancement represents a key advancement in the preparation of the azide-functionalized 3a-RGD derivative, providing a robust and high-yielding entry to integrin-targeted conjugates for downstream nanomedicine applications.

In conclusion, through the development of a microwave-assisted metathesis protocol and the refinement of the azide-linker attachment step via buffered solvent systems, my PhD work contributed significant improvements to the scalability, yield and, overall, robustness of the synthetic route toward functionalised azabicycloalkane-based RGD cyclopeptides, thereby strengthening their applicability as versatile platforms for tumour-targeted drug delivery.

2.5 cRGD-Decorated Nanoparticles for Targeted Drug Delivery

The next step of this PhD work was the development of integrin-targeted nanoparticle-based delivery systems. In particular, the ability of the 3a-RGD scaffold to act as a selective $\alpha_v\beta_3/\alpha_v\beta_5$ integrin-targeting moiety makes it an ideal candidate for surface functionalization of nanocarriers aimed at improving tumor-selective drug accumulation and cellular internalization. Within this framework, this chapter focuses on the design, synthesis and evaluation of cRGD-nanoparticle bioconjugates, exploiting three complementary platforms. The first part describes the development of 3a-RGD-decorated liposomes, engineered as multifunctional theranostic systems for targeted delivery of doxorubicin toward aggressive cancer models, with results fully reported in the associated published work. The second part explores the conjugation of 3a-RGD to chitosan-based polymeric nanoparticles, representing an alternative nanocarrier strategy. The third and final part focuses on the development of a dual-targeting silk fibroin-based nanosystem, in which the 3a-RGD ligand is combined with glucose functionalization to integrate tumor targeting with enhanced barrier-crossing capability. Overall, this chapter highlights the versatility of the 3a-RGD ligand as a modular targeting element for the development of integrin-directed nanomedicine platforms.

2.5.1 3a-RGD functionalized liposomal nanocarriers

A part of the work described in this chapter is published in the following article:

- Ovejero-Paredes, Karina et al. (Zupi, Marco) “Microfluidic-assisted synthesis of integrin-targeting cRGD liposomes as a scalable strategy for theranostic applications in triple-negative breast cancer.”, *Eu J Pharma Sci*, vol. 213 (2025), 107216. doi: 10.1016/j.ejps.2025.107216

Liposomes are among the most widely investigated nanocarriers in drug delivery owing to their high biocompatibility, structural versatility and ability to encapsulate both hydrophilic and hydrophobic therapeutic agents. In oncology, liposomal formulations have been

successfully employed to improve the pharmacokinetic profile of cytotoxic drugs, reduce systemic toxicity and enhance tumor accumulation through passive targeting mechanisms such as the EPR effect. However, the development of actively targeted liposomes requires additional surface functionalization with ligands capable of recognizing tumor-specific receptors.

In collaboration with Prof. Marco Filice's research group at the *Universidad Complutense de Madrid*, a liposomal nanosystem was developed as a delivery platform for doxorubicin and functionalized with 3a-RGD to enable the active targeting of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors, which are overexpressed in aggressive tumors such as triple-negative breast cancer (TNBC), the focus of this study.

Liposome nanoparticles preparation and characterization

Liposomes were prepared by comparing two different synthetic strategies: the conventional *thin-film hydration* method and an innovative *microfluidic-assisted approach*¹¹⁰.

The lipid formulation consisted of:

- **Dimyristoylphosphatidylcholine** (DMPC), selected for its ability to form stable and biocompatible nanoparticles;
- **Dibenzocyclooctyne-modified phospholipids** (DBCO-PEG2000-DSPE), enabling subsequent functionalization through click-chemistry;
- **Gadolinium-containing lipids** (Gd-DTPA-BSA), which provide contrast enhancement for magnetic resonance imaging (MRI), allowing the *in vivo* tracking of liposomes.
- **Rhodamine-labelled phospholipid** (18:1 Liss Rhod PE), used to exploit rhodamine fluorescence for monitoring nanoparticle internalization during *in vitro* experiments.

For the *thin-film hydration* the lipid mixture was dissolved in chloroform. After solvent evaporation, a lipid film was formed and subsequently rehydrated in aqueous solution, yielding liposomes upon sonication. Although effective, this method is time-consuming and often associated with limited reproducibility and scalability. To overcome these limitations, a microfluidic-based synthesis was optimized.

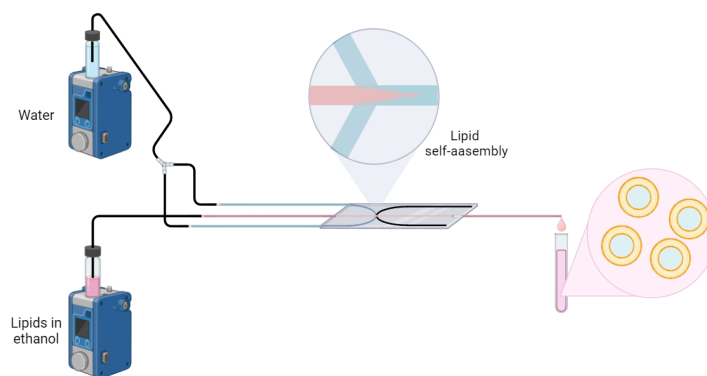


Figure 24 – Overview of the liposomal synthesis by microfluidics techniques

Microfluidic technology enables precise control over nanoparticle assembly through regulation of parameters such as total flow rate (TFR), solvent composition and temperature, resulting in liposomes with narrow size distribution and enhanced batch-to-batch reproducibility¹¹⁰. Different TFR values (0.5 -1.5 mL/min) and temperatures (25 °C and 37 °C) were investigated, demonstrating that liposomes produced at 25 °C were relatively insensitive to TFR variation, while at 37 °C smaller particles were obtained at lower flow rates. Importantly, polydispersity indexes below 0.3 were achieved at optimized conditions, confirming the suitability of the microfluidic strategy for producing monodisperse liposomal formulations (*figure 25*). Given its advantages in terms of speed, scalability and reproducibility, the microfluidic approach was selected for all subsequent experiments.

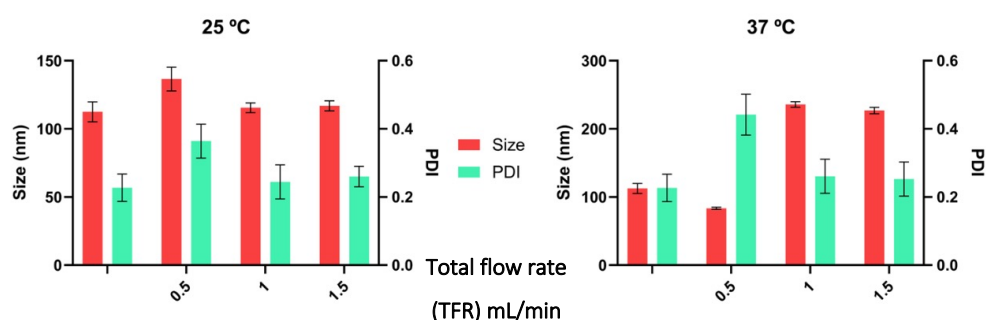


Figure 25 – Comparison of the average particle size and PDI of synthesized liposomes obtained.

Following liposome preparation, doxorubicin was encapsulated through a remote loading strategy based on transmembrane buffer gradients. Liposomes were initially resuspended in diammonium phosphate buffer, followed by exchange of the external phase with HEPES-buffered saline. Incubation with doxorubicin hydrochloride at a *drug-to-lipid* molar ratio of 1:3 enabled efficient active loading. This method resulted in an encapsulation efficiency exceeding 65%, corresponding to approximately 0.15 mg DOX per mg of lipid.

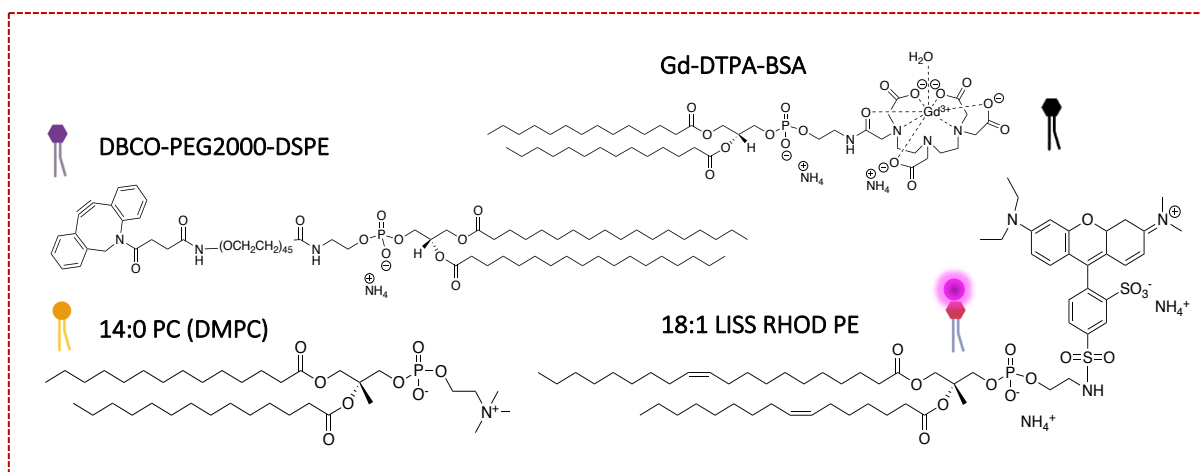
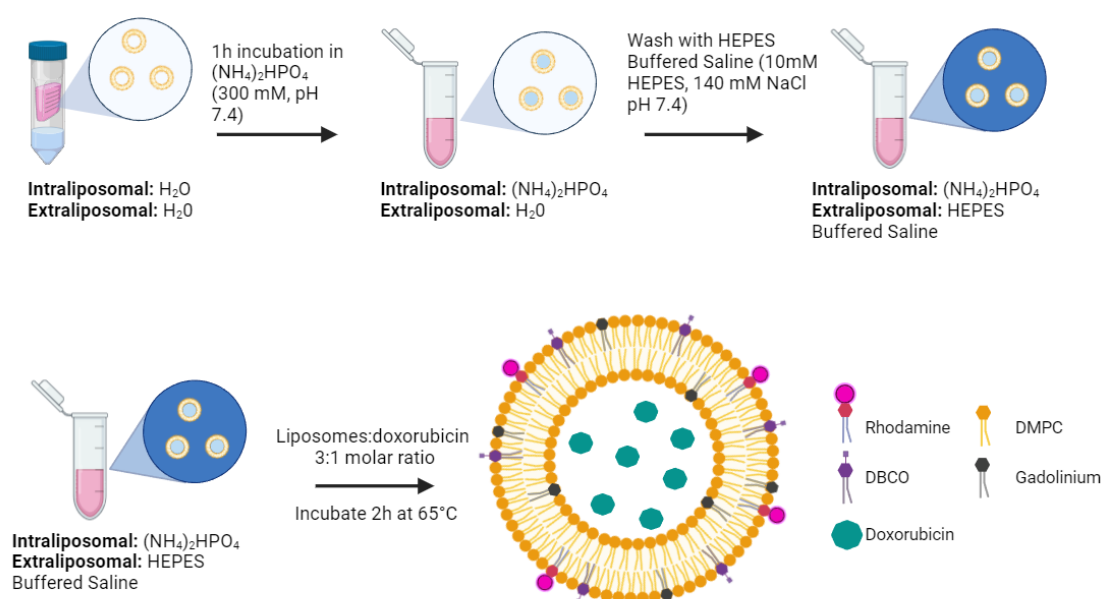


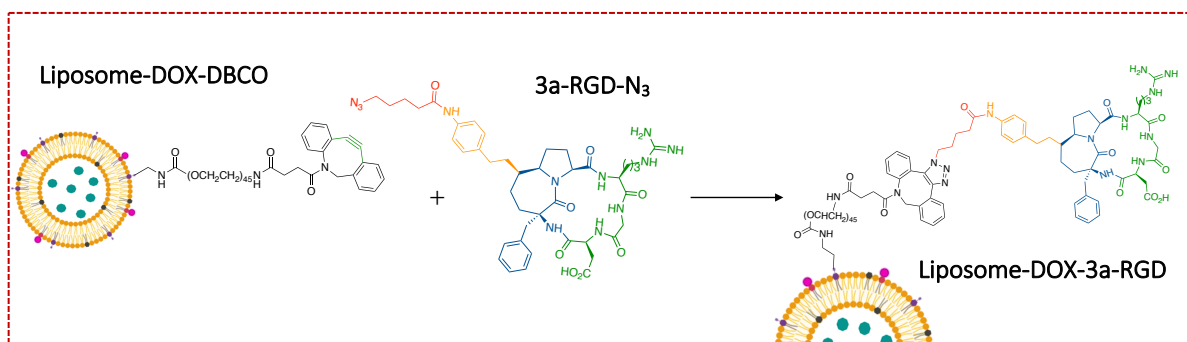
Figure 26 – Structure of lipids use for liposomes preparation



Scheme 16 – Intra/extra liposomal buffer exchange procedure and remote loading of Doxorubicin into the liposomes

Conjugation of 3a-RGD ligand to the liposomal surface

After the obtainment of the liposomes charge with doxorubicin, the liposomal surface was functionalized with the integrin-binding ligand **3a-RGD**. The conjugation strategy relied on **copper-free click chemistry**, specifically a *strain-promoted azide-alkyne cycloaddition* (SPAAC) reaction¹¹¹. This biorthogonal approach was selected because it avoids the use of copper catalysts, which are commonly required in classical azide-alkyne cycloadditions but may induce cytotoxicity and limit applicability in biological environments. SPAAC reactions are therefore particularly suitable for nanoparticle functionalization intended for biomedical applications.



Scheme 17 – Liposome functionalization using Strain-Promoted Alkyne-Azide Cycloaddition (SPAAC)

For this purpose, liposomes were formulated incorporating DBCO-modified phospholipids which provide strained cyclooctyne groups exposed on the outer surface of the lipid bilayer and act as reactive handles for the subsequent conjugation. This reaction was performed by dissolving 3a-RGD-N₃ in pure methanol and subsequently diluting it in sodium bicarbonate buffer. Prior to conjugation, liposomes-DOX were purified using *Amicon ultrafiltration units* in order to remove unencapsulated drug and exchange HEPES buffer. The purified liposomes were then resuspended directly in the prepared peptide solution and incubated for 2 hours at room temperature. To quantify the efficiency of surface functionalization, the supernatant collected after the conjugation step was analyzed by reverse-phase HPLC and compared with the initial peptide solution. Analyses were performed on a Gemini® NX-C18 column (250 × 4.6 mm) using a gradient starting from 80% water (0.1% TFA) and 20% acetonitrile, increasing to 80% acetonitrile over 6 minutes, with detection at **255 nm**. The functionalization yield was calculated by comparing the peak areas of the peptide before and after the reaction, according to the following relationship:

$$\text{cRGD surface functionalization (\%)} = \frac{(\text{cRGD offered} - \text{cRGD in supernatant})}{\text{cRGD offered}} \times 100$$

This analysis revealed an average surface modification efficiency of approximately 50%, confirming the successful decoration of the liposomal surface with the integrin-targeting ligand.

Imaging applications of 3a-RGD decorated liposomes

An important aspect of this liposomal platform was its development as a theranostic nanosystem, combining therapeutic delivery with diagnostic imaging. To this end, gadolinium-containing lipids were incorporated into the formulation to enable MRI, while

rhodamine-labelled phospholipids provided fluorescence imaging functionality. MRI phantom studies were performed at different gadolinium concentrations (5 - 150 $\mu\text{g/mL}$), demonstrating a strong enhancement of signal intensity in T1-weighted imaging. Relaxometric analysis revealed longitudinal and transverse relaxation rates ($r_1 = 6.66 \text{ mM}^{-1}\text{s}^{-1}$, $r_2 = 18.84 \text{ mM}^{-1}\text{s}^{-1}$), with an r_2/r_1 ratio below 3, indicating preferential behaviour as a positive T1 contrast agent. These values are comparable with clinically used nanoparticle-based contrast agents. In parallel, fluorescence imaging confirmed a linear increase in signal intensity as a function of liposome concentration, supporting the dual-modality imaging potential of the formulation. Together, these results validate the suitability of 3a-RGD-liposomes for combined therapeutic delivery and non-invasive tracking (*figure 27*).

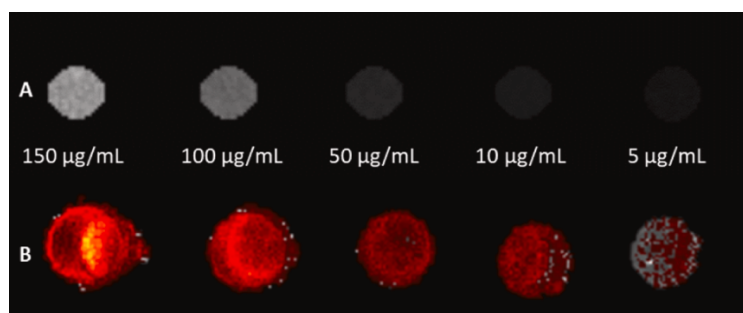


Figure 27 – Phantom images of the synthesized liposomes at different Gd concentrations by MRI (A) and fluorescence imaging (B) techniques.

In vitro cellular uptake studies

Following their synthesis and characterization, the targeting efficiency and enhanced cellular uptake of cRGD-DOX-liposomes were investigated. The biological evaluation of 3a-RGD-decorated liposomes focused on their ability to selectively target cells overexpressing $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Immunostaining assays using a specific primary antibody against these integrins were performed on four different cancer cell lines (MDA-MB-231, triple-negative breast cancer; A375, human malignant melanoma; MG-63, human osteosarcoma; and PANC-1, human pancreatic carcinoma) to evaluate integrin expression. The results showed that $\alpha_v\beta_3$ integrins were strongly expressed only in MDA-MB-231 TNBC cells, whereas A375, MG-63 and PANC-1 cells did not show significant overexpression level (*figure 28*).

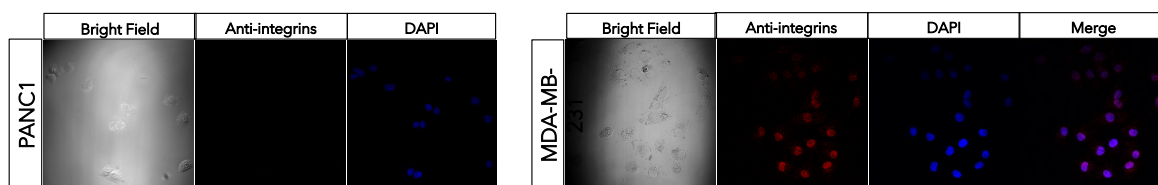


Figure 28 – $\alpha_v\beta_3$ integrins expression in PANC-1 (left) and MDA-MB-231 (right) cell lines. In blue the cellular nucleus stained with DAPI, in red the antibodies anti- $\alpha_v\beta_3$

Cellular uptake studies using confocal microscopy revealed significantly enhanced internalization of cRGD-functionalized liposomes compared to non-targeted liposomes in MDA-MB-231 cells (*figure 29*). Competitive inhibition experiments, in which cells were pre-incubated with free cRGD peptide, resulted in markedly reduced uptake, confirming that internalization occurred through integrin-mediated endocytosis.

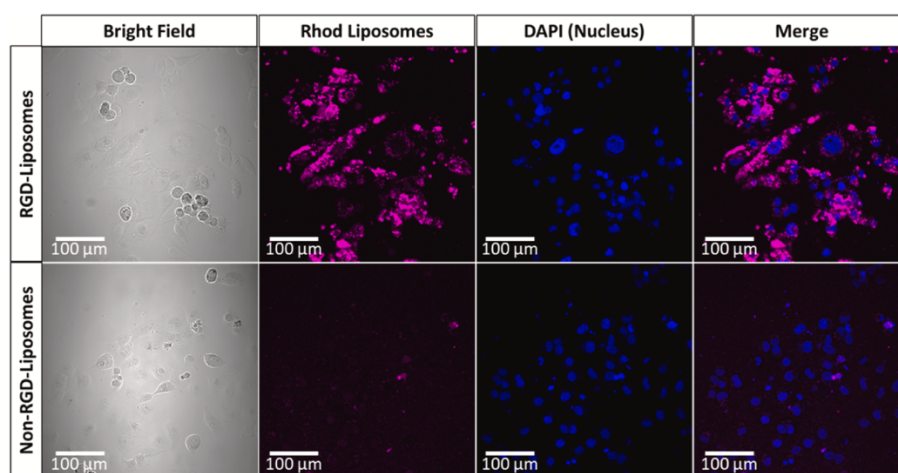


Figure 29 – Functionalized and non-functionalized liposomes internalization in **MDA-MB-231 cell line**. In blue the cellular nucleus stained with DAPI, in pink the synthesized fluorescent liposomes.

Notably, uptake was also evaluated in 3D spheroid models, which more closely mimic the tumor microenvironment. Targeted liposomes demonstrated improved penetration and approximately 1,4-fold higher fluorescence intensity within spheroids, particularly at the periphery, compared to untargeted controls (*figure 30*).

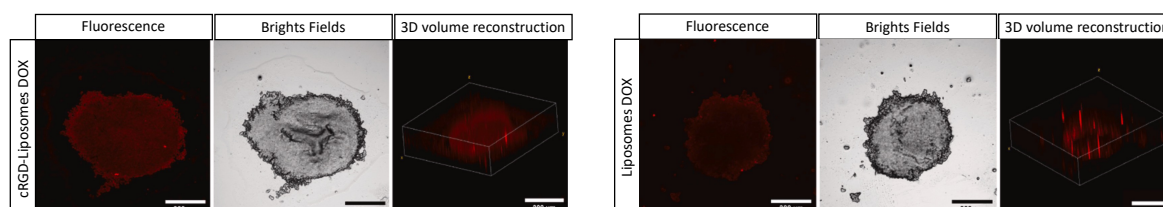


Figure 30 – Confocal Laser scanning Microscopy images of 3D spheroids of MDA-MB-231 cells incubated with cRGD-liposomes and not functionalized liposomes.

After assessing the improved targeting and cellular uptake, the corresponding enhancement of DOX activity in cancer cells was investigated. MTT assays performed after 48 h incubation showed that cRGD-targeted liposomes produced a > 20-fold increase in toxicity compared to untargeted liposomes in MDA-MB-231 cells ($IC_{50} \approx 0.51 \mu M$). In contrast, no significant targeting advantage was observed in integrin-negative cell lines, highlighting the specificity of the system (*figure 31*). Finally, *wound-healing assays* demonstrated that cRGD-liposomes were also capable of inhibiting TNBC cell migration at sub-toxic DOX concentrations, suggesting potential anti-metastatic activity beyond cytotoxic effects.

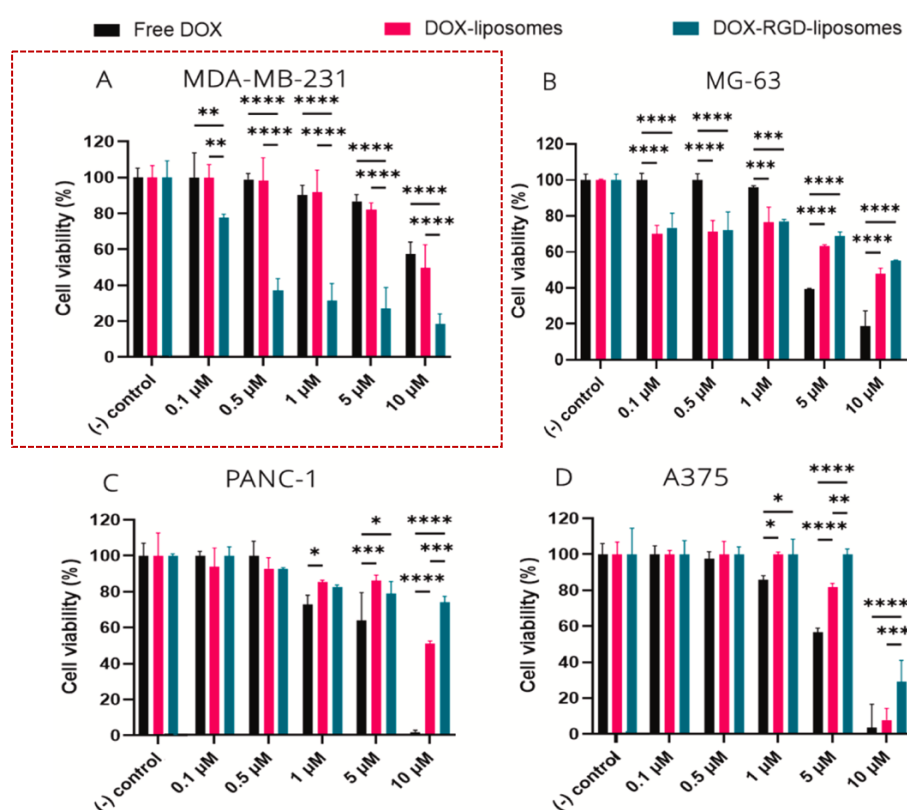


Figure 31 – Cell viability assays in 2D models: free doxorubicin (DOX), DOX-liposomes and DOX-RGD-liposomes when incubated for 48h in MDA-MB-231 (A), MG-63 (B), PANC-1 (C) and A375 (D) cell lines. $n = 3$ wells per condition. Only the MDA-MB-231 (A) cell lines overexpress the integrin receptor on their cell surface.

Overall, these results demonstrate that 3a-RGD-liposomes produced via microfluidic synthesis represent a robust and scalable theranostic platform for integrin-targeted drug delivery. The combination of efficient doxorubicin loading, selective $\alpha_v\beta_3$ -mediated uptake, enhanced cytotoxicity in TNBC models and dual MRI/fluorescence imaging capabilities supports their further development as precision nanomedicine tools for difficult-to-treat cancers.

2.5.2 3 α -RGD-based chitosan nanoparticles delivery systems

Chitosan-based nanoparticles (CS-NPs) have emerged as highly versatile platforms for drug delivery due to their biocompatibility, biodegradability, low immunogenicity and ease of chemical functionalization. As a cationic polysaccharide, chitosan readily forms nanoscale systems through mild ionotropic gelation or polyelectrolyte complexation, allowing the encapsulation or surface display of a wide range of therapeutic and diagnostic agents. An additional, and particularly relevant, feature of chitosan is its intrinsic mucoadhesive character, which arises from electrostatic interactions between protonated amino groups and negatively charged mucosal surfaces¹¹². This property makes chitosan nanoparticles especially attractive for non-invasive administration routes, such as the intranasal route, where prolonged residence time and enhanced epithelial interaction can significantly improve drug absorption. From a translational perspective, nasal administration represents a promising strategy to bypass the blood-brain barrier (BBB), enabling direct *nose-to-brain* transport through the olfactory and trigeminal pathways¹¹³. In this context, chitosan nanoparticles offer further advantages, as they have been reported to transiently open tight junctions and promote paracellular transport, potentially enhancing the permeation of macromolecules and nanocarriers across epithelial barriers. These characteristics make chitosan-based systems particularly suitable for the development of targeted delivery platforms aimed at central nervous system applications, where overcoming the BBB remains one of the major challenges¹¹⁴.

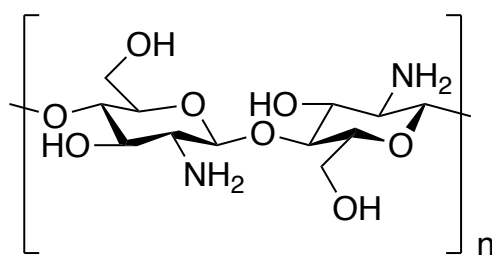


Figure 32 – Chitosan's monomeric unit

Within this framework, and in collaboration with Prof. Sara Perteghella's research group at the *University of Pavia*, a chitosan-based nanoparticle system was developed as a potential *nose-to-brain* delivery platform. The nanosystem was functionalized with the 3 α -RGD targeting motif to enable active recognition of $\alpha_v\beta_3/\alpha_v\beta_5$ integrin receptors, which are highly

overexpressed in a wide range of tumor cells and tumor-associated vasculature. The incorporation of the 3a-RGD ligand was primarily intended to promote selective targeting and enhanced internalization into cancer cells via integrin-mediated endocytosis. At the same time, given the involvement of $\alpha_v\beta_3$ integrins in cellular adhesion and epithelial transport processes, this functionalization may also contribute to improved interaction with epithelial cells and facilitate nanoparticle translocation across the nasal mucosal barrier.

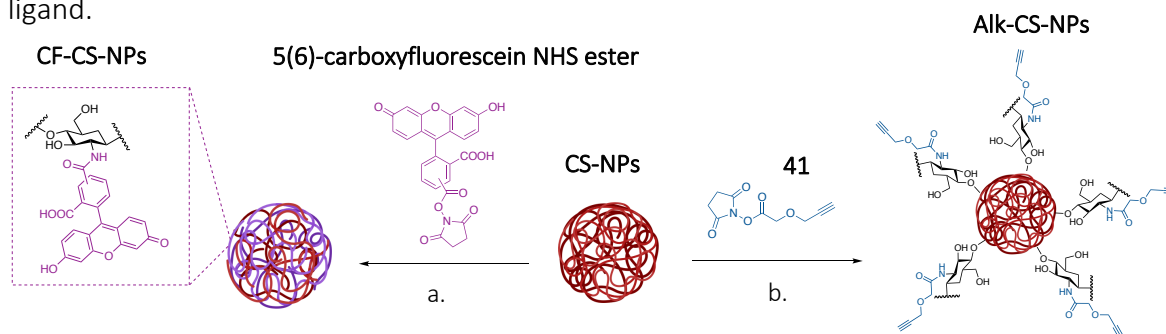
Chitosan nanoparticles preparation

Chitosan-coated PLGA nanoparticles were prepared using a modified emulsification/solvent evaporation method, optimized to promote the formation of a stable polymer-lipid interface. Briefly, poly(lactic-co-glycolic acid) (PLGA) and oleic acid (OA) were dissolved in ethyl acetate to generate the organic phase. In parallel, chitosan hydrochloride (Chitosan-HCl, degree of deacetylation 0.8) was dissolved in Milli-Q water to obtain the aqueous phase. A molar ratio of 1.4:1 (OA:chitosan) was selected to favor hydrophobic interactions between the fatty acid and the polysaccharide, thereby enabling the *in situ* formation of chitosan-oleate complexes. The organic phase was then slowly added to the aqueous chitosan solution under continuous magnetic stirring, resulting in the formation of a coarse oil-in-water emulsion. To reduce droplet size and enhance emulsion homogeneity, the system was subjected to probe sonication for 10 minutes. Subsequently, the organic solvent was allowed to evaporate under ambient conditions, leading to nanoparticle solidification and the formation of chitosan-coated PLGA nanoparticles stably dispersed in the aqueous medium.

Conjugation of 3a-RGD ligand to the chitosan nanoparticles surface

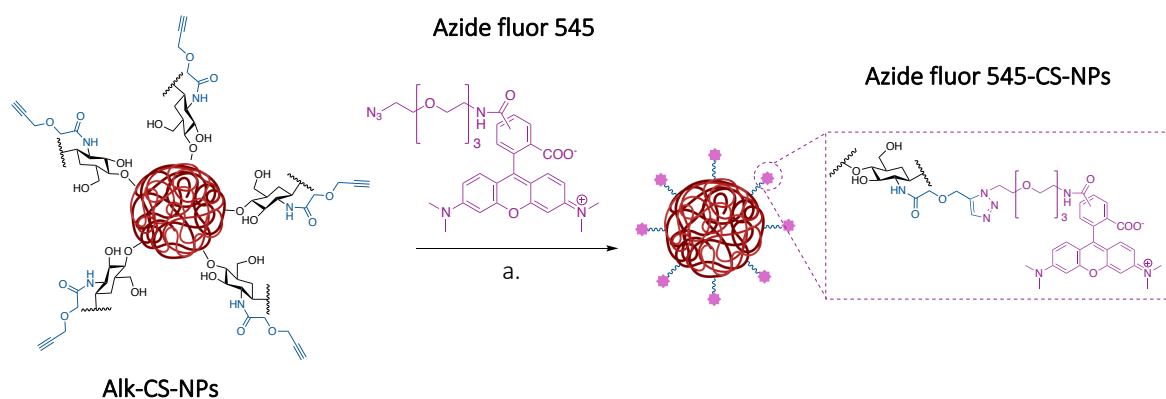
To translate the 3a-RGD targeting concept into a mucoadhesive nanosystem, chitosan nanoparticles were functionalized through a stepwise strategy conceived to be modular, chemically controlled and compatible with downstream quantification and biological testing. Surface modification of CS-NPs was first performed through N-hydroxysuccinimide (NHS) ester chemistry, exploiting the reactivity of surface-exposed amino groups. Two parallel derivatization strategies were implemented. In the first, nanoparticles were reacted with **5(6)-carboxyfluorescein NHS ester (CF-NHS)**, generating fluorescently labelled nanoparticles primarily intended for method validation and transport monitoring. In the

second approach, nanoparticles were functionalized with an **alkyne-linker NHS activated (41)** to introduce reactive handles suitable for *Cu(I)-catalyzed azide-alkyne cycloaddition* (CuAAC) (*scheme 18*). This latter pathway produced a “click-ready” intermediate that enabled the subsequent installation of azide-functionalized moieties, including the 3a-RGD ligand.



Scheme 18 – a) CF-NHS, PBS pH 7.4, 24 h, rt; b) **41**, PBS pH 7.4, 24 h, rt

Within this framework, a preliminary validation of the click chemistry on the nanoparticle surface was carried out by reacting a fraction of the alkyne-functionalized nanoparticles (**Alk-CS-NPs**) with **azide fluor 545**, a fluorescent probe bearing an azide group. This step was designed to provide direct evidence of the efficiency of the CuAAC transformation under the selected conditions, while also establishing a reference system for the estimation of the nanoparticle functionalization degree. In particular, the fluorescence associated with this model system enabled a comparative evaluation with 3a-RGD-functionalized nanoparticles prepared under analogous conditions, allowing a semi-quantitative assessment of surface ligand density (*scheme 19*).

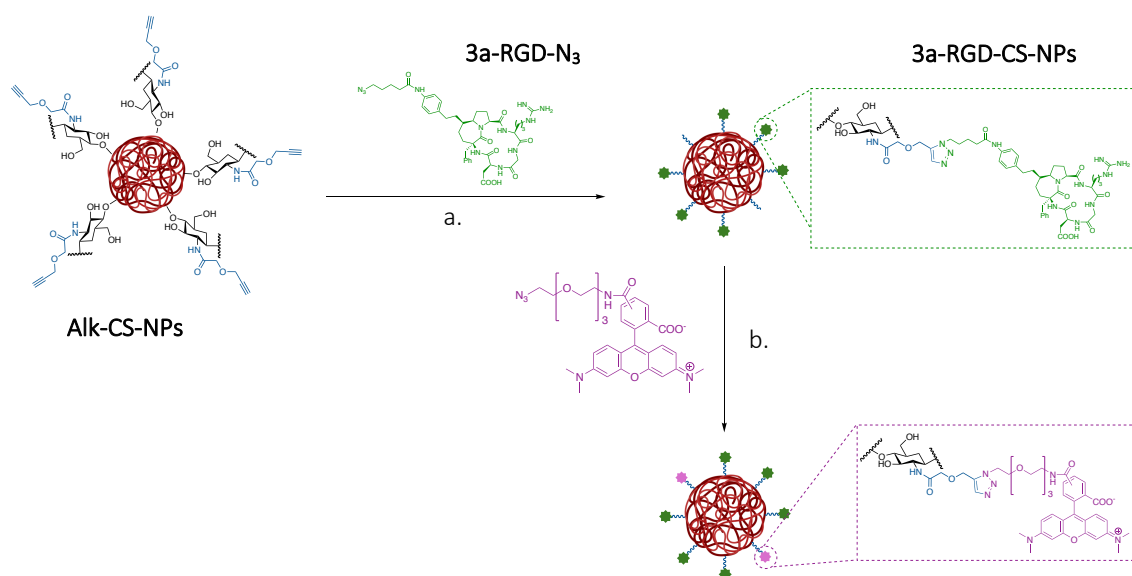


Scheme 19 – Azide fluor 545, PBS pH 7.4, 72 h, rt

A critical methodological consideration in this work concerned the removal of residual low-molecular-weight species following nanoparticle functionalization. Free fluorophores,

coupling reagents or unreacted ligands can significantly bias both fluorimetric measurements and biological assays, particularly in permeability studies where signal attribution must be unambiguously associated with nanoparticle transport. For this reason, purification was treated as a key experimental parameter rather than a routine post-reaction step. After each functionalization stage, nanoparticle suspensions were subjected to extended dialysis (24-72 h) with frequent buffer exchange, and the dialysate was monitored at spectrofluorimeter until fluorescence levels became negligible. This procedure was consistently applied across all nanoparticle batches, including after CuAAC conjugation, to minimize the possibility that detected fluorescence signals could arise from freely diffusing molecules rather than nanoparticle-associated fluorophores.

The installation of the targeting ligand was subsequently achieved via CuAAC between azide-functionalized **3a-RGD-N₃** and the alkyne-bearing nanoparticle surface (*scheme 20*). This strategy allows highly selective and efficient conjugation under mild conditions, ensuring preservation of nanoparticle integrity while enabling controlled surface functionalization.



Scheme 20 – a) **3a-RGD-N₃**, Ascorbate Na, CuSO₄ · 5H₂O, PBS pH 7.4, 72 h, rt; b) **Azide fluor 545**, Ascorbate Na, CuSO₄ · 5H₂O PBS pH 7.4, 72 h, rt

Following peptide conjugation, an additional control experiment was performed to evaluate the presence of residual unreacted alkyne functionalities on the nanoparticle surface. To this end, a fraction of the 3a-RGD-functionalized nanoparticles was further subjected to CuAAC reaction with Azide Fluor 545. This post-functionalization step provided direct insight

into the extent of surface saturation, as any residual fluorescence signal could be attributed to the presence of unoccupied reactive sites. At the same time, this approach enabled the preparation of a nanoparticle population simultaneously bearing the targeting ligand and a fluorescent reporter, suitable for subsequent tracking and biological evaluation.

Overall, this approach provided a practical *proof-of-concept* for anchoring 3a-RGD to CS-NPs through a reproducible chemical sequence, while maintaining reaction conditions sufficiently mild to preserve nanoparticle integrity.

The extent of nanoparticle functionalization was subsequently estimated using fluorimetric analysis after purification. The analysis supports the successful functionalization of chitosan nanoparticles through both NHS-mediated derivatization and subsequent CuAAC click chemistry. Comparative analysis of functionalization efficiency revealed a markedly higher fluorophore loading for NHS-mediated conjugation (7.4 nmol/mg) compared to CuAAC-based click functionalization (1.0 nmol/mg), corresponding to an approximately 7-fold difference. When compared to the theoretical feed ratio (40 nmol/mg), the effective degree of functionalization achieved via click chemistry corresponds to 2-3%. The lower-than-theoretical fluorescence values suggest that only a fraction of the available sites undergo reaction, likely due to steric hindrance and limited surface accessibility. Importantly, the minimal residual fluorescence observed, under limit of detection, after reacting 3a-RGD-CS-NPs with the azide fluorophore indicates that most of the alkyne groups are consumed during ligand conjugation. Overall, these results confirm that the adopted strategy enables effective surface functionalization, while highlighting that the process does not reach complete site saturation, which is consistent with the heterogeneous nature of nanoparticle surfaces.

In vitro preliminary biological evaluation of functionalized CS-NPs

Preliminary cytocompatibility of the nanoparticle formulations was evaluated using RPMI 2650 nasal epithelial cells, a cell line commonly used as an *in vitro* model of the nasal mucosa. Cell viability was assessed through an MTT assay following 24 h exposure to nanoparticles. Across all formulations, nanoparticles were generally well tolerated at concentrations up to 75 µg/mL, whereas exposure to 300 µg/mL resulted in a marked

reduction in cell viability. At the intermediate concentration of 150 $\mu\text{g/mL}$, the cytotoxic response appeared more dependent on nanoparticle surface functionalization, suggesting that surface chemistry and potentially cellular uptake mechanisms may become increasingly relevant as nanoparticle dose increases (*figure 33*).

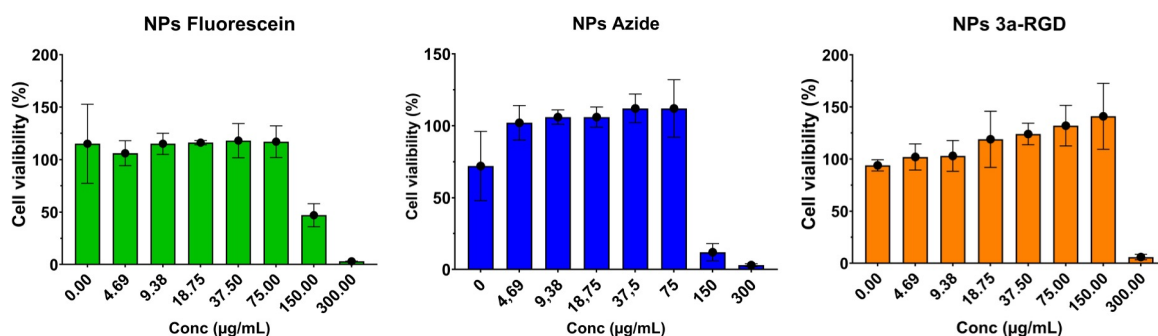


Figure 33 – Effects of develop NPs systems on cell viability. RPMI 2650 cells were seeded in 96-well plates and allowed to grow for 48 h, followed by 24 h treatment with NPs Fluorescein, NPs Azide e NPs 3a-RGD at concentrations ranging from 4.69 μM to 300 μM . Cell viability was assessed using the MTT assay. Data are representative of a single independent experiment and expressed as percentage of viable cells relative to untreated controls for each cell line.

Based on these observations, 75 $\mu\text{g/mL}$ was selected as a working concentration for permeability studies, representing a compromise between maintaining cell viability and ensuring detectable signal generation. Nanoparticle-associated transport was then investigated using a Transwell permeability model, monitoring fluorescence in the basolateral compartment over time. Under these experimental conditions, all nanoparticle formulations produced a time-dependent increase in basolateral fluorescence relative to control, indicating measurable transport across the epithelial layer (*figure 34*).

However, the magnitude of this increase varied substantially between formulations. Nano-RGD at 75 $\mu\text{g/mL}$ produced a modest but reproducible increase in signal (approximately +12 - 16% between 30 and 120 min), while Nano-3a-RGD at 150 $\mu\text{g/mL}$ exhibited a slightly stronger effect ($\sim +9\%$ at 60 min rising to $\sim +22\%$ at 120 min). A similar trend was observed for Nano-Azide at 75 $\mu\text{g/mL}$ (approximately +11-19%). In contrast, Nano-fluorescein at 75 $\mu\text{g/mL}$ generated the most pronounced increase, reaching approximately +64% after 120 min. This observation is consistent with the substantially higher fluorophore loading measured for this formulation and likely reflects signal amplification due to fluorophore density rather than necessarily enhanced nanoparticle transport.

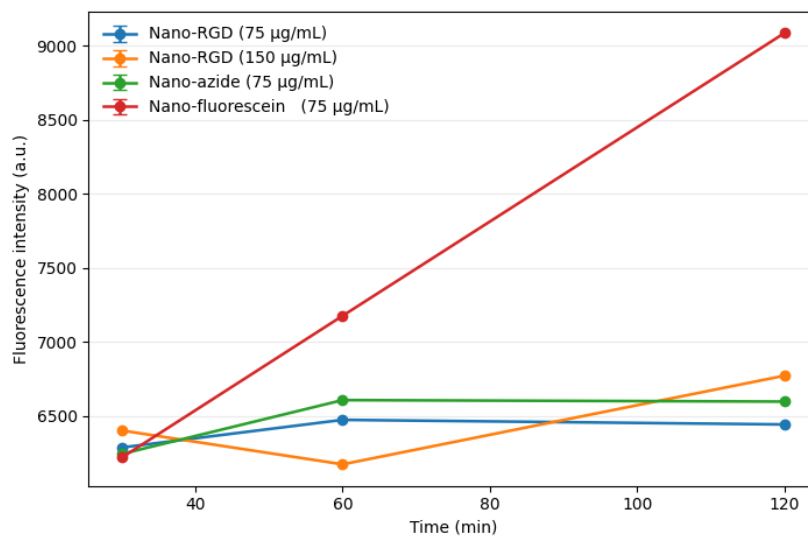


Figure 34 – Time-dependent permeability of chitosan-based nanoparticles across RPMI 2650 epithelial cell monolayers evaluated in a Transwell system.

Overall, the results obtained in this chapter demonstrate the feasibility of producing 3a-RGD-functionalized chitosan nanoparticles through a controlled click-chemistry strategy, while maintaining nanoparticle stability and compatibility with biological evaluation. Although the present data remain preliminary, the work establishes a reproducible functionalization workflow and identifies experimentally justified concentration ranges suitable for epithelial transport studies. At the same time, the findings highlight several methodological aspects, particularly the influence of fluorophore loading, purification efficiency and detection parameters on fluorescence-based transport measurements, that must be carefully addressed in future studies. Further work will therefore focus on improving ligand conjugation efficiency, implementing calibration-based quantification of nanoparticle transport and investigating the potential contribution of integrin-mediated uptake mechanisms in the interaction between 3a-RGD-functionalized nanoparticles and nasal epithelial barriers.

2.5.3 3a-RGD-based silk fibroin nanoparticles delivery systems

In the previous sections, the 3a-RGD ligand has been demonstrated to act as a high affinity targeting moiety for $\alpha_v\beta_3/\alpha_v\beta_5$ integrins, enabling selective interaction with tumor cells. Building on these findings, the present work focuses on the application of this scaffold to silk fibroin nanoparticle (SFNs)-based delivery systems.

The study presented in this section, carried out in collaboration with Prof. Marina Torre's research group at *University of Piemonte Orientale*, can be considered a direct evolution of the previously published work on cRGD-functionalized silk fibroin nanoparticles (E. Bari, M. Serra *et al.*, "*Silk Fibroin Nanoparticle Functionalization with Arg-Gly-Asp Cyclopentapeptide Promotes Active Targeting for Tumor Site-Specific Delivery*"), in which RGD decoration was shown to enhance active targeting toward integrin-overexpressing tumor cells through receptor-mediated endocytosis. In that work, the functionalization strategy enabled selective internalization in cancer cells, while maintaining the favorable physicochemical and biological properties of silk fibroin.

However, although effective in promoting tumor cell uptake, the previously developed system did not address one of the major challenges in cancer nanomedicine, the delivery of therapeutics to tumors located behind biological barriers such as the BBB. Therefore, the present work introduces a second targeting element, namely glucose, with the aim of enabling interaction with glucose transporters GLUT-1 expressed on brain endothelial cells and thus promoting nanoparticle translocation across the BBB¹¹⁵. The resulting nanosystem is therefore based on a **dual-targeting strategy**, in which glucose is intended to mediate barrier crossing, while 3a-RGD retains its role in promoting tumor-specific uptake. This approach represents a conceptual advancement from single-ligand targeting systems toward more complex, multistep delivery platforms.

Silk fibroin nanoparticles preparation and characterization

Silk fibroin was isolated from *Bombyx mori* cocoons through a multistep purification process, consisting in boiling step with Na_2CO_3 aqueous solution following by was washing with distilled water and drying a room temperature, in which the effective removal of sericin represents a critical prerequisite to ensure material reproducibility and biocompatibility.

Incomplete degumming during the boiling step can significantly affect both the physicochemical properties and the biological response of the final system; therefore, particular attention was paid to obtaining a purified fibroin matrix free of residual impurities. Following the previous processing steps, fibroin was brought into aqueous *lithium bromide* solution that promote the transition toward a processable protein conformation (from *silk II* to *silk I*), enabling the preparation of homogeneous aqueous formulations with controlled concentration. This step is particularly important, as the conformational state of fibroin directly influences its ability to undergo subsequent self-assembly.

Fluorescent labelling was introduced, before nanoparticles formation, to allow reliable tracking of the material in biological environments. A key aspect of this step was the control over the degree of functionalization: only a limited fraction of available reactive groups was targeted in order to balance signal detectability with preservation of the native structural and assembly properties of fibroin: excessive labelling could interfere with protein folding and nanoparticles formation, while insufficient labelling would compromise analytical sensitivity; to select the appropriate reaction conditions was first evaluate the number of available lysine residues and then fluorophore marker, the fluorescein isothiocyanate (FITC), was added to target approximately 10% of these residues.

Nanoparticle formation was achieved through a desolvation-driven self-assembly process, which constitutes another critical stage of the procedure. The labelled fibroin solution was added dropwise to acetone under magnetic stirring (*1:5 fibroin-to-acetone ratio*), promoting a controlled conformational transition toward the β -sheet-rich *Silk II* structure, which is essential for nanoparticle stabilization and for defining their mechanical strength and colloidal properties. Finally, extensive dialysis purification and freeze-drying steps were implemented to remove unreacted species and residual solvents, yielding stable fluorescent silk fibroin nanoparticles suitable for further biological application.

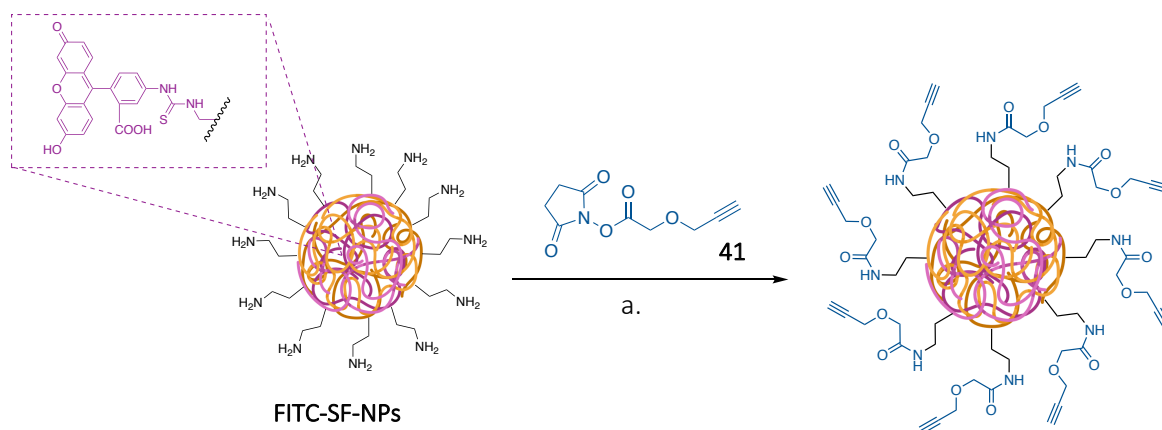
Conjugation of 3a-RGD ligand to the silk fibroin nanoparticles surface

The surface functionalization of silk fibroin nanoparticles represents the central technological advancement of this work and was design to enable both visualization and targeted delivery. The strategy adopted builds upon the methodology previously developed

for RGD conjugation (see Chapter [2.5.1](#) and [2.5.2](#)), while introducing key modifications to allow controlled dual functionalization.

As a preliminary step, nanoparticles were labelled with a fluorescent probe, fluorescein isothiocyanate (FITC), to allow their detection and quantification in subsequent *in vitro* experiments. This modification exploits the reactivity of the isothiocyanate group toward primary amines present on the lysine residues of silk fibroin, resulting in the formation of stable thiourea linkages without significantly affecting nanoparticle integrity.

Following fluorescent labelling, the nanoparticle surface was further engineered to introduce reactive handles for ligand conjugation. This was accomplished through covalent modification of residual lysine amino groups using an *NHS-activated alkyne-bearing linker* (compound **41**), previously employed for CS-NP derivatization, enabling the formation of stable amide bonds and the introduction of alkyne functionalities suitable for subsequent click chemistry reactions (*scheme 21*). The efficiency of this process is crucial, as it directly determines the density of functional groups available on the nanoparticle surface and, consequently, the extent of ligand decoration.

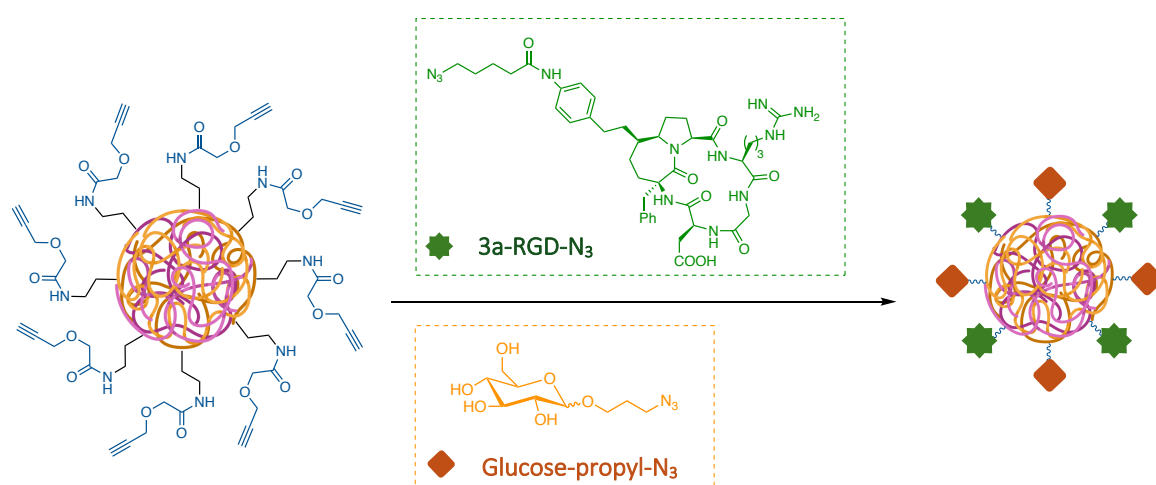


Scheme 21 – a) **41, PBS pH 7.4, 24 h, rt**

Following activation, nanoparticles were purified to remove unreacted reagents and by-products, yielding a stable intermediate compatible with further chemical modification.

The second stage involved the introduction of targeting ligands via CuAAC click chemistry, following an approach analogous to that previously employed for chitosan-based nanoparticles. This strategy allows highly selective and efficient conjugation under mild

conditions, ensuring preservation of nanoparticle integrity. Activated nanoparticles were functionalized either with glucose alone and with a combination of glucose and 3a-RGD, enabling the preparation of both single- and dual-functionalized systems. For the dual-functionalized nanoparticles, **glucose-propyl-N₃** (3-Azidopropyl D-glucopyranoside) and **3a-RGD-N₃** were introduced in controlled proportions to achieve a balanced surface composition (*scheme 22*). The reaction was catalyzed by a Cu(I)-generating system, promoting efficient cycloaddition and uniform ligand distribution across the nanoparticle surface. This modular approach enables the simultaneous incorporation of multiple targeting elements while maintaining precise control over surface functionalization.



Scheme 22 – 3a-RGD-N₃, Glucose-propyl-N₃, Ascorbate Na, CuSO₄ · 5H₂O, PBS pH 7.4

Following conjugation, nanoparticles were purified through repeated washing cycles to remove unreacted ligands, catalyst residues and low molecular weight by-products. Final lyophilization ensured the recovery of stable, dry nanoparticle formulations suitable for storage and subsequent technological and biological evaluation. Particle size distribution analysis confirmed that the developed silk fibroin nanoparticles fall within the expected nanometric range, consistent with a well-controlled self-assembly process. Importantly, no significant variations in particle size were observed following surface functionalization, indicating that the modification strategy did not adversely affect the structural integrity or colloidal characteristics of the system. This result suggests that the functionalization approach is compatible with the preservation of key physicochemical properties, maintaining nanoparticle uniformity and stability, which are essential for reliable performance in subsequent applications.

In vitro preliminary biological evaluation of SF-NPs

The *in vitro* biological validation aimed to assess the effectiveness of the SFNs prepared in terms of both cellular uptakes, using tumor cells that overexpress integrin receptors (glioblastoma cells, U87) or do not (HeLa), and their ability to cross the BBB in an *in vitro* model (using hCMEC/D3 cells). Overall, the *in vitro* evaluation of the developed nanosystems provides a comprehensive validation of the proposed dual-targeting strategy, highlighting the distinct and complementary contributions of glucose and 3a-RGD functionalization.

First, the cytocompatibility of the prepared SFNs was evaluated on all cell lines used for *in vitro* validation (HeLa, U87 and hCMEC/D3) using the MTT assay. As shown in *figure 35*, all nanoparticle formulations exhibited a favorable cytocompatibility profile across the investigated concentration range, with no significant differences among the different formulations ($p = 0.6$). This indicates that neither surface functionalization nor the presence of glucose and 3a-RGD moieties introduces additional cytotoxicity. Conversely, cell line-dependent differences were observed (*figure 35*). At 0.4 mg/mL, HeLa cells showed significantly lower cytocompatibility compared to U87 and hCMEC/D3 cells ($p < 0.05$). At 0.8 mg/mL, both HeLa and U87 cells (with no significant difference between them) exhibited significantly reduced viability compared to hCMEC/D3 cells ($p < 0.05$). Finally, a concentration-dependent decrease in cell viability was observed for all samples (*figure 36*), consistent with the statistically significant effect of concentration ($p < 0.001$). The reduction in viability at higher concentrations can therefore be attributed to nonspecific nanoparticle accumulation and cellular burden rather than ligand-specific effects.

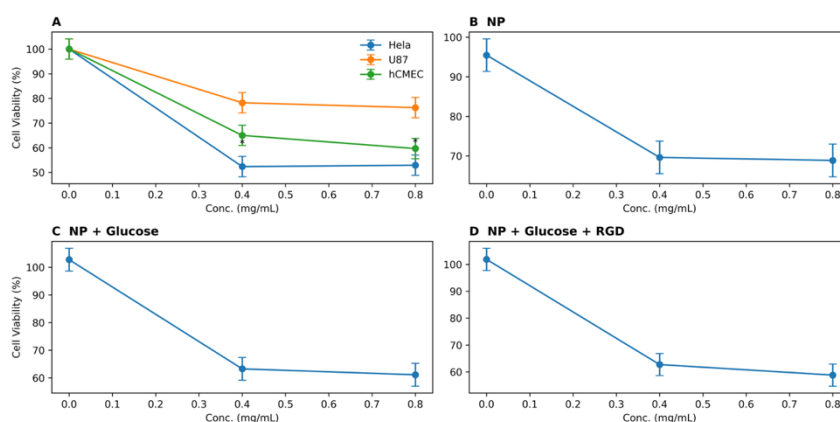


Figure 35 – Cytocompatibility of SFNs. (A) Interaction plot showing the cell line-dependent response (HeLa, U87, and hCMEC/D3) as a function of concentration.

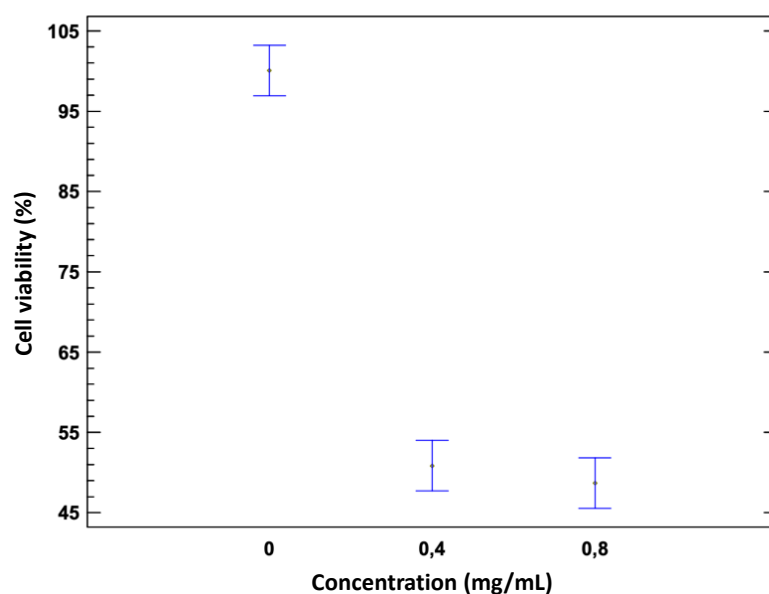


Figure 36 – Concentration-dependent decrease in cell viability. Data are reported as mean values $\pm$ LSD, $n=3$. The overlapping of Fisher's LSD bars indicates a non-significant difference ($p > 0.05$).

The cellular uptake results (figure 37) demonstrate that nanoparticle internalization is significantly influenced by multiple factors. In particular, the uptake was both time- and concentration-dependent ($p < 0.001$), with a progressive increase observed at higher concentrations and longer incubation times. A marked cell line-dependent behavior was also observed with hCMEC/D3 cells showing limited internalization under all tested conditions, whereas HeLa and U87 cells exhibited significantly higher uptake, with U87 cells displaying the highest internalization levels.

In addition, uptake was strongly affected by nanoparticle surface functionalization. Specifically, 3a-RGD-functionalized nanoparticles showed a significant increase in intracellular fluorescence compared to non-functionalized controls, consistent with the activation of integrin-mediated endocytosis pathways. This effect was particularly pronounced in U87 glioblastoma cells, in agreement with their known integrin overexpression. Notably, dual-functionalized nanoparticles exhibited the highest uptake overall, suggesting a synergistic effect in which glucose may enhance initial nanoparticle-cell interactions without impairing integrin recognition.

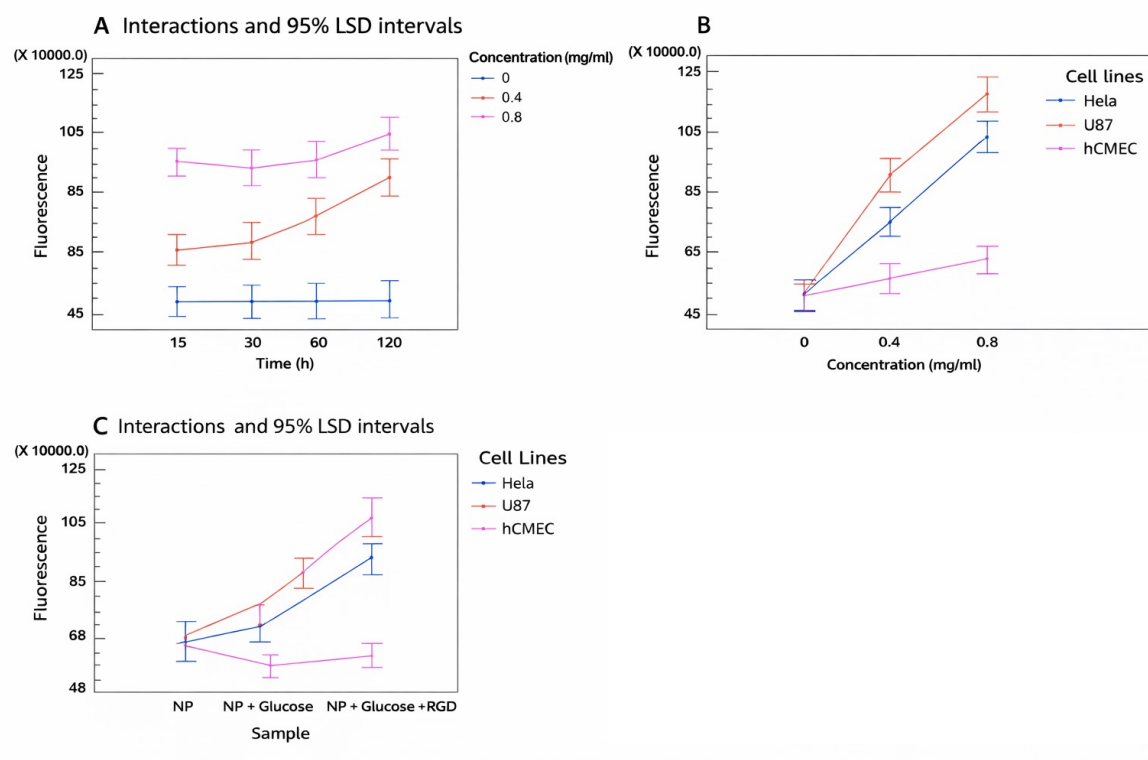


Figure 37 – Effect of nanoparticle surface functionalization on cellular uptake in different cell lines. (A) Uptake as a function of concentration and incubation time. (B) Uptake as a function of concentration and cell line. (C) Uptake as a function of nanoparticle formulation and cell line. Data are reported as mean values $\pm$ LSD, $n=3$. The overlapping Fisher's LSD bars indicates no statistically significant differences ($p > 0.05$).

A further level of insight is provided by the transwell experiments, which model nanoparticle transport across the BBB using hCMEC/D3 cells (*figure 38*).

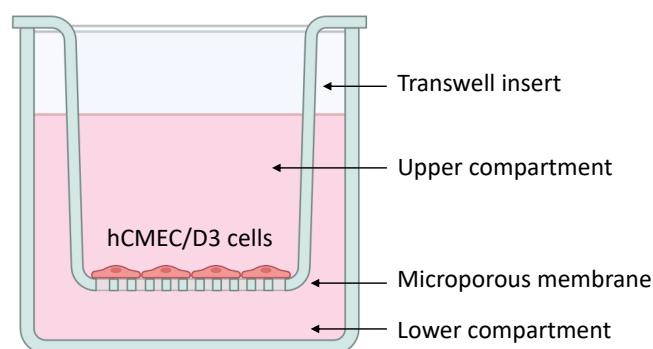


Figure 38 – Schematic representation of a transwell permeable support system seeded with hCMEC/D3 cells. The endothelial cells form a confluent monolayer on the porous membrane, mimicking the blood–brain barrier interface. The system is divided into an apical (upper) and basolateral (lower) compartment, enabling the evaluation of nanoparticle transport across the endothelial barrier under controlled *in vitro* conditions.

The results reveal how time did not have a significant effect on nanoparticle transport ($p = 0.3$), whereas both the nanoparticle formulation and the compartment in which

fluorescence was measured (apical vs. basolateral) were significant factors ($p < 0.001$). Non-functionalized SFNs largely remained in the apical compartment, with no significant difference between apical and basolateral fluorescence. In contrast, glucose-functionalized SFNs exhibited enhanced translocation to the basolateral compartment, with significantly higher fluorescence compared to the apical side, supporting the hypothesis of glucose transporter-mediated transport. The addition of RGD to glucose-functionalized nanoparticles did not significantly alter barrier crossing, confirming that RGD's main function is tumor targeting rather than endothelial transport (*figure 39*).

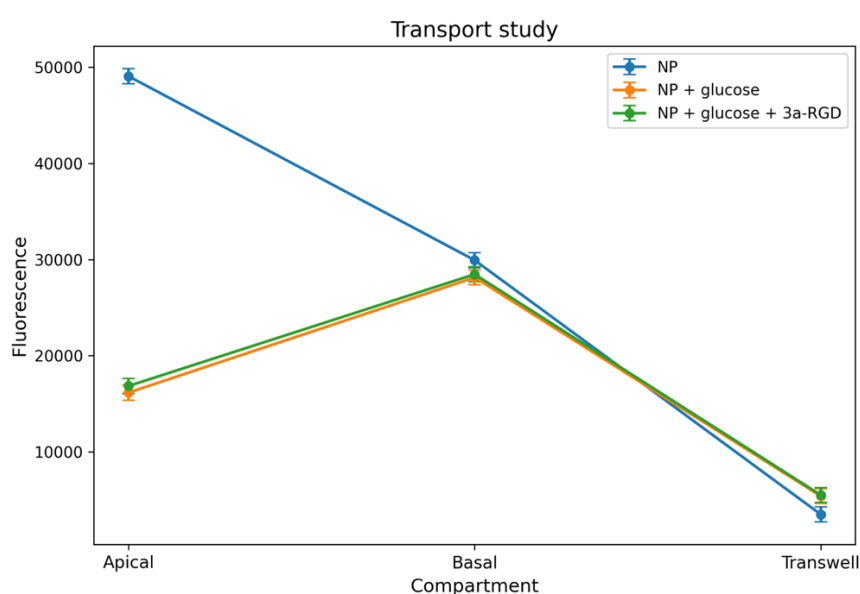


Figure 39 – Transwell evaluation of SFN transport across an *in vitro* BBB model. Effect of nanoparticle surface functionalization on transport from apical to basolateral compartment. Data are reported as mean values $\pm$ LSD, $n=3$. The overlapping Fisher's LSD bars indicates no statistically significant differences ($p > 0.05$).

Overall, these results support a sequential targeting mechanism in which glucose facilitates BBB crossing and 3a-RGD promotes tumor cell internalization. The superior performance of the dual-functionalized system demonstrates the effectiveness of combining these two targeting strategies within a single nanocarrier.

2.6 cRGD-Naphthalene diimide (NDI) Conjugates

Naphthalene diimide (NDI) derivatives represent one of the most extensively investigated classes of small molecules in the field of nucleic acid secondary structure recognition. Owing to their rigid, planar aromatic core and their strong π - π stacking capability, NDIs have been widely reported as highly effective ligands for the stabilization of G-quadruplex (G4) structures¹¹⁶. These non-canonical DNA conformations, located in key genomic regions such as telomeres and oncogene promoters, have attracted significant interest as promising therapeutic targets in anticancer drug discovery. Indeed, the ability of NDI-based ligands to selectively interact with G4s can interfere with crucial biological processes, including telomerase activity and transcriptional regulation, ultimately resulting in strong cytotoxic effects in tumor cells^{117–119}. However, despite their remarkable binding affinity and biological potential, many potent G4-stabilizing ligands, including NDIs, suffer from a major limitation: their lack of selectivity toward cancer cells over healthy tissues. This issue is primarily related to their systemic distribution and off-target interactions, which can lead to undesired toxicity and narrow therapeutic windows. Therefore, the development of strategies able to enhance the selective delivery of these cytotoxic agents represents a critical step toward their possible translation into more effective and safer anticancer therapeutics. In this context, targeted drug-delivery strategies that exploit tumor-associated receptors have emerged as powerful tools to improve the cellular uptake and specificity of NDIs¹²⁰. In particular, conjugation of NDI compounds with cyclic RGD derivatives could represent an effective strategy to enable receptor-mediated internalization and selective tumor accumulation¹²¹.

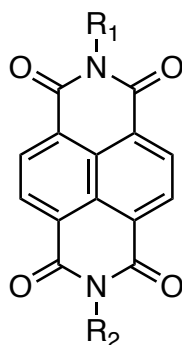


Figure 40 - Naphthalene diimide (NDI) generic structure

2.6.1 Design and Synthesis of Cleavable NDI-3a-RGD Conjugates

The work presented in this chapter builds upon previous studies reported in Dr. Bisbano's PhD thesis and subsequently published in *Scientific Reports*, carried out in collaboration with Prof. Doria's research group at *University of Pavia*^{55,107,121}. In that work, a proof-of-concept NDI-cRGD conjugate was developed by covalently linking a tetrasubstituted NDI scaffold to a cyclic RGD peptide through a non-cleavable linker, combining G-quadruplex stabilizing activity with integrin-targeting capability. *In vitro* studies demonstrated enhanced selective uptake and biological activity in integrin-overexpressing glioma cells, while preserving the intrinsic G4-binding properties of the NDI core. Building on these results, this work extends the NDI-3a-RGD platform through the design of cleavable conjugates with programmable release and potentially improve pharmacokinetic and pharmacodynamic profiles. In this strategy, the linker plays a central role as a *molecular switch*, remaining stable during circulation while enabling selective processing following cellular uptake. To systematically explore the impact of linker chemistry and NDI structure, we selected three cleavable linkers and three NDI scaffolds to obtain derivatives spanning different polarity/size and different release mechanisms: (1) **Alkyne-Val-Cit-PAB-PNP**, (2) **DBCO-PEG2-Val-Cit-PAB-PNP**, and (3) a non-self-immolative linker bearing a **DBCO moiety** and a **GGFG** (*Gly-Gly-Phe-Gly*) tetrapeptide as protease-cleavage site (*figure 41*).

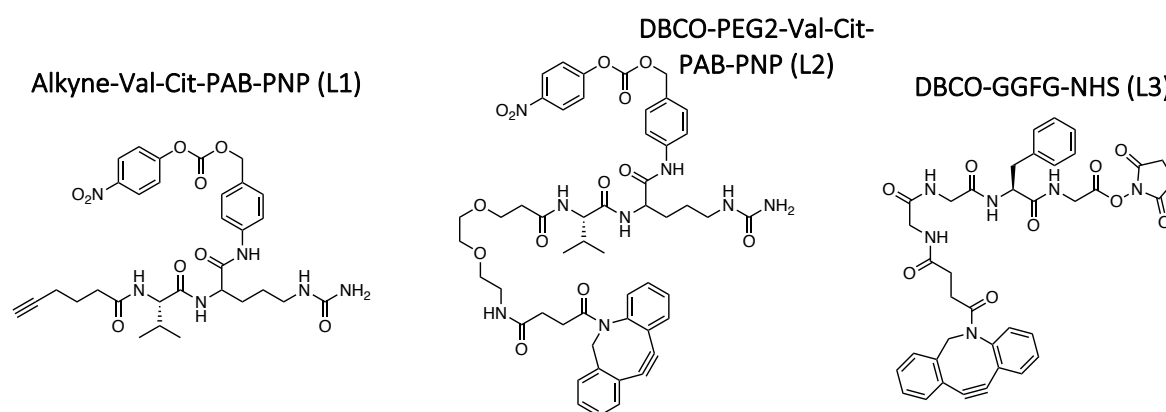
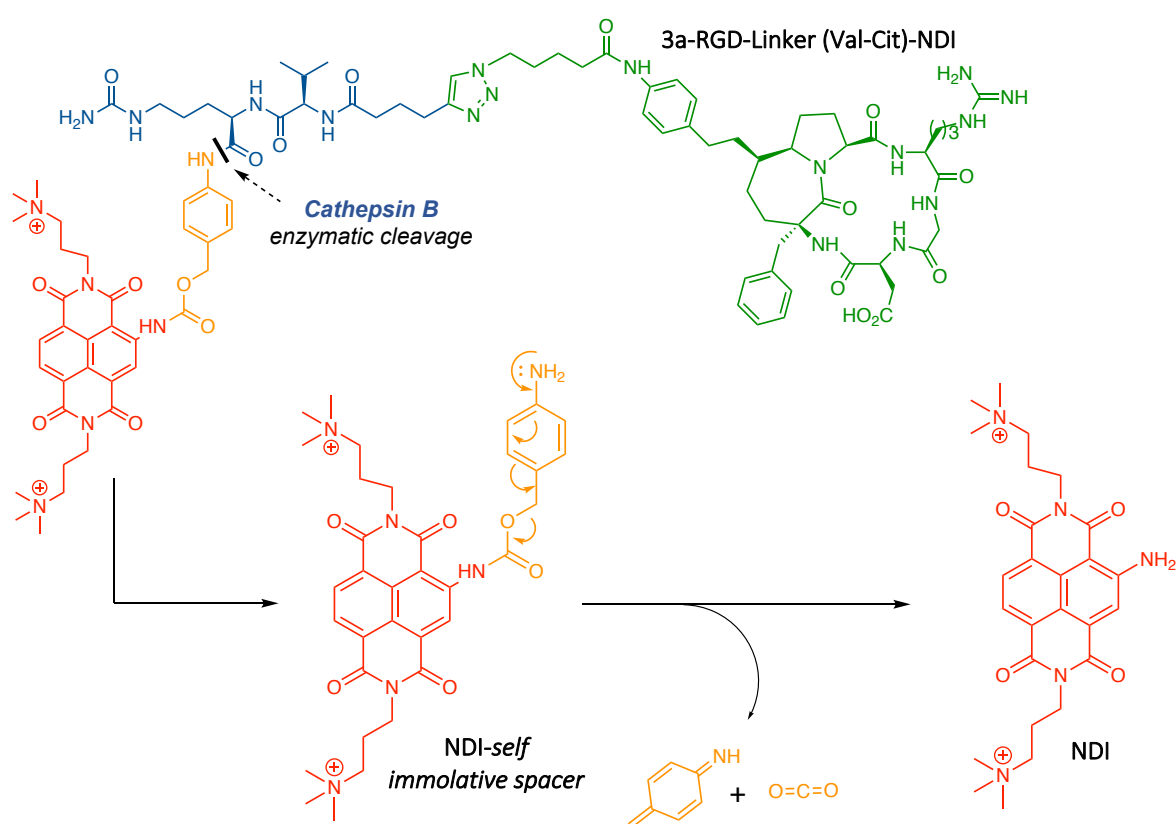


Figure 41 –Chemical structures of *Alkyne-Val-Cit-PAB-PNP*, *DBCO-PEG2-Val-Cit-PAB-PNP* and *DBCO-GGFG-NHS* linkers (from left to right)

Despite their structural differences, all three linkers share key common features: a protease-cleavable peptide motif to enable enzyme-triggered release and a conjugation handle for modular coupling to the NDI payloads and 3a-RGD. The first two selected linkers feature the

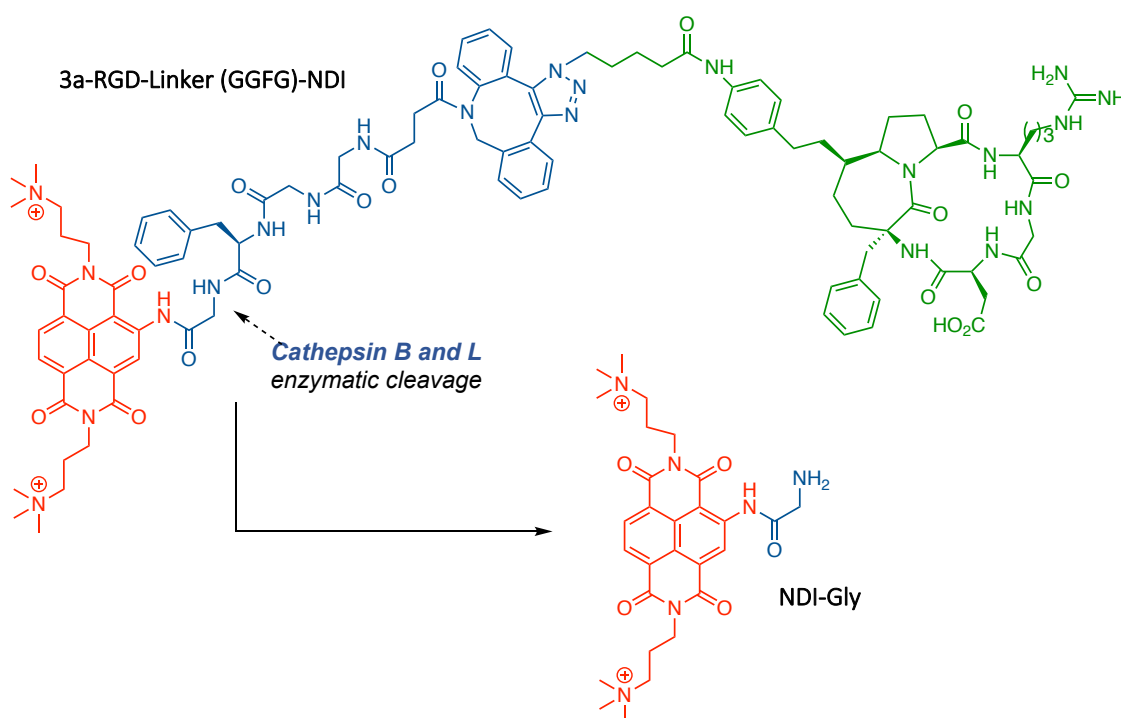
Valine-Citrulline-*para*-aminobenzoic (PAB) motif which is a prototypical lysosomal/protease-cleavable system widely used in ADC chemistry: proteolytic cleavage of the Val-Cit dipeptide by lysosomal enzyme **cathepsin B**, overexpressed in several cancer cells line, can trigger self-immolation of the PAB spacer (elimination), thereby releasing the payload in its “native” form ,without a residual peptide scar, while maintaining good stability in human plasma (*scheme 23*); however, species-dependent instability (e.g., mouse plasma carboxylesterases) and premature extracellular processing (e.g., by neutrophil elastase) are known caveats and should be considered when interpreting *in vivo* data and cross-species comparisons¹²².



Scheme 23 – Release mechanism of NDI payload after enzymatic cleavage of Val-Cit motif

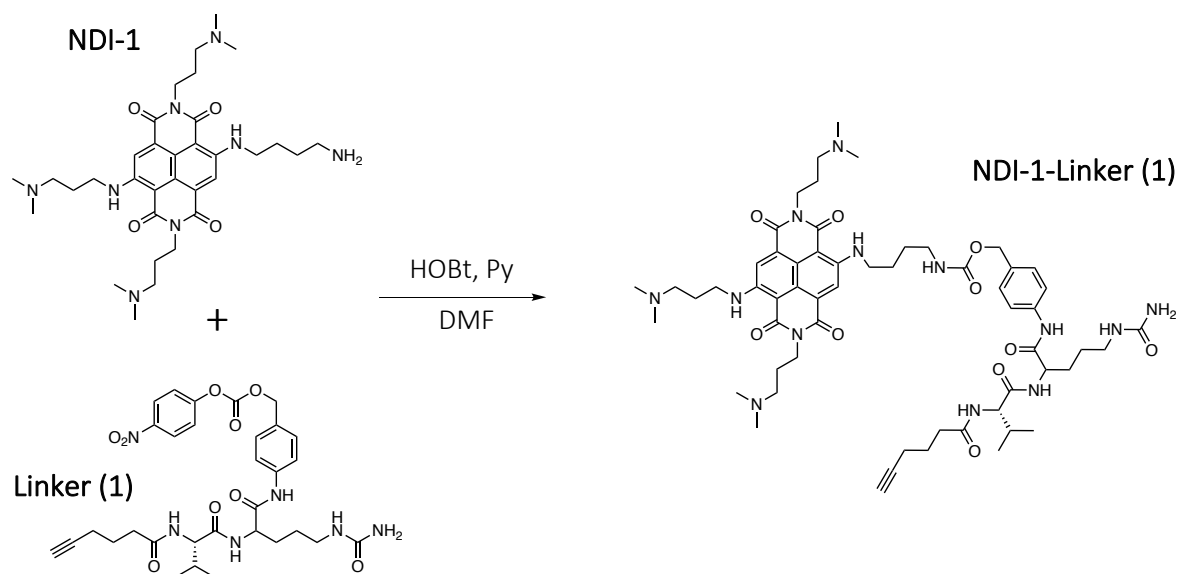
In parallel, the GGFG tetrapeptide represents an alternative lysosomal-cleavable sequence that has also been successfully used in clinically relevant linker systems due to his high stability in systemic circulation; in its *NON-self-immolative* implementation, cleavage is expected to generate a residual fragment on the payload (*scheme 24*), which may influence both activity and physicochemical behaviour and thus provides an additional insight into structure-activity relationships⁵⁵.

From a synthetic standpoint, both Val-Cit-PAB linkers were employed in their PNP-carbonate activated form, because the p-nitrophenyl (PNP) carbonate is a highly activated leaving group enabling efficient coupling to amines under mild conditions; additionally, the DBCO-bearing variants were selected to enable copper-free SPAAC conjugation with azide-bearing targeting vectors, in order to reduce copper-related side issues, while PEG2 was introduced to mitigate the intrinsic hydrophobicity of the cyclooctyne/aromatic domains and improve overall solubility and handling in mixed aqueous/organic media.

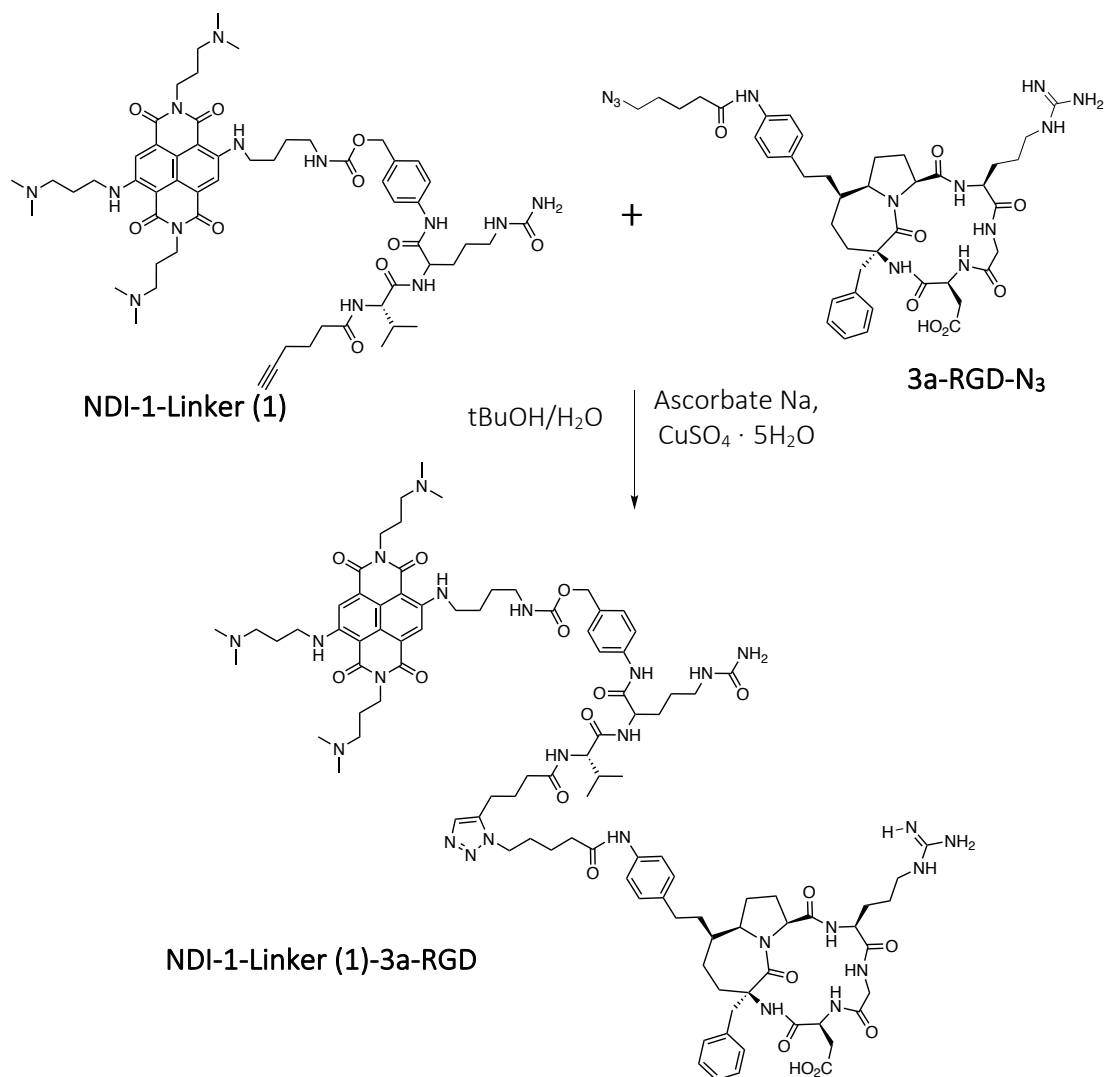


Scheme 24 – Release mechanism of NDI payload after enzymatic cleavage of Gly-Gly-Phe-Gly motif

As a first *proof-of-concept*, **NDI-1** was selected as the initial payload scaffold and coupled to Alkyne-Val-Cit-PAB-PNP. Briefly, NDI-1 and Alkyne-Val-Cit-PAB-PNP linker were dissolved in anhydrous DMF and reacted at room temperature in the presence of pyridine and HOBt, following an HOBt/pyridine-assisted coupling protocol adapted from the literature for activated-carbonate/amine condensations¹²³ (*scheme 25*).



Scheme 25 – Synthesis of NDI-1-Linker (1), γ : 62%



Scheme 26 – Synthesis of NDI-1-Linker (1)-3a-RGD, γ : 18%

The reaction was monitored by analytical HPLC and showed complete consumption of the starting material after 24h at RT. Purification by preparative HPLC afforded the intermediate **NDI-1-Linker (1)** in 62% isolated yield and HRMS analysis confirmed the chemical identity by the expected molecular mass, providing orthogonal validation to the HPLC purity profile. In the second step, the targeting vector was introduced via a CuAAC “click reaction”, a Huisgen (2+3) cycloaddition, between the terminal alkyne of **NDI-1-L(1)** and **3a-RGD-N₃**. (*Scheme 26*). The reaction was performed in a biphasic-compatible solvent system (tert-BuOH/H₂O 50:50, v/v) chosen to ensure complete solubilization of both components (hydrophobic/aromatic NDI-linker and highly polar peptide). NDI-1-L(1) and 3a-RGD-N₃ were reacted in the presence of CuSO₄ and sodium ascorbate. After 24 h stirring at RT, HPLC monitoring showed complete disappearance of the NDI-linker peak and formation of a new dominant peak corresponding to the desired conjugate, together with residual unreacted 3a-RGD-N₃ (*figure 42*).

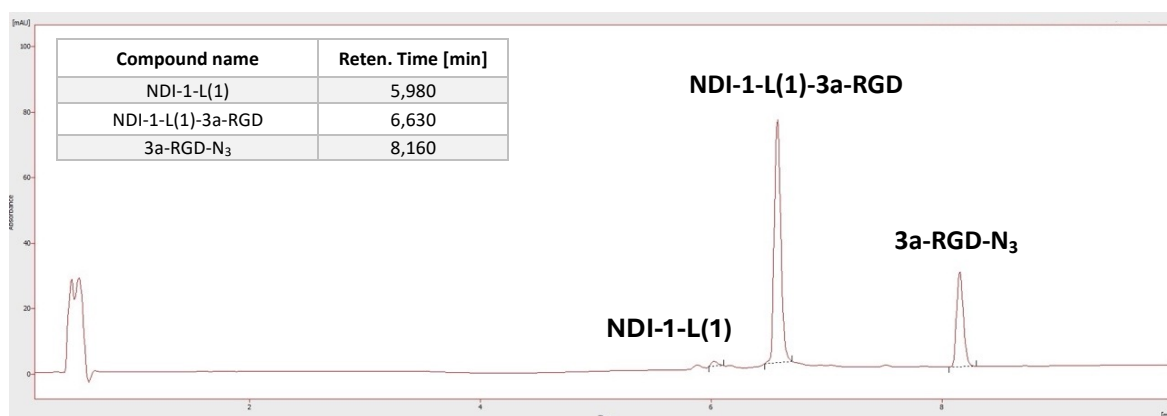
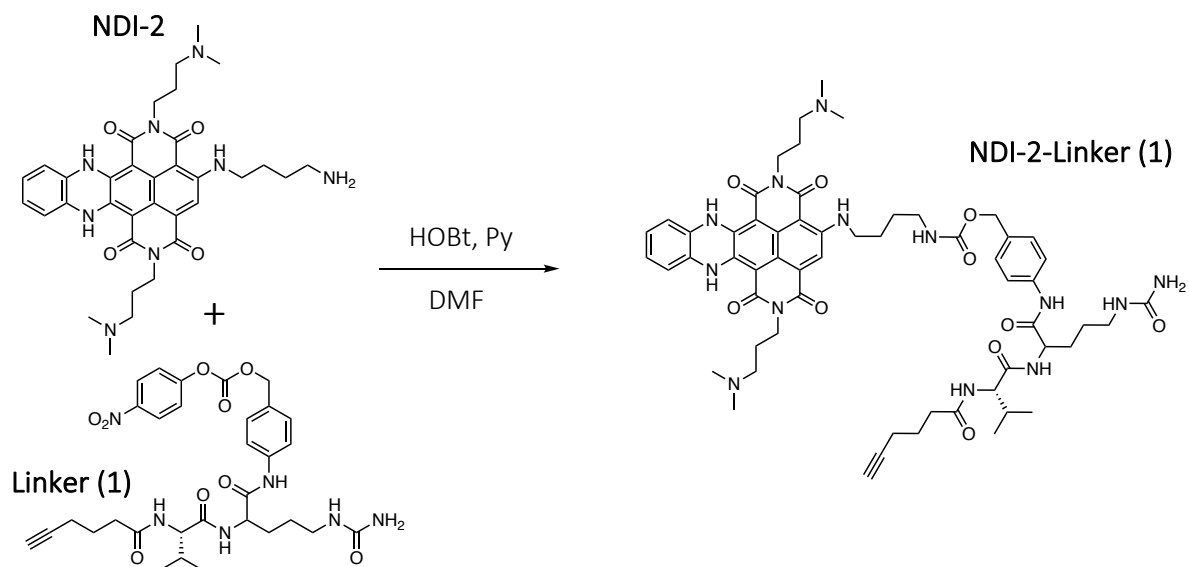
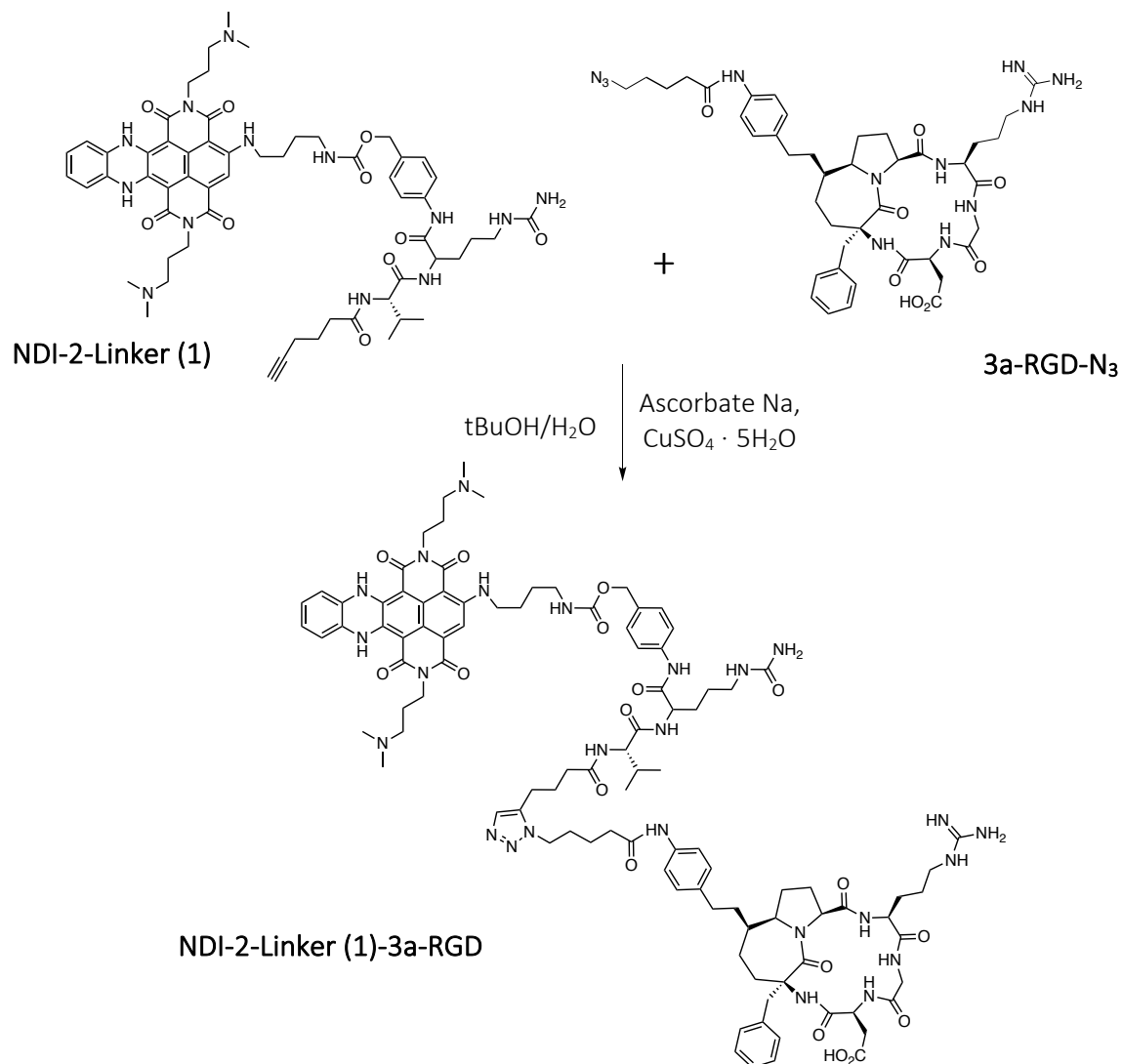


Figure 42 – HPLC chromatogram of 3a-RGD-N₃ and NDI-1-L(1) coupling reaction after 24 hours.

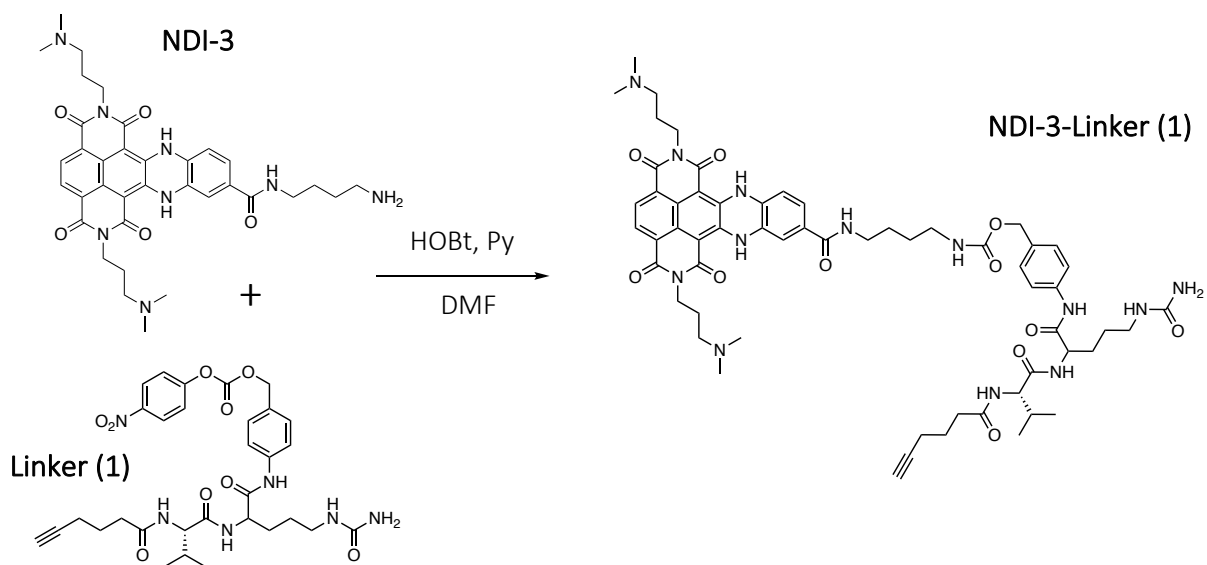
The crude mixture was purified by preparative HPLC to afford the final construct **NDI-1-L1-3a-RGD** (MW = 1979.37) with a 18% yield, and HRMS confirmed the expected molecular weight. The same two-step sequence (activated-carbonate coupling followed by CuAAC) was subsequently applied to the other two selected NDI scaffolds (**NDI-2** and **NDI-3**, *scheme 27-30*), providing analogous outcomes in terms of reaction completion and structural confirmation, thereby validating the overall modular strategy for assembling cleavable NDI-cRGD conjugates suitable for downstream biological and structure-activity investigations.



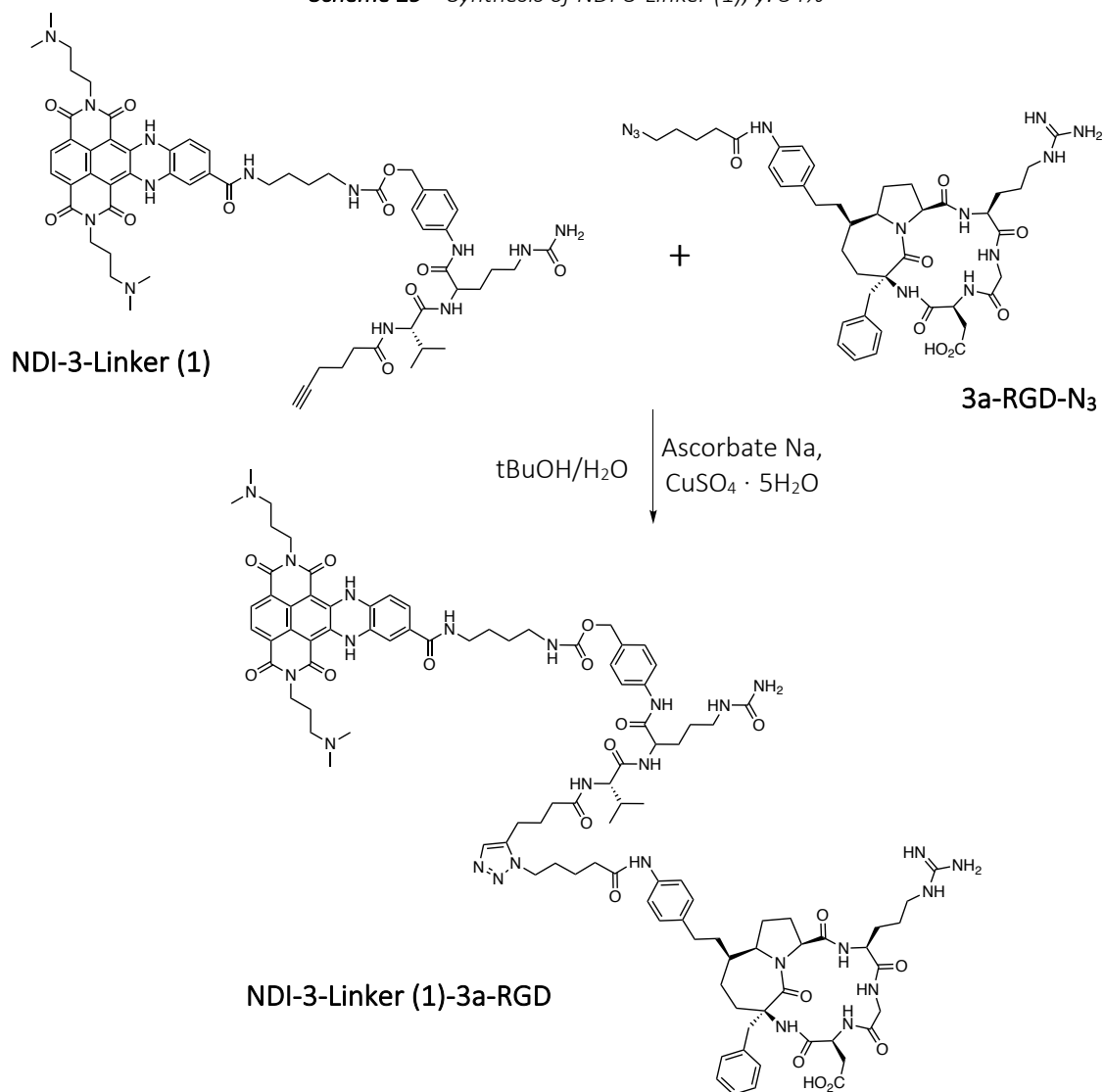
Scheme 27 – Synthesis of NDI-2-Linker (1), γ : 38%



Scheme 28 – Synthesis of NDI-2-Linker (1)-3a-RGD, γ : 27%



Scheme 29 – Synthesis of NDI-3-Linker (1), γ : 54%



Scheme 30 – Synthesis of NDI-3-Linker (1)-3a-RGD, γ : 31%

2.6.2 *In vitro* preliminary biological evaluation of NDI-3a-RGD Conjugates

The *in vitro* cytotoxicity of the synthesized **NDI-3a-RGD** conjugates (NDI1-L(1)-3a-RGD, NDI2-L(1)-3a-RGD and NDI3-L(1)-3a-RGD), carried out in collaboration with prof. Paolillo's research group at *University of Pavia*, was evaluated by MTS assay on U251 glioblastoma cells, characterized by high expression of $\alpha_v\beta_3$ integrins, and on normal human astrocytes (NHA), used as a non-tumoral control. This experimental design was specifically intended to assess whether conjugation with the 3a-RGD targeting moiety could enhance tumor selectivity, in analogy with previously reported cRGD-based systems, the preliminary MTS data obtained after 48 hours treatment indicate that the synthesized 3a-RGD-NDI conjugates retain a measurable antiproliferative activity in U251 glioblastoma cells, while exhibiting a markedly attenuated effect in NHA (*figure 43*).

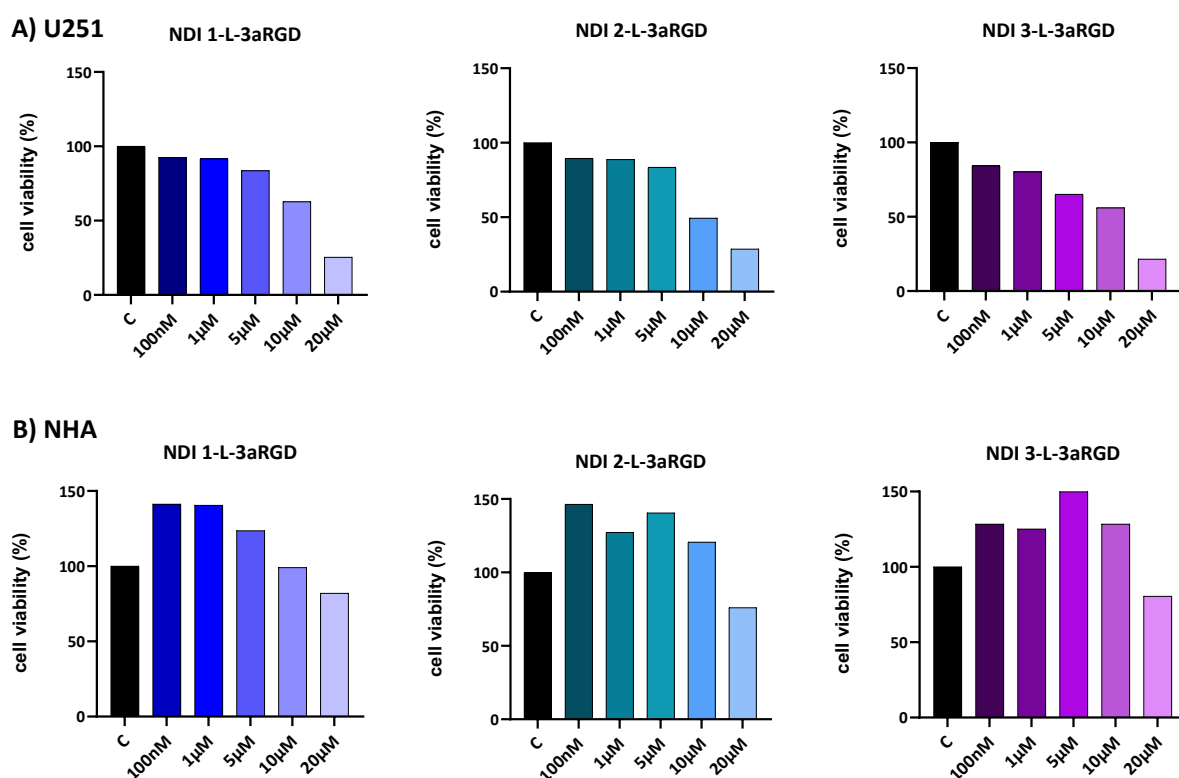


Figure 43 – Effects of NDI 1/2/3-L(1)-3a-RGD compounds on cell viability. U251 and astrocytes cells were seeded in 96-well plates and allowed to grow for 48 h, followed by 48 h treatment with NDI 1-L(1)-3a-RGD, NDI 2-L(1)-3a-RGD, and NDI 3-L(1)-3a-RGD at concentrations ranging from 100 nM to 20 μ M. Cell viability was assessed using the MTS assay. Data are representative of a single independent experiment and expressed as percentage of viable cells relative to untreated controls for each cell line.

This differential biological response is consistent with the design rationale of the system, as U251 cells are known to overexpress $\alpha_v\beta_3$ integrin receptors, which can mediate the selective internalization of RGD-containing constructs, whereas the non-tumoral astrocyte model is expected to display a significantly lower level of integrin-dependent uptake.

In this context, the reduced sensitivity observed in NHA cells suggests that conjugation with the 3a-RGD targeting moiety may effectively limit nonspecific cytotoxicity, contributing to an improved selectivity profile compared to unconjugated NDI systems, which are typically characterized by broad and non-discriminatory activity (*figure 44*).

The overall trend consistently supports a receptor-mediated mechanism of action. In particular, the more pronounced reduction in viability observed in U251 cells, together with the preservation of viability in astrocytes, suggests that the 3a-RGD motif is not merely acting as a passive structural element, but rather plays an active role in promoting preferential interaction with tumor cells. At the same time, the variability observed among the different NDI derivatives indicates that the biological performance of these conjugates is not exclusively governed by the targeting unit but is also strongly influenced by the structural features of the NDI scaffold and the linker architecture. Such differences may reflect variations in cellular uptake efficiency or the ability of the system to release or expose the active NDI moiety in a biologically relevant form.

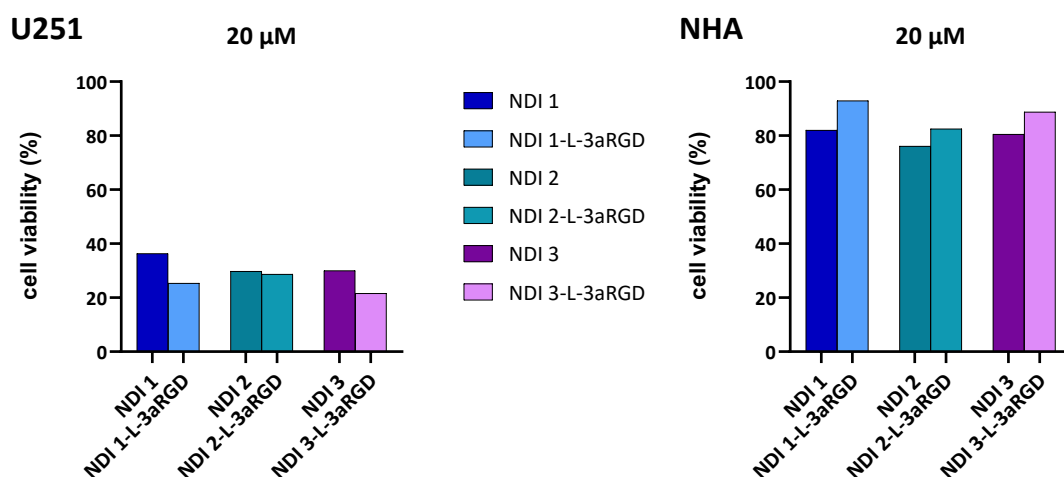


Figure 44 – Effects of NDI 1/2/3 and NDI 1/2/3-L-3a-RGD compounds on cell viability at 20 μM. Astrocytes and U251 cells were treated with NDI 1, NDI 2, NDI 3 and their corresponding L-3a-RGD-conjugated compounds (NDI 1-L(1)-3a-RGD, NDI 2-L(1)-3a-RGD, NDI 3-L(1)-3a-RGD) at a target concentration of 20 μM. Cell viability was assessed by MTS assay. Data are derived from a single independent experiment and expressed as the percentage of viable cells relative to untreated controls.

On this basis, the current results can be regarded as a promising proof of concept supporting the validity of the 3a-RGD-based targeting strategy. However, further experimental work is required to consolidate these findings, including the acquisition of additional biological replicates to ensure statistical robustness, the extension of viability studies to multiple exposure times, and the investigation of linker-dependent payload release under biologically relevant conditions. Ultimately, these studies will be essential to identify the most effective construct, evaluating the impact of different linkers, and to guide the subsequent development of optimized NDI-3a-RGD conjugates incorporating cleavable linker systems with improved therapeutic potential.

2.7 Novel bicyclolactam peptidomimetic scaffolds

A part of the work described in this chapter is published in the following article:

- Rubes, Davide; Zupi, Marco et Al. "A Concise Ring-Closing Enyne Metathesis Approach for the Preparation of Functionalized Bicyclolactam Scaffolds" and are discussed in detail in the following sections", *Eur. J. Org. Chem.* **2024**, doi: e202400599

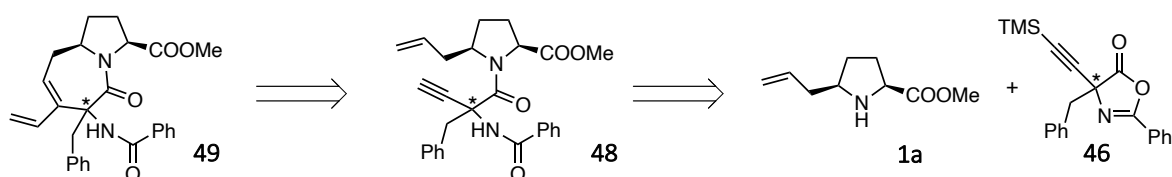
The final part of this PhD work focused on the development of a concise, flexible and robust synthetic strategy for the preparation of new "easy-to-functionalize" bicyclolactam scaffolds, capable of expanding the chemical space accessible around the RGD pharmacophore. As discussed in the previous chapters of this thesis (see [Chapter 2.2](#)), the rational design of integrin-targeting ligands strongly benefits from the introduction of conformational constraints moiety capable of maintain the bioactive spatial conformation of the RGD recognition motif. In particular, the replacement of flexible dipeptide segments with rigid small-molecule scaffolds has proven to be an effective strategy to improve affinity, selectivity and pharmacological robustness of integrin antagonists. Within this framework, 7,5-fused azabicyclolactam scaffolds have emerged as highly efficient reverse-turn inducers.

Building on the development of modular synthetic strategies for 7,5-fused azabicyclolactam scaffolds, functionalized in position 5 and 6 onto the seven-membered lactam ring, the next logical step was to expand the structural diversity accessible around this peptidomimetic core^{101,104,106}. Among the accessible azabicycloalkane (Aba) derivatives, the C4 position of the 7,5-fused azabicycloalkane scaffold emerged as a particularly attractive site for functionalization, enabling a comprehensive overview of this class of compounds. From a structural standpoint, substitution at C4 is expected to exert minimal perturbation on the backbone conformation responsible for reverse-turn induction, while simultaneously providing a new vector for the introduction of functional groups. This feature is particularly relevant in the context of targeted drug delivery and bioconjugation strategies, where additional attachment points are required to install linkers, probes or payloads without compromising receptor binding. In addition, access to C4-functionalized scaffolds offers the opportunity to systematically investigate how peripheral modifications influence

physicochemical properties such as solubility, lipophilicity and metabolic stability, independently of the core pharmacophore. For these reasons and motivated by the need to further extend the versatility of azabicycloalkane-based peptidomimetics, the following section focuses on the exploration of synthetic routes toward C4-functionalized azabicycloalkane scaffolds, with the aim of opening new perspectives for scaffold optimization and downstream applications. Furthermore, the presence of a functional group directly bound to the bicyclic scaffold enables the construction of more complex structures, which can be exploited in the total synthesis of natural products or in drug discovery.

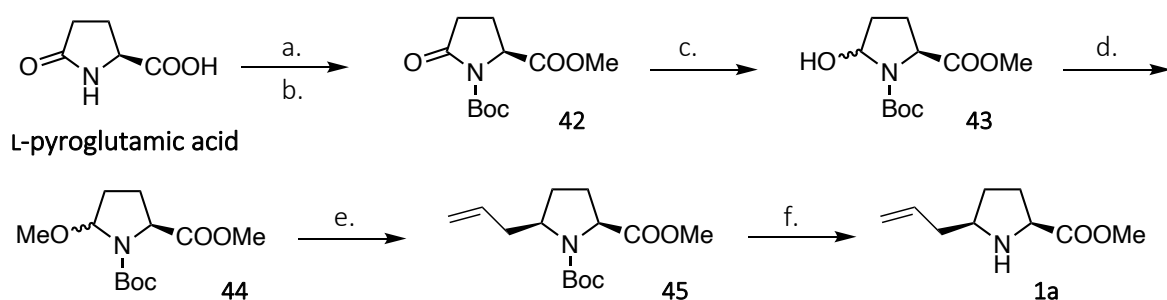
2.7.1 Synthesis of C4-functionalized azabicycloalkane amino acids

The synthetic investigation toward C4-functionalized azabicycloalkane scaffolds began with retrosynthetic analysis for the preparation of suitable dipeptide precursor, the compound **48**. This enyne derivative could be traced back to 5-allylproline methyl ester (**1a**) and ethynyl oxazolone (**46**) by farther retrosynthetic analysis (*scheme 31*).



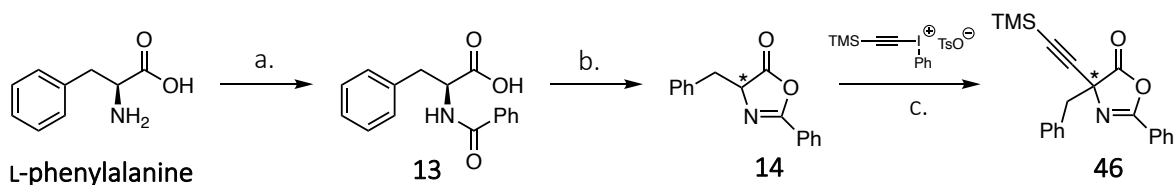
Scheme 31 – Retrosynthetic analysis for the preparation of C4 functionalized aba scaffold

Starting from L-pyrroglutamic acid, the synthesis of the allyl-proline derivative was obtained through a multistep sequence (*scheme 32*), according to literature, that afforded the desired product as a mixture of diastereoisomers¹⁰⁴. Importantly, the major diastereoisomer **1a** corresponding to the (2S,5R) configuration was isolated in 49% yield, while the minor (2S,5S) isomer was obtained in 17% yield, enabling the use of a stereochemically defined precursor in the following steps.



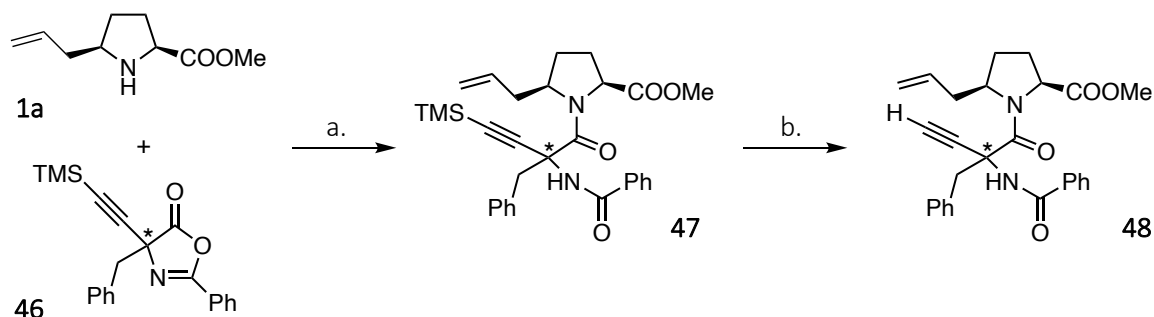
Scheme 32 – a) SOCl_2 , MeOH, 0 °C, y: 99%; b) $(\text{Boc})_2\text{O}$, DMAP, DCM, rt, y:66%; c) LiEt_3BH , THF, -78 °C, y: 99%; d) $\text{CH}(\text{OCH}_3)_3$, PPTS, MeOH, rt, y:99%; e) Allyl-TMS, InCl_3 , DCM, 0 °C, y: 40%; f) TFA, DCM **1a** y: 49%, **1b** y: 17%

In parallel, the complementary alkyne-bearing amino acid fragment was prepared through the reaction of the phenylalanine-derived azlactone **14** with trimethylsilyl-ethynyl(phenyl)iodonium p-toluenesulfonate¹²⁴. This transformation allowed the efficient introduction of a terminal ethynyl group at the α -position of the amino acid, furnishing the desired alkynylated derivative **46** in 80% yield (*scheme 33*).



Scheme 33 – a) $BnCl$, $NaOH$ 6N, $0^{\circ}C$ 2 h $\rightarrow$ HCl 6N; b) DCC , DCM , $0^{\circ}C$; TMS -ethynyl(phenyl)iodonium p -toluenesulfonate, $DIPEA$, DCM , y : 80%

The condensation reaction was initially performed in refluxing dichloromethane (DCM) in the presence of catalytic 4-dimethylaminopyridine ($DMAP$), smooth conversion was observed, providing the trimethylsilyl (TMS)-protected dipeptide **47** in 68% yield after 48 h. Further optimization revealed that switching the solvent to THF and extending the reaction time to 96 h at $45^{\circ}C$ led to a significant improvement in yield, which increased to 96% (*scheme 34*).

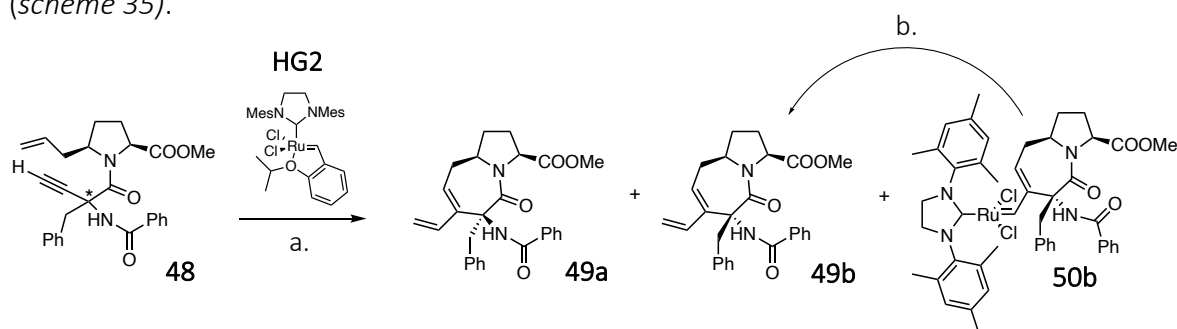


Scheme 34 – a) $DMAP$, THF , $50^{\circ}C$, y : 96%; b) $TBAF$, THF , $-78^{\circ}C$, y : 97%.

In contrast, attempts to shorten the reaction time by microwave irradiation ($95^{\circ}C$ for 3 h) resulted in lower yields (lower than 60%), attributed to degradation of azlactone **47** and formation of a complex mixture of polar by-products, likely arising from opening of the oxazolone ring. Subsequent removal of the TMS protecting group using tetrabutylammonium fluoride ($TBAF$) in THF at $-78^{\circ}C$ furnished dipeptide **48** as a mixture of diastereoisomers in excellent 97% yield. To assess the preparative applicability of this methodology, both the condensation and deprotection steps were performed on gram scale delivering dipeptide **48** in unchanged overall yields.

2.7.2 Scaffold formation via Ring-Closing Enyne Metathesis

Once the enyne dipeptide precursor was obtained, the synthetic route advanced to RCEYM, the crucial transformation for assembling the 7,5-fused azabicycloalkane scaffold. In this strategy, the RCEYM not only enables ring formation but also directly transfers the substitution pattern encoded in the precursor to the C4 position of the final scaffold. Given the presence of a sterically demanding quaternary stereocenter, the reaction conditions were carefully optimized to ensure efficient cyclization. Second-generation Hoveyda-Grubbs catalysts (HG2) was identified, after a screening between different ruthenium-based catalysts, as the most effective promoters of the intramolecular metathesis, providing high chemoselectivity and minimizing undesired by-product. Under the optimized conditions, the enyne dipeptide underwent smooth cyclization to afford the C4-functionalized azabicycloalkane scaffold in 55% overall yield, isolated as a mixture of diastereoisomers (*scheme 35*).



Scheme 35 – a) HG2 20-25%, toluene, 80°C, 72h y: 49a + 49b 55%; 50b 10% → b) Ethylene, toluene, 80°C, 24h

During the reaction, the formation of a ruthenium-carbene intermediate was observed; this complex exhibited an exceptional stability allowed chromatographic purification representing, to the best of our knowledge, the first time that a complex Ru-carbene, with comparable chromatographic and long-term stability, was isolated during or at the end of RCEYM processes. Conversion of this carbene intermediate into the desired bicyclic product was efficiently achieved by conducting the final stage of the reaction under an ethylene atmosphere, adopting the so-called “Mori conditions”¹²⁵. This operational modification proved essential for maximizing material recovery (78% final yield) and highlights the importance of catalyst control in RCEYM-based processes. The RCEYM reaction furnishes a C4-functionalized bicyclic core that retains the rigid architecture required for reverse-turn induction while providing a versatile handle for subsequent derivatization.

2.7.3 Computational studies

Computational investigations were carried out in collaboration with Prof. Belvisi's group at *University of Milan* to evaluate the ability of the newly synthesized C4-functionalized scaffolds to act as effective reverse-turn inducers. To this aim, the conformational behavior of the two diastereomeric Aba derivatives synthesized was analysed using simplified dipeptide models (**C1/2a** and **C1/2b**), in which the N- and C-termini were capped with benzoyl and methylamide groups, respectively (*figure 45*).

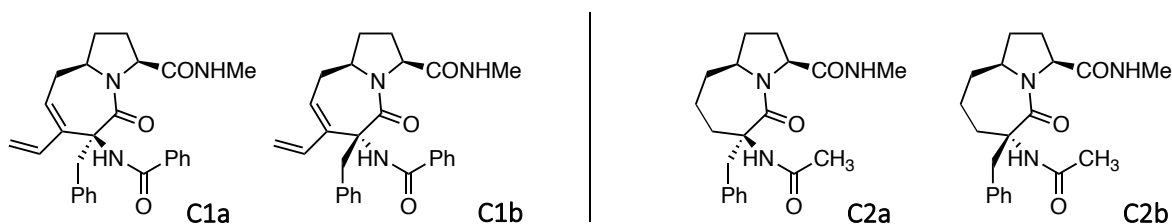


Figure 45 – Chemical structure of bicyclic dipeptide mimics used in molecular mechanics studies

This model system was chosen to enable a reliable comparison with saturated Aba analogues (**C2a** and **C2b**) and to allow a consistent evaluation of turn-related geometrical parameters. Conformational searches were performed using molecular mechanics calculations in an implicit aqueous environment, and the resulting low-energy conformers were analyzed in terms of backbone torsion angles (ϕ and ψ), the virtual torsion angle β ($\text{C}_i\text{-C}_{\alpha i+1}\text{-C}_{\alpha i+2}\text{-N}_{i+3}$), and the presence of intramolecular hydrogen bonds characteristic of γ - or β -turn motifs. The propensity to adopt reverse-turn conformations was quantified by calculating the percentage of conformers displaying β values below 60° , a widely accepted criterion for turn formation. The results indicate that only the Aba scaffolds bearing the amino and carboxyl termini on the same face of the bicyclic framework (i.e., **C1a** and **C2a**) exhibit a significant tendency to adopt turn conformations. In particular, the unsaturated scaffold **C1a** displayed a high percentage, higher than 85%, of conformers compatible with reverse-turn geometries, comparable to, and in some respects exceeding, that observed for the corresponding saturated analogue **C2a**. Analysis of hydrogen-bonding patterns further revealed that both γ -turn and type II' β -turn motifs are accessible, with a slightly increased stabilization of β -turn conformations in the unsaturated system.

At the adopted level of theory, the global minimum energy conformation of **C1a** corresponds to a β -turn geometry, while the lowest-energy γ -turn conformation lies approximately 1

kcal·mol⁻¹ higher in energy. Notably, the unsaturated Aba scaffold exhibits a greater flexibility in the orientation of the benzyl substituent at C3 compared to its saturated counterpart. Two main conformations were identified, differing in whether the benzyl group is oriented toward “Bn-in” or away from “Bn-out” the bicyclic core (*figure 46*). Comparison with experimental NOESY data supports the coexistence of both arrangements in solution and suggests that the “Bn-in” conformation may contribute to the distinctive NMR features observed for compound **49a**.

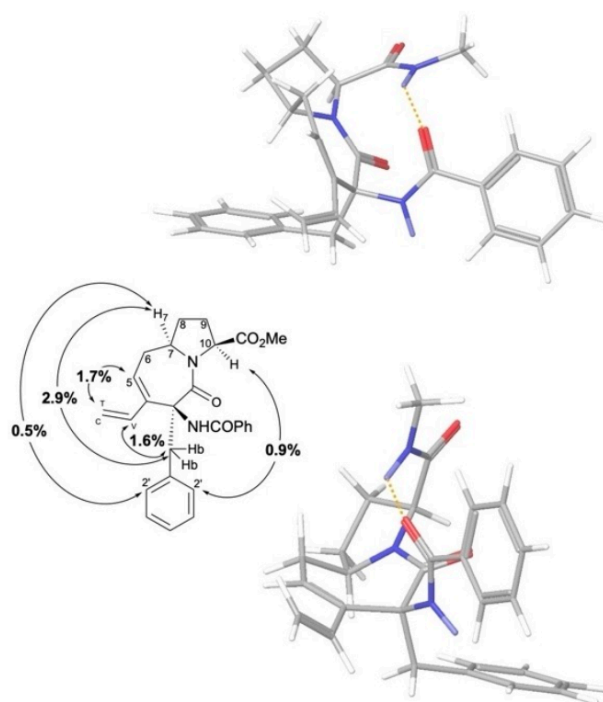


Figure 46 – Relevant NOE correlations exhibited by **49a** (left), lowest energy conformer of C1a displaying the “Bn-in” conformation (right, top) and lowest energy conformer of C1a displaying the “Bn-out” conformation (right, bottom).

Overall, these computational studies confirm that the newly developed C4-functionalized unsaturated Aba scaffold retains, and in some cases enhances, the reverse-turn inducing properties previously reported for saturated 7,5-fused azabicycloalkane scaffolds. This finding is particularly relevant in the context of peptidomimetic design, as it demonstrates that introduction of a diene functionality does not compromise the conformational control exerted by the bicyclic framework, while simultaneously providing a versatile handle for further chemical elaboration.

2.7.4 Functionalization of the diene system

To demonstrate the synthetic versatility of the newly developed C4-functionalized scaffold, a series of post-functionalization reactions involving the embedded diene system were investigated. Through the introduction of targeted chemical modifications, the scaffold could be further elaborated to access structurally more complex constrained dipeptide mimics, potentially valuable in medicinal chemistry applications or as intermediates for the construction of more elaborate molecular architectures.

As an initial proof of concept, the reactivity of the diene system was evaluated through a Diels-Alder cycloaddition reaction. If successful, this transformation would allow the stereoselective access to tricyclic architectures of type **51** and **52**, which can be viewed as simplified analogues of the *Cephalotaxus alkaloid core*. Related tricyclic frameworks are also present in a variety of biologically relevant molecules, including selected steroid derivatives, analogues of the alkaloid *clavizepine* and *paclitaxel-mimicking alkaloids*^{126–129} (figure 47).

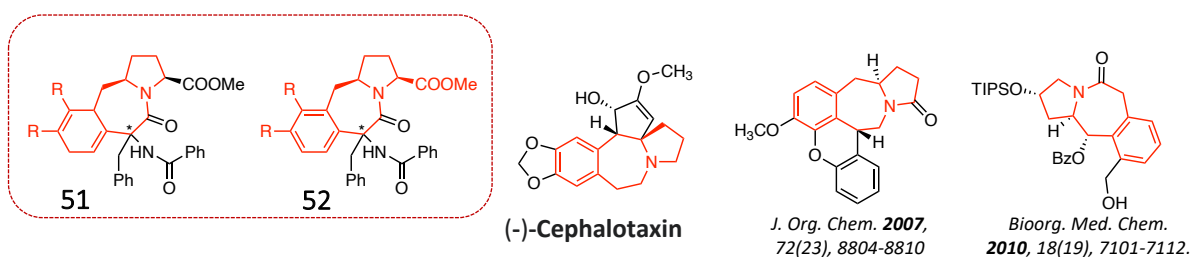
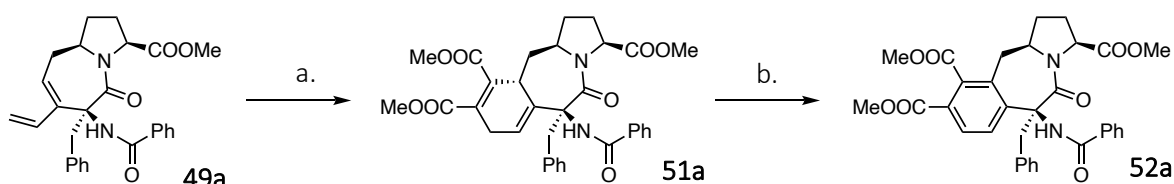


Figure 47 – tricyclic moiety in alkaloid derivatives compounds

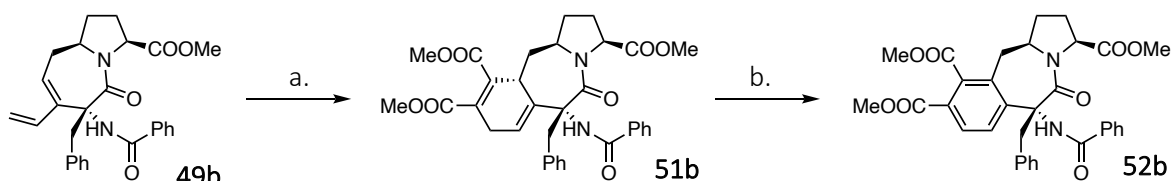
The reaction was carried out using acetylenedicarboxylate, a symmetric dienophile, in order to avoid the formation of regioisomers, and afforded the tricyclic derivative **51a** in 70% yield as a single diastereoisomero. Subsequent aromatization of the newly formed diene moiety using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) enabled the efficient conversion into the corresponding benzapinone derivative **52a**, which was isolated in 98% yield.



Scheme 36 – a) acetylenedicarboxylate, toluene, 80 °C, 24 h, y: 70%; (b) DDQ, toluene, 80 °C, overnight, y: 98%

Notably, application of the same reaction sequence to the (R)-configured Aba scaffold **49b** provide the formation of **52b** in 65% overall yield (scheme 37), without the need for isolation

of the DA intermediate. This outcome suggests that the configuration at C3 does not significantly influence the intrinsic reactivity of the scaffold in these transformations.

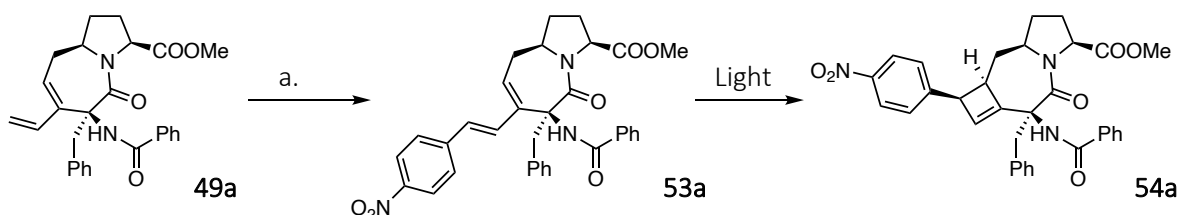


Scheme 37 – a) acetylenedicarboxylate, toluene, 80°C, 24 h → (b) DDQ, toluene, 80 °C, overnight, y: 65%

Since ABA scaffolds bearing an S-configured stereocenter at C3 displayed superior *turn-inducing properties* compared to their R-configured analogues (as indicated by computational studies), subsequent functionalization efforts were primarily focused on compound **49a**.

To investigate and expand the reactivity of the diene system, **49a** was submitted to a microwave-assisted Heck coupling reaction with 1-bromo-4-nitrobenzene, employing PdEnCat[®] 30, a polyurea-encapsulated palladium(II) acetate catalyst. Reaction conditions previously optimized by our group for the functionalization of structurally related cyclic diene systems and sterically hindered vinyl or allyl substrates were employed^{101,104,106}. Under these conditions, the expected aryl-substituted diene **53a** was initially formed as a single E-isomer; however, its isolated yield did not exceed 10%. The major product obtained after chromatographic purification (67% yield) was instead the cyclobutene derivative **54a**, arising from electrocyclic rearrangement of **53a**. This conversion can be rationalized, from a mechanistic point of view, as a photochemically induced 4π electrocyclic ring closure. Upon excitation of the conjugated diene π system, promotion of an electron from the π HOMO to the π* LUMO changes the symmetry of the frontier molecular orbitals, rendering a disrotatory closure symmetry-allowed according to the Woodward-Hoffmann rules^{130,131}. Opposite rotation of the terminal p orbitals enables constructive orbital overlap and formation of the new C-C σ bond, while preserving the stereochemical information encoded in the rigid bicyclic scaffold. Accordingly, the experimentally observed *cis* relationship between the newly generated stereocenters is fully consistent with a *disrotatory photochemical electrocyclization*¹³². Recent computational studies further suggest that photoexcitation primarily redistributes electron density toward the terminal carbons,

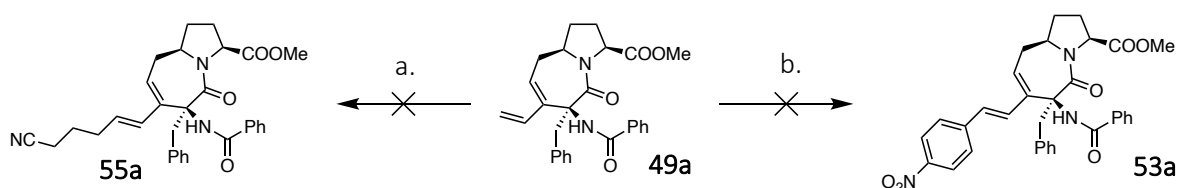
whereas the final C-C bond formation occurs after relaxation to the ground state through nonadiabatic decay, providing additional support for this mechanistic interpretation.



Scheme 38 – a) 4-bromo-nitrobenzene, PdEnCat 30, Bu₄NOAc, EtOH, 120 °C, mw irradiation, γ : 46%

In agreement with this hypothesis, exclusion of direct sunlight during purification allowed isolation of **53a** in an improved yield of 46%.

By contrast, attempts to engage diene **49a** in cross-metathesis reactions with either aromatic (4-nitrostyrene) or aliphatic (5-hexenenitrile) partners, using Grubbs 2nd generation (G2) or HG2 catalysts, were unsuccessful. Even under forcing conditions, such as increased catalyst loading or elevated reaction temperature, the starting material was largely recovered unchanged.

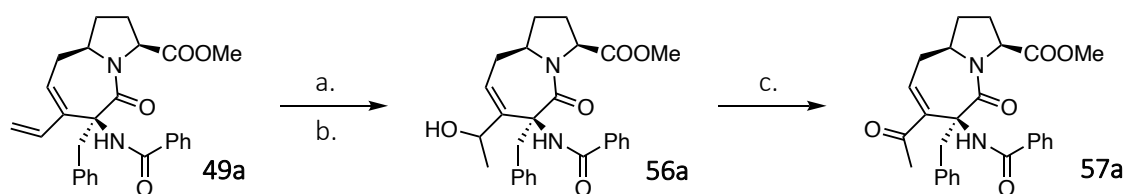


Scheme 39 – a) *p*-Nitrostyrene, 20% HGII, toluene, 80 °C; b) 5-Hexenenitrile, 20% HGII, toluene, 80 °C

Following the limited reactivity observed in cross-metathesis transformations, alternative strategies were explored to expand the functional diversity of the Aba scaffold. Attention was devoted to the introduction of a hydroxyl functionality at the exocyclic double bond, as such a group would provide a chemically versatile handle for further derivatization and downstream scaffold modification. On this basis, classical hydroboration and oxymercuration-reduction protocols were evaluated.

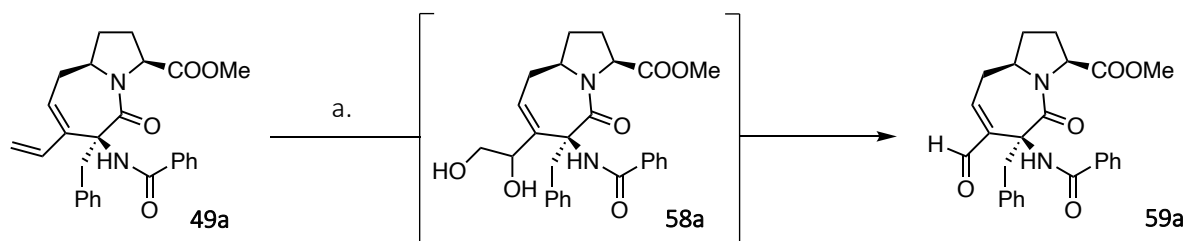
Initial attempts to introduce the hydroxyl group via hydroboration proved unsuccessful, as compound **49a** remained unreactive toward both borane and 9-borabicyclo[3.3.1]nonane (9-BBN). In contrast, more complex behavior was observed under oxymercuration–reduction conditions using aqueous Hg(OAc)₂ followed by NaBH₄. The mercuration step led to complete consumption of the starting material and formation of a highly polar

intermediate, detected as a single spot by TLC. However, subsequent reduction resulted in partial regeneration of **49a** together with the formation of a minor new species. ^1H -NMR analysis of the isolated product, obtained in yield lower than 10% was consistent with hydration of the exocyclic double bond to give the corresponding secondary alcohol. Notably, further NMR monitoring revealed rapid reversion of this intermediate to the parent diene within 24 h, highlighting the instability of the hydroxylated derivative under the reaction and isolation conditions. Several attempts to trap or stabilize this intermediate, including in situ acetylation and oxidation using pyridinium chlorochromate (PCC) or Dess-Martin periodinane, proved unsuccessful. Nevertheless, when the crude mixture obtained after oxymercuration-reduction was subjected to liquid/liquid extraction followed by oxidation with PCC, the corresponding ketone **57a** could be isolated in 35% overall yield, together with recovery of approximately 50% of unreacted **49a**.



Scheme 40 – a) $\text{Hg}(\text{OAc})_2$, $\text{THF}/\text{H}_2\text{O}$, rt, 24 h $\rightarrow$ b) NaBH_4 , NaOH $\rightarrow$ c) PCC , DCM , rt, y: 35% over 3 steps

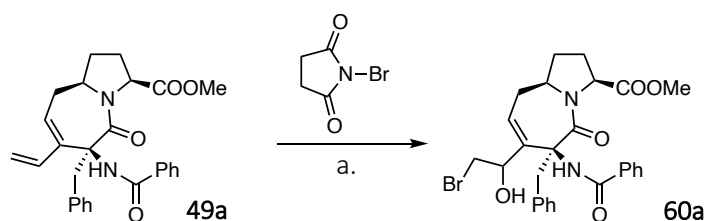
In addition, selective oxidative cleavage of the exocyclic double bond was achieved using $\text{OsO}_4/\text{NaIO}_4$, in the so-called *Lemieux-Johnson oxidation*, affording aldehyde **59a** in 49% yield¹³³. Collectively, these results indicate that while direct installation of a stable hydroxyl group at the exocyclic position is challenging, the diene system remains amenable to oxidative transformations that enable access to synthetically useful oxygenated derivatives.



Scheme 41 – a) OsO_4 , NaIO_4 , Et_3N , $\text{THF}/t\text{-Butanol}$, $0\text{ }^\circ\text{C}$, 2.5 h, y: 49%

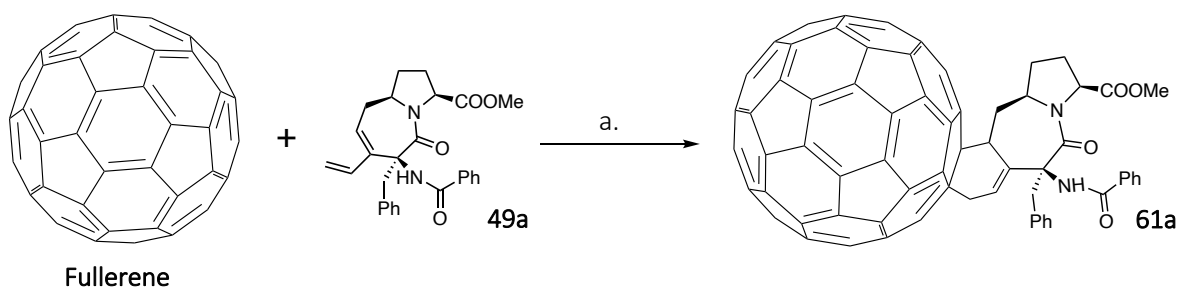
To further broaden the functional space accessible from the diene system, the simultaneous installation of two distinct functional groups was explored through halohydrin formation. This transformation was specifically evaluated in view of the potential conversion of the

resulting halohydrin into the corresponding epoxide, which would provide a highly versatile attachment point for subsequent scaffold derivatization. Accordingly, treatment of compound **49a** with N-bromosuccinimide (NBS) in dichloromethane at 0 °C led to the clean formation of bromohydrin **60a** in 82% yield as a single diastereoisomer (*scheme 42*). Although the reaction proceeded with high efficiency and stereochemical selectivity, the available NMR data did not allow an unambiguous assignment of the newly generated stereocenter.



Scheme 42 – a) NBS, DCM, 20 °C, 20 min, y: 82%

Finally, to further demonstrate the general applicability of the proposed functionalization strategy beyond the nanosystems described in the previous sections, we investigated its extension to fullerene-based nanomaterials. This study was conceived primarily as a *proof of concept* to assess whether the diene-containing scaffold could enable the direct covalent modification of chemically distinct nanoparticle platforms without the need for preinstalled linkers or auxiliary functional handles. Fullerene C₆₀ was selected as a representative substrate because of its well-established reactivity toward cycloaddition processes and its growing interest as a scaffold for the development of functional nanomaterials, including biologically relevant conjugates with sugars and peptides^{134,135}. Gratifyingly, the DA reaction between **49a** and Buckminster fullerene (C₆₀) in refluxing toluene proceeded smoothly, affording the monoadduct **61a** in a satisfactory isolated yield of 61% highlights the versatility and potential impact of our approach in diverse scientific domains (*scheme 43*).



Scheme 43 – toluene, 80 °C, 24 h, y: 61%

In conclusion, this work presents an efficient synthetic strategy based on ring-closing enyne metathesis for the preparation of novel C4-functionalized 7,5-fused azabicycloalkane scaffolds. This methodology enables the synthesis of constrained dipeptide mimics in high yields while introducing a synthetically versatile diene moiety on the seven-membered lactam ring. This functionality proved particularly valuable for *late-stage modification*, allowing the installation of substituents at C4 that serve as useful attachment points for subsequent conjugation reactions. Notably, Diels-Alder cycloadditions provided access to previously unexplored tricyclic architectures based on the azabicycloalkane scaffold. Finally, the efficient functionalization of fullerene achieved with these scaffolds paving the way for potential further development of novel fullerene-based nano-pharmaceutical systems.

3. EXPERIMENTAL SECTION

3.1 General Remarks

Chemical reagents and solvents: All chemicals were of reagent grade and used without further purification. The solvents were purified according to the guidelines described in *Purification of Laboratory Chemicals* (Armarego, W. L. F., Chai, C. L. L. *In Purification of Laboratory Chemicals*, 7th ed.; Butterworth-Heinemann: Oxford, UK, 2013). All solvents were freshly distilled from the appropriate drying agent. THF and diethyl ether were distilled from sodium/benzophenone; dichloromethane, toluene and triethylamine were distilled from CaH_2 ; Chloroform is distilled from CaCl_2 . Methanol is distilled after treatment with sodium from Magnesium and Iodine. When necessary, before use, solvents were dried by reflux distillation under a nitrogen atmosphere.

Reactions setup: Glassware for all reactions was flame-dried and cooled under inert atmosphere prior to use. Reactions requiring anhydrous conditions were performed under atmosphere of anhydrous nitrogen. Liquid reagents and solvents were introduced using oven-dried syringes in septa-sealed flasks under an inert atmosphere. Yields were calculated for compounds purified by crystallization, flash chromatography or preparative HPLC, and judged to be homogeneous by TLC, NMR and mass spectrometry (HRMS).

Microwave assisted reactions setup: Reactions was performed on a Biotage® Initiator plus microwave system use a 20 mL microwave reactions vials.

Thin Layer Chromatography (TLC): Reactions have been monitoring using silica gel 60 F₂₅₄ plates with a thickness of 0.25 mm (Merck); spots were visualized using the following detection methods:

- *Molybdic reagent:* prepared by dissolving 21 g of $(\text{NH}_4)\text{MoO}_4$, 1 g of CeSO_4 and 31 mL of H_2SO_4 in 470 mL of H_2O .
- *TMED reagent:* prepared by dissolving 12.5 g of tetramethyl diaminodiphenylmethane in 50 mL of AcOH and 25 mL of H_2O ; subsequently, a KI solution (25 g in 500 mL of H_2O) and 7.5 mL of saturated ninhydrin solution were added.
- *Ninhydrin ethanolic solution:* 1.5 g of Ninhydrin is dissolved in 100 mL of Ethanol (96%); subsequently 3 ml of acetic acid were added.

- *Iodine chamber*
- *UV lamp at 254 nm*

Flash chromatography: purification techniques by flash chromatography were carried out following the general procedure of Still (*J. Org. Chem.* 1978, 93, 14), using SiO₂ 270-400 mesh (Merck).

HPLC analysis and purification: Except where otherwise indicated, HPLC analyses were performed using an Agilent system SERIES 1260 with XSelect® HSS C18 column (2.5 µm, 4.6x 50 mm) and the following method was used: flow 1.4 mL/min, isocratic gradient over 2 min 95% of H₂O + 0.1% TFA (5% CH₃CN), gradually to 40% aqueous solvent over 6 min, then isocratic flow for 4 min (λ = 256 nm). HPLC purifications were carried out on an Agilent Technologies 1260 Infinity preparative HPLC provided with a diode array UV-vis detector, using a Waters XSelect® CSH Prep C18 OBDTM column (5 µm, 100 x 30 mm) at a 30 mL/min flow rate.

¹H-NMR and ¹³C-NMR: the spectra were recorded on a Bruker ULTRA-SHIELD-400 spectrometer operating in Fourier transform (FT) mode, equipped with a 9.4 T magnet, a BBO probe with Z-gradients, and a CP-MAS probe. The instrument was equipped with a 60-position automatic sample changer and operated using TopSpin™ 3.6 software. Spectral processing and integration were performed using iNMR software. The abbreviations **s**, **d**, **t**, **q**, **sept**, **br s**, and **m** stand for the resonance multiplicities *singlet*, *doublet*, *triplet*, *quartet*, *septet*, *broad singlet*, and *multiplet*, respectively. In the peak listing of ¹³C spectra, abbreviations **s** and **t** refer to zero and two protons attached to the carbons, respectively, as determined by DEPT-135 experiments. Phase-sensitive 2D-NOESY experiments were performed at 298 K by using *noesygpph pulse program* from the Bruker library (mixing time of 0.5 s or 0.8 s).

Mass spectrometry: The HRMS spectra were acquired on a X500B QTOF System (SCIEX, Framingham, MA 01701 USA) equipped with the Twin Sprayer ESI probe and coupled to an ExionLCTM system (SCIEX). The SCIEX OS software 2.1.6 was used as an operating platform. Mobile phase: methanol with 0.1% formic acid, injection: 10 µL, concentration of samples: 0.1 mg/mL.

Optical rotational power: Optical rotations $[\alpha]_D$ were measured in a cell of 5 cm path length and 1 mL capacity with a Jasco DIP-1000 polarimeter.

IR: Infrared spectra were recorded on a Perkin-Elmer ATR-FTIR 1600 series spectrometer using neat samples.

Computational studies: Each compound was subjected to a Monte Carlo/Energy Minimization (MC/EM) conformational search within the framework of the Schrodinger MacroModel version 11.9, using the MacroModel implementation of the Amber all-atom force field (denoted AMBER*) and the implicit water GB/SA solvation model. The torsional space of each molecule was randomly varied with the usage-directed Monte Carlo conformational search of Chang, Guida, and Still (*J. Am. Chem. Soc.* **1989**, 111, 4379-4386). A ring-closure bonds was defined in the seven-membered ring of the 7,5-fused bicyclic lactam. Ester and amide bonds were constrained to their most stable conformations. For each search, 20.000 starting structures were generated and minimized until the gradient was less than 0.05 kJ/Åmol using the truncated Newton-Raphson method. Duplicate conformations and those with an energy greater than 5 kcal/mol above the global minimum were discarded.

Chemical Nomenclature and Structure Drawing: Unless otherwise stated, the IUPAC names of all compounds described in this thesis were generated using ChemDraw Professional (version 16.0.1.4, build 61). Chemical structures were prepared using the same software and are presented using the ACS Document 1996 drawing style unless otherwise indicated.

3.2 Abbreviations

A

Aba - Azabicycloalkane
ACN – Acetonitrile
AcOH – Acetic acid
AcOEt – Ethyl acetate
ADC – Antibody–drug conjugate
ADCC – Antibody-dependent cellular cytotoxicity
ADMIDAS – Adjacent to metal ion-dependent adhesion site
AMBER – Assisted model building with energy refinement force field
ANOVA – Analysis of variance
AZIDE FLUOR 545 – 5-Carboxytetramethylrhodamine-azide

B

BBB – Blood–brain barrier
9-BBN – 9-borabicyclo[3.3.1]nonane
BnOH – Benzyl alcohol
Boc – tert-Butoxycarbonyl
(Boc)₂O – Di-tert-butyl dicarbonate
BOP-Cl – Bis(2-oxo-3-oxazolidinyl)phosphinic chloride
BTMSA – Bis(trimethylsilyl)acetylene

C

CAMs – cell-adhesion molecules
Cbz – Benzyloxycarbonyl

(Cbz)₂O – Dibenzyl decarbonate
CDC – Complement-dependent cytotoxicity
CEYM – Cross-enyne metathesis
CF-NHS – Carboxyfluorescein N-hydroxysuccinimide ester
CS-NPs – Chitosan nanoparticles
CuAAC – Copper-catalyzed azide–alkyne cycloaddition

D

DAPI – 4',6-diamidino-2-phenylindole
DA – Diels-Alder
DAR – drug to antibody ratio
DBCO – Dibenzocyclooctyne
DBBQ – 2,5-di-tert-butyl-1,4-benzoquinone
DCC – N,N'-dicyclohexylcarbodiimide
DCM – Dichloromethane
DDQ – 2,3-dichloro-5,6-dicyano-1,4-benzoquinone
DIPEA – N,N-diisopropylethylamine
DLS – Dynamic light scattering
DMAP – 4-dimethylaminopyridine
DMEM – Dulbecco's Modified Eagle Medium
DMF – N,N-dimethylformamide

DMPC – 1,2-dimyristoyl-sn-glycero-3-phosphocholine

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

DOX – Doxorubicin

DPPE – Ethylenebis(diphenylphosphine)

DSPE – 1,2-distearoylphosphatidylethanolamine

E

ECM – Extracellular matrix

EDC – 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

EDT – ethane-1,2-dithiol

EGFR – Epidermal growth factor receptor

EMT – Epithelial–mesenchymal transition

EPR – Enhanced permeability and retention

ERK – Extracellular signal-regulated kinase

ESI – Electrospray ionization

F

FAK – Focal adhesion kinase

FBS – Fetal bovine serum

FGF – Fibroblast growth factor

FITC – Fluorescein isothiocyanate

Fmoc – 9-fluorenylmethoxycarbonyl

FT – Fourier transform

G

G2 – Grubbs catalyst 2nd generation

G4 – G-quadruplex

GB/SA – Generalized Born/surface area solvation model

Gd-DTPA-BSA – diethylenetriaminepentaacetic acid-bis(stearylamide) (gadolinium salt)

GGFG - Gly-Gly-Phe-Gly

H

HATU – Hexafluorophosphate

azabenzotriazole tetramethyl uronium

HEPES – 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid

HER – Human epidermal growth factor

HG2 – Hoveyda-Grubbs catalyst 2nd generation

HOBt – 1-hydroxybenzotriazole

HOMO – Highest occupied molecular orbital

HPLC – High-performance liquid chromatography

HRMS – High-resolution mass spectrometry

I

IG – immunoglobulin

IR – Infrared spectroscopy

L

LC – Liquid chromatography

LiHMDS – Lithium bis(trimethylsilyl)amide

LIMBS – ligand-associated metal-binding site

Liss Rhod PE – Lissamine rhodamine PE -
1,2-dioleoyl-sn-glycero-3-
phosphoethanolamine-N-(lissamine
rhodamine B sulfonyl) (ammonium salt)
LUMO – Lowest unoccupied molecular
orbital

M

mAbs – monoclonal antibodies
MAP – Mitogen-activated protein kinase
MC/EM – Monte Carlo energy
minimization
MDR – Multidrug resistance
MES – 2-(N-morpholino)ethanesulfonic
acid
MIDAS – Metal ion-dependent adhesion
site
MMP – Matrix metalloproteinase
MRI – Magnetic resonance imaging
MTS – 3-(4,5-dimethylthiazol-2-yl)-5-(3-
carboxymethoxyphenyl)-2-(4-
sulfophenyl)-2H-tetrazolium
MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-
diphenyltetrazolium bromide
MW – Microwave

N

NDI – Naphthalene diimide
NHA – Normal human astrocytes
NHS – N-hydroxysuccinimide
NMM – N-methylmorpholine
NMR – Nuclear magnetic resonance

NOE – Nuclear overhauser effect
NPs – Nanoparticles

O

OA – Oleic acid

P

PABC – p-aminobenzyl carbamate
PAB – para-aminobenzoic
PBS – Phosphate-buffered saline
Pbf - 2,2,4,6,7-
pentamethyldihydrobenzofuran-5-
sulfonyl
PCC – Pyridinium chlorochromate
PDB – Protein data bank
PDCs – Peptide drug conjugates
Pd₂(dba)₃· CHCl₃ –
Tris(dibenzylideneacetone)dipalladium(0)
-chloroform adduct
PdEnCat[®] – Palladium acetate,
microencapsulated in polyurea matrix
PEG – Polyethylene glycol
Pbf – 2,2,4,6,7-
pentamethyldihydrobenzofuran-5-
sulfonyl
PFA – Paraformaldehyde
PLA – Polylactic acid
PLGA – Poly(lactic-co-glycolic acid) PIPKIγ
– phosphatidylinositol-4-phosphate 5-
kinase type γ
PNP – para-Nitrophenol
PPTS – Pyridinium para-Toluenesulfonate

PTX – Paclitaxel

PyBroP[®] – Bromo-tris-pyrrolidino-
phosphonium hexafluorophosphate

R

RCM – Ring-closing metathesis

RCEYM – Ring-closing enyne metathesis

RGD – Arginine–glycine–aspartic acid

RNA – Ribonucleic acid

RP-HPLC – Reverse phase HPLC

RT – Room temperature

S

SAR - Structure-activity relationship

SD – Standard deviation

SF-NPs / SFNs – Silk fibroin nanoparticles

siRNA – Short interfering RNA

SMDCs – Small molecule drug conjugates

SPAAC – strain-promoted azide-alkyne
cycloaddition

SPECT – single-photon emission
computed tomography

T

TBAF – Tetrabutylammonium fluoride

TFA – Trifluoroacetic acid

TFR – Total flow rate

TGF – Transforming growth factor

THF - Tetrahydrofuran

TLC – Thin-layer chromatography

TMS – Trimethylsilyl iodide

TNBC – Triple-negative breast cancer

TIS – Triisopropylsilane

U

uPAR – urokinase plasminogen activator
receptor

UV – Ultraviolet

V

VEGF – vascular endothelial growth
factor

Z

Z (Cbz) – Benzyloxycarbonyl protecting
group

3.3 Synthetic procedures

3.3.1 *Synthesis of 3a-RGD*

Synthesis of (2S,5R)-methyl 1-(3-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-benzylhexa-1,5-dien-2-yl)-5-ethynylpyrrolidine-2-carboxylate (**19a**)

To a solution of **18a** (645.1 mg, 1.51 mmol) and **12** (231.2 mg, 1.51 mmol) in dry THF (30.2 mL, 0.05 M), bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOP-Cl) (387.4 mg, 1.51 mmol) and NaHCO₃ (127.0 mg, 1.51 mmol) were added. The reaction mixture was heated to 50 °C. After 72 hours the solvent was removed under reduced pressure and the crude residue was purified by flash chromatography (Hexane/AcOEt 65:35 → 50:50) to obtain pure compound **19a** as a white amorphous solid (428.5 mg, 51%). TLC: Hexane/AcOEt 50:50, R_f = 0.41

Spectroscopic and mass data:

¹H NMR (CDCl₃, 400 MHz, mixture of diastereoisomers and rotamers) δ 1.59–1.81 (m, 1.3H), 1.90–2.48 (m, 4.7H), 2.52–2.74 (m, 0.6H) 2.84–3.46 (m, 2.4H), 3.54–4.00 (m, 4H), 4.18–4.30 (m, 1H), 4.32–4.57 (m, 2.6H), 4.65–4.76 (m, 0.4H), 5.04–5.32 (m, 2.3H), 5.64–5.93 (m, 1.4H), 6.03–6.25 (m, 0.3H), 7.02–7.15 (m, 1H), 7.16–7.27 (m, 4H), 7.29–7.37 (m, 2H), 7.38–7.47 (m, 2H), 7.54–7.66 (m, 2H), 7.75–7.84 (m, 2H);

¹³C{¹H} NMR (CDCl₃, 100 MHz, mixture of diastereoisomers and rotamers) δ 26.8 (br, t), 34.6 (br, t), 40.0 (t), 40.3 (t), 40.5 (t), 47.6, 50.1, 50.6, 52.7, 61.9 (br), 65.4 (br, s), 66.8 (t), 74.1 (br), 82.5 (s), 119.8 (t), 119.9 (t), 120.4, 125.4, 125.6, 127.2, 127.4, 127.5 (2C), 128.1, 128.2, 128.4, 128.8, 130.1 (br), 130.9, 132.8, 133.0, 136.1 (br, s), 136.9 (s), 141.7 (s), 144.1 (s), 144.3 (2C, s), 155.1 (s), 170.3 (s), 170.6 (s), 172.3 (s);

IR (neat, cm⁻¹) 3375, 3286, 3030, 2948, 2254, 2116, 1784, 1754, 1719, 1632, 1495, 1391, 1167, 1077;

HRMS (ESI) calculated for C₃₅H₃₄N₂NaO₅⁺ [M+Na]⁺ m/z 585.2360, found 585.2349.

Synthesis of tert-butyl (4-vinylphenyl)carbamate (**25**)

To a solution of **4-vinylaniline** (1.0 g, 8.39 mmol) in dry DCM (8.4 mL, 1.0 M) under nitrogen atmosphere, was added (Boc)₂O (2.38 g, 10.9 mmol). After 12 hours the solvent was removed under reduced pressure. The crude residue was purified by flash chromatography (Hexane/AcOEt 95:05) to obtained pure compound **25** as a white amorphous solid (1.72 g, 93%). TLC: Hexane/AcOEt 95:05, R_f = 0.27

The spectroscopic data of compound were matched with literature values.

Synthesis of methyl (3*S*,6*R*,9*aR*)-6-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-6-benzyl-9-((*E*)-4-((tert-butoxycarbonyl)amino)styryl)-5-oxo-2,3,5,6,7,9*a*-hexahydro-1*H*-pyrrolo[1,2-*a*]azepine-3-carboxylate (**26a**)

A mixture of **19a** (88 mg, 0.156 mmol), styrene derivative tert-butyl(4-vinylphenyl)carbamate (68 mg, 0.313 mmol) and HG2 catalyst were solubilized in toluene (20.0 mL, 0.008 M) into a 20 mL microwave reaction vials equipped with a stir bar. The vial was sealed and irradiated in a microwave reactor at 120 °C for 45 min under stirring. After cooling to room temperature, the reaction mixture was filtered through a sintered glass funnel (G4) to remove the precipitate and then concentrated under reduced pressure. Purification by flash column chromatography on silica gel (Hexane/EtOAc 70:30 → 50:50) afforded compound **26a** as a white amorphous solid (72.6 mg, 61%). TLC: Hexane/AcOEt 70:30, R_f = 0.25

Spectroscopic data:

¹H NMR (CDCl₃, 400 MHz) δ 1.55 (s, 9H), 2.04–2.24 (m, 3H), 2.57–2.70 (m, 1H), 2.86 (d, J = 17.6 Hz, 1H), 3.42 (d, J = 14.1 Hz, 1H), 3.54 (dd, J = 8.1, 17.6 Hz, 1H), 3.70 (d, J = 14.1 Hz, 1H), 3.76 (s, 3H), 4.18–4.29 (m, 2H), 4.46–4.57 (m, 1H), 4.76 (d, J = 6.7 Hz, 1H), 4.88 (br s, 1H), 6.10 (br d, J = 7.5 Hz, 1H), 6.56 (d, J = 16.0 Hz, 1H), 6.61 (d, J = 16.0 Hz, 1H), 6.69 (s, 1H), 7.06 (br s, 2H), 7.15–7.26 (m, 3H), 7.29–7.46 (m, 9H), 7.53 (d, J = 7.2 Hz, 1H), 7.62 (d, J = 7.3 Hz, 1H), 7.79 (d, J = 7.5 Hz, 2H);

¹³C{¹H} NMR (CDCl₃, 100 MHz) (CDCl₃, 100 MHz) δ 26.8 (t), 28.8 (3C), 33.9 (2C, t), 36.1 (t), 47.6, 52.8, 62.4, 62.8, 63.0 (s), 66.8 (t), 81.1 (s), 119.0 (2C), 120.3 (2C), 124.6, 125.6, 125.8, 127.3, 127.4 (3C), 127.5, 128.0 (2C), 128.1, 128.8 (2C), 129.5, 129.7 (2C), 132.1 (s), 136.9 (s), 137.3 (s), 138.5 (s), 141.6 (s), 141.7 (s), 144.3 (s), 144.5 (s), 153.1 (s), 154.7 (s), 172.3 (s), 172.7 (s);

IR (neat, cm⁻¹) 3368, 3029, 2979, 2932, 2848, 1716, 1630, 1587, 1519, 1491, 1411, 1314, 1222, 1154, 1070, 1051.

Synthesis of methyl (3S,6R,9S,9aR)-6-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-benzyl-9-(4-((tert-butoxycarbonyl)amino)phenethyl)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepine-3-carboxylate (27a)

To a solution of **26a** (266.7 mg, 0.351 mmol) in dry MeOH (7 mL, 0.05 M) under N₂, PtO₂·H₂O (platinum oxide hydrate) was added (16 mg, 6% weight). The reaction mixture was stirred at room temperature for 6 h. The reaction mixture was filtered through a pad of Celite, and the organic phase was evaporated under reduced pressure. The crude mixture was purified by flash chromatography (Hexane/AcOEt 65:35 → 60:40) to obtain pure compound **27a** as a white amorphous solid (219.3 mg, 82%). TLC: Hexane/AcOEt 60:40, R_f = 0.35

Spectroscopic data:

¹H NMR (DMSO-d₆, 80 °C, 400 MHz) δ 1.49 (s, 9H), 1.63–2.18 (m, 11H), 2.35–2.45 (m, 1H), 2.58–2.69 (m, 1H), 3.18 (br s, 1H), 3.33 (d, J = 12.5 Hz, 1H), 3.53 (s, 3H), 4.11 (dd, J = 3.8, 8.6 Hz, 1H), 4.27 (t, J = 5.9 Hz, 1H), 4.31–4.46 (m, 2H), 4.54 (dd, J = 5.8, 10.5 Hz, 1H), 6.26 (br s, 1H), 6.82 (br s, 2H), 7.10 (d, J = 8.4 Hz, 2H), 7.13–7.19 (m, 3H), 7.31–7.38 (m, 4H), 7.39–7.45 (m, 2H), 7.65 (t, J = 8.2 Hz, 2H), 7.87 (d, J = 8.2 Hz, 2H), 8.89 (s, 1H);

¹³C{¹H} NMR (DMSO-d₆, 80 °C, 100 MHz) δ 26.7 (t), 27.0 (t), 27.6 (t), 27.7 (t), 29.1, 30.5 (t), 33.2 (t), 36.5 (t), 38.6, 47.9, 52.2, 62.6, 63.7, 64.7 (s), 66.2 (t), 79.7 (s), 119.5 (2C), 120.8 (2C), 125.6, 125.7, 127.3, 127.8 (2C), 127.9 (2C), 128.4 (2C), 128.7 (2C), 129.1 (2C), 130.3 (2C), 136.7 (s), 137.0 (s), 138.1 (s), 141.8 (s, 2C), 144.8 (s), 144.9 (s), 153.8 (s), 154.4 (s), 171.6 (s), 172.5 (s);

IR (neat, cm⁻¹) 3364, 3029, 2978, 2932, 2877, 1719, 1626, 1594, 1521, 1486, 1448, 1412, 1366, 1313, 1225, 1155, 1077, 1050.

Synthesis of methyl (3S,6R,9S,9aR)-6-amino-6-benzyl-9-(4-((tert-butoxycarbonyl)amino)phenethyl)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepine-3-carboxylate (28a)

To a solution of compound **27a** (308.6 mg, 0.40 mmol) in dry THF (4 mL, 0.1 M) under nitrogen atmosphere, piperidine (48 μ L, 0.49 mmol) was added. After 6 hours the solvent was evaporated under reduced pressure and the crude mixture was purified by flash chromatography (AcOEt 100% $\rightarrow$ AcOEt/MeOH 95:05) to obtain compound **28a** as a white amorphous solid (180.8 mg, 85%). TLC: Hexane/AcOEt 60:40 for starting material, R_f = 0; AcOEt/MeOH 95:05 for product, R_f = 0.34

Synthesis of (3S,6R,9S,9aR)-6-benzyl-6-(((benzyloxy)carbonyl)amino)-9-(4-((tert-butoxycarbonyl) amino)phenethyl)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepine-3-carboxylic acid (30a)

Compound **28a** (180.8 mg, 0.34 mmol) was dissolved in dry THF (6.75 mL, 0.05 M) under nitrogen atmosphere and cooled to 0 °C. The reaction mixture was added with (Cbz)₂O (124 μ L, 0.5 mmol) and, after 2 hours, was warmed to room temperature. After 16 hour the reaction mixture was added with a 1.6 M LiOH solution in methanol with 10% (v/v) water. (850 μ L, 1.36 mmol). The reaction mixture was heated to 60 °C. After 16 hours the solvent was evaporated under reduced pressure, the residue was recovered with water, the pH was adjusted to 2 by addition of a HCl aqueous solution and the aqueous layer was extracted three times with DCM. The organic phase was dried with anhydrous Na₂SO₄, filtered and the solvent was evaporated under reduced pressure. The crude mixture was purified by flash chromatography (AcOEt 100% $\rightarrow$ AcOEt/MeOH 95:05) to obtain compound **30a** as a white amorphous solid (223 mg, 99%). TLC: AcOEt/MeOH 90:10, R_f = 0.45

Spectroscopic data:

¹H NMR (CDCl₃, 400 MHz) δ 1.54 (s, 9H), 1.56–1.67 (m, 73 2H), 1.69–1.80 (m, 1H), 1.81–2.14 (m, 6H), 2.32–2.47 (m, 2H), 2.66–2.76 (m, 1H), 2.89 (br s, 1H), 3.38 (d, J = 13.9 Hz, 1H), 3.61 (d, J = 13.9 Hz, 1H), 4.29 (br s, 1H), 4.43 (d, J = 7.2 Hz, 1H), 5.08 (d, J = 12.4 Hz, 1H), 5.24 (d, J = 12.4 Hz, 1H), 6.56 (br s, 2H), 6.86 (d, J = 6.9 Hz, 2H), 7.07 (d, J = 8.4 Hz, 2H), 7.14–7.30 (m, 5H), 7.32–7.45 (m, 5H), 10.85 (br s, 1H);

¹³C{¹H} NMR (CDCl₃, 100 MHz) δ 26.1 (t), 26.7 (t), 26.8 (t), 28.8 (3C), 30.1 (t), 30.3 (t), 32.3 (t), 32.4 (t), 36.5, 64.4, 65.3 (s), 66.6 (t), 81.0 (s), 119.4 (2C), 127.6, 128.5, 128.8 (2C), 128.9 (2C), 129.1 (2C), 129.7 (2C), 136.1 (s), 136.2 (s), 136.8 (s), 137.2 (s), 153.5 (s), 154.6 (s), 172.0 (s), 174.9 (s);

IR (neat, cm⁻¹) 3600–2600 (broad band), 3354, 2961, 2929, 2856, 1716, 1624, 1593, 1520, 1485, 1447, 1412, 1366, 1313, 1227, 1156, 1075, 1049, 1026.

Synthesis of methyl N²-(((9H-fluoren-9-yl)methoxy)carbonyl)-N^ω-((2,2,6,7-tetramethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)-L-arginylglycinate (31**)**

To a solution of Z-Arg(Pbf)-OH (500 mg, 0.77 mmol) in dry THF (7.7mL, 0.10 M), under nitrogen atmosphere, NMM (170 μL, 1.54 mmol) was added. Isobutyl chloroformate (120 μL, 0.93 mmol) was added at 0 °C and, after 20 minutes, H₂N-Gly-OMe*HCl (116 mg, 0.93 mmol) was also added to the solution. The reaction mixture was warmed at room temperature and left stirring for 16 hours. Afterwards the reaction was evaporated under reduced pressure. The residue was recovered with AcOEt and was washed with 1 M HCl and the with NaHCO₃ saturated solution. The organic phase was dried with anhydrous Na₂SO₄, filtered, and the solvent was evaporated under reduced pressure. The crude mixture was purified by flash chromatography (DCM/Acetone 70:30) to obtain compound **31** as a white amorphous solid (369 mg, 67%). TLC: DCM/Acetone 70:30, R_f = 0.35

Spectroscopic and mass data:

¹H NMR (400 MHz, CDCl₃) δ 1.45 (s, 6H), 1.58–1.69 (m, 2H), 1.77 (m, 1H), 1.97 (m, 1H), 2.09 (s, 3H), 2.53 (s, 3H), 2.60 (s, 3H), 2.93 (s, 2H), 3.25 (m, 1H), 3.36 (m, 1H), 3.70 (s, 3H), 3.92 (dd, J = 5.5, 17.9 Hz, 1H), 4.09 (dd, J = 6.2, 18.0 Hz, 1H), 4.16 (t, J = 7.0 Hz, 1H), 4.30–4.46 (m, 3H), 6.09 (br s, 2H), 6.34 (br s, 2H), 7.26 (t, J = 7.5 Hz, 2H), 7.38 (t, J = 7.5 Hz, 2H), 7.53–7.61 (m, 2H), 7.67 (br s, 1H), 7.75 (d, J = 7.5 Hz, 2H);

¹³C{¹H} NMR (CDCl₃, 100 MHz) δ 12.5, 17.9, 19.3, 25.1 (t), 28.6, 30.2 (t), 40.2 (t), 41.1 (t), 43.2 (t), 47.1, 52.3, 54.0, 67.1 (t), 86.4 (s), 117.6 (s), 199.9, 124.7 (s), 125.1, 127.1, 127.7, 132.3 (s), 132.5 (s), 128.4 (s), 141.2 (s), 143.7 (s), 143.8 (s), 156.5 (s), 158.9 (s), 170.8 (s), 172.8 (s)

IR (neat, cm⁻¹) 3436, 3314, 2927, 1715, 1663, 1616, 1548, 1448, 1239, 1209, 1089, 1032.

HRMS (ESI) calculated for C₃₇H₄₆N₅O₈S⁺ [M+H]⁺ m/z 720.3062, found 720.3057

Synthesis of methyl N^ω-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)-L-arginylglycinate (**32**)

To a solution of dipeptide **31** (250 mg, 0.347 mmol) in THF (3.5 mL, 0.1 M) piperidine (51 μL, 0.52 mmol) was added and the mixture stirred at rt for 5 h. The solvent was evaporated under reduced pressure, and the crude mixture was purified by flash chromatography (DCM/Acetone 70:30 → DCM/MeOH 80:20) to obtain compound **32** as white amorphous solid (172,7 mg, 99%). TLC: DCM/Acetone 70:30 for monitoring the starting material and DCM/MeOH 80:20 for monitoring the product, R_f = 0.4

The spectroscopic data of compound were matched with literature values.

Synthesis of methyl N²-((3S,6R,9S,9aR)-6-benzyl-6-(((benzyloxy)carbonyl)amino)-9-(4-((tert-butoxycarbonyl)amino)phenethyl)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepine-3-carbonyl)-N^ω-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)-L-arginylglycinate (**33**)

Compound **30a** (50 mg, 0.076 mmol) and compound **32** (75 mg, 0.15 mmol) were dissolved in dry THF (1.5 mL, 0.05 M). The reaction mixture was added with HATU (57 mg, 0.15 mmol) and DIPEA (40 μL, 0.23 mmol) and was left stirring for 12 hours. Afterwards, the solvent was evaporated under reduced pressure and purified by flash chromatography (AcOEt/Hexane 90:10) to obtain compound **33** as a white amorphous solid (76 mg, 99%). TLC: AcOEt/MeOH 90:10 for starting material; AcOEt/Hexane 90:10 for product, R_f = 0.34

Spectroscopic data:

¹H NMR (CDCl₃, 400 MHz) δ 1.46 (s, 6H), 1.54 (s, 9H), 1.55–2.15 (m, 11H), 2.09 (s, 3H), 2.23 (br s, 1H), 2.31–2.43 (m, 2H), 2.49–2.66 (m, 2H), 2.53 (s, 3H), 2.61 (s, 3H), 2.86–2.94 (m, 1H), 2.95 (s, 2H), 3.26 (br s, 1H), 3.38 (d, J = 13.8 Hz, 1H), 3.56 (d, J = 13.8 Hz, 1H), 3.68 (s, 3H), 3.90 (dd, J = 5.6, 17.6 Hz, 1H), 4.01 (dd, J = 5.6, 17.6 Hz, 1H), 4.35 (br s, 2H), 4.53 (d, J = 6.9 Hz, 1H), 5.03 (d, J = 12.3 Hz, 1H), 5.24 (d, J = 12.3 Hz, 1H), 5.95 (br s, 1H), 6.15 (br s, 2H), 6.68 (br s, 1H), 6.77 (br s, 1H), 6.84 (d, J = 6.7 Hz, 2H), 7.08 (d, J = 7.9 Hz, 2H), 7.12–7.25 (m, 5H), 7.32–7.45 (m, 5H), 7.48 (br s, 1H), 7.82 (br s, 1H);

¹³C{¹H} NMR (CDCl₃, 100 MHz) δ 12.9, 18.3, 19.7, 25.9 (br t), 26.3 (br t), 26.9 (t), 27.7 (2C, br t), 28.8 (3C), 29.0 (2C), 29.9 (t), 30.1 (t), 30.8 (t), 33.5 (t), 38.1, 39.0, 39.7 (br t), 41.4 (t), 43.6 (t), 52.6, 63.4 (br), 64.3, 65.1 (s), 66.6 (t), 81.0 (s), 86.9 (s), 118.0 (s), 120.9, 125.1 (s), 127.5,

128.4 (2C), 128.6 (2C), 128.8 (2C), 128.9 (2C), 129.4 (2C), 129.9 (2C), 132.9 (s), 135.9 (s), 136.3 (s), 137.1 (s), 137.8 (s), 138.9 (s), 154.4 (s), 155.1 (s), 156.5 (s), 159.3 (s), 166.2 (s), 170.9 (s), 171.2 (s), 172.7 (s), 173.4 (s);

IR (neat, cm^{-1}) 3600–2600 (broad band), 3329, 2963, 2928, 2855, 1720, 1664, 1613, 1522, 1449, 1411, 1367, 1313, 1304, 1261, 1234, 1157, 1090, 1049, 1026.

Synthesis of tert-butyl (S)-4-(((3S,6R,9S,9aR)-6-benzyl-9-(4-((tert-butoxycarbonyl) amino) phenethyl)-3-(((S)-1-((2-methoxy-2-oxoethyl)amino)-1-oxo-5-(3-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)guanidino)pentan-2-yl)carbamoyl)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepin-6-yl)amino)-3-(((benzyloxy)carbonyl)amino)-4-oxobutanoate (34**)**

To a solution of compound **33** (177,6 mg, 0.156 mmol) in dry MeOH (3.3 mL, 0.05 M), under H_2 (balloon), $\text{Pd}(\text{OH})_2/\text{C}$ (17,8 mg, 10% weight) was added. The reaction mixture was stirred at room temperature overnight. Afterwards, it was filtered through a pad of Celite and the organic phase was evaporated under reduced pressure. The crude mixture was dissolved in dry THF (3.3 mL, 0.05 M) under nitrogen atmosphere and was added with Z-L-Asp(OtBu)-OH (101 mg, 0.313 mmol), HATU (119 mg, 0.313 mmol) and DIPEA (84 μL , 0.47 mmol). After 7 hours, the solvent was evaporated under reduced pressure. The crude mixture was purified by flash chromatography (AcOEt/hexane 90:10) to obtain compound **34** as a white amorphous solid (200 mg, 98% over 2 steps). TLC: the first step was monitored with AcOEt/Hexane 90:10; the second step was monitored with AcOEt/MeOH 90:10 for starting materials and AcOEt/Hexane 90:10 for product, $R_f = 0.35$

Spectroscopic data:

^1H NMR (CDCl_3 , 400 MHz) δ 1.29–1.43 (m, 3H), 1.38 (s, 9H), 1.40 (s, 6H), 1.47 (s, 9H), 1.50–1.61 (m, 2H), 1.64–1.76 (m, 2H), 1.78–2.06 (m, 7H), 1.98 (s, 3H), 2.27–2.38 (m, 1H), 2.40 (s, 3H), 2.42–2.45 (m, 1H), 2.46 (s, 3H), 2.54–2.63 (m, 1H), 2.69 (dd, $J = 5.5, 15.8$ Hz, 1H), 2.79 (br s, 1H), 2.86–3.03 (m, 4H), 3.41 (d, $J = 13.6$ Hz, 1H), 3.51 (d, $J = 13.6$ Hz, 1H), 3.60 (s, 3H), 3.74–3.89 (m, 2H), 4.10–4.19 (m, 1H), 4.20–4.27 (m, 1H), 4.32–4.43 (m, 2H), 4.97 (d, $J = 12.6$ Hz, 1H), 5.05 (d, $J = 12.6$ Hz, 1H), 6.39 (br s, 1H), 6.63 (br s, 1H), 6.92 (d, $J = 6.1$ Hz, 2H), 7.04 (d, $J = 8.4$ Hz, 2H), 7.10–7.22 (m, 3H), 7.22–7.38 (m, 7H), 7.77 (d, $J = 8.4$ Hz, 1H), 7.84–7.92 (m, 2H), 8.23 (t, $J = 5.6$ Hz, 1H), 9.18 (s, 1H);

¹³C{¹H} NMR (CDCl₃, 100 MHz) δ 12.3, 17.9, 19.3, 25.0 (br t), 26.1 (t), 27.1 (br t), 27.3 (br t), 27.9 (3C), 28.3 (3C), 28.5 (2C), 29.2 (t), 29.5 (t), 29.6 (t), 32.3 (t), 36.5 (t), 38.7, 51.2, 52.2, 52.9, 60.0 (br), 64.0, 65.3 (s), 67.1 (t), 80.2 (s), 81.5 (s), 86.6 (s), 117.7 (s), 119.4 (2C), 124.9 (s), 127.5, 127.9 (2C), 128.2, 128.5 (6C), 130.0 (2C), 133.3 (s), 134.7 (s), 136.0 (s), 136.2 (s), 136.4 (s), 139.3 (s), 153.3 (s), 155.2 (s), 156.8 (s), 159.5 (s), 169.9 (s), 170.2 (s), 171.0 (s), 171.7 (s), 173.0 (s), 173.5 (s);

IR (neat, cm⁻¹) 3600–2600 (broad band), 3336, 2977, 2928, 2869, 1723, 1665, 1616, 1525, 1453, 1411, 1367, 1294, 1235, 1158, 1108, 1088, 1051.

Synthesis of tert-butyl (S)-4-(((3S,6R,9S,9aR)-6-benzyl-3-(((S)-1-(2-(benzyloxy)-2-oxoethyl)amino)-1-oxo-5-(3-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)guanidino) pentan-2-yl)carbamoyl)-9-(4-((tert-butoxycarbonyl)amino) phenethyl)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepin-6-yl)amino)-3-(((benzyloxy) carbonyl)amino)-4-oxobutanoate (35)

To a solution of compound **34** (257.5 mg, 0.197 mmol) in dry THF (2 mL, 0.10 M), under nitrogen atmosphere, BnOH (2 mL, 0.1 M), Ti(iPrO)₄ (29 µL, 0.1 mmol) and 4 Å molecular sieves (300 mg) were added. The reaction mixture was heated to 90 °C and was left stirring for 16 hours. Afterwards, the mixture was filtered through a pad of Celite and the organic phase was evaporated under reduced pressure. The residue was recovered with DCM and was washed two times with a 1 M HCl aqueous solution. The organic phase was dried with anhydrous Na₂SO₄, filtered and the solvent was evaporated under reduced pressure. The crude mixture was purified by flash chromatography (Hexane/AcOEt 60:40 → AcOEt/Hexane 80:20) to obtain compound **35** as a white amorphous solid (257 mg, 94 %). TLC: DCM/MeOH 95:05, R_f = 0.38

Spectroscopic data:

¹H NMR (DMSO-d₆, 60 °C, 400 MHz) δ 1.31–1.44 (m, 3H), 1.39 (s, 9H), 1.40 (s, 6H), 1.48 (s, 9H), 1.53–1.64 (m, 2H), 1.68–2.03 (m, 9H), 2.00 (s, 3H), 2.30–2.39 (m, 1H), 2.43 (s, 3H), 2.44–2.49 (m, 1H), 2.48 (s, 3H), 2.53–2.62 (m, 1H), 2.70 (dd, J = 5.5, 15.8 Hz, 1H), 2.74–2.81 (m, 1H), 2.89–3.01 (m, 4H), 3.41 (d, J = 13.7 Hz, 1H), 3.53 (d, J = 13.7 Hz, 1H), 3.81–3.95 (m, 2H), 4.14–4.22 (m, 1H), 4.23–4.29 (m, 1H), 4.30–4.42 (m, 2H), 4.99 (d, J = 12.6 Hz, 1H), 5.04 (d, J = 12.6 Hz, 1H), 5.11 (s, 2H), 6.42 (br s, 1H), 6.54 (br s, 1H), 6.95 (d, J = 6.3 Hz, 2H), 7.04 (d, J

= 8.4 Hz, 2H), 7.13–7.22 (m, 3H), 7.24–7.39 (m, 12H), 7.54 (br s, 1H), 7.73 (d, $J = 8.1$ Hz, 1H), 7.81–7.89 (m, 1H), 8.10 (t, $J = 5.7$ Hz, 1H), 8.97 (s, 1H);

$^{13}\text{C}\{^1\text{H}\}$ NMR (DMSO- d_6 , 60 °C, 100 MHz) δ 12.0, 17.4, 76.18.7, 25.2 (t), 26.1 (t), 26.2 (t), 26.8 (t), 27.8 (3C), 28.2 (5C), 28.6 (t), 28.9 (t), 29.3 (t), 29.6 (t), 32.3 (t), 34.5 (t), 37.0, 38.7, 37.6 (t), 40.8 (t), 42.7 (t), 52.3, 53.1, 61.8, 63.5, 64.9 (s), 65.7 (t), 65.9 (t), 78.8 (s), 80.1 (s), 86.1 (s), 116.2 (s), 118.6 (2C), 124.3 (s), 126.5, 127.4 (2C), 127.6, 127.8 (2C), 127.8 (2C), 127.9, 128.2 (2C), 128.2 (2C), 128.3 (2C), 129.6 (2C), 131.5 (s), 134.5 (s), 135.6 (s), 135.9 (s), 136.3 (s), 136.8 (s), 137.2 (s), 152.9 (s), 155.6 (s), 156.1 (s), 157.5 (s), 169.2 (s), 169.4 (s), 169.5 (s), 170.7 (s), 171.3 (s), 171.8 (s);

IR (neat, cm^{-1}) 3600–2600 (broad band), 3327, 2970, 2927, 2869, 1722, 1663, 1615, 1523, 1454, 1411, 1367, 1236, 1156, 1108, 1089, 1051, 1028.

Synthesis of compound **37**:

To a solution of compound **35** (137 mg, 0.10 mmol) in dry MeOH (2 mL, 0.05 M), under H_2 (balloon), $\text{Pd}(\text{OH})_2/\text{C}$ (13.7 mg, 10% weight) was added. The reaction mixture was stirred at room temperature for 5 h. Afterwards, it was filtered through a pad of Celite and the organic phase was evaporated under reduced pressure. The crude mixture was dissolved in dry THF (25 mL, 0.004 M) under nitrogen atmosphere and was added with HATU (75 mg, 0.197 mmol) and DIPEA (35 μL , 0.197 mmol). After 24 hours, the solvent was evaporated under reduced pressure and the crude mixture was purified by flash chromatography (AcOEt/MeOH 90:10) to obtain compound **37** as a white amorphous solid (106 mg, 91% over 2 step). TLC AcOEt/MeOH 70:30, $R_f = 0.45$

Synthesis of 3a-RGD:

Compound **37** (55 mg, 0.048 mmol) was dissolved in a 95:2.5:2.5 TFA/TIS/ H_2O (5 mL, 0.010 M) at 0 °C and the reaction mixture was left stirred at room temperature for 5 h. Afterwards, the solvent was evaporated under reduced pressure (the residual TFA was evaporated under nitrogen flow). The crude mixture was dissolved in 10% v/v DMF in water solution and purified by Biotage® Isolera One chromatography system using an EcoFlex C18 (4 g, 50 μm , spherical) cartridge at 10 mL/min flow, with H_2O + 0.1% TFA and ACN as eluents. The column was conditioned with 2 CV of 95:5 H_2O /0.1% TFA: CH_3CN , then eluted with a gradient of 95:5 H_2O + 0.1% TFA/ACN to 40:60 over 7 CV, followed by 40:60 100% ACN over 1.5 CV.

Appropriate fractions were combined and concentrated to obtain **3a-RGD** (di-trifluoroacetate salt) as a white amorphous solid (32 mg, 70%).

Spectroscopic and mass data:

^1H NMR (D_2O , 60 °C, 400 MHz) δ 1.68–1.79 (m, 3H), δ 1.81–2.32 (m, 9H), δ 2.42 (m, 1H), δ 2.52 (m, 1H), δ 2.62–2.78 (m, 2H), δ 2.91 (m, 1H), δ 3.11 (dd, J = 6.7, 17.1 Hz, 1H), δ 3.17 (m, 1H), δ 3.25 (dd, J = 6.8, 17.0 Hz, 1H), δ 3.30–3.45 (m, 2H), δ 3.52 (d, J = 13.4 Hz, 1H), δ 3.80 (d, J = 13.4 Hz, 1H), δ 3.88 (d, J = 14.7 Hz, 1H), δ 4.29 (d, J = 14.6 Hz, 1H), δ 4.41 (dd, J = 8.1, 10.1 Hz, 1H), δ 4.65 (dd, J = 4.7, 8.0 Hz, 1H), δ 5.16 (t, J = 6.5 Hz, 1H), δ 7.34–7.40 (m, 2H), δ 7.56–7.77 (m, 7H);

$^{13}\text{C}\{^1\text{H}\}$ NMR (D_2O , 60 °C, 100 MHz) δ 23.8 (t), δ 25.1 (t), δ 27.7 (t), δ 28.9, δ 29.5 (t), δ 32.0, δ 33.6, δ 39.1, δ 40.2 (t), δ 44.0 (t), δ 50.1, δ 51.7, δ 51.8, δ 65.6 (s), δ 112.0 (s), δ 114.9 (s), δ 117.8 (s), δ 122.9, δ 124.2, δ 127.4, δ 127.6, δ 128.5, δ 129.8, δ 130.0, δ 132.2, δ 135.5 (s), δ 143.6 (s), δ 156.7 (s), δ 162.4 (s), δ 162.7 (s), δ 163.1 (s), δ 170.5 (s), δ 171.9 (s), δ 173.8 (s), δ 174.0 (s), δ 174.8 (s);

IR (neat, cm^{-1}): 3372, 2926, 2855, 1733, 1717, 1683, 1639, 1515, 1456, 1375, 1338, 1299, 1246, 1205, 1146, 1069;

HRMS (ESI) calculated for $\text{C}_{37}\text{H}_{50}\text{N}_9\text{O}_7^+$ $[\text{M}+\text{H}]^+$ m/z 732.38277, found 732.38379

Synthesis of methyl 5-azidopentanoate (38)

To a solution of **5-bromopentanoic acid** (1.00 g, 5.52 mmol) in dry MeOH (6.9 mL, 0.80 M) at 0 °C, SOCl_2 (1.20 mL, 16.6 mmol) was added dropwise under nitrogen atmosphere. After 30 minutes, the reaction has warmed to rt. After 1 hour, the solvent was evaporated under reduced pressure, and the crude residue was recovered with 7 mL of AcOEt. The organic layer was washed three times with 5 mL of a NaHCO_3 saturated aqueous solution, three times with 5 mL of H_2O and once with 4 mL of brine. The organic phase was dried with anhydrous Na_2SO_4 , filtered, and the solvent was removed under reduced pressure. The crude residue of compound 50 was dissolved in dry DMF (7.3 mL, 0.75 M) under nitrogen atmosphere and NaN_3 (717 mg, 11.0 mmol) was added. The reaction was heated to 80°C through microwave irradiation for 20 minutes. The residue was recovered with AcOEt and was washed three times with a NaHCO_3 saturated aqueous solution, three times with H_2O and once with brine. The organic phase was dried with anhydrous Na_2SO_4 , filtered, and the

solvent was removed under reduced pressure. The crude residue was purified by flash chromatography (toluene/AcOEt 95:05) to obtain compound **38** as a pale-yellow oil (840.8 mg, 97% over two steps). TLC: toluene/AcOEt 95:05, R_f = 0.37

The **spectroscopic data** of compound **38** were matched with literature values:

^1H NMR (CDCl_3 , 400 MHz) δ 1.59–1.68 (m, 2H), 1.69–1.78 (m, 2H), 2.36 (t, J = 7.1 Hz, 2H), 3.30 (t, J = 6.7 Hz, 2H), 3.68 (s, 3H);

$^{13}\text{C}\{^1\text{H}\}$ NMR (CDCl_3 , 100 MHz) δ 22.5 (t), 28.7 (t), 33.8 (t), 51.4 (t), 52.0, 174.0 (s).

Synthesis of 5-azidopentanoic acid (**39**)

To a solution of compound **38** (840.8 mg, 5.36 mmol) in dry MeOH (5.4 mL, 1.0 M) under nitrogen atmosphere, a 3 M aqueous solution of LiOH (3.6 mL, 10.72 mmol) was added. After 2 hours the solvent was removed under reduced pressure, the residue was recovered with water, the pH was adjusted to 2 by addition of a 1M HCl aqueous solution and the aqueous layer was extracted three times with AcOEt. The organic phase was dried with anhydrous Na_2SO_4 , filtered, and the solvent was evaporated under reduced pressure to afford compound **38** as a colourless oil without the need of further purifications (767.2 mg, quantitative). TLC analysis toluene/AcOEt 95:05 for monitoring the starting materials and AcOEt/MeOH 90:10 for monitoring product, R_f = 0.55

The **spectroscopic data** of compound **39** were matched with literature values:

^1H NMR (CDCl_3 , 400 MHz) δ 1.62–1.79 (m, 4H), 2.42 (t, J = 7.1 Hz, 2H), 3.32 (t, J = 6.5 Hz, 2H), 9.70 (br s, 1H);

$^{13}\text{C}\{^1\text{H}\}$ NMR (CDCl_3 , 100 MHz) δ 22.2 (t), 28.6 (t), 33.8 (t), 51.4 (t), 180.0 (s).

Synthesis of 2,5-dioxopyrrolidin-1-yl 5-azidopentanoate (**40**)

To a solution of compound **39** (767.2 mg, 5.36 mmol) in dry DCM (11 mL, 0.5 M) under nitrogen atmosphere, N-hydroxysuccinimide (680 mg, 5.9 mmol) and EDC*HCl (1.5 g, 8 mmol) were added. After 1 hour, H_2O was added, the organic phase was evaporated under reduced pressure and the mixture was extracted three times with AcOEt. The organic phases were dried with anhydrous Na_2SO_4 , filtered, and the solvent was evaporated under reduced

pressure to afford compound **40** without the need of further purifications (1.28 g, 97%). TLC: Hexane/AcOEt 60:40, R_f = 0.30

The **spectroscopic data** of compound **40** were matched with literature values:

^1H NMR (CDCl_3 , 400 MHz) δ 1.68–1.79 (m, 2H), 1.82–1.91 (m, 2H), 2.68 (t, J = 7.1 Hz, 2H), 2.82–2.90 (m, 4H), 3.36 (t, J = 6.6 Hz, 2H);

$^{13}\text{C}\{^1\text{H}\}$ NMR (CDCl_3 , 100 MHz) δ 22.3 (t), 26.0 (t), 28.3 (t), 30.8 (t), 51.2 (t), 168.6 (s), 170.0 (s).

3.3.2 *Synthesis of 3a-RGD-N₃*

The chromatograms reported in the [Chapter 2.4.2](#) were acquired using the following methods to monitor the progress of the azide-linker coupling reaction under the different experimental conditions investigated during the optimization study.

General HPLC monitoring conditions

Analytical HPLC monitoring was performed using a **VWR Hitachi Chromaster** system equipped with a 5160 pump, 5260 autosampler, 5310 column oven, and 5430 diode array detector (DAD). Data acquisition and processing were carried out using **Clarity VA** software (version 9.0.1.19). All analyses were performed on a **Kinetex C18 column** (2.6 μm particle size, 100 Å pore size, 100 $\times$ 4.6 mm). The column temperature was maintained at 30 °C. Detection was carried out at a wavelength of 214 nm.

For experiments involving in **Et₃N/DMF media**: the mobile phase consisted of solvent A (water containing 0.1% trifluoroacetic acid, TFA) and solvent B (acetonitrile). The elution program was as follows: 95% A at the start of the run, followed by a linear gradient to 40% A over 18 min; the composition was then changed to 5% A and maintained for 3 min to wash the column, after which the initial conditions (95% A) were restored to re-equilibrate the system before the subsequent injection. The flow rate was set to 1.0 mL/min.

For experiments involving **phosphate-buffered** reaction media: the mobile phase consisted of solvent A (water containing 0.1% trifluoroacetic acid, TFA) and solvent B (acetonitrile). The elution program was as follows: 95% A at the start of the run, followed by a linear gradient to 40% A over 22 min; the composition was then changed to 5% A and maintained

for 3 min to wash the column, after which the initial conditions (95% A) were restored to re-equilibrate the system before the subsequent injection. The flow rate was set to 1.0 mL/min.

For experiments involving **MES/HEPES buffer** reaction media: the mobile phase consisted of solvent A (water containing 0.1% trifluoroacetic acid, TFA) and solvent B (acetonitrile). The elution program was as follows: 90% A at the start of the run, followed by a linear gradient to 20% A over 32 min; the composition was then changed starting from 20% A and followed by linear gradient to 90% A over 5 minutes in order to wash the column for re-equilibrate the system before the subsequent injection. The flow rate was set to 0.5 mL/min.

Optimized synthesis of **3a-RGD-N₃**:

The **3a-RGD** (29 mg, 0.03 mmol) was dissolved in a DMF solution (300 μ L) containing compound **40** (21.8 mg, 0.09 mmol). Subsequently, 300 μ L of 100 mM **HEPES buffer** (pH 8.0) was added. The reaction mixture was stirred for 24 h and monitored by HPLC. Upon completion, the solvent was removed under a nitrogen stream. The crude product was then diluted with water containing 0.1% CF₃COOH and purified by preparative HPLC (see *general remarks*). The purified product was then freeze-dried to give the pure trifluoroacetate salt of the desired **3a-RGD-N₃** derivative as a white foam (26 mg, 0.027 mmol, 90% yield).

Spectroscopic and mass data:

¹H NMR (CD₃OD, 400 MHz) δ 1.43–1.52 (m, 3H), δ 1.61–1.86 (m, 13H), δ 2.10–2.22 (m, 2H), δ 2.41 (t, 2H), δ 2.46 (m, 1H), δ 2.66–2.72 (m, 3H), δ 2.78 (m, 1H), δ 2.95 (dd, *J* = 7.2, 16.8 Hz, 1H), δ 3.08 (m, 1H), δ 3.14 (m, 1H), δ 3.35–3.38 (m, 3H), δ 3.44 (d, *J* = 13.1 Hz, 1H), δ 3.57 (d, *J* = 13.1 Hz, 1H), δ 3.96–4.12 (m, 2H), δ 4.33 (m, 1H), δ 4.92 (t, *J* = 6.6 Hz, 1H), δ 7.05–7.13 (m, 2H), δ 7.17 (d, *J* = 8.1 Hz, 2H), δ 7.20–7.31 (m, 3H), δ 7.43 (d, *J* = 8.3 Hz, 2H);

¹³C{¹H} NMR (CD₃OD, 100 MHz) δ 21.6, δ 22.6, δ 24.7, δ 24.9, δ 25.1, δ 27.5, δ 27.8, δ 28.0, δ 29.3, δ 29.4, δ 29.7, δ 30.4, δ 35.8, δ 40.4, δ 50.0, δ 50.5, δ 50.8, δ 51.8, δ 52.8, δ 53.0, δ 53.2, δ 110.4, δ 111.3, δ 113.2, δ 114.1, δ 116.0, δ 117.0, δ 118.9, δ 119.9, δ 120.3, δ 128.0, δ 128.3, δ 130.0, δ 135.9, δ 136.2, δ 138.3, δ 157.0, δ 157.2, δ 157.4, δ 157.8, δ 158.2, δ 159.1, δ 168.6, δ 170.1, δ 170.5, δ 172.8, δ 173.1, δ 173.4, δ 173.6.

HRMS (ESI) calculated for C₄₂H₅₇N₁₂O₈⁺ [M+H]⁺ *m/z* 857.44168, found 857.44159

3.3.3 *Synthesis of NDI-Linker-3a-RGD and biological evaluation*

Chemical synthesis of the new conjugate NDI-cRGD

All the solvents and reactants for the synthesis were purchased from Merck and Fluorochem and were used as supplied without further purification. HPLC analyses were performed using an Agilent system SERIES 1260 with XSelect® HSS C18 column (2.5 μ m, 4.6 $\times$ 50 mm) and the following method was used: flow 1.4 mL/min, isocratic gradient over 2 min 95% of H₂O + 0.1% TFA (5% CH₃CN), gradually to 40% aqueous solvent over 6 min, then isocratic flow for 4 min (λ = 256 nm). HPLC purifications were carried out on an Agilent Technologies 1260 Infinity preparative HPLC provided with a diode array UV-vis detector, using a Waters XSelect® CSH Prep C18 OBD™ column (5 μ m, 100 $\times$ 30 mm) at a 30 mL/min flow rate. Using acidic water (0.1% of TFA) and acetonitrile as eluents, the following methods were employed for purifying the crude. Method A: 2 min 80% acidic water, followed by a gradient step of 18 min reaching 40% water phase (λ = 254 nm, 500 nm, 600 nm). Method B: 2 min 95% acidic water, followed by a gradient step of 14 min reaching 40% water phase (λ = 254 nm, 500 nm, 600 nm). UPLC-MS data were recorded, using a surveyor UPLC system (Thermo Finnigan, San Jose, CA, USA) equipped with a BEH Acquity UPLC column (1.7 μ m) 2.1 $\times$ 50 mm, and an LCQ ADV MAX ion-trap mass spectrometer, with an ESI ion source¹.

Synthesis of NDI 1-Linker (1)

Compound **NDI 1** (4 mg, 0.00657 mmol), **linker 1** (5 mg, 0.00783 mmol), HOBt (1 mg, 0.0074 mmol), and pyridine (14 μ L, 0.18 mmol) were dissolved in 3 mL of DMF. The resulting solution was stirred overnight and monitoring in HPLC. The final product was isolated using a preparative RP-HPLC following method A to afford the final compound as a dark blue amorphous solid. (4.6 mg, 62%)

HRMS (ESI⁺) calculated for C₅₈H₈₄N₁₃O₁₀⁺ [M+H]⁺ m/z 1122.6459, found 1122.6511.

Synthesis of NDI 2-Linker (1)

Compound **NDI 2** (4 mg, 0.00638 mmol), **linker 1** (5 mg, 0.00783 mmol), HOBt (1 mg, 0.0074 mmol), and pyridine (14 μ L, 0.18 mmol) were dissolved in 3 mL of DMF. The resulting solution was stirred overnight. The final product was isolated using a preparative RP-HPLC following method A to afford the final compound as a dark green amorphous solid. (2.7 mg, 38%)

HRMS (ESI) calculated for $C_{59}H_{76}N_{13}O_{10}^+$ $[M+H]^+$ m/z 1126.5833, found 1126.5831.

Synthesis of NDI 3-Linker (1)

Compound **NDI 3** (4 mg, 0.00657 mmol), **linker 1** (5 mg, 0.00783 mmol), HOBt (1 mg, 0.0074 mmol), and pyridine (14 μ L, 0.18 mmol) were dissolved in 3 mL of DMF. The resulting solution was stirred overnight. The final product was isolated using a preparative RP-HPLC following method A to afford the final compound as a violet amorphous solid. (3.8 mg, 54%)

HRMS (ESI) calculated for $C_{60}H_{76}N_{13}O_{11}^+$ $[M+H]^+$ 1154.5782, found 1154.5763.

Synthesis of NDI 1-Linker (1)-3a-RGD

NDI 1-Linker (1) (4.8 mg, 4.3 μ mol) and 3a-RGD- N_3 (3.7 mg, 4.3 μ mol) were dissolved in 500 μ L of a 1:1 $H_2O:tBuOH$ mixture. Subsequently, 1 eq. of sodium ascorbate (0.85 mg, 4.3 μ mol) and 1 eq. of $CuSO_4 \cdot 5H_2O$ (1.1 mg, 4.3 μ mol) were simultaneously added and the solution was left under stirring at room temperature for 24 h. The crude mixture was purified by preparative RP-HPLC following method B, obtaining compound **NDI 1-Linker (1)-3a-RGD** as a dark blue solid (1.5 mg, 18%).

HRMS (ESI) calculated for $C_{100}H_{140}N_{25}O_{18}^+$ $[M+H]^+$ m/z 1979,0803, found 1979,0863.

HRMS (ESI) calculated for $C_{100}H_{141}N_{25}O_{18}^{2+}$ $[M+2H]^{2+}$ m/z 990.0438, found 990.0488.

Synthesis of NDI 2-Linker (1)-3a-RGD

NDI 2-Linker (1) (2.2 mg, 1.95 μmol) and 3a-RGD- N_3 (1.7 mg, 1.95 μmol) were dissolved in 500 μL of a 1:1 H_2O : $t\text{BuOH}$ mixture. Subsequently, 2 eq. of sodium ascorbate (0.8 mg, 3.9 μmol) and 2 eq. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (1. mg, 3.9 μmol) were simultaneously added and the solution was left under stirring at room temperature for 24 h. The crude mixture was purified by preparative RP-HPLC following method B, obtaining compound **NDI 2-Linker (1)-3a-RGD** as a dark green solid (1.0 mg, 27%).

HRMS (ESI) calculated for $\text{C}_{101}\text{H}_{133}\text{N}_{25}\text{O}_{18}^{2+}$ $[\text{M} + 2\text{H}]^{2+}$ m/z 992.5179, found 992.5101.

Synthesis of NDI 3-Linker (1)-3a-RGD

NDI 3-Linker (1) (3mg, 2.7 μmol) and 3a-RGD- N_3 (2.3 mg, 2.7 μmol) were dissolved in 500 μL of a 1:1 H_2O : $t\text{BuOH}$ mixture. Subsequently, 2 eq. of sodium ascorbate (1 mg, 5.3 μmol) and 2 eq. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (1.3 mg, 5.3 μmol) were simultaneously added and the solution was left under stirring at room temperature for 24 h. The crude mixture was purified by preparative RP-HPLC following method B, obtaining compound **NDI 3-Linker (1)-3a-RGD** as a violet solid (1.7 mg, 31%).

HRMS (ESI) calculated for $\text{C}_{102}\text{H}_{132}\text{N}_{25}\text{O}_{19}^+$ $[\text{M} + \text{H}]^+$ 2011.0126, found 2011.0146.

In vitro Studies

Cell Culture

U251 human glioblastoma cell line was obtained from the European Collection of Authenticated Cell Cultures (ECACC, Merck Life Science, Milan, Italy) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 2 mM glutamine, and penicillin-streptomycin (10,000 U/ml) (all from Sigma-Aldrich, St. Louis, MO, USA). Normal Human Astrocytes (NHA) were purchased from Clinisciences (Nanterre, France) and maintained under the same culture conditions as U251 cells. All cell lines were maintained in a humidified incubator at 37°C with 5% CO₂. Forty-eight hours before the experiments, the culture medium was replaced with DMEM supplemented with 1% (v/v) fetal bovine serum for all cell lines.

Cell Viability Assay

Cell viability was assessed after 48 h treatment with NDI 1, 2, and 3 compounds and NDI 1-, 2-, and 3-linker-3a-RGD at concentrations of 100 nM, 1 µM, 5 µM, 10 µM, and 20 µM, using the MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay; Promega Corporation, Madison, WI, USA). Cells were seeded at a density of 3.5×10^3 cells/well in 96-well plates, maintained in culture for 48 h in medium supplemented with 1% FBS, and then treated as indicated for an additional 48 h. After treatment, MTS solution (90% culture medium and 10% MTS reagent) was added to each well and, following 1.5 h of incubation at 37°C, absorbance was measured at 490 nm using a Synergy HT plate reader (BioTek, Swindon, UK). All experiments were performed two times in duplicate. Data are expressed as the percentage of viable cells in experimental wells relative to untreated control wells.

Statistics and analysis

All experiments were performed at least in triplicate ($n = 3$), and data are presented as mean $\pm$ standard deviation (SD). Statistical analysis was carried out using GraphPad Prism. For comparison between multiple groups, one-way analysis of variance (one-way ANOVA) followed by Tukey's post hoc test was applied. In experiments involving two independent variables (e.g., concentration and formulation), two-way ANOVA followed by appropriate multiple comparison tests was used.

3.3.4 Synthesis of C4-functionalized azabicyclo[5.3.0]alkanone amino acids

Synthesis of methyl (2S,5R)-5-allyl-1-(2-benzamido-2-benzyl-4-(trimethylsilyl)but-3-ynoyl)pyrrolidine-2-carboxylate (**47**)

A solution of compound **46** (1.036 g, 2.98 mmol, 1.0 equiv) and **1a** (578 mg, 3.42 mmol, 1.15 equiv) in THF (0.1 M) was treated with a catalytic amount of DMAP under nitrogen. The mixture was stirred at 50 °C for 96 h. The solvent was removed under reduced pressure, and the crude product was purified by flash chromatography (Hexane/EtOAc 95:5 → 70:30) to afford **47** as a white solid (1.480 g, 96%). TLC: hexane/EtOAc 70:30, R_f = 0.33

Spectroscopic and mass data:

^1H NMR (400 MHz, CDCl_3) δ 0.20–0.25 (m, 9H), 1.57–1.88 (m, 1.3H), 1.94–2.44 (m, 4H), 2.80–2.92 (m, 0.3H), 3.02–3.25 (m, 1H), 3.42 (d, J = 13.7 Hz, 0.4H), 3.58–3.68 (m, 0.4H), 3.75–3.95 (m, 2.4H), 4.02 (d, J = 13.7 Hz, 0.7H), 4.20–4.32 (m, 0.7H), 4.47 (t, J = 8.8 Hz, 0.3H), 4.64–4.75 (m, 0.4H), 4.86–5.24 (m, 2.3H), 5.39–5.45 (m, 0.4H), 5.54–5.94 (m, 1.1H), 6.07 (s, 0.4H), 6.24 (d, J = 7.9 Hz, 0.3H), 6.93 (s, 0.1H), 7.05–7.13 (m, 0.4H), 7.15–7.26 (m, 4H), 7.29–7.32 (m, 0.6H), 7.35–7.55 (m, 3.4H), 7.61–7.78 (m, 2H).

$^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, CDCl_3) δ -0.4, -0.3, 28.4 (t), 28.5 (t), 30.2 (t), 38.8 (t), 40.3 (t), 51.5, 59.3 (s), 60.4, 60.8, 61.0, 94.2 (s), 101.0 (s), 101.7 (s), 116.5 (t), 126.9, 127.0, 127.3 (2C), 127.8, 128.0, 128.5, 130.5, 131.3, 131.9, 133.3 (s), 134.9, 135.5, 136.3 (s), 165.6 (s), 166.0 (s), 172.2 (s).

IR (neat, cm^{-1}): 3354, 2952, 2161, 1750, 1735, 1670, 1637, 1471, 1390, 1273, 1170.

HRMS (ESI) calculated for $\text{C}_{30}\text{H}_{36}\text{N}_2\text{O}_4\text{SiNa}^+ [\text{M}+\text{Na}]^+$ m/z 539.2337, found 539.2322.

Synthesis of methyl (2S,5R)-5-allyl-1-(2-benzamido-2-benzylbut-3-ynoyl)pyrrolidine-2-carboxylate (**48**)

A solution of **47** (1.480 g, 2.86 mmol, 1.0 equiv) in THF (0.06 M) was cooled to -78 °C under nitrogen, then treated with TBAF (1.0 M in THF, 3.15 mL, 3.15 mmol, 1.1 equiv). The reaction mixture was allowed to warm to 0 °C and stirred until complete consumption of the starting

material. The reaction was quenched with NH_4Cl solution and extracted three times with EtOAc. The combined organic phases were dried over Na_2SO_4 , filtered and concentrated in vacuo. Purification by flash chromatography (hexane/EtOAc 60:40) afforded **48** as a white amorphous solid (1.235 g, 97%). TLC: Hexane/EtOAc 70:30, R_f = 0.26

Spectroscopic and mass data:

^1H NMR (400 MHz, CDCl_3) δ 1.55–1.83 (m, 1H), 1.46–2.46 (m, 4H), 2.69–2.89 (m, 1H), 2.92–3.12 (m, 0.6H), 3.13–3.21 (m, 0.5H), 3.24 (s, 1H), 3.48–3.67 (m, 0.8H), 3.72–3.91 (m, 2.4H), 3.96–4.33 (m, 1.3H), 4.38 (t, J = 8.8 Hz, 0.3H), 4.58–4.73 (m, 0.5H), 4.99–5.31 (m, 2.5H), 5.63–5.90 (m, 1H), 6.10 (d, J = 7.3 Hz, 0.2H), 6.83 (s, 0.3H), 7.01–7.11 (m, 0.5H), 7.14–7.27 (m, 3.5H), 7.29–7.55 (m, 4H), 7.59–7.74 (m, 1.8H), 7.79 (d, J = 7.5 Hz, 0.6H).

$^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, CDCl_3) δ 28.3 (t), 30.4 (t), 38.6 (t), 41.4 (t), 51.8, 58.3 (s), 60.9, 61.1, 80.1 (s), 116.8 (t), 127.2, 128.1, 128.5 (3C), 130.8, 131.6, 133.7 (s), 135.3 (s), 165.7 (s, 2C), 172.2 (s).

IR (neat, cm^{-1}): 3346, 3279, 3063, 2950, 2111, 1748, 1661, 1637, 1473, 1395, 1277, 1171.

HRMS (ESI) calculated for $\text{C}_{27}\text{H}_{28}\text{N}_2\text{O}_4\text{Na}^+$ $[\text{M}+\text{Na}]^+$ m/z 467.1941, found 467.1930.

Synthesis of methyl (3S,6S,9aR)-6-benzamido-6-benzyl-5-oxo-7-vinyl-2,3,5,6,9,9a-hexahydro-1H-pyrrolo[1,2-a]azepine-3-carboxylate (49a)

To a solution of **48** (100 mg, 0.225 mmol, 1.0 equiv) in toluene, under nitrogen and at 80 °C, a solution of Hoveyda-Grubbs second-generation catalyst (HG2, 28 mg, 0.045 mmol, 0.2 equiv) in toluene (18 mL) was added by syringe pump over 72 h, so that the final concentration after catalyst addition was 0.01 M. The nitrogen atmosphere was then replaced with ethylene and the reaction mixture was stirred for an additional 8 h. The solvent was removed and the crude was purified by flash chromatography (Hexane/EtOAc 60:40 $\rightarrow$ 40:60) to afford **49a** as a light brown amorphous solid (43 mg, 43%). TLC: Hexane/EtOAc 40:60, R_f = 0.25

Spectroscopic and mass data:

^1H NMR (400 MHz, CDCl_3) δ 1.71–1.82 (m, 2H), 1.85–1.95 (m, 2H), 2.30 (dd, J = 8.5, 16.8 Hz, 1H), 2.84 (m, 1H), 3.05 (m, 1H), 3.45 (d, J = 13.0 Hz, 1H), 3.49 (d, J = 13.0 Hz, 1H), 3.72 (s,

3H), 4.47 (d, $J = 8.2$ Hz, 1H), 5.09 (dd, $J = 1.8, 10.6$ Hz, 1H), 5.47 (dd, $J = 1.8, 16.9$ Hz, 1H), 6.22 (dd, $J = 2.7, 8.4$ Hz, 1H), 6.69 (dd, $J = 10.6, 16.8$ Hz, 1H), 6.91 (s, 1H), 7.14–7.20 (m, 2H), 7.24–7.28 (m, 3H), 7.38 (t, $J = 7.5$ Hz, 2H), 7.46 (t, $J = 7.4$ Hz, 1H), 7.76 (d, $J = 7.4$ Hz, 2H).

$^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, CDCl_3) δ 27.2 (t), 32.3 (t), 34.3 (t), 43.9 (t), 52.3, 56.8, 62.7, 67.4 (s), 115.5 (t), 127.1, 127.5, 127.7, 128.1, 128.4, 130.1, 131.3, 134.8 (s), 135.0 (s), 138.2 (s), 138.9, 166.8 (s), 169.8 (s), 172.5 (s).

$[\alpha]^{25}_{\text{D}} = -81.4$ ($c = 0.025$, CHCl_3).

IR (neat, cm^{-1}): 3318, 2950, 1738, 1626, 1483, 1412, 1282, 1172, 1074.

HRMS (ESI) calculated for $\text{C}_{27}\text{H}_{29}\text{N}_2\text{O}_4^+$ $[\text{M}+\text{H}]^+$ m/z 445.2122, found 445.2111.

Synthesis of methyl (3*S*,6*R*,9*aR*)-6-benzamido-6-benzyl-5-oxo-7-vinyl-2,3,5,6,9,9*a*-hexahydro-1*H*-pyrrolo[1,2-*a*]azepine-3-carboxylate (**49b**)

Compound **49b** was obtained together with **49a** from ring-closing enyne metathesis of **48** under the same conditions described above: slow addition of HG2 (20 mol%) in toluene at 80 °C over 72 h, followed by exposure to ethylene for 8 h and chromatographic purification (hexane/EtOAc 60:40 → 40:60). **49b** was isolated as a light brown amorphous solid (41 mg, 41%). TLC: Hexane/EtOAc 50:50, $R_f = 0.25$

Spectroscopic and mass data:

^1H NMR (400 MHz, CDCl_3) δ 1.47 (m, 1H), 1.71 (m, 1H), 1.84 (dd, $J = 6.7, 13.0$ Hz, 1H), 1.92–2.13 (m, 3H), 2.99 (d, $J = 12.2$ Hz, 1H), 3.79 (s, 3H), 3.91 (d, $J = 12.2$ Hz, 1H), 4.16 (td, $J = 5.9, 10.7$ Hz, 1H), 4.66 (d, $J = 9.4$ Hz, 1H), 4.94 (dd, $J = 1.8, 10.6$ Hz, 1H), 5.38 (dd, $J = 1.8, 16.8$ Hz, 1H), 6.11 (dd, $J = 4.1, 9.8$ Hz, 1H), 6.34 (dd, $J = 10.6, 16.8$ Hz, 1H), 6.84 (s, 1H), 7.20–7.25 (m, 2H), 7.27–7.32 (m, 3H), 7.41 (t, $J = 7.5$ Hz, 2H), 7.50 (t, $J = 7.5$ Hz, 1H), 7.74 (d, $J = 7.4$ Hz, 2H).

$^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, CDCl_3) δ 26.7 (t), 32.3 (t), 32.8 (t), 44.7 (t), 52.0, 59.1, 62.7, 67.3 (s), 115.1 (t), 126.4, 127.1 (2C), 127.8, 128.5, 131.3, 131.6, 134.0 (s), 134.3 (s), 137.3, 139.8 (s), 166.7 (s), 169.7 (s), 173.0 (s).

$[\alpha]^{25}_{\text{D}} = -80.9$ ($c = 0.025$, CHCl_3)

IR (neat, cm^{-1}): 3306, 3027, 2941, 1739, 1638, 1610, 1598, 1433, 1287, 1170, 1074.

HRMS (ESI) calculated for $\text{C}_{27}\text{H}_{29}\text{N}_2\text{O}_4^+$ $[\text{M}+\text{H}]^+$ m/z 445.2122, found 445.2109.

Synthesis of ruthenium-diene complex (50b)

When the RCEYM of **48** was conducted without application of an ethylene atmosphere, the ruthenium-diene complex **50b** was isolated as a side product in variable yield (10–20%). TLC: Hexane/EtOAc 40:60, R_f = 0.48

Spectroscopic and mass data:

^1H NMR (400 MHz, CDCl_3) δ -0.04 (ddd, J = 4.0, 10.3, 12.9 Hz, 1H), 1.62 (m, 1H), 1.73–1.81 (m, 2H), 1.85 (m, 1H), 1.99 (m, 1H), 2.28–2.43 (m, 12H), 2.52 (s, 6H), 3.27 (d, J = 12.1 Hz, 1H), 3.58 (d, J = 12.1 Hz, 1H), 3.76 (s, 3H), 3.87 (ddd, J = 5.5, 10.4, 10.8 Hz, 1H), 4.10 (s, 4H), 4.60 (d, J = 9.6 Hz, 1H), 5.87 (dd, J = 4.0, 9.5 Hz, 1H), 6.75–6.80 (m, 2H), 6.82 (s, 1H), 6.85–7.11 (m, 4H), 7.18–7.26 (m, 5H), 7.43 (t, J = 7.4 Hz, 1H), 7.70 (d, J = 8.1 Hz, 2H), 16.1 (s, 1H).

$^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, CDCl_3) δ 19.2, 21.2, 26.6 (t), 32.2 (t), 32.8 (t), 47.0 (t), 51.8 (t), 52.0, 57.6, 62.2, 69.7 (s), 124.9, 126.7, 127.2, 128.1, 128.2, 129.2, 129.4, 131.6, 132.4, 132.8 (s), 134.4 (s), 138.5 (3C, s), 163.7 (s), 170.7 (s), 172.0 (s), 172.8 (s), 209.6 (s).

$[\alpha]^{25}_{\text{D}}$ = +139.8 (c = 0.019, CHCl_3).

IR (neat, cm^{-1}): 3304, 2949, 2919, 1744, 1719, 1623, 1418, 1365, 1260, 1172, 1026.

HRMS (ESI) calculated for $\text{C}_{47}\text{H}_{52}\text{ClN}_4\text{O}_4\text{Ru}^+$ $[M]^+$ m/z 873.2715, found 873.2721.

Synthesis of trimethyl (3S,6S,10aR,11aR)-6-benzamido-6-benzyl-5-oxo-2,3,5,6,8,10a,11,11a-octahydro-1H-benzo[d]pyrrolo[1,2-a]azepine-3,9,10-tricarboxylate (51a)

To a solution of **49a** (30 mg, 0.067 mmol, 1.0 equiv) in toluene (0.1 M) was added dimethyl acetylenedicarboxylate (17 μL , 0.135 mmol, 2.0 equiv). The reaction mixture was heated at 80 °C for 24 h, until complete consumption of the starting material. The solvent was evaporated and the crude purified by flash chromatography (hexane/EtOAc 40:60) to afford **51a** as a white amorphous solid (27 mg, 70%). TLC: Hexane/EtOAc 50:50, R_f = 0.25

Spectroscopic and mass data:

^1H NMR (400 MHz, CDCl_3) δ 1.55 (ddd, J = 4.6, 10.0, 12.5 Hz, 1H), 1.80 (m, 1H), 1.99–2.14 (m, 2H), 2.25 (m, 1H), 2.38 (td, J = 7.0, 12.4 Hz, 1H), 3.14 (ddd, J = 1.8, 4.3, 22.9 Hz, 1H), 3.38

(ddd, $J = 2.3, 5.7, 22.9$ Hz, 1H), 3.47 (m, 1H), 3.59 (d, $J = 13.7$ Hz, 1H), 3.77 (s, 3H), 3.79 (s, 6H), 4.10 (m, 1H), 4.28 (d, $J = 13.7$ Hz, 1H), 4.38 (t, $J = 7.4$ Hz, 1H), 6.99 (dd, $J = 1.8, 5.7$ Hz, 1H), 7.03–7.09 (m, 2H), 7.19–7.24 (m, 3H), 7.40 (t, $J = 7.4$ Hz, 2H), 7.48 (t, $J = 7.4$ Hz, 1H), 7.71–7.77 (m, 3H).

$^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, CDCl_3) δ 22.3 (t), 23.8 (t), 26.0 (t), 32.3, 32.9 (t), 36.0 (t), 47.5, 47.6, 47.7, 50.4, 56.4, 62.8 (s), 120.1, 122.3, 122.4, 123.4, 123.6, 125.3, 126.5, 128.9 (s, 2C), 131.1 (s, 2C), 132.9 (s), 161.9 (s), 162.5 (s), 163.3 (s), 164.2 (s), 167.5 (s).

$[\alpha]^{25}_{\text{D}} = -111.9$ ($c = 0.800$, CHCl_3).

IR (neat, cm^{-1}): 3341, 2951, 1716, 1666, 1646, 1431, 1259, 1072.

HRMS (ESI) calculated for $\text{C}_{33}\text{H}_{35}\text{N}_2\text{O}_8^+$ $[\text{M}+\text{H}]^+$ m/z 587.2388, found 587.2372.

Synthesis of trimethyl (3S,6S,11aR)-6-benzamido-6-benzyl-5-oxo-2,3,5,6,11,11a-hexahydro-1H-benzo[d]pyrrolo[1,2-a]azepine-3,9,10-tricarboxylate (52a)

To a solution of **51a** (21 mg, 0.036 mmol, 1.0 equiv) in toluene (0.1 M) was added DDQ (13 mg, 0.056 mmol, 1.6 equiv). The reaction mixture was heated at 80 °C and stirred for 12 h. After solvent removal, the crude was purified by flash chromatography (Toluene/EtOAc 40:60) to afford **52a** as a white amorphous solid (20 mg, 98%). TLC: Toluene/EtOAc 60:40, $R_f = 0.23$

Spectroscopic and mass data:

^1H NMR (400 MHz, CDCl_3) δ 1.77 (m, 1H), 1.84–2.00 (m, 3H), 2.88 (m, 1H), 2.95 (d, $J = 15.9$ Hz, 1H), 3.54–3.64 (m, 2H), 3.69 (s, 3H), 3.79 (d, $J = 13.2$ Hz, 1H), 3.91 (s, 3H), 3.94 (s, 3H), 4.46 (d, $J = 8.5$ Hz, 1H), 6.85 (d, $J = 7.3$ Hz, 2H), 7.16–7.25 (m, 3H), 7.34 (s, 1H), 7.39 (t, $J = 7.3$ Hz, 2H), 7.48 (t, $J = 7.4$ Hz, 1H), 7.75 (d, $J = 7.9$ Hz, 2H), 7.95 (s, 2H).

$^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, CDCl_3) δ 27.3 (t), 32.7 (t), 36.9 (t), 48.7 (t), 52.3, 52.5, 52.6, 56.9, 62.6, 69.1 (s), 126.3 (s), 127.3, 127.8, 127.9, 128.2, 128.4, 130.0, 130.3, 131.6, 134.0 (s), 134.2 (s), 135.9 (s, 2C), 145.3 (s), 165.7 (s), 167.3 (s), 169.4 (s), 170.0 (s), 172.1 (s).

$[\alpha]^{25}_{\text{D}} = +8.34$ ($c = 0.173$, CHCl_3).

IR (neat, cm^{-1}): 3355, 2952, 2917, 2849, 1722, 1650, 1634, 1434, 1274, 1195, 1131, 1079.

HRMS (ESI) calculated for $\text{C}_{33}\text{H}_{33}\text{N}_2\text{O}_8^+$ $[\text{M}+\text{H}]^+$ m/z 585.2231, found 585.2222.

Synthesis of trimethyl (3S,6R,11aR)-6-benzamido-6-benzyl-5-oxo-2,3,5,6,11,11a-hexahydro-1H-benzo[d]pyrrolo[1,2-a]azepine-3,9,10-tricarboxylate (**52b**)

To a solution of **49b** (35 mg, 0.0787 mmol, 1.0 equiv) in toluene (0.1 M) was added dimethyl acetylenedicarboxylate (19 μ L, 0.157 mmol, 2.0 equiv), and the mixture was heated at 80 °C for 24 h. After complete consumption of starting material, the solvent was removed, the crude was redissolved in toluene (0.1 M), treated with DDQ (28 mg, 0.126 mmol, 1.6 equiv), and heated at 80 °C for 8 h. The solvent was evaporated and the crude purified by flash chromatography (hexane/EtOAc 35:65) to afford **52b** as a white amorphous solid (30 mg, 65%, two steps). TLC: Hexane/EtOAc 30:70, R_f = 0.34

Spectroscopic and mass data:

^1H NMR (400 MHz, CDCl_3) δ 1.86–2.00 (m, 2H), 2.03–2.20 (m, 2H), 2.63 (dd, J = 9.2, 15.8 Hz, 1H), 2.80 (d, J = 15.8 Hz, 1H), 3.19 (d, J = 12.7 Hz, 1H), 3.81 (s, 3H), 3.87 (d, J = 12.7 Hz, 1H), 3.90 (s, 3H), 3.95 (s, 3H), 4.04 (m, 1H), 4.67 (d, J = 8.9 Hz, 1H), 6.63 (d, J = 7.5 Hz, 2H), 7.14–7.27 (m, 4H), 7.39–7.48 (m, 3H), 7.54 (t, J = 7.5 Hz, 1H), 7.72–7.81 (m, 3H).

$^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, CDCl_3) δ 26.7 (t), 33.0 (t), 36.5 (t), 48.5 (t), 52.2, 52.5, 52.6, 59.1, 62.7, 68.6 (s), 126.4 (s), 127.2, 127.7, 128.3, 128.4, 128.7, 128.8, 130.7, 132.0, 133.2 (s), 133.5 (s), 135.4 (s), 135.6 (s), 144.7 (s), 165.7 (s), 166.8 (s), 169.3 (s), 169.8 (s), 172.8 (s).

$[\alpha]^{25}_{\text{D}} = -125.9$ (c = 0.216, CHCl_3).

IR (neat, cm^{-1}): 3312, 2951, 1722, 1659, 1622, 1602, 1434, 1273, 1153, 1078, 1023.

HRMS (ESI) calculated for $\text{C}_{33}\text{H}_{33}\text{N}_2\text{O}_8^+$ $[\text{M}+\text{H}]^+$ m/z 585.2231, found 585.2214.

Synthesis of methyl (3S,6S,9aR)-6-benzamido-6-benzyl-7-(4-nitrostyryl)-5-oxo-2,3,5,6,9,9a-hexahydro-1H-pyrrolo[1,2-a]azepine-3-carboxylate (**53a**)

A solution of **49a** (20 mg, 0.045 mmol, 1.0 equiv) in EtOH (0.10 M), under nitrogen, was treated with 4-bromonitrobenzene (10 mg, 0.049 mmol, 1.1 equiv), PdEnCat 30 (5.6 mg, 0.002 mmol, 0.05 equiv), and Bu_4NOAc (41 mg, 0.135 mmol, 3.0 equiv) in a sealed microwave vial. The mixture was heated at 120 °C under microwave irradiation for 20 min; the heating cycle was repeated three times until complete consumption of starting material. The solvent was removed and the crude purified by flash chromatography (hexane/EtOAc

30:70), avoiding direct sunlight exposure, to afford **53a** as a yellow amorphous solid (12 mg, 46%). TLC: Hexane/EtOAc 40:60, R_f = 0.15

Spectroscopic and mass data:

^1H NMR (400 MHz, CDCl_3) δ 1.85–2.03 (m, 3H), 2.12 (m, 1H), 2.50 (dd, J = 9.0, 18.1 Hz, 1H), 2.92 (dd, J = 10.8, 18.0 Hz, 1H), 3.49 (d, J = 13.6 Hz, 1H), 3.62 (m, 1H), 3.74 (s, 3H), 3.91 (d, J = 13.6 Hz, 1H), 4.51 (m, 1H), 6.36 (dd, J = 2.9, 8.0 Hz, 1H), 6.81 (d, J = 15.6 Hz, 1H), 7.14–7.20 (m, 2H), 7.25–7.28 (m, 3H), 7.36–7.43 (m, 4H), 7.46–7.53 (m, 3H), 7.75 (d, J = 7.5 Hz, 2H), 8.17 (d, J = 8.8 Hz, 2H).

$^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, CDCl_3) δ 27.3 (t), 32.5 (t), 35.0 (t), 43.1 (t), 52.4, 56.3, 62.8, 67.7 (s), 124.0, 126.9, 127.0, 127.7, 127.9, 128.3, 128.4, 128.5, 129.9, 131.5, 134.6 (s), 135.2 (s), 135.8, 138.2 (s), 144.1 (s), 146.6 (s), 167.6 (s), 169.5 (s), 172.4 (s).

$[\alpha]^{25}_{\text{D}}$ = +83.3 (c = 0.500, CHCl_3)

IR (neat, cm^{-1}): 3329, 2951, 2920, 1737, 1651, 1628, 1511, 1411, 1338, 1196, 1173, 1074.

HRMS (ESI) calculated for $\text{C}_{33}\text{H}_{32}\text{N}_3\text{O}_6^+$ $[\text{M}+\text{H}]^+$ m/z 566.2286, found 566.2280

Synthesis of methyl (3S,6S,8R,8aR,9aR)-6-benzamido-6-benzyl-8-(4-nitrophenyl)-5-oxo-2,3,5,6,8,8a,9,9a-octahydro-1H-cyclobuta[d]pyrrolo[1,2-a]azepine-3-carboxylate (54a)

Compound **54a** was obtained from **49a** under the same Heck reaction conditions used for **53a**, but without precautions to avoid sunlight during purification. Under these conditions, the major product isolated was **54a** as a yellow amorphous solid (17 mg, 67%). TLC: Hexane/EtOAc 40:60, R_f = 0.42

Spectroscopic and mass data:

^1H NMR (400 MHz, CDCl_3) δ 1.29 (m, 1H), 1.60 (dd, J = 5.8, 13.6 Hz, 1H), 1.77 (m, 1H), 2.01 (m, 1H), 2.13 (m, 1H), 2.31 (m, 1H), 3.46 (d, J = 14.0 Hz, 1H), 3.70 (s, 3H), 3.77 (m, 1H), 4.25 (d, J = 14.0 Hz, 1H), 4.32 (d, J = 4.5 Hz, 1H), 4.45–4.54 (m, 2H), 6.20 (s, 1H), 6.96–7.03 (m, 2H), 7.16–7.23 (m, 3H), 7.36 (d, J = 8.5 Hz, 2H), 7.44 (t, J = 7.6 Hz, 2H), 7.52 (t, J = 7.6 Hz, 1H), 7.82 (d, J = 7.8 Hz, 2H), 7.99 (s, 1H), 8.19 (d, J = 8.4 Hz, 2H).

¹³C{¹H} NMR (100 MHz, CDCl₃) δ 27.2 (t), 33.5 (t), 34.9 (t), 36.1 (t), 45.4, 46.2, 52.4, 59.2, 63.2, 66.1 (s), 123.4, 127.0, 127.3, 128.3, 128.6, 129.1, 129.2, 129.4, 131.5, 135.2 (s), 145.5 (s), 146.8 (s), 147.8 (s), 165.9 (s), 168.4 (s), 172.1 (s).

[α]²⁵_D = +86.8 (c = 0.250, CHCl₃).

IR (neat, cm⁻¹): 3356, 2951, 2921, 1742, 1663, 1623, 1507, 1474, 1398, 1340, 1196, 1170, 1074.

HRMS (ESI) calculated for C₃₃H₃₂N₃O₆⁺ [M+H]⁺ m/z 566.2286, found 566.2283.

Synthesis of methyl (3S,6S,9aR)-7-acetyl-6-benzamido-6-benzyl-5-oxo-2,3,5,6,9,9a-hexahydro-1H-pyrrolo[1,2-a]azepine-3-carboxylate (**57a**)

To a solution of **49a** (20 mg, 0.045 mmol, 1.0 equiv) in THF/H₂O (0.05 M) was added Hg(OAc)₂ (43 mg, 0.135 mmol, 3.0 equiv), and the mixture was stirred at room temperature for 12 h. The reaction was cooled to 0 °C and treated with 540 μL of a solution of NaBH₄ (0.5 M) in NaOH (3 M). After immediate formation of metallic mercury, the pH was neutralized with AcOH, the mixture was filtered, diluted with brine, and extracted three times with DCM. After solvent removal, the crude was redissolved in DCM (0.05 M) and treated with NaOAc (8 mg, 0.098 mmol, 2.0 equiv) and PCC (30 mg, 0.139 mmol, 3.0 equiv). After 30 min, the mixture was filtered through Celite, washed with DCM and Et₂O, concentrated, and purified by flash chromatography (Hexane/EtOAc 30:70 → 20:80) to afford **57a** as a white amorphous solid (7 mg, 35%). TLC: Hexane/EtOAc 30:70, R_f = 0.18

Spectroscopic and mass data:

¹H NMR (400 MHz, CDCl₃) δ 1.79–1.98 (m, 4H), 2.35–2.47 (m, 4H), 2.87 (m, 1H), 3.05 (ddd, J = 2.2, 10.6, 17.3 Hz, 1H), 3.50 (d, J = 12.9 Hz, 1H), 3.75 (s, 3H), 4.01 (d, J = 12.9 Hz, 1H), 4.59 (m, 1H), 6.84–6.93 (m, 2H), 7.12–7.17 (m, 2H), 7.29–7.34 (m, 3H), 7.38 (t, J = 7.4 Hz, 2H), 7.47 (t, J = 7.3 Hz, 1H), 7.70 (d, J = 7.4 Hz, 2H).

¹³C{¹H} NMR (100 MHz, CDCl₃) δ 27.2 (t), 29.1, 32.2 (t), 34.3 (t), 44.1 (t), 52.4, 55.8, 62.7, 66.6 (s), 127.2, 127.7, 128.3, 128.4, 130.5, 131.4, 134.5 (s), 135.0 (s), 139.4, 140.3 (s), 166.8 (s), 168.8 (s), 172.6 (s), 201.8 (s).

[α]²⁵_D = –69.5 (c = 0.600, CHCl₃).

IR (neat, cm^{-1}): 3027, 2922, 2850, 1739, 1684, 1653, 1530, 1364, 1262, 1216, 1025.

HRMS (ESI) calculated for $\text{C}_{27}\text{H}_{28}\text{N}_2\text{O}_5\text{Na}^+$ $[\text{M}+\text{Na}]^+$ m/z 483.1890, found 483.1882.

Synthesis of methyl (3S,6S,9aR)-6-benzamido-6-benzyl-7-formyl-5-oxo-2,3,5,6,9,9a-hexahydro-1H-pyrrolo[1,2-a]azepine-3-carboxylate (59a)

A suspension of **49a** (20 mg, 0.045 mmol, 1.0 equiv) in water (1.0 M) was cooled to 0 °C and treated with NaIO_4 (192 mg, 0.898 mmol, 20 equiv) and 0.85 mL of a solution prepared from OsO_4 in tert-butanol (1.1 mL), Et_3N (26 μL), and THF (600 μL). After 2.5 h, the solvent was removed and the crude purified by flash chromatography (Hexane/EtOAc 30:70 $\rightarrow$ 20:80) to afford **59a** as an amorphous solid (10 mg, 49%). TLC: Hexane/EtOAc 30:70, R_f = 0.17

Spectroscopic and mass data:

$[\alpha]^{25}_{\text{D}} = -79.7$ (c = 0.140, CHCl_3).

IR (neat, cm^{-1}): 3361, 2953, 2925, 2853, 2729, 1737, 1627, 1436, 1282, 1174, 1079.

^1H NMR (400 MHz, CDCl_3) δ 1.69–1.82 (m, 3H), 1.90 (m, 1H), 2.34 (dd, J = 7.8, 16.5 Hz, 1H), 2.47 (dd, J = 8.6, 17.5 Hz, 1H), 3.15 (dd, J = 10.3, 17.5 Hz, 1H), 3.30 (d, J = 12.5 Hz, 1H), 3.75 (s, 3H), 4.12 (d, J = 12.5 Hz, 1H), 4.61 (d, J = 8.1 Hz, 1H), 7.00–7.12 (m, 4H), 7.23–7.28 (m, 3H), 7.39 (t, J = 7.5 Hz, 2H), 7.47 (t, J = 7.0 Hz, 1H), 7.75 (d, J = 7.7 Hz, 2H), 9.42 (s, 1H).

$^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, CDCl_3) δ 27.1 (t), 32.1 (t), 34.6 (t), 42.8 (t), 52.4, 56.1, 62.7, 65.4 (s), 127.3, 127.7, 128.1, 128.3, 130.5, 131.4, 134.2 (s), 134.6 (s), 140.5 (s), 157.0, 166.1 (s), 168.8 (s), 172.3 (s), 194.9.

HRMS (ESI) calculated for $\text{C}_{26}\text{H}_{27}\text{N}_2\text{O}_5^+$ $[\text{M}+\text{H}]^+$ m/z 447.1914, found 447.1909.

Synthesis of methyl (3S,6S,9aR)-6-benzamido-6-benzyl-7-(2-bromo-1-hydroxyethyl)-5-oxo-2,3,5,6,9,9a-hexahydro-1H-pyrrolo[1,2-a]azepine-3-carboxylate (60a)

To a solution of **49a** (30 mg, 0.068 mmol, 1.0 equiv) in DCM (0.1 M), under nitrogen and at -20 °C, was added NBS (13 mg, 0.074 mmol, 1.1 equiv). The mixture was stirred for 20 min. The solvent was removed and the crude purified by flash chromatography (Hexane/EtOAc 45:55) to afford **60a** as an amorphous solid (30 mg, 82%). TLC: Hexane/EtOAc 50:50, R_f = 0.25

Spectroscopic and mass data:

¹H NMR (400 MHz, CDCl₃) δ 1.85 (m, 1H), 1.94–2.10 (m, 2H, 1H exchanges with D₂O), 2.28 (m, 1H), 2.57 (dd, J = 5.9, 18.1 Hz, 1H), 2.74 (m, 1H), 3.40–3.54 (m, 2H), 3.57–3.64 (m, 2H), 3.70 (s, 3H), 4.15 (m, 1H), 4.21 (m, 1H), 4.57 (d, J = 6.6 Hz, 1H), 5.90 (d, J = 5.0 Hz, 1H), 7.25 (br s, 6H), 7.39 (t, J = 7.4 Hz, 2H), 7.45 (t, J = 7.4 Hz, 1H), 8.11 (d, J = 7.6 Hz, 2H).

¹³C{¹H} NMR (100 MHz, CDCl₃) δ 27.3 (t), 31.5 (t), 32.8 (t), 35.3 (t), 48.2 (t), 52.3, 54.8, 62.4, 66.6 (s), 74.8, 125.7, 127.3, 128.0, 128.2 (2C), 128.3 (s), 130.3, 131.0 (2C, 1 s), 135.8 (s), 169.7 (s), 173.0 (s), 177.0 (s).

[α]²⁵_D = –190.5 (c = 0.116, CHCl₃).

IR (neat, cm^{–1}): 3148, 2941, 1737, 1646, 1446, 1274, 1198, 1173, 1026.

HRMS (ESI) calculated for C₂₇H₂₆BrN₂O₄⁺ [M–H₂O+H]⁺ m/z 523.1227; found 523.1220.

HRMS (ESI) calculated for C₂₇H₂₉N₂O₅⁺ [M–HBr+H]⁺ m/z 461.2071; found 461.2074.

Synthesis of Aba-decorated C₆₀ fullerene (61a)

A solution of **49a** (30 mg, 0.068 mmol, 1.0 equiv) and **fullerene C₆₀** (49 mg, 0.068 mmol, 1.0 equiv) in toluene (0.005 M) was heated at 80 °C for 24 h. The solvent was evaporated and the crude purified by flash chromatography (100% toluene → toluene/acetone 95:5) to afford **61a** as a black amorphous solid (48 mg, 61%). TLC: DCM/acetone 99:1, R_f = 0.37

Spectroscopic and mass data:

¹H NMR (400 MHz, CDCl₃) δ 1.99 (m, 1H), 2.04–2.14 (m, 2H), 2.20 (m, 1H), 2.67 (ddd, J = 7.5, 12.3, 12.4 Hz, 1H), 3.17 (td, J = 4.2, 12.4 Hz, 1H), 3.72 (s, 3H), 4.03 (d, J = 13.6 Hz, 1H), 4.18–4.39 (m, 4H), 4.46 (dd, J = 7.5, 11.0 Hz, 1H), 4.57 (dd, J = 6.2, 8.2 Hz, 1H), 7.29–7.41 (m, 9H), 7.64–7.73 (m, 3H).

¹³C{¹H} NMR (100 MHz, CDCl₃) δ 27.1 (t), 31.0 (t), 36.8 (t), 38.9 (t), 39.5 (t), 49.8, 52.5, 57.1, 61.3, 64.5 (s), 66.4 (s), 69.2 (s), 126.9, 127.2, 128.2, 128.3, 128.4, 130.8, 131.3, 135.0 (s, C₆₀), 135.5 (s, C₆₀), 135.6 (s), 135.7 (s, C₆₀), 135.9 (s, C₆₀), 136.0 (s), 138.2 (s), 140.0–147.7 (38 partially overlapped C₆₀ signals), 154.6 (s, C₆₀), 156.6 (s, C₆₀), 158.0 (s, C₆₀), 158.9 (s, C₆₀), 166.7 (s), 169.8 (s), 172.5 (s).

$[\alpha]_D^{25} = -338.3$ ($c = 0.005$, CHCl_3).

IR (neat, cm^{-1}): 3375, 3028, 2923, 2851, 1741, 1634, 1404, 1262, 1172, 1025.

HRMS (ESI) calculated for $\text{C}_{87}\text{H}_{29}\text{N}_2\text{O}_4^+$ $[\text{M}+\text{H}]^+$ m/z 1165.2122; found 1165.2151.

3.3.5 *Synthesis of 3a-RGD-decorated liposomes and biological evaluation*

Materials for liposome preparation

DSPE-PEG(2000)-DBCO (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[dibenzo-cyclooctyl(polyethylene glycol)-2000] (ammonium salt)), DMPC (14:0 PC, 1,2-dimyristol-sn-glycero-3-phosphocholine), Rhodamine (18:1 Liss Rhod PE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt)), and Gadolinium (DTPA-BSA, DTPA-bis(stearylamide) (gadolinium salt)) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Doxorubicin Hydrochloride was purchased from Sigma-Aldrich (Darmstadt, Germany). All chemicals have analytical grade and were obtained from Sigma-Aldrich and VWR chemicals. Throughout the process, all distilled water was filtered prior to using, with hydrophilic 0.22 μ m 13 mm syringe filters (Millex-GP). All purification steps of the liposomes were performed using 100 kDa Amicon filters. During purification, all centrifugal cycles were performed at 4000 rpm, 10°C for 15 minutes and washing of the liposomes occurred throughout three complete centrifugal cycles.

Synthesis of liposome

Thin-film hydration method

A lipid mix comprising DMPC, DBCO, Gadolinium, and Rhodamine was dissolved in chloroform at a molar ratio of 84:10:5:1, respectively. Subsequently, the solvent was removed through evaporation using a rotatory evaporator (Büchi R-215, Buchegg, Switzerland) under the conditions of 120 millibar, 280 rpm, for 10 min at 40 °C, followed by a final step under a nitrogen flux to ensure complete lipid desiccation. The resulting lipid film was rehydrated and resuspended in filtered distilled water, subjected to sonication at 40 °C for 1 h, and then underwent purification and washing with filtered distilled water.

Microfluidic method

To compare synthetic methods and improve the synthesis efficiency, liposomes were also prepared using the Dolomite (Royston, UK) microfluidic flow control device and software. Employing the same phospholipid composition (DMPC, DBCO, Gadolinium and Rhodamine)

and ratio (84:10:5:1, respectively), different temperatures and flow rates were studied to investigate the impact of changing the synthesis conditions on the size and polydispersity of the liposomes. The phospholipids were dissolved in ethanol ($\geq 99.8\%$ v/v) at a 2mg/ mL lipid concentration. The pre-prepared lipid phase was in one of the chambers, and the water was inserted to the second chamber. The lipid and aqueous phases were injected into a 5-input hydrophilic chip 3D with 150 μm channels diameter. Maintaining a constant flow rate ratio (FRR; 1:4, ethanol:water ratio) but changing the total flow rate (TFR; 0.5, 1 and 1.5 ml/min), different formulations were synthesized at 25 and 37 °C.

Doxorubicin encapsulation by remote loading strategy

The encapsulation of doxorubicin salt within plain liposomes was achieved through a remote loading strategy, involving the creation of distinct buffer gradients across the phospholipidic membrane. To facilitate the ion gradient essential for drug encapsulation, intra and extraliposomal buffers underwent exchange. In brief, liposomes were re-suspended in diammonium phosphate buffer (300 mM $(\text{NH}_4)_2\text{HPO}_4$, pH 7.4) and incubated on a roller for 1 hour at room temperature. Subsequently, the liposomes were subjected to purification with Amicon filters, washed with HEPES buffered saline (140 mM NaCl, 10 mM HEPES, pH 7.4) (HBS) to substitute the extra-liposomal solution, and filtered using a 0.22 μm 13 mm syringe filter (hydrophilic). The pH values of the utilized buffers were adjusted with 0.5 - 3.0 M HCl and 0.05 - 1.0 M NaOH. Meanwhile, Doxorubicin HCl (DOX) was dissolved in diammonium phosphate buffer (300 mM, pH 7.4) to achieve a final concentration of 0.1 mg/mL. The DOX solution was added to the liposomes at a drug-to- lipid molar ratio of 1:3 and incubated for 2 h at 65 °C in a shaker incubator operating at 300 rpm. Subsequently, the liposomes DOX underwent purification and washing with HBS. To assess the yield of DOX encapsulation, while purifying and washing liposomes DOX with HBS, the supernatant obtained from the initial centrifugal cycle, conducted prior to liposome washing, was preserved. Following the completion of the washing process, liposomes DOX were recovered from the filters and re-suspended in HBS. To assess encapsulation efficiency, the supernatant from the liposomes and the 0.1 mg/mL DOX solution initially administered to the liposomes were subjected to analysis using high-pressure liquid chromatography (HPLC), considering the dilution factor of the DOX solution during incubation with the liposomes. To

quantify DOX encapsulated in the liposomes, the following HPLC method (Wei et al., 2008) was applied. In brief, the mobile phase consisted of a mixture of methanol-water (60:40) containing 0.1% formic acid (pH 3) and the analysis were carried out using a Gemini® 5 µm NX-C18 110 Å LC Column (250 x 4.6 mm, Phenomenex, Alcobendas, Spain) at 35 °C with a flow rate of 1 mL/min. All samples were analyzed at 480 nm, being approximately 5 min the retention time of the DOX. Previously, all samples were evaporated at 50 °C and rehydrated in the same volume with the mobile phase, then they were analyzed as described previously. The obtained chromatogram was used to determine encapsulation of DOX in the liposomes. Each prepared sample was quantified by extrapolation with a calibration curve (R^2 : 0.997) prepared by using free DOX at known concentrations. Each sample was analyzed in triplicate. Toward this scope, a comparison was made between the peak area of DOX in the liposome supernatant and the initial total amount of drug provided at the onset of the remote loading process. The encapsulation efficiency was quantified using formula:

$$\text{Encapsulated DOX (\%)} = \frac{(\text{DOX offered} - \text{DOX in supernatant})}{\text{DOX offered}} \times 100$$

The HPLC equipment used was a LC-4000 HPLC system equipped with an interface box (LC–NetII/ADC), a photo diode-array detector (MD-4010), a RHPLC pump (PU-4180) and a column oven (CO-4061) (JASCO, USA). The software used for the analysis and peak integration was ChromNAV, a software system from JASCO–HPLC analysis (Tokyo, Japan).

Functionalization of liposomes DOX with 3a-RGD

The cRGD peptide was linked to the surface of the liposomes by a *SPAAC reaction*. **3a-RGD** was dissolved in pure methanol and added to a sodium bicarbonate buffer (12 mM, pH 8.5) at a 5-times molar excess with respect to the DBCO lipid previously quantified as described below. Meanwhile, the liposomes DOX were purified in Amicon filters after which they were resuspended in the prepared cRGD-peptide solution and incubated for 2 h at room temperature on a roller incubator. Finally, the cRGD-liposomes DOX were purified and filtered using HBS. To assess the yield of surface modification of the cRGD-peptide on the liposomes, the supernatant of the liposomes at the end of the *SPAAC reaction* as well as the initial cRGD-solution offered to the liposomes were analysed using HPLC. Briefly, the

analyses were carried out using a Gemini® 5 µm NX-C18 110 Å LC Column (250 ×4.6 mm, Phenomenex, Alcobendas, Spain) with a flow rate of 1 mL/min and a gradient condition starting for the initial 2 min with 80% H₂O + 0.1% CF₃COOH and 20% acetonitrile. Then, the gradient was increased to 80% acetonitrile during 6 min. Before the analysis, all samples were centrifuged and then measured as described above at 255 nm. The obtained chromatogram was used to determine cRGD surface modification rate of the liposomes by comparing cRGD representative peaks, being its retention time around 8 min. Each prepared sample was quantified by extrapolation with a calibration curve (R²: 0.998) prepared by using free cRGD-peptide at known concentrations. Each sample was analyzed in triplicate. The amount of cRGD-peptide immobilized over liposomes' surface was quantified by comparing the respective peak areas of the initial offered solution of 3a-RGD-N₃ peptide vs the liposome supernatant recovered at the end of the reaction. The yield of liposomes DOX functionalization with cRGD-peptide was determined using the following formula:

$$\text{cRGD surface functionalization (\%)} = \frac{(\text{cRGD offered} - \text{cRGD in supernatant})}{\text{cRGD offered}} \times 100$$

Physicochemical characterization

Hydrodynamic size and zeta-potential with dynamic light scattering (DLS)

Produced liposomes, including plain, liposomes DOX and cRGD-liposomes DOX, were analyzed in their hydrodynamic size distribution and surface z-potential using a Malvern Nano ZS-90 DLS instrument (ZEN3600, Worcestershire, UK) and analysed with Zetasizer Software (v7.04). For zeta-potential measurements, liposomes DOX and cRGD-liposomes DOX were diluted in a solution of KNO₃ (0.01 M), with pH was adjustments carried out using KOH (0.05 M – 3 M) and HNO₃ (0.5 M). Zeta-potential was measured in triplicate at various pH values ranging from 2 to 11.

Lipid concentration

For the quantification of lipids in the synthesized liposomes, a Stewart assay was conducted (Stewart, J. C. M. (1980). "Colorimetric determination of phospholipids with ammonium ferrothiocyanate." *Analytical Biochemistry*, 104(1), 10–14.). Briefly, samples were

evaporated overnight at 50 °C, followed by resuspension in chloroform and subsequent addition of ammonium ferrothiocyanate in a 1:1 (v/v) ratio. The resulting solutions were vortexed and centrifuged (1000 rpm, 10 min) leading to the separation of two phases. The chloroform layer was then analyzed in triplicate at a fixed wavelength of 488 nm using a JASCO V-730 spectrophotometer. The lipid concentration was determined by referencing the measurements against a previously established standard curve of these liposomes ($R^2 = 0.994$).

Magnetic resonance and fluorescence imaging phantoms

The magnetic properties of the synthesized liposomes, attributed to the presence of Gd, were studied through magnetic resonance imaging (MRI) at BioImaC (ICTS BioImagen Complutense), a node of the ICTS ReDIB (<https://www.redib.net/>), using a 1 Tesla benchtop MRI scanner (ICON 1T-MRI; Bruker BioSpin GmbH, Ettlingen, Germany). The ICON 1T-MRI, featuring a permanent magnet and a gradient system with a capability of supplying 450 mT/m gradient strength, employed a linear polarized solenoid RF-coil (inner diameter 61x52 mm² and length 90 mm). MRI data were collected using Paravision 6.0.1 software (Bruker, BioSpin). The primary MRI protocol involved a two-dimensional T1-weighted experiment. Coronal anatomical sections were acquired using a spin echo sequence with a repetition time of 750 ms, and echo time of 7.5 ms, one average, a field of view of 35x11 mm², a slice thickness of 1.5 mm, and a total of 5 slices. The matrix size was 350x110 (resolution 0.200 x 0.200 x 1.50 mm), and the total acquisition time was approximately 1 min. Additionally, due to the incorporation of the fluorescent lipid Liss Rhod PE (560/583 nm ex/em), fluorescent images at varying Gd-lipid concentrations were obtained using the IVIS Imaging System 200 series (Xenogen). Both imaging techniques were performed to samples at different known Gd concentrations: 5, 10, 50, 100 and 150 µg/mL.

To evaluate the efficiency of the synthesized liposomes as contrast agents, relaxation time measurements were also carried out in a MINISPEC MQ60 (Bruker) at 37 °C and a magnetic field of 1.5 T. The samples were prepared at different Gd concentrations and the relaxivity values (r_1 and r_2) were calculated by the linear fitting of the relaxation rates $R_{1,2}$ ($1/T_{1,2}$) values for each Gd concentration and blank solution.

In vitro studies

Cell lines and culture conditions

All cell lines were incubated at 37 °C and 5% CO₂. Cell lines A375 and MG63 were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotics (Penicillin/Streptomycin). PANC-1 cells were cultured in DMEM with 10% FBS, 1% Glutamine, and 1% antibiotics (Pen/Strep). Finally, MDA-MB-231 cells were cultured in DMEM - F12, containing 10% FBS, 1% non-essential amino acids (NEAA), 1% sodium pyruvate, and 1% antibiotics (Pen/Strep). 3D spheroids of MDA-MB-231 were generated as described by *Keller et al.* (Towards optimized breast cancer 3D spheroid mono- and co-culture models for pharmacological research and screening. *Journal of Cellular Biotechnology*. **2019**;5(2):89-101). Briefly, in 96-well spheroid microplates (Corning), 30,000 MDA-MB-231 cells per well were seeded and 2.5% v/v of basement membrane extract (BME/Cultrex,) were added before centrifugating for 6 min at 500 rcf. The achieved spheroids were cultured 3 days in the specific medium previously described before their use.

Integrin expression evaluation

Total of 3x10⁵ cells of A375, MDA-MB-231, PANC-1 and MG-63 cell lines were seeded in a 6-well culture plate. After 24 h, cells were fixed with 4% PFA pH 7.4 for 10 min at 37 °C and 5% CO₂. After washing 3 times with PBS 1X for 5 min each, a blocking solution of 5% Serum Goat in PBS 1X was added for 60 min at room temperature. After the blocking solution, the incubation of the primary antibody was carried out. Briefly, the CD51/CD61 (Integrin $\alpha_v\beta_3$) Monoclonal Antibody (23C6) (ThermoFisher Scientific) was incubated with the cells in 0.1% Serum Goat for 3 hours at RT. Cells were washed 3 times with PBS 1X for 5 min each and incubated with the secondary antibody. In this case, the Alexa Fluor 647 Goat Anti Rabbit IgG (H+L) antibody (ThermoFisher Scientific, REF: A-21,245) was incubated with the cells in 0.1% Serum Goat for 45 min at RT. After that, cells were washed 3 times with PBS 1X for 5 min each. Cell nucleus was stained with DAPI (0.1% in media without phenol red) for 15 min. A control without the primary antibody was also studied to compare the specific binding of the secondary antibody to the primary one. Images were obtained using a 20X objective in a confocal laser scanning microscope Zeiss LSM 780 (Ex/Em 647/670 nm for secondary

antibody and 405/457 nm for DAPI) under 37 °C and 5% CO₂ conditions and they were processed with ImageJsoftware.

Cellular uptake

3 x 10⁵ MDA-MB-231 cells were seeded in a 6-well culture plate. After 24 h of cellular growing, cells were incubated other 24 h with the functionalized and non-functionalized liposomes (with or without 3a-RGD but both without DOX) in a 0.01 mg/mL final lipid concentration. Then, cell nucleus was stained with DAPI (0.1% in media without phenol red) for 15 min. Images were obtained using a 20X objective in a confocal laser scanning microscope Zeiss LSM 780 (Ex/Em 560/583 nm for Rhodamine and 405/457 nm for DAPI) under 37 °C and 5% CO₂ conditions and they were processed with ImageJ software. Additionally, another 6-well culture plate was seeded with 3 x 10⁵ MDA-MB-231 cells per well and allowed to grow for 24 h. Cells were then pre-incubated for 1 h with free cRGD peptide at a final concentration of 0.25 mg/mL. After this blocking step, the medium was replaced with fresh medium containing either functionalized or non-functionalized liposomes (with or without cRGD) at a final lipid concentration of 0.01 mg/mL. Following a 4-hour incubation, images were acquired using a Zeiss LSM 780 confocal laser scanning microscope with a 20x objective (Ex/Em 560/583 nm for Rhodamine), under standard culture conditions (37 °C, 5% CO₂). Image processing was performed using ImageJ software. Finally, cellular uptake was also evaluated in 3D models. MDA-MB- 231 spheroids were incubated for 4 h with either cRGD-functionalized or non-functionalized liposomes. Images were acquired using a Nikon A1-R confocal microscope equipped with a 10 ×objective (Ex/Em 560/ 583 nm for Rhodamine), under standard culture conditions (37 °C, 5% CO₂). Image processing and fluorescence quantification were carried out using ImageJ software.

Cell viability

The MTT toxicity assay was carried out in the A375, MDA-MB-231, PANC-1 and MG-63 cell lines. DMEM without phenol red was used for the MTT assay to avoid interferences. Cells were seeded in a 96-wells plate to yield circa 80% confluency at the day of incubation with the liposomes. The cells were incubated for 24, 48, and 72 h (37 °C, 5% CO₂) with either liposomes DOX or cRGD-liposomes DOX at a DOX concentration ranging from 0.1-10 µM.

After incubation with the liposomes, the supernatants were discarded, replaced with 100 μ L of medium and 10 μ L of MTT solution (5 mg/ mL) was added. After a 3 hours incubation (37 °C, 5% CO₂), the formazan salts generated were dissolved with DMSO and plates were analysed using the SPECTROstarNano (BMG Labtech INC). The same protocol was followed to perform the MTT cell viability assay in spheroids.

Cell migration

MDA-MB-231 cells (1.5×10^4 cells in 25 μ L of medium) were seeded into each chamber of Allegro silicone inserts (Stencell™, Idylle Labs, Paris, France) placed in a 24-well plate. After 24 h of incubation to allow cell attachment and monolayer formation, the silicone inserts were carefully removed to create a defined cell-free gap. Cells were then treated with free DOX, liposomes DOX, or cRGD-liposomes DOX at concentrations of 0.1 μ M for 24 h. Wound healing was monitored by imaging the same fields at 0, 8, 24, and 48 h using a Motic AE31E inverted trinocular microscope and comparing the remaining uncovered areas during the time.

Statistics analysis

All experiments were performed at least in triplicate ($n = 3$), and data are presented as mean $\pm$ standard deviation (SD). Statistical analysis was carried out using GraphPad Prism. For comparison between multiple groups, one-way analysis of variance (one-way ANOVA) followed by Tukey's post hoc test was applied. In experiments involving two independent variables (e.g., concentration and formulation), two-way ANOVA followed by appropriate multiple comparison tests was used. A p-value < 0.05 was considered statistically significant. Statistical significance levels were defined as follows:

$p < 0.05$ (*),

$p < 0.01$ (**),

$p < 0.001$ (***),

$p < 0.0001$ (****).

3.3.6 Synthesis of 3a-RGD-decorated chitosan NPs and biological evaluation

General Methods

All reagents and solvents were purchased from commercial suppliers and used as received unless otherwise specified. Phosphate-buffered saline (PBS, pH 7.4) and Milli-Q water were used for all nanoparticle preparations and purification steps. Sonication was performed using a bath sonicator to ensure homogeneous nanoparticle dispersion. Centrifugation steps were carried out at 13 000 rpm at 25 °C for 15 minutes for each cycle. Lyophilization was performed to obtain dry nanoparticle powders for storage.

Preparation of chitosan-coated PLGA nanoparticles

Chitosan-coated PLGA nanoparticles were prepared using an emulsification/solvent evaporation method. Briefly, poly(lactic-co-glycolic acid) (PLGA) and oleic acid (OA) were dissolved in ethyl acetate to form the organic phase, while chitosan hydrochloride (Chitosan-HCl, degree of deacetylation 0.8) was dissolved in Milli-Q water to obtain the aqueous phase. A molar ratio of 1.4:1 (OA:chitosan) was employed to promote hydrophobic interactions and enable the in situ formation of chitosan–oleate complexes. The organic phase containing PLGA and OA was then added dropwise to the aqueous chitosan solution under continuous magnetic stirring, leading to the formation of a coarse emulsion. This emulsion was subsequently subjected to probe sonication (Fisherbrand™) for 10 minutes (15 s on/15 s off cycles, 30% amplitude) to reduce droplet size and improve homogeneity. Following emulsification, the organic solvent was removed by evaporation at room temperature, resulting in the formation of chitosan-coated nanoparticles dispersed in the aqueous phase. The resulting nanoparticle suspension was stored at 4 °C until further use.

Functionalization of chitosan-coated PLGA nanoparticles

Chitosan nanoparticles (CS-NPs) were provided as a suspension in phosphate-buffered saline (PBS, pH 7.4) at a concentration of 9 mg·mL⁻¹. All reactions were performed under magnetic stirring following preliminary sonication to ensure homogeneous dispersion. Purification steps were carried out by dialysis using Milli-Q water or PBS as specified.

Fluorescent Functionalization of CS-NPs (CF-NHS)

CS-NPs (500 μL , 4.5 mg) were sonicated and transferred to a reaction vial under vigorous stirring. A solution of 5(6)-carboxyfluorescein N-hydroxysuccinimide ester (CF-NHS, MW = 473.39 $\text{g}\cdot\text{mol}^{-1}$) was prepared by dissolving 1 mg in 27.5 μL of DMF. An aliquot of this stock solution (1.17 μL , 90 nmol, corresponding to 20 $\text{nmol}\cdot\text{mg}^{-1}$ relative to nanoparticles) was added to the nanoparticle suspension. The reaction mixture was stirred for 24 h at room temperature. The resulting functionalized nanoparticles were purified by dialysis against Milli-Q water (100 mL) for 48 h with solvent replacement every 2 h. Completion of purification was confirmed by fluorimetric analysis of the dialysate. The purified nanoparticles were recovered and lyophilized.

Functionalization of CS-NPs with Alkyne-Containing NHS Linker

CS-NPs (3 mL, 27 mg) were sonicated and placed under vigorous stirring. A stock solution of the compound **41** (MW = 211.17 $\text{g}\cdot\text{mol}^{-1}$) was prepared by dissolving 1 mg in 25 μL of DMF. An aliquot (2.85 μL , 540 nmol, corresponding to 20 $\text{nmol}\cdot\text{mg}^{-1}$) was added to the nanoparticle suspension. The reaction was allowed to proceed for 24 h under stirring. The resulting nanoparticles were purified by dialysis against PBS (pH 7.4, 100 mL) for 24 h with solvent changes every 2 h. The recovered suspension (2.3 mL) was divided into aliquots for subsequent click reactions.

Click Functionalization with Azide Fluor 545

An aliquot of alkyne-functionalized CS-NPs (corresponding to 5.87 mg) was sonicated and stirred. Azide Fluor 545 was dissolved in DMF (1 mg in 100 μL), and an aliquot (14.8 μL , 234.8 nmol, 40 $\text{nmol}\cdot\text{mg}^{-1}$) was added to the nanoparticle suspension. $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$ (2.94 μL , 0.01 M, 29.35 nmol) and sodium ascorbate (11.74 μL , 0.01 M, 117.4 nmol) were added. The reaction mixture was stirred for 72 h at room temperature. Purification was carried out by dialysis against Milli-Q water (100 mL) for 72 h with solvent replacement every 2 h. The purified nanoparticles were recovered and lyophilized.

Click Functionalization with 3a-RGD Peptide

Alkyne-functionalized CS-NPs were reacted with 3a-RGD-N₃ (MW = 852.44 g·mol⁻¹) using a ratio of 40 nmol·mg⁻¹ (total: 845.2 nmol). The peptide was dissolved in methanol to obtain a 10 mg·mL⁻¹ stock solution (3.3 mg in 330 µL), and an aliquot (72 µL) was added to the nanoparticle suspension. After sonication and stirring, CuSO₄·5H₂O (10.57 µL, 0.01 M, 105.65 nmol) and sodium ascorbate (42.3 µL, 0.01 M, 422.6 nmol) were added. The reaction was allowed to proceed for 72 h under stirring. Purification was performed by dialysis against Milli-Q water (100 mL) for 72 h with solvent changes every 2 h. The nanoparticles were then recovered and lyophilized.

Post-Functionalization Dialysis and Secondary Fluorescent Labeling

RGD-functionalized CS-NPs were further dialyzed twice against PBS (pH 7.4) to restore physiological conditions. The resulting suspension (1.5 mL, corresponding to 21.13 mg of nanoparticles) was sonicated, and an aliquot (300 µL) was used for additional fluorescent labeling. Azide Fluor 545 (5.34 µL from a stock solution of 1 mg in 100 µL DMF, 84.52 nmol, corresponding to 20 nmol·mg⁻¹) was added to the suspension. CuSO₄·5H₂O (2.14 µL, 0.01 M, 21.13 nmol) and sodium ascorbate (2.14 µL, 0.01 M, 21.13 nmol) were then introduced. The reaction mixture was stirred for 72 h, followed by purification via dialysis against Milli-Q water (100 mL) for 72 h with solvent replacement every 2 h. The final nanoparticles were recovered and lyophilized.

In vitro studies

Cell viability

The MTT toxicity assay was carried out in RPMI 2650 cells (human nasal epithelial cell line). Cells were cultured in Dulbecco's Modified Eagle Medium, high glucose (DMEM-HG), supplemented with antibiotics. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. RPMI 2650 cells were seeded in 96-well plates at a density of 1.0 × 10⁴ cells per well and allowed to adhere for 48 h under standard culture conditions. Nanoparticle formulations (fluorescein-labeled nanoparticles, azide-functionalized nanoparticles, and RGD-functionalized nanoparticles) were dispersed in DMEM-HG (without phenol red and without fetal bovine serum) and tested at concentrations of 300, 150, 75, 37.5, 18.75, 9.38,

and $4.69 \mu\text{g}\cdot\text{mL}^{-1}$. Following incubation with nanoparticles for 24 h, the culture medium was removed and replaced with 100 μL of MTT solution ($0.5 \text{ mg}\cdot\text{mL}^{-1}$). After 3 h of incubation at 37 °C, the MTT solution was discarded, and the resulting formazan crystals were dissolved in 100 μL of DMSO. Absorbance was measured using a microplate reader at 570 nm with a reference wavelength of 670 nm. Untreated cells were used as control (100% viability). All experiments were performed in triplicate.

In Vitro Permeability Assay (Transwell Model)

RPMI 2650 cells were seeded in Transwell® inserts (24-well format) at a density of 1.6×10^5 cells per insert and cultured under submerged conditions for 7 days, with culture medium present in both apical and basolateral compartments. Subsequently, the cells were maintained at an air-liquid interface (ALI) for an additional 3 weeks by removing the medium from the apical compartment while maintaining medium in the basolateral compartment. The culture medium was replaced every 2-3 days. For permeability experiments, nanoparticle formulations were applied to the apical compartment (200 μL), while 600 μL of fresh culture medium was added to the basolateral compartment. The following formulations were tested:

- RGD-functionalized nanoparticles (150 and $75 \mu\text{g}\cdot\text{mL}^{-1}$)
- Fluorescein-labeled nanoparticles ($75 \mu\text{g}\cdot\text{mL}^{-1}$)
- Azide-functionalized nanoparticles ($75 \mu\text{g}\cdot\text{mL}^{-1}$)

At predetermined time points (30, 60, and 120 min), 300 μL aliquots were collected from the basolateral compartment and replaced with an equal volume of fresh medium. Fluorescence intensity was measured using a microplate reader (BMG Labtech) with the following excitation/emission settings:

- Fluorescein: $\lambda_{\text{ex}} = 492 \text{ nm}$, $\lambda_{\text{em}} = 526 \text{ nm}$
- Azide Fluor 545: $\lambda_{\text{ex}} = 545 \text{ nm}$, $\lambda_{\text{em}} = 590 \text{ nm}$

Statistical analysis

All experiments were performed in triplicate ($n = 3$), and data are reported as mean $\pm$ standard deviation (SD). Statistical analyses were carried out using GraphPad Prism (or equivalent software). Differences between multiple groups were evaluated using one-way

analysis of variance (one-way ANOVA) followed by Tukey's post hoc test. For experiments involving multiple variables (e.g., time and formulation), two-way ANOVA followed by appropriate multiple comparison tests was applied.

3.3.7 *Synthesis of 3a-RGD-decorated silk fibroin NPs and biological evaluation*

General Methods

All reagents and solvents were purchased from commercial suppliers and used as received unless otherwise specified. Phosphate-buffered saline (PBS, pH 7.4) and Milli-Q water were used for all nanoparticle preparations and purification steps. Sonication was performed using a bath sonicator to ensure homogeneous nanoparticle dispersion. Centrifugation steps were carried out at 13 000 rpm at 25 °C for 15 minutes for each cycle. Lyophilization was performed to obtain dry nanoparticle powders for storage.

Preparation of silk fibroin nanoparticles

Preparation of FITC-labelled Silk Fibroin Nanoparticles

Silk fibroin was extracted from 5 g of *Bombyx mori* cocoons, which were cut into small pieces (1 × 1 cm), washed under running water, and degummed by boiling in 2 L of 0.02 M aqueous Na₂CO₃ for 30 minutes with occasional stirring. The fibroin was then rinsed with distilled water to remove residual salts and sericin and dried at room temperature. Once completely dry, the fibroin was solubilized in aqueous LiBr solution (100% w/v) at 65 °C for 4 hours, with stirring every 30 minutes to promote solubilization and the conformational transition from Silk II to Silk I. The resulting solution was filtered through a 70 µm easySTRAINER (VWR International, Milan, Italy) to remove solid residues, transferred into a dialysis membrane (3.5 kDa cut-off; Spectrum Laboratories, Milan, Italy), and dialyzed against distilled water for 72 hours at room temperature. The final fibroin concentration, determined gravimetrically after complete water evaporation, was typically between 6-9% w/v.

For fluorescent labeling, fibroin was conjugated with FITC to prepare fluorescent SFNs. The number of available lysine residues was first calculated and FITC was added to target approximately 10% of these residues. Fibroin was diluted to 1.67% w/v in 0.1 M carbonate/bicarbonate buffer (pH 9) and supplemented with FITC stock solution (9.8 × 10⁻³ M in DMSO) at a 1:2 fibroin:FITC molar ratio. The reaction proceeded overnight at room temperature, protected from light.

Fluorescent SFNs were then prepared by diluting the labeled fibroin to 1.5% w/v and adding it dropwise to acetone under magnetic stirring at a fibroin:acetone ratio of 1:5 to induce the conformational transition from Silk I to Silk II. The resulting nanoparticle suspension was dialyzed against distilled water for 72 hours using cellulose dialysis tubes (3.5 kDa cut-off; Spectrum Laboratories, Milan, Italy), followed by lyophilization (Christ Epsilon 2-16D LSCplus) at -50 °C and 0.8 mbar. The dry nanoparticles were stored at room temperature until use.

Preparation of Alkyne-Functionalized Silk Fibroin Nanoparticles

FITC-labeled silk fibroin nanoparticles (SF-NPs, 97 mg) were suspended in PBS (9.7 mL, pH 7.4) to obtain a concentration of 10 mg·mL⁻¹. The suspension was sonicated and stirred vigorously at room temperature. The compound **41** was dissolved in DMF (1 mg in 25 µL), and an aliquot (10.21 µL, 1940 nmol, corresponding to 20 nmol·mg⁻¹ relative to nanoparticles) was added to the nanoparticle suspension. The reaction mixture was stirred at room temperature for 24 h. Upon completion, the mixture was divided into aliquots and centrifuged. The supernatant was discarded, and the pellet was redispersed in Milli-Q water and sonicated. This washing cycle was repeated three times. The purified nanoparticles were lyophilized to afford alkyne-functionalized silk fibroin nanoparticles (76 mg).

CuAAC Functionalization with Glucose-N₃ and 3aRGD-N₃ (Dual-Functionalized Nanoparticles)

Alkyne-functionalized nanoparticles (30 mg) were suspended in PBS (1.5 mL, pH 7.4) to obtain a concentration of 20 mg·mL⁻¹. The suspension was sonicated and stirred vigorously. **Glucose-propyl-N₃** (3-Azidopropyl D-glucopyranoside, 75 nmol) was added from a methanolic stock solution (33.1 mg in 2.5 mL MeOH; 1.48 µL added). Subsequently, 3a-RGD-N₃ (75 nmol) was introduced from a methanolic stock solution (2.2 mg in 2.0 mL MeOH; 58 µL added). The CuAAC reaction was initiated by addition of CuSO₄·5H₂O (15 µL, 0.01 M, 150 nmol) and sodium ascorbate (15 µL, 0.01 M, 150 nmol). The reaction mixture was stirred at room temperature for 64 h. After completion, the nanoparticles were purified by centrifugation followed by redispersion in Milli-Q water and sonication. The washing procedure was repeated three times. The final product was lyophilized and stored at 5 °C.

Physicochemical characterization

Particle size distribution

Particle size distribution was measured after dispersing each freeze-dried sample in deionized water at 0.1 mg/mL and sonicating for 1 min. Measurements were performed using a NanoSight NS300 (Malvern Panalytical, Grovewood Rd, UK) with Nanoparticle Tracking Analysis (NTA) technology. Each batch was analyzed in triplicate, with five 90-second captures per measurement.

In vitro studies

Cell viability

The MTT toxicity assay was carried out in the U87, HeLa and hCMEC/D3 cells lines. cells were seeded in 96-well plates (3000 cells/well) and cultured for 72 h at 37 °C in a humidified 5% CO₂ atmosphere using their respective complete culture media. HeLa and U87 cells were maintained in DMEM high glucose (DMEM HG) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin, and 1% (v/v) amphotericin. hCMEC/D3 cells were cultured in complete endothelial growth medium consisting of Endothelial Basal Medium-2 (EBM-2, Lonza, Milan, Italy) supplemented with 10% fetal calf serum (heat-inactivated at 56 °C for 30 min), 1% penicillin-streptomycin, 1% chemically defined lipid concentrate (Gibco), ascorbic acid (5 µg/mL), HEPES (10 mM), and basic fibroblast growth factor (bFGF, 1 ng/mL). To promote adhesion, hCMEC/D3 cells were seeded on collagen I-coated multiwell plates. Cells were then treated with 100 µL of culture medium containing SFNs at concentrations of 0.8 and 0.4 mg/mL. After 24 h of incubation, cell metabolic activity was evaluated using the MTT assay, as previously described.

Cellular uptake

U87, HeLa and hCMEC/D3 cells were seeded in 96-well black plates (7500 cells per well) in 250 µL of cell suspension using the respective complete culture media described above and incubated for 4 days at 37 °C in a humidified 5% CO₂ atmosphere. The culture medium was then replaced with serum-free, phenol red-free medium containing SFNs. Nanoparticles were tested at final concentrations of 0.2 and 0.4 mg/mL and incubated for 30, 60 and 120

min. At the end of the incubation period, the medium was removed, cells were washed three times with PBS, and intracellular fluorescence (FITC-labelled fibroin) was measured using a plate reader (Victor Nivo, PerkinElmer, Waltham, MA, USA; excitation 480 nm, emission 530 nm). Untreated cells were used as controls.

A linear generalized analysis of variance (ANOVA) model was applied to analyze normally distributed data, and differences between groups were assessed using Fisher's LSD post hoc test (STATGRAPHICS XVII, Statpoint Technologies, Inc., Warrenton, VA, USA). Cell line, sample type, concentration, and incubation time were considered fixed factors, while fluorescence intensity was used as the response variable.

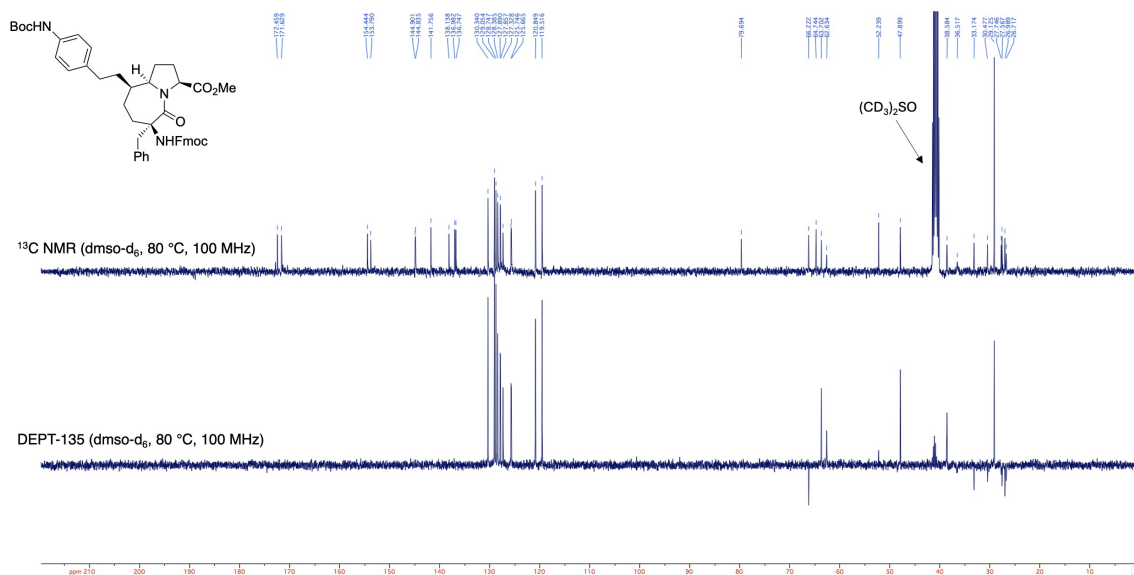
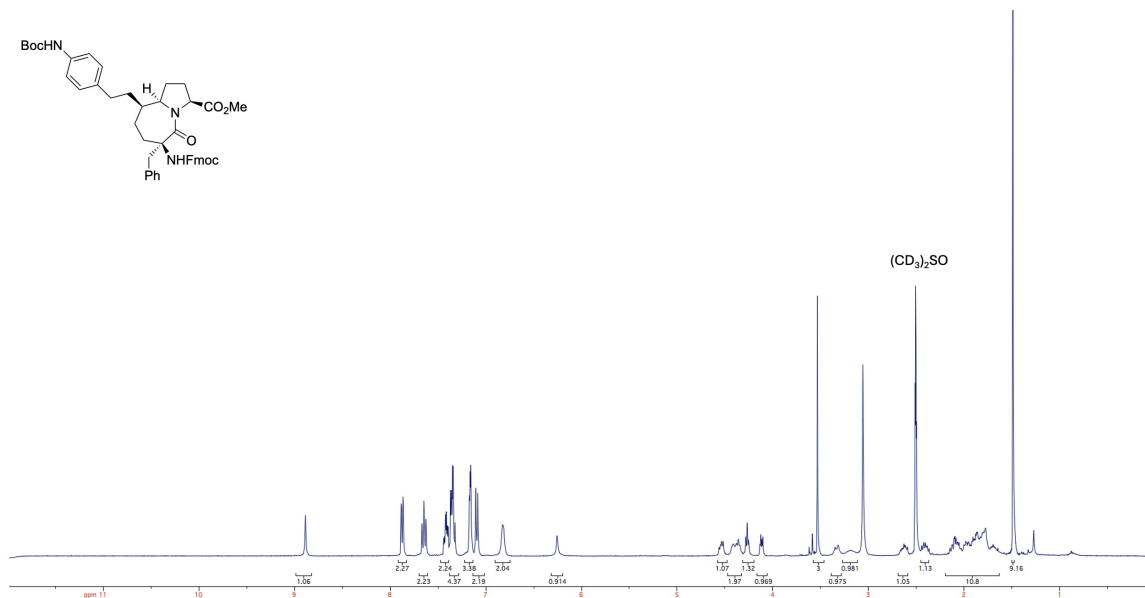
In Vitro Permeability Assay (Transwell Model)

hCMEC/D3 cells were seeded onto Transwell® inserts (3 µm pore size, 0.33 cm²) previously coated with type I collagen to promote cell adhesion. A total of 70,000 cells per insert were cultured in complete endothelial growth medium (see above) for 5 days, until the formation of tight junctions was confirmed by measuring the transendothelial electrical resistance (TEER), which exceeded 40 Ω·cm². SFN samples were then added to the apical compartment (400 µL) at a concentration of 0.4 mg/mL. Each Transwell insert was placed into a well of a 24-well plate, and 1 mL of complete culture medium (see above) was added to the basolateral compartment. After 1 and 4 h of incubation, fluorescence was measured in both the apical and basolateral compartments to assess nanoparticle transport across the endothelial monolayer using a plate reader (Victor Nivo, PerkinElmer, Waltham, MA, USA; excitation 480 nm, emission 530 nm).

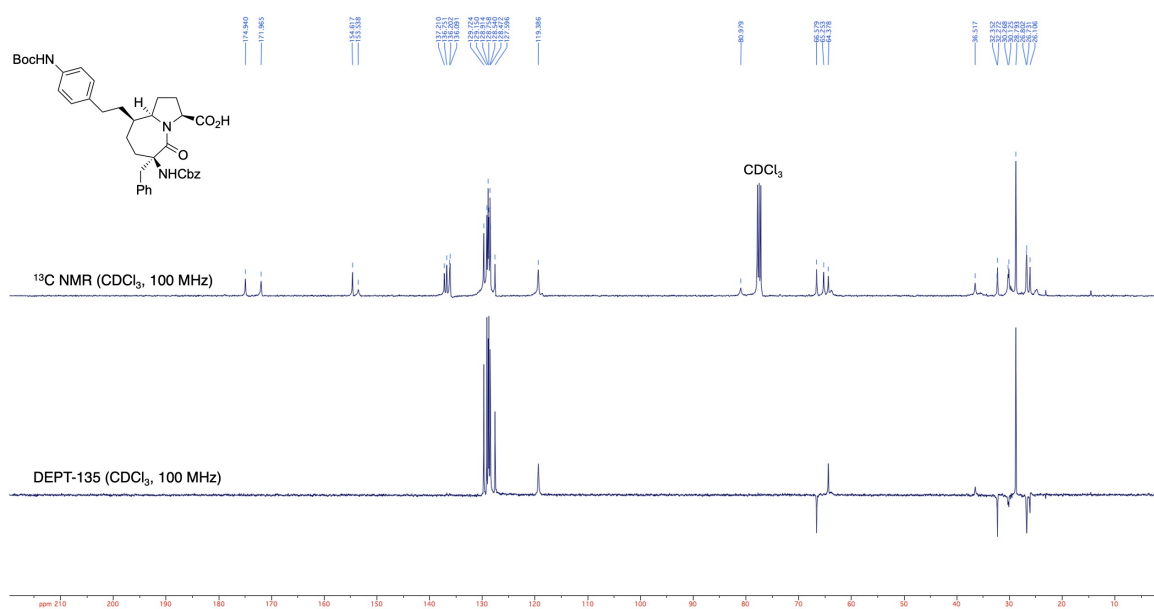
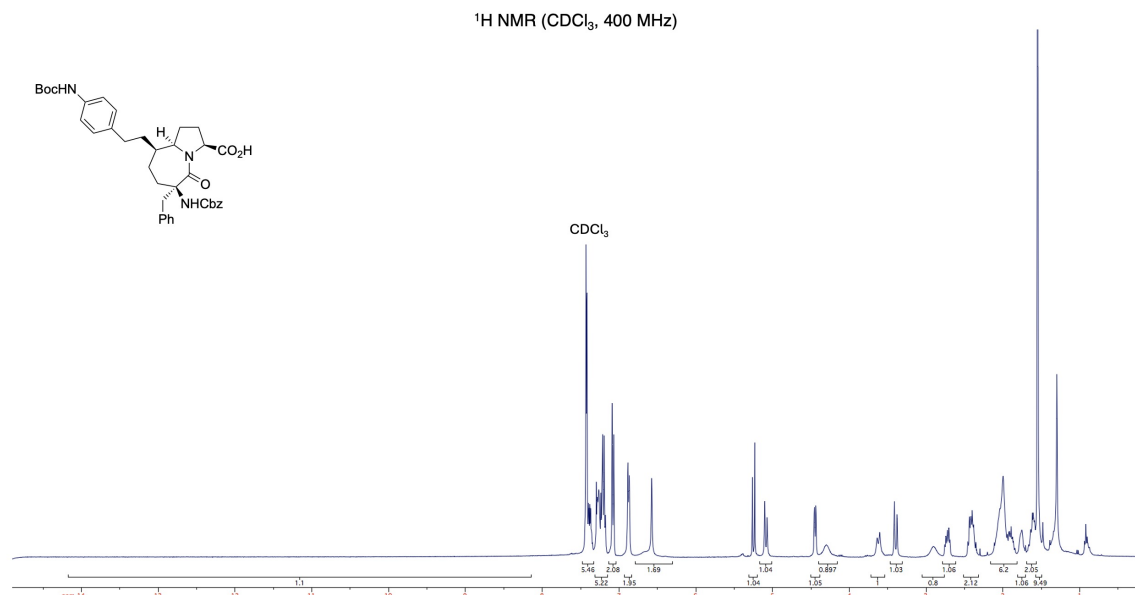
Statistics analysis

A linear generalized analysis of variance (ANOVA) model was applied to analyze normally distributed data, and differences between groups were assessed using Fisher's LSD post hoc test (STATGRAPHICS XVII, Statpoint Technologies, Inc., Warrenton, VA, USA). Sample type, the compartment where fluorescence was measured, and incubation time were considered fixed factors, while fluorescence intensity was used as the response variable.

Methyl (3S,6R,9S,9aR)-6-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-benzyl-9-(4-((tert-butoxycarbonyl)amino)phenethyl)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepine-3-carboxylate (27a)

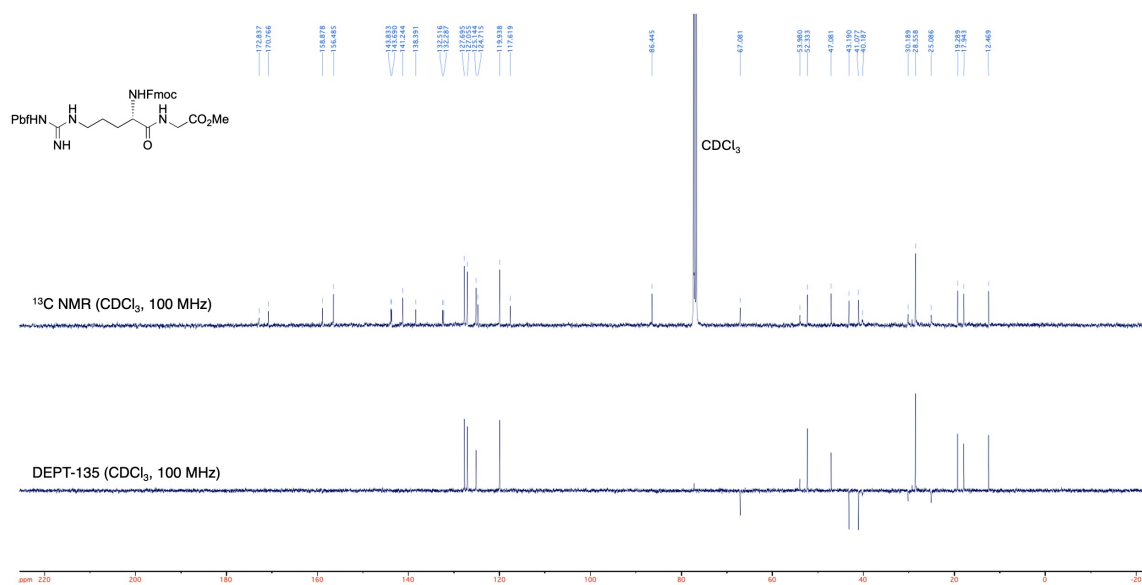
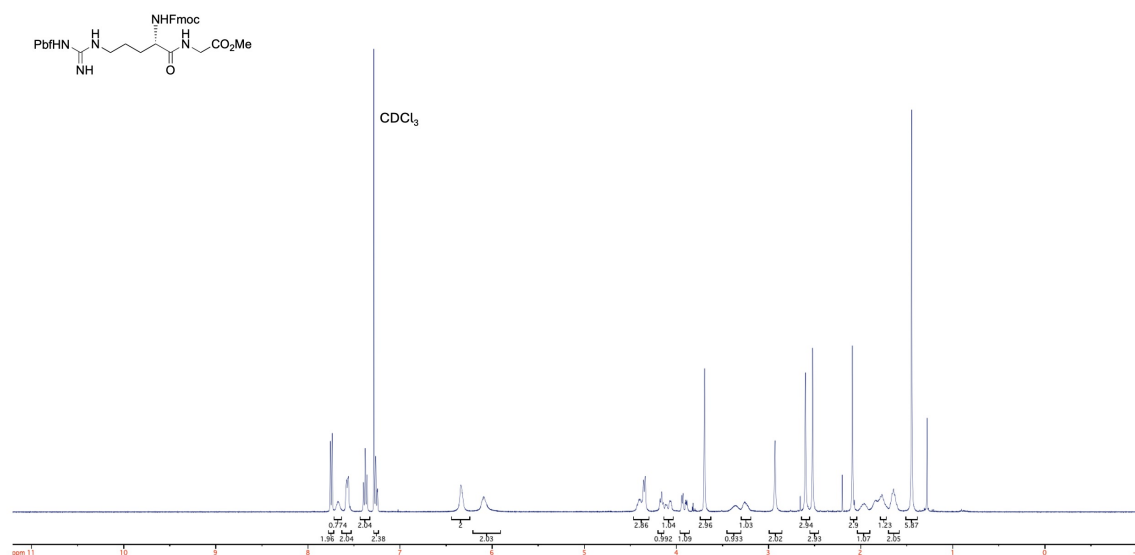
¹H NMR (dms_o-d₆, 80 °C, 400 MHz)

(3S,6R,9S,9aR)-6-benzyl-6-(((benzyloxy)carbonyl)amino)-9-(4-((tert-butoxycarbonyl)amino)phenethyl)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepine-3-carboxylic acid (30a)

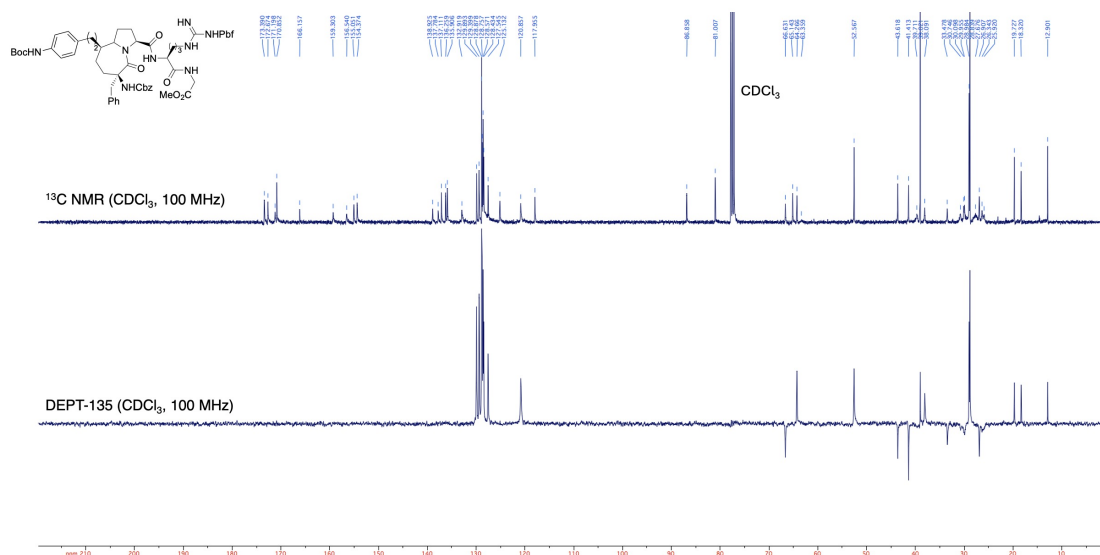
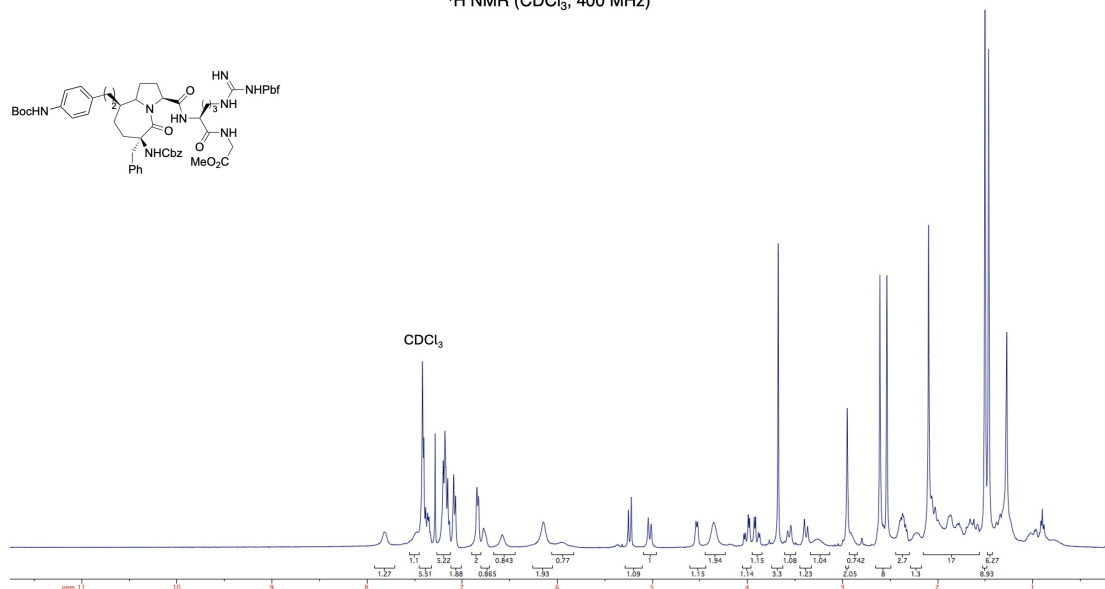


Methyl N²-(((9H-fluoren-9-yl)methoxy)carbonyl)-N^ω-((2,2,6,7-tetramethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)-L-arginylglycinate (31)

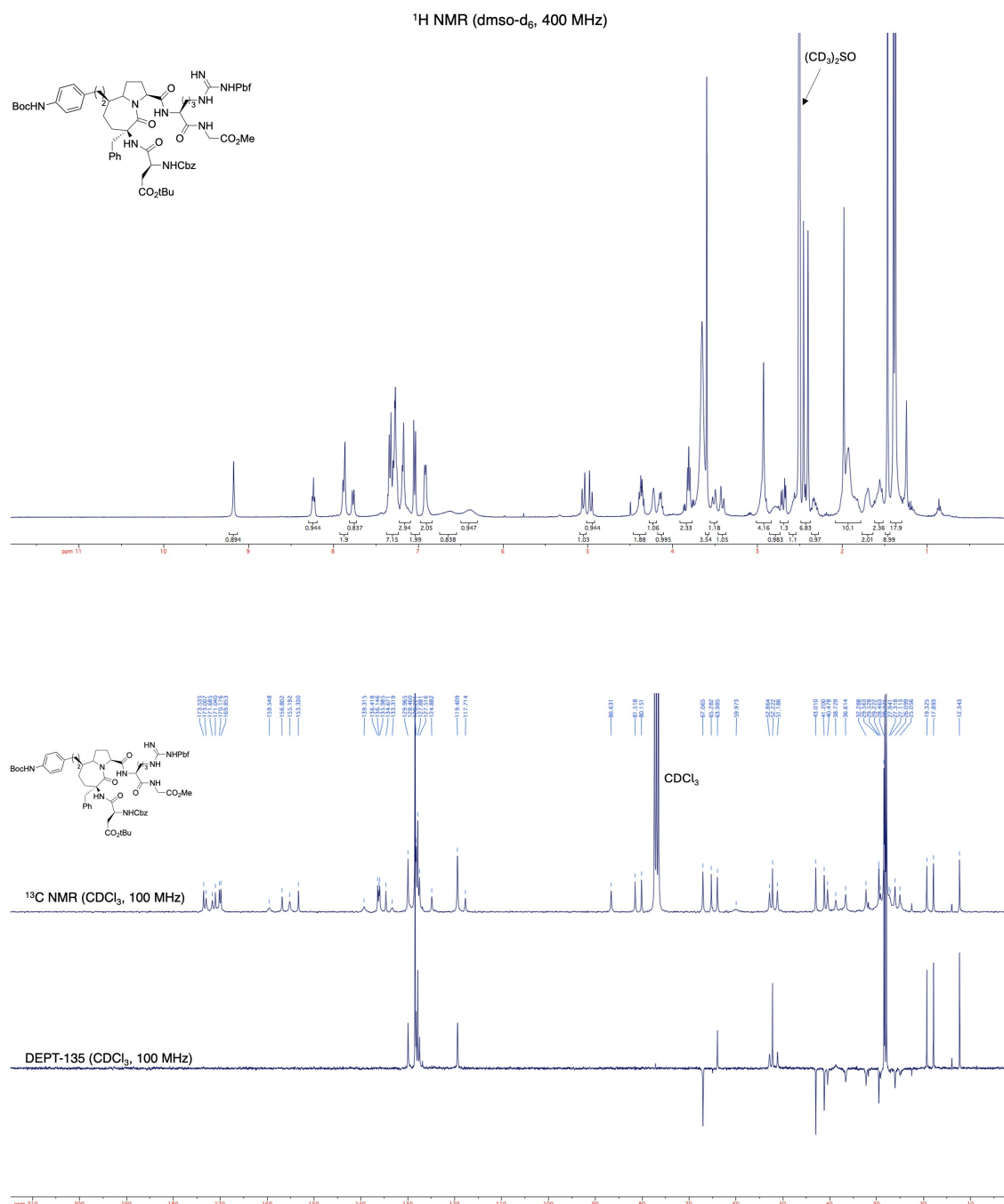
¹H NMR (CDCl₃, 400 MHz)



Methyl N²-((3S,6R,9S,9aR)-6-benzyl-6-(((benzyloxy)carbonyl)amino)-9-(4-((tert-butoxycarbonyl)amino)phenethyl)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepine-3-carbonyl)-N^ω-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)-L-arginylglycinate (33)

¹H NMR (CDCl₃, 400 MHz)

tert-butyl (S)-4-(((3S,6R,9S,9aR)-6-benzyl-9-(4-((tert-butoxycarbonyl) amino) phenethyl)-3-(((S)-1-((2-methoxy-2-oxoethyl)amino)-1-oxo-5-(3-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)guanidino)pentan-2-yl)carbamoyl)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepin-6-yl)amino)-3-(((benzyloxy)carbonyl)amino)-4-oxobutanoate (34)



¹H NMR (dmsd-d₆, 60 °C, 400 MHz)

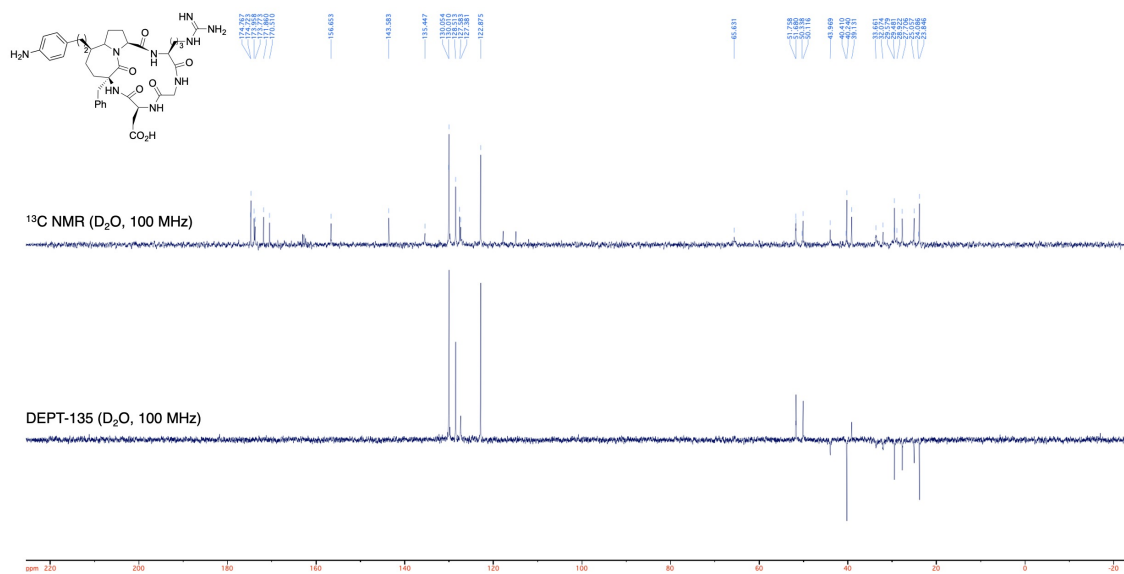
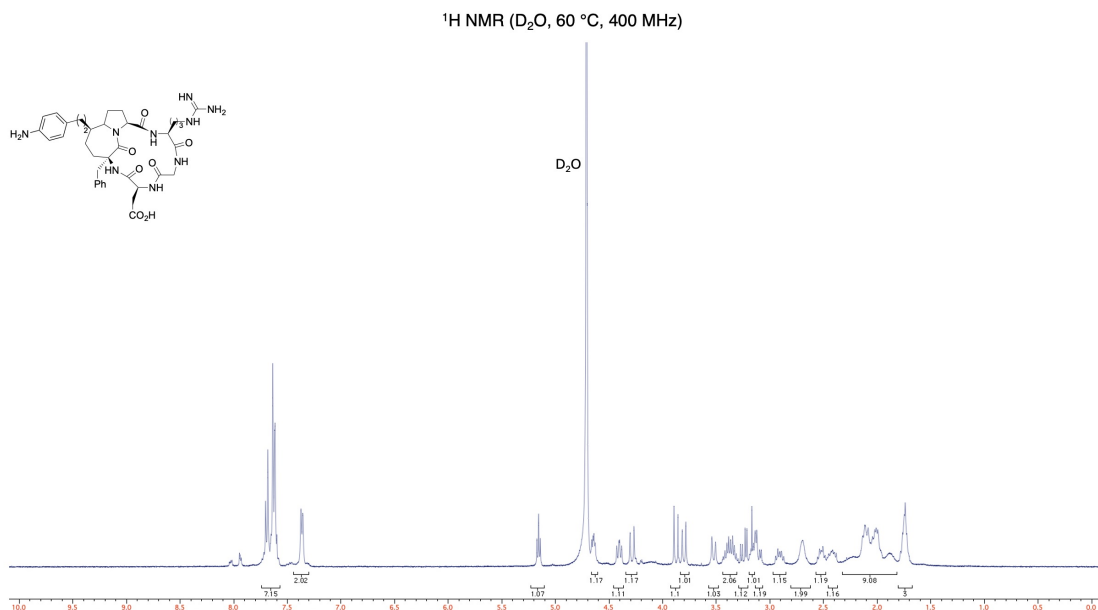
(CD₃)₂SO

¹³C NMR (dmsd-d₆, 60 °C, 100 MHz)

(CD₃)₂SO

DEPT-135 (dmsd-d₆, 60 °C, 100 MHz)

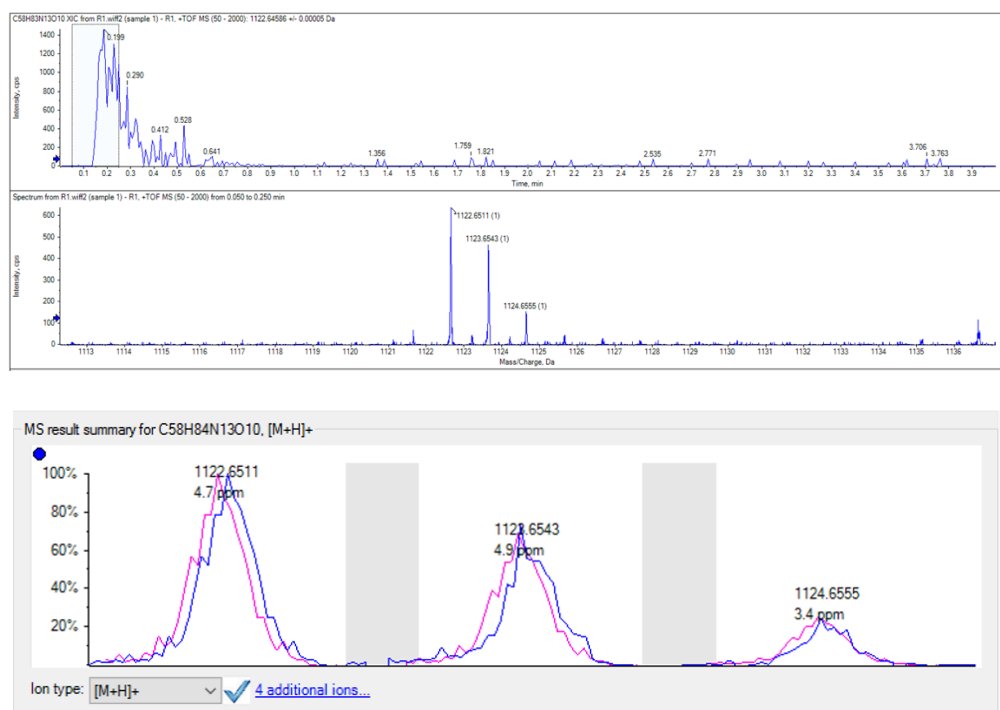
3aRGD



3.4.2 Synthesis of NDI-Linker-3a-RGD

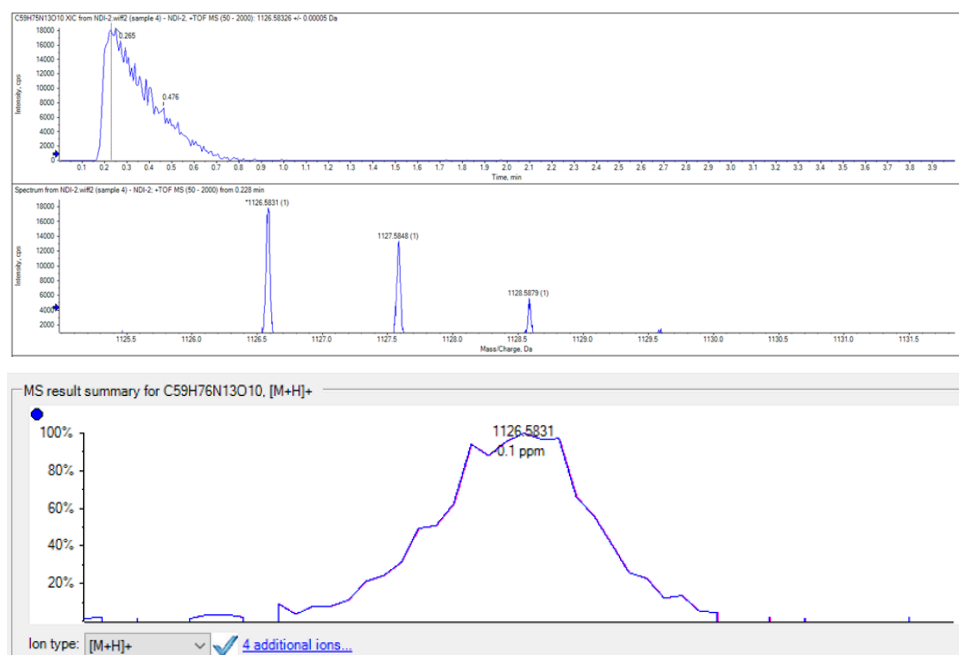
NDI 1-Linker (1)

HRMS analysis



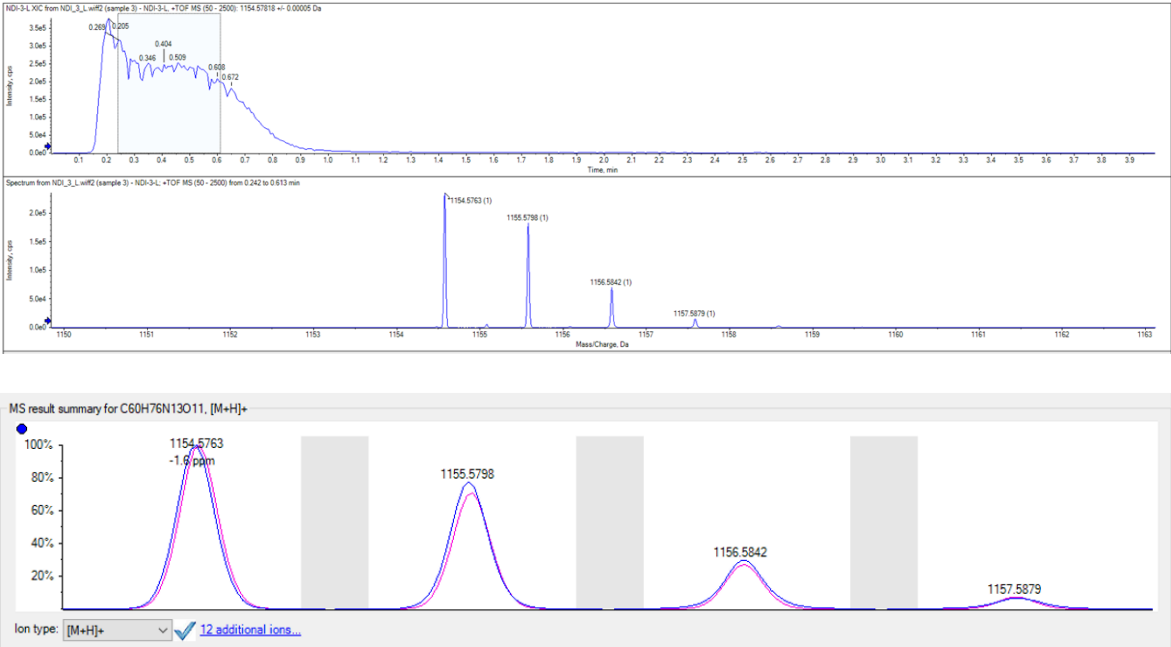
NDI 2-Linker (1)

HRMS analysis



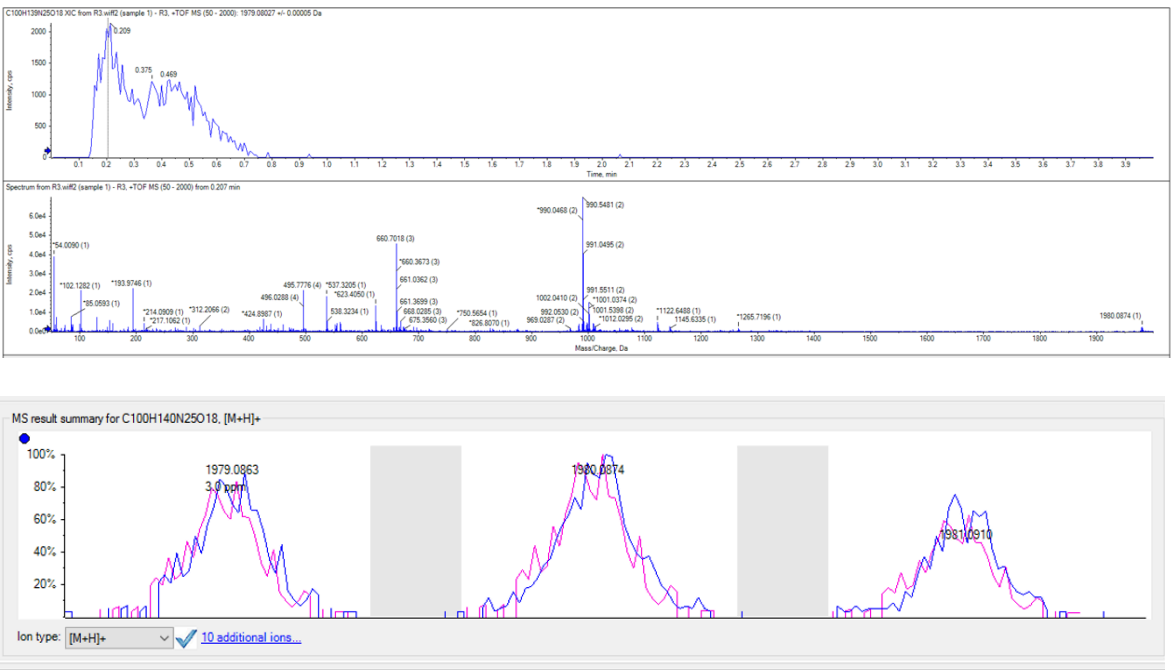
NDI 3-Linker (1)

HRMS analysis

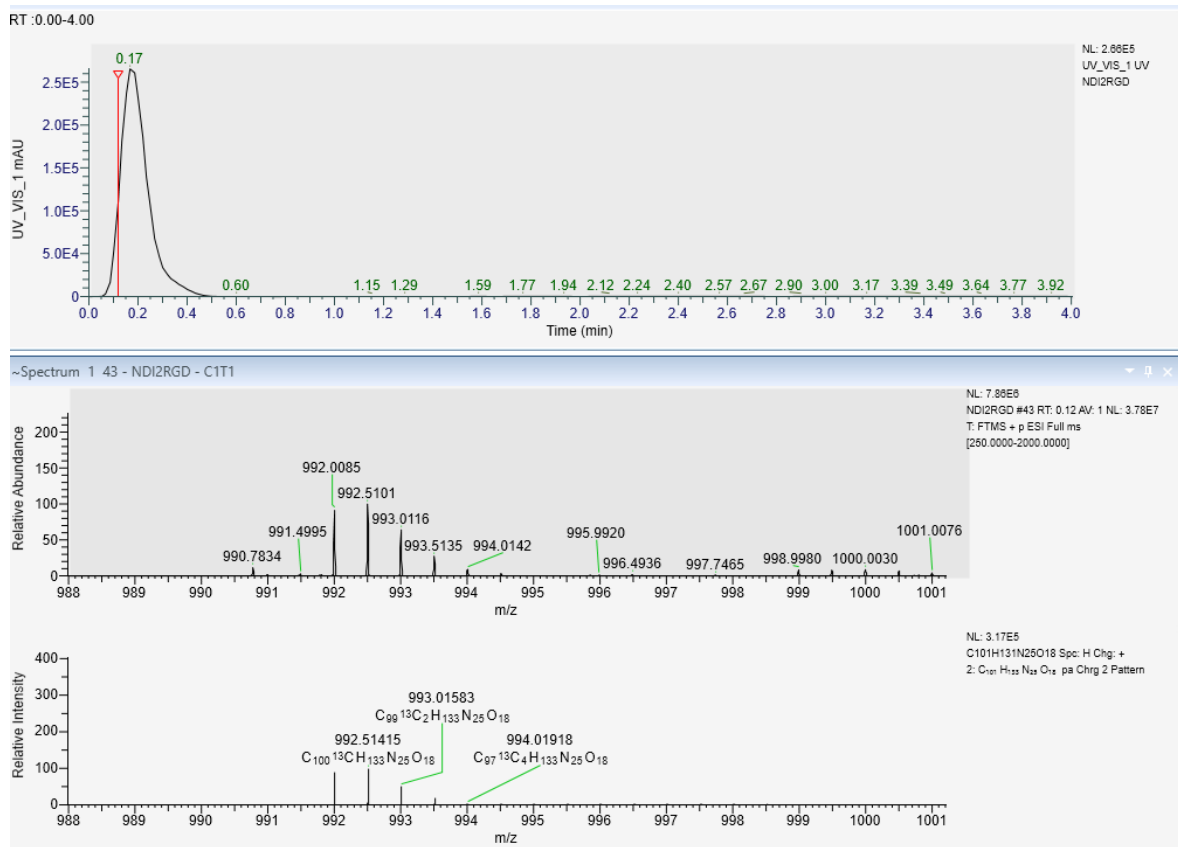


NDI 1-Linker (1)-3a-RGD

HRMS analysis

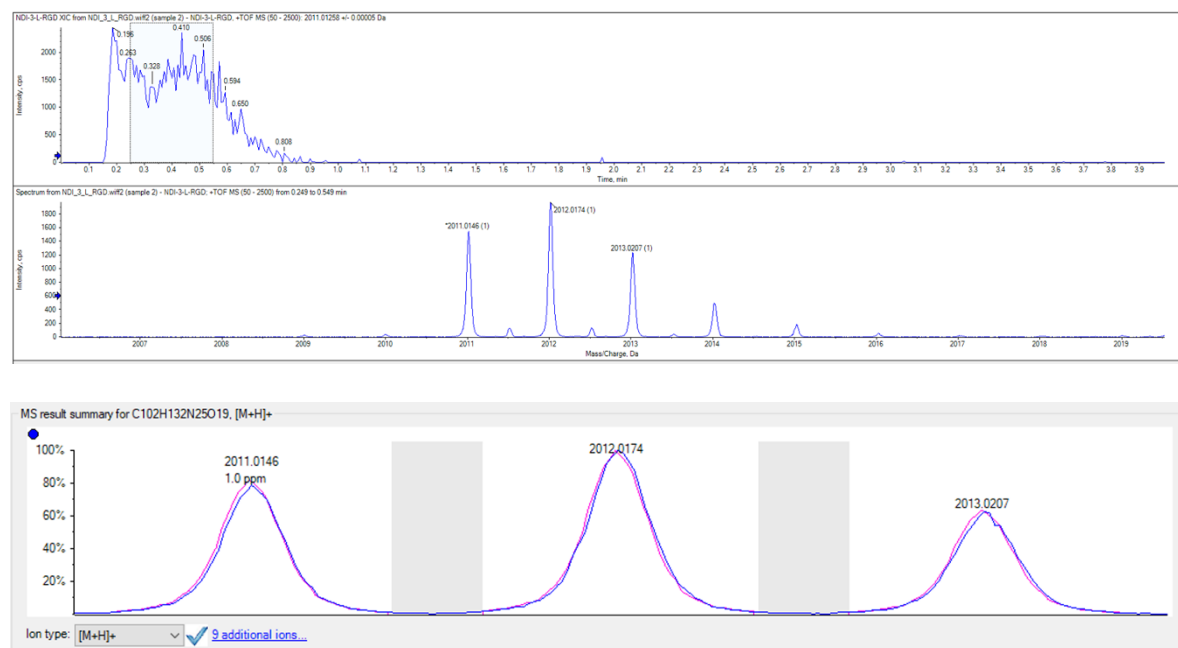


NDI 2-Linker (1)-3a-RGD



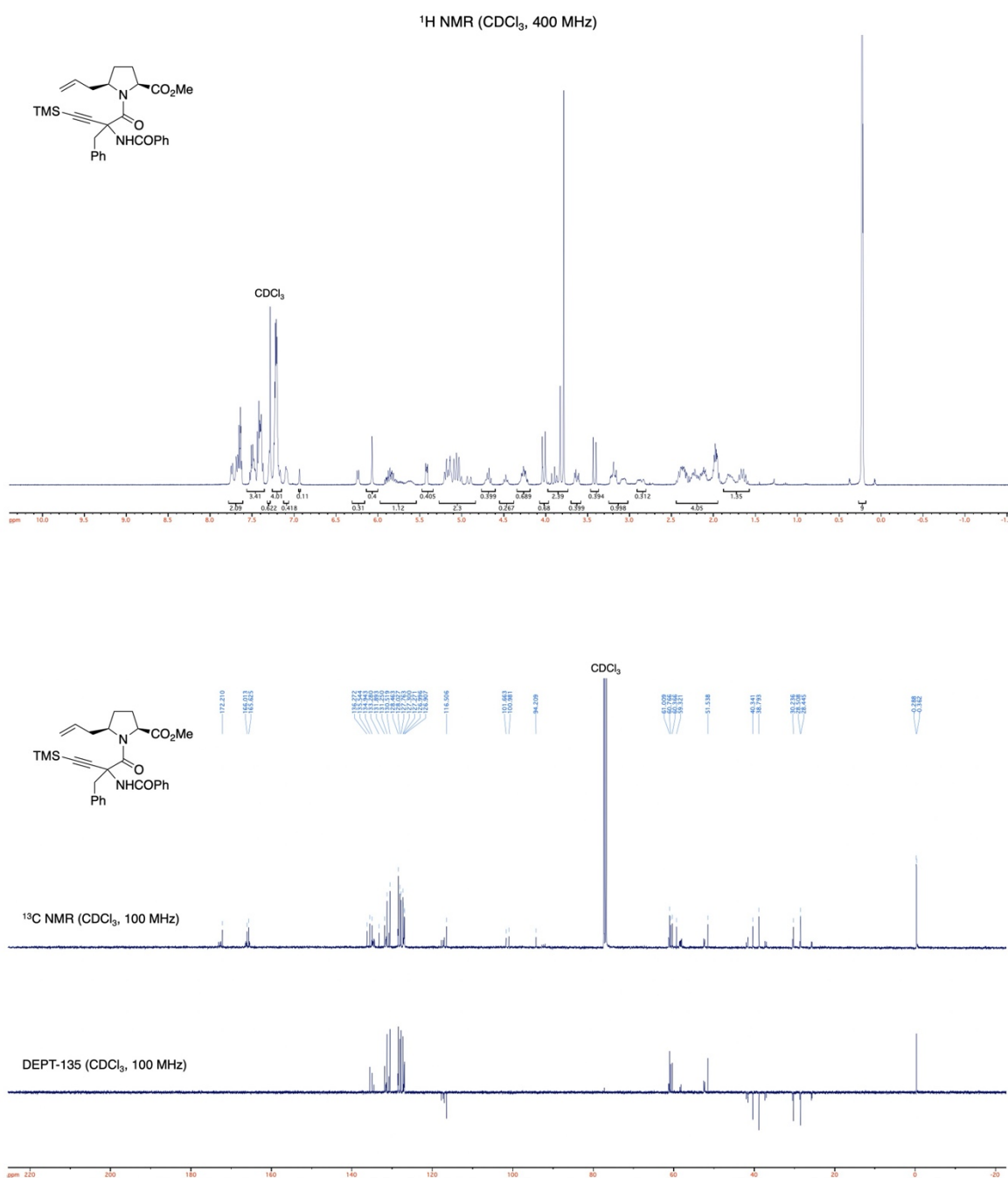
NDI 3-Linker (1)-3a-RGD

HRMS analysis

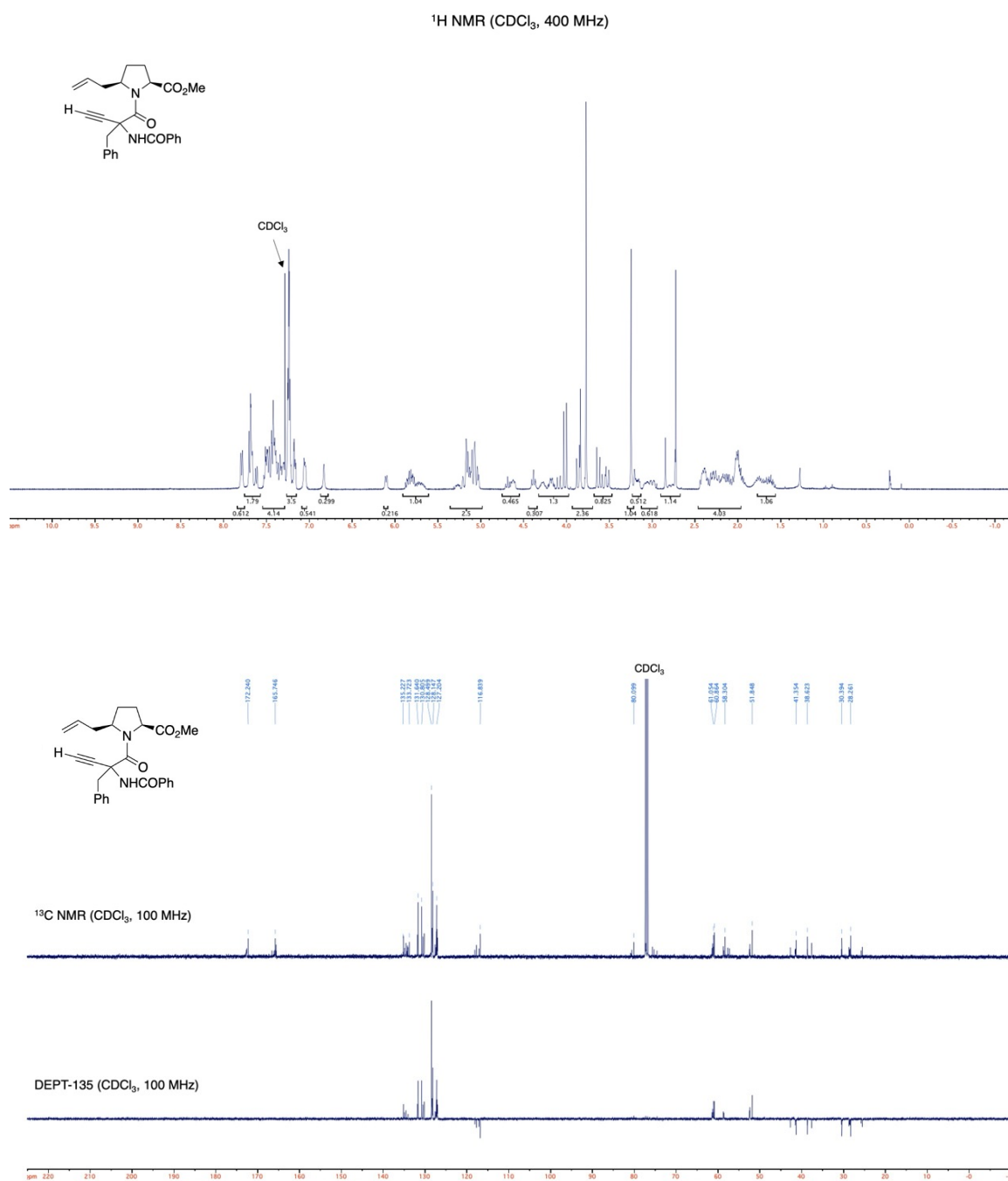


3.4.3 Synthesis of C4-functionalized azabicyclo[5.3.0]alkanone amino acids

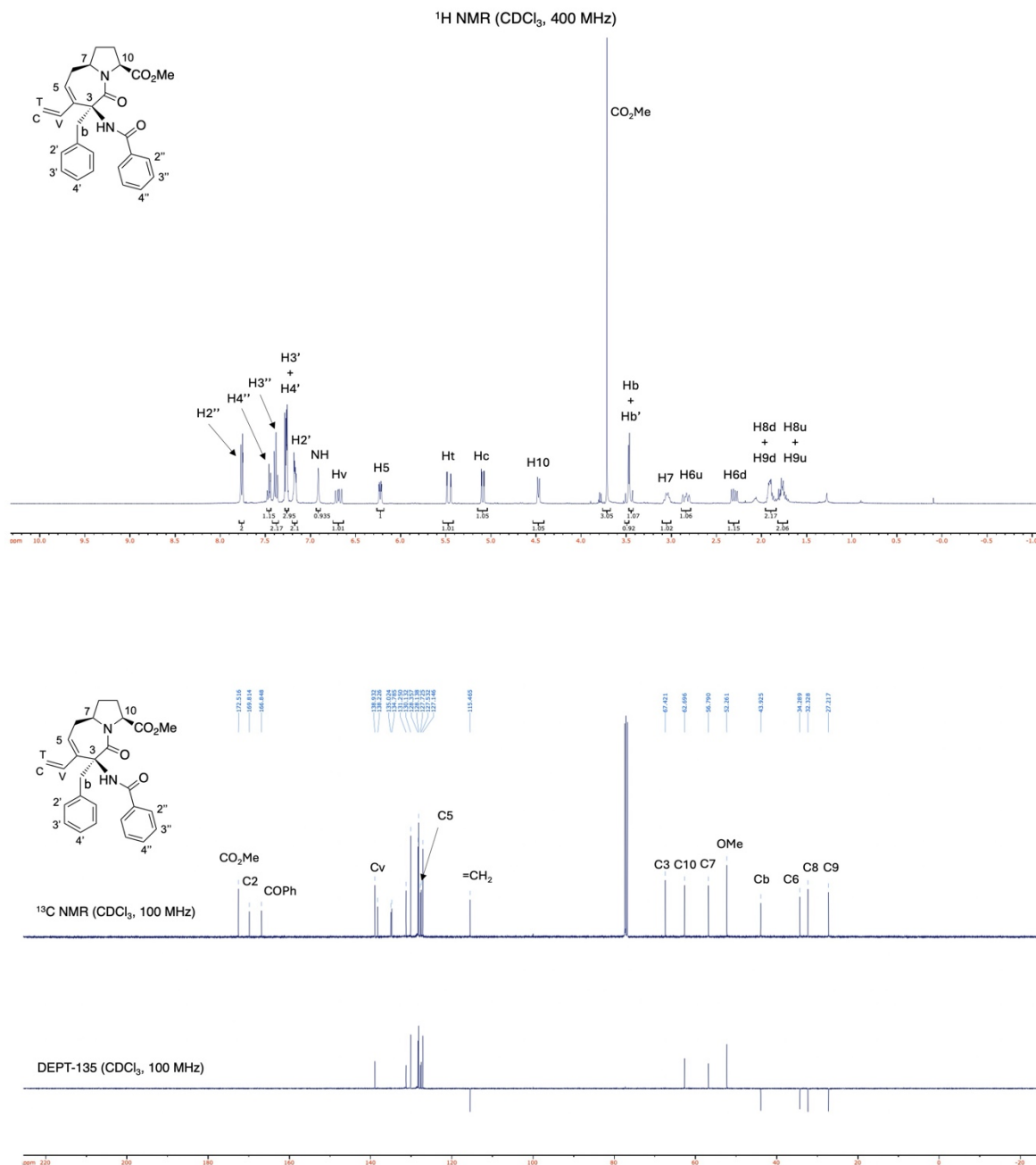
Methyl (2S,5R)-5-allyl-1-(2-benzamido-2-benzyl-4-(trimethylsilyl)but-3-ynoyl)pyrrolidine-2-carboxylate (47)

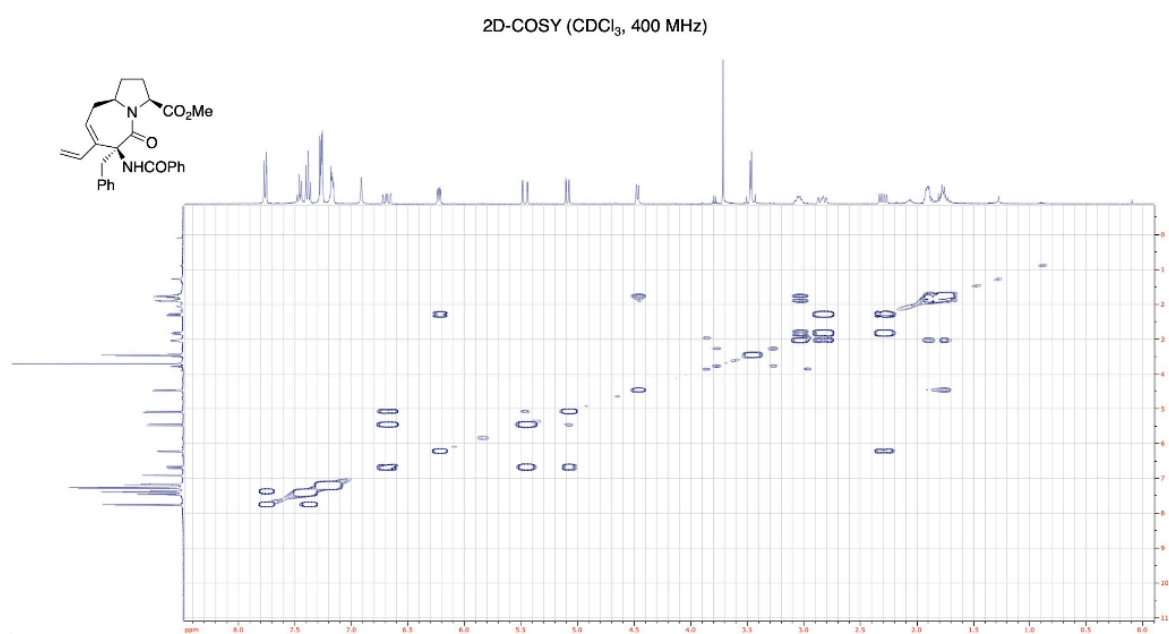
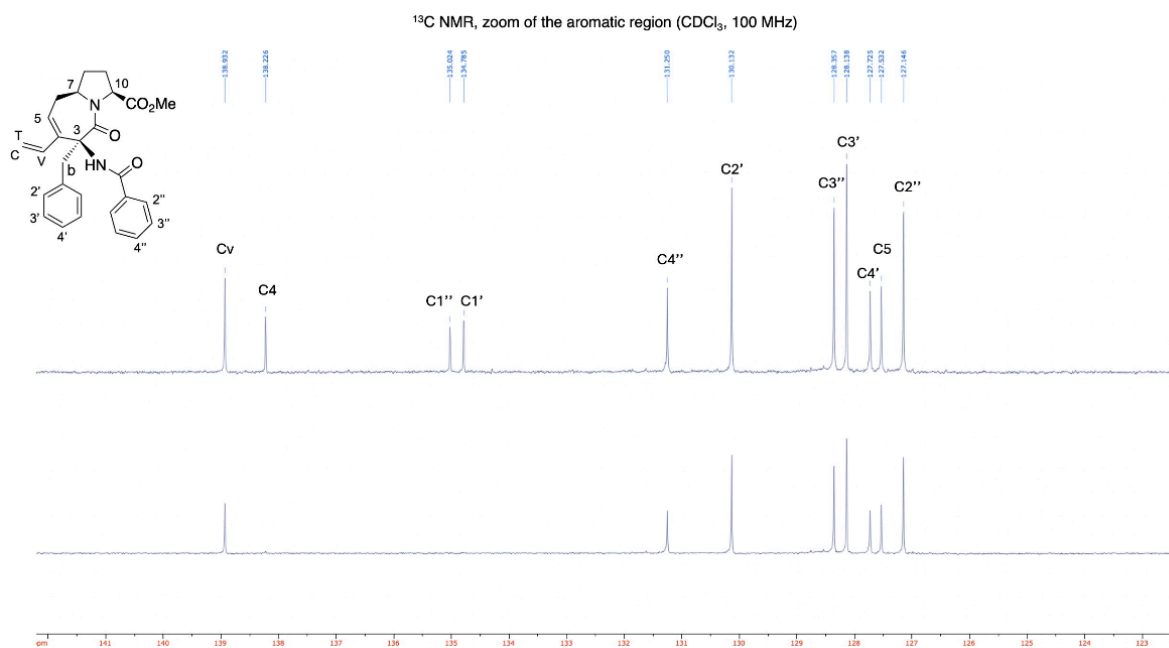


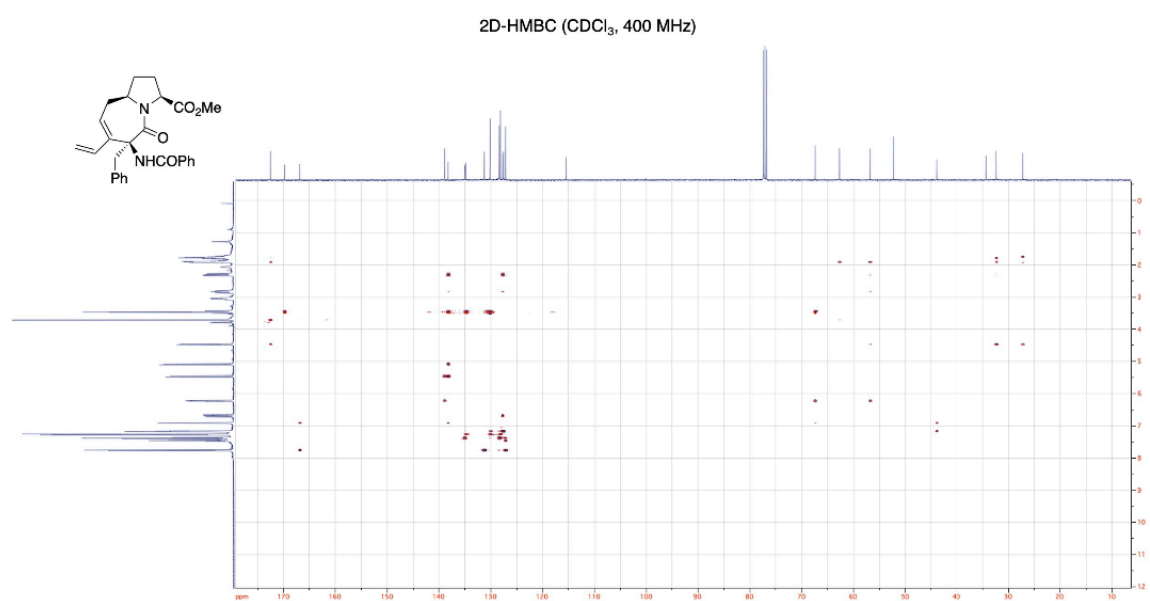
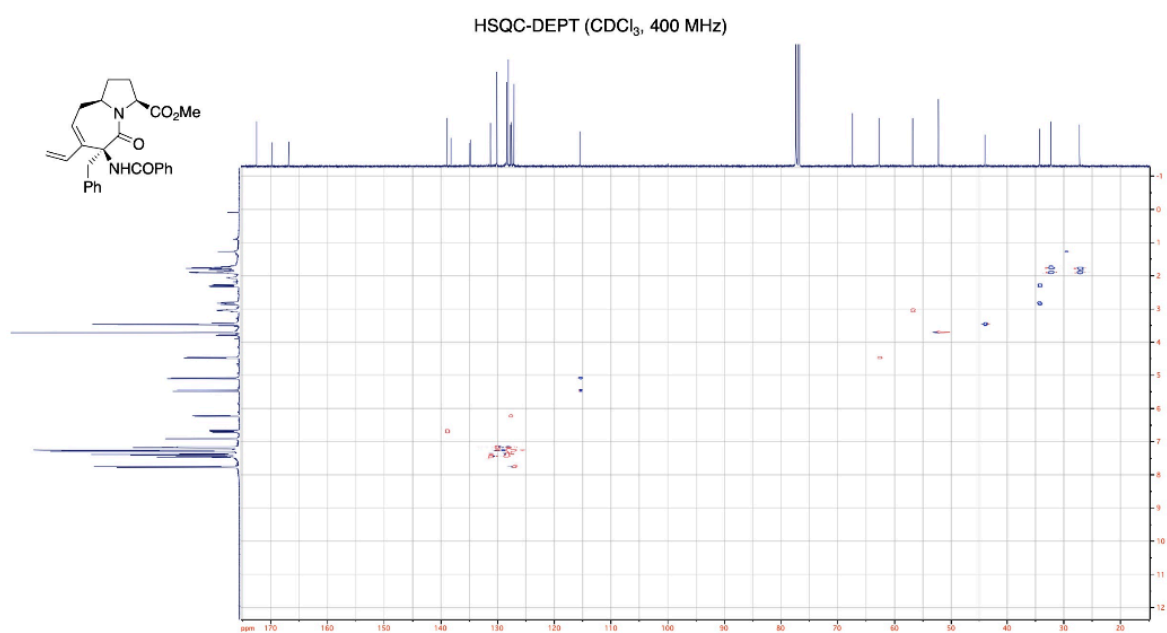
Methyl (2S,5R)-5-allyl-1-(2-benzamido-2-benzylbut-3-ynoyl)pyrrolidine-2-carboxylate (48)

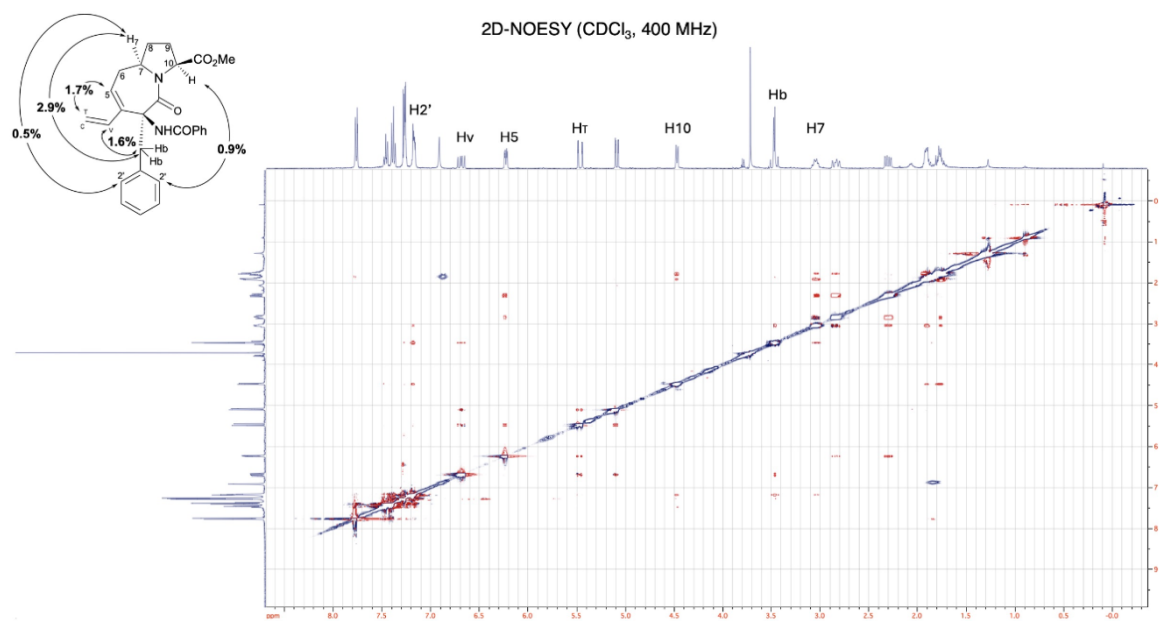


Methyl (3S,6S,9aR)-6-benzamido-6-benzyl-5-oxo-7-vinyl-2,3,5,6,9,9a-hexahydro-1H-pyrrolo[1,2-a]azepine-3-carboxylate (49a)

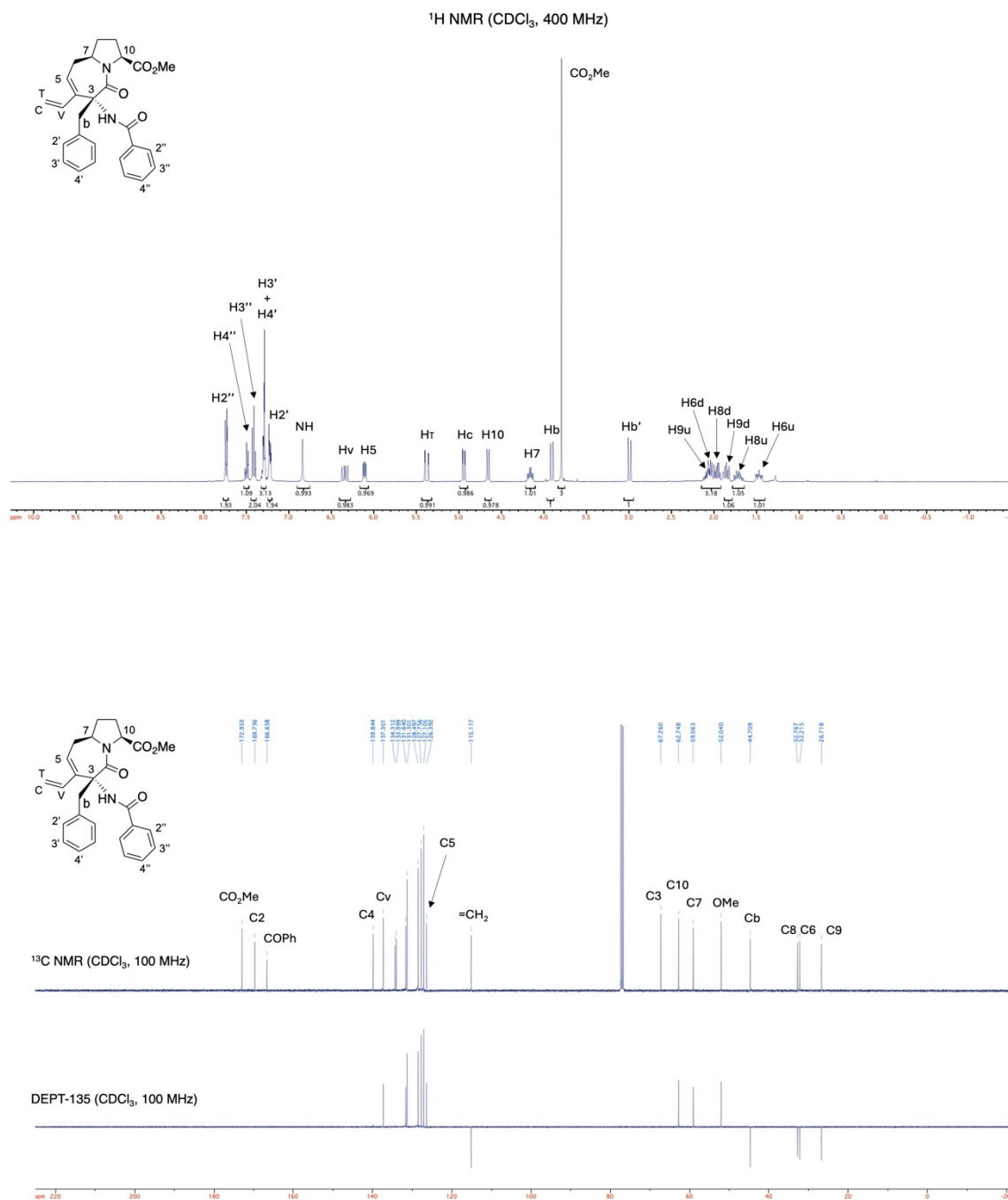


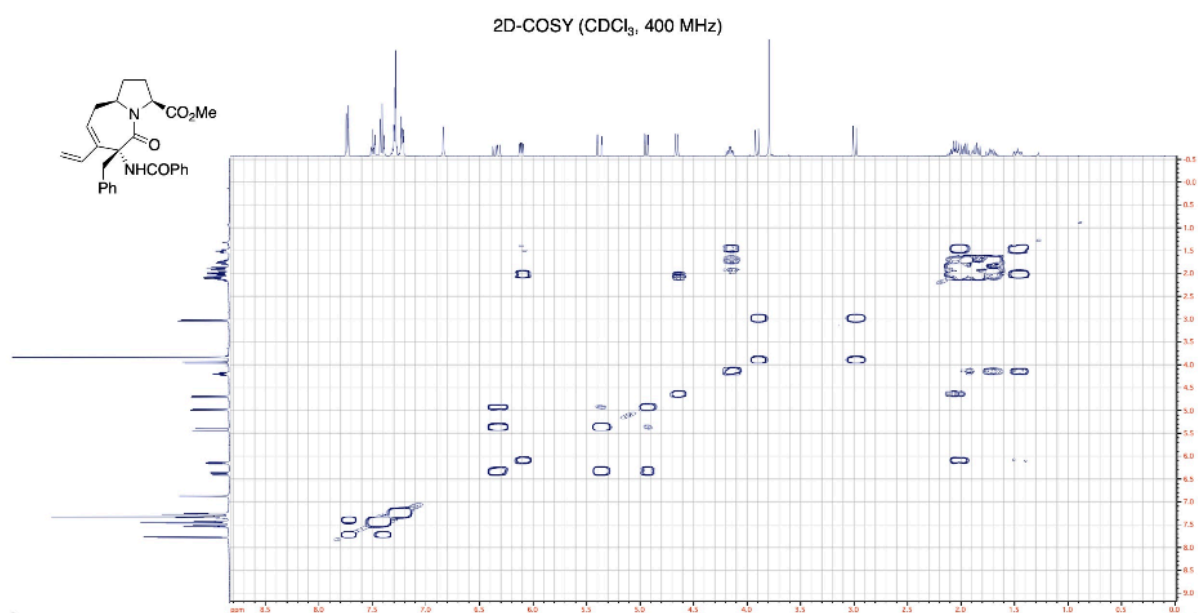
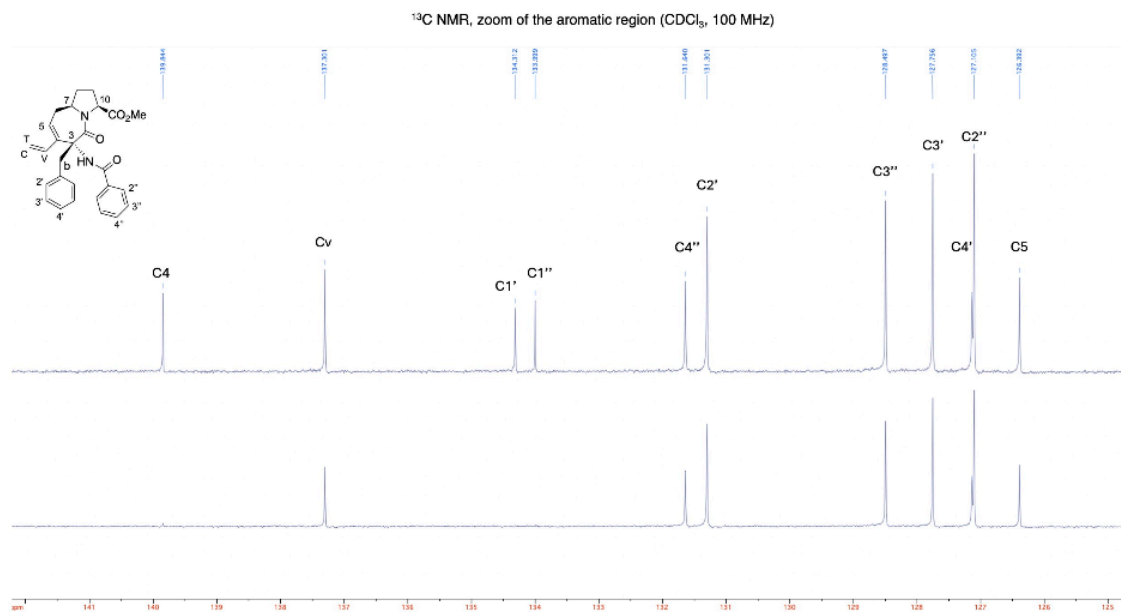


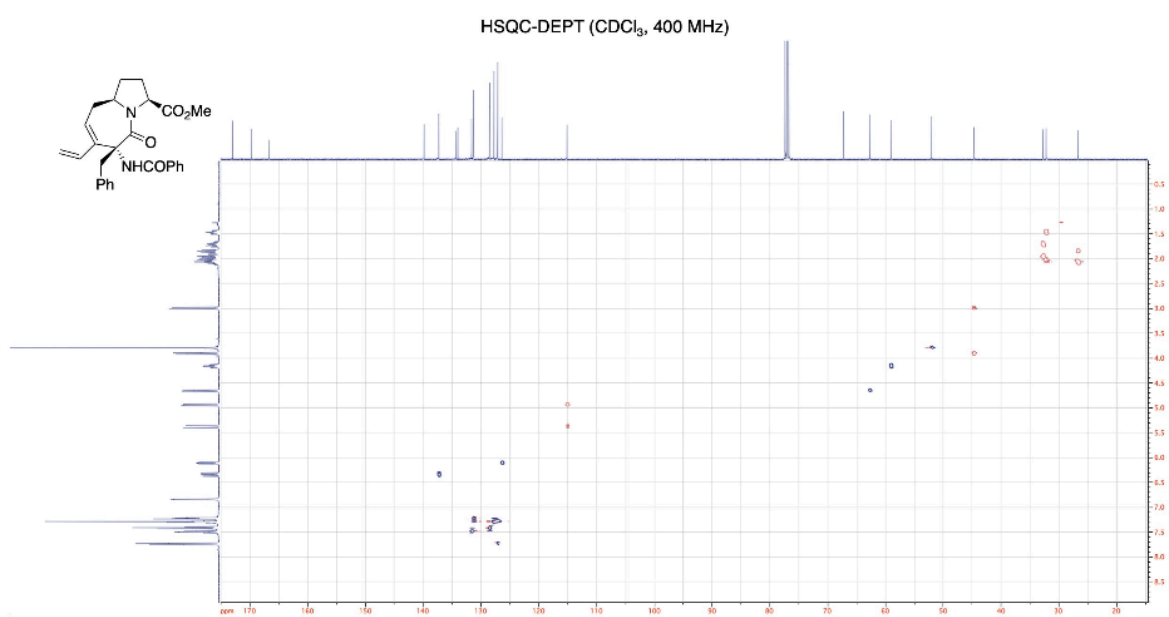
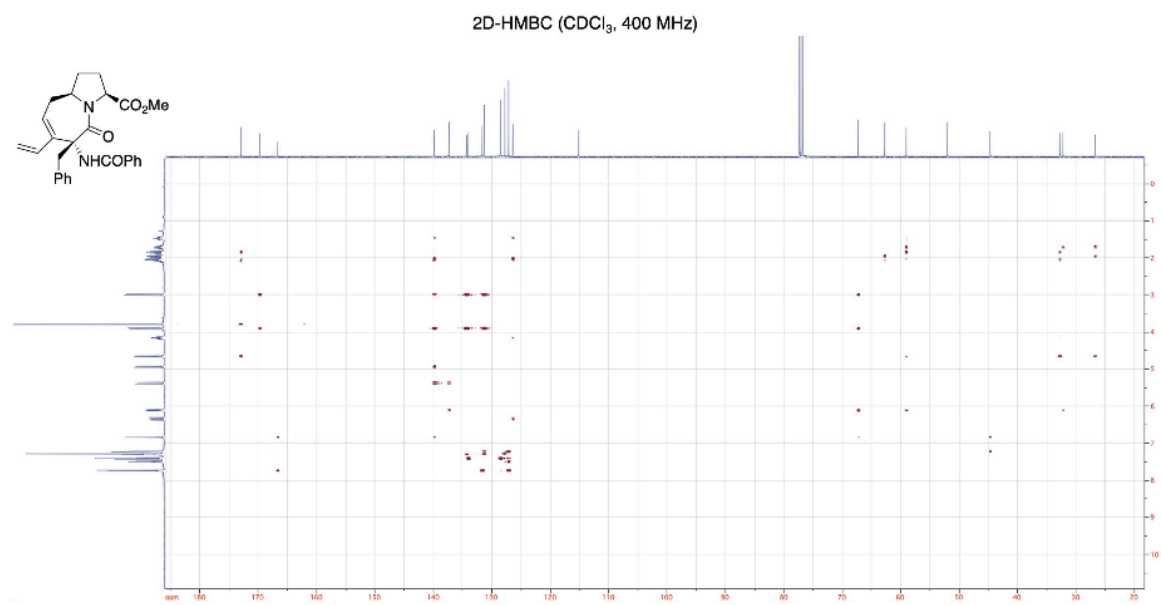


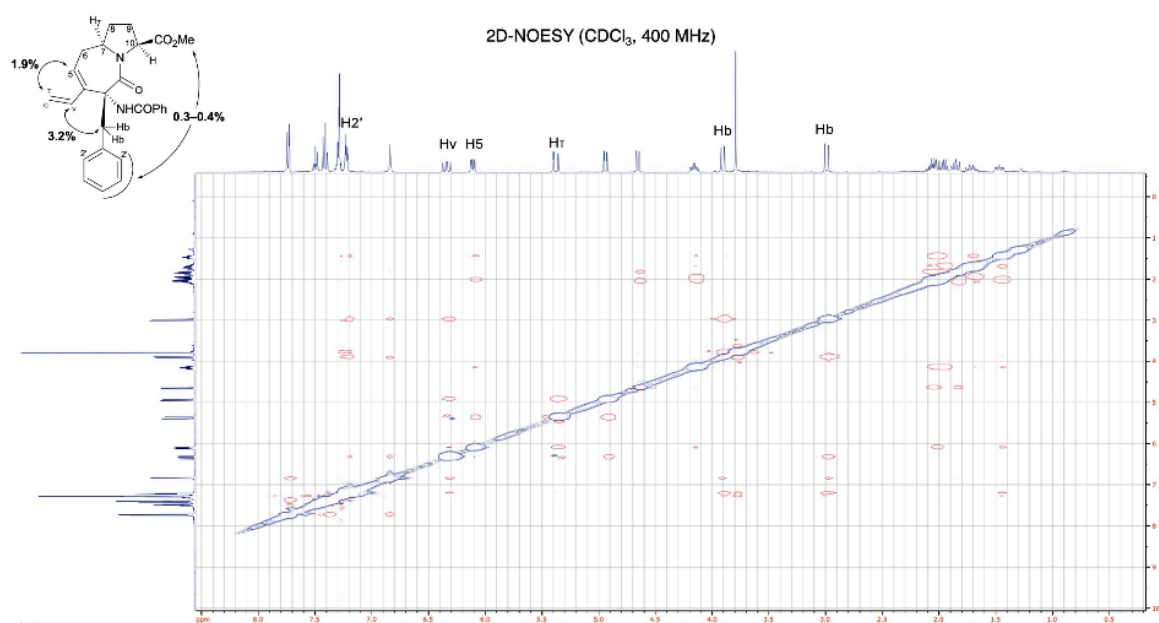


Methyl (3S,6R,9aR)-6-benzamido-6-benzyl-5-oxo-7-vinyl-2,3,5,6,9,9a-hexahydro-1H-pyrrolo[1,2-a]azepine-3-carboxylate (49b)



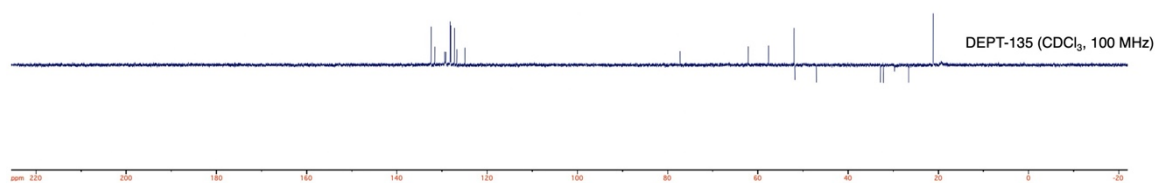
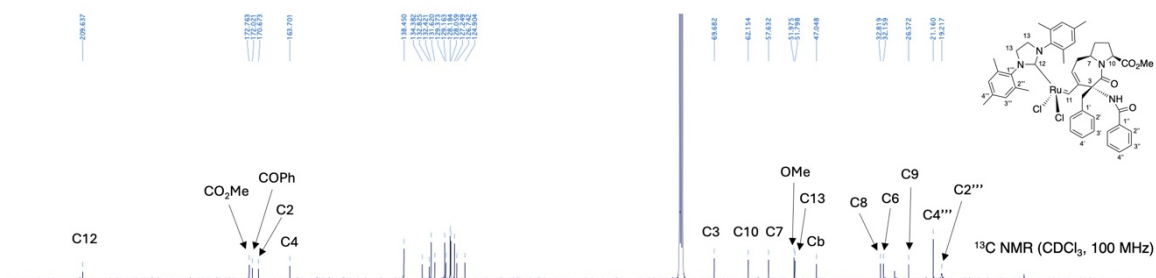
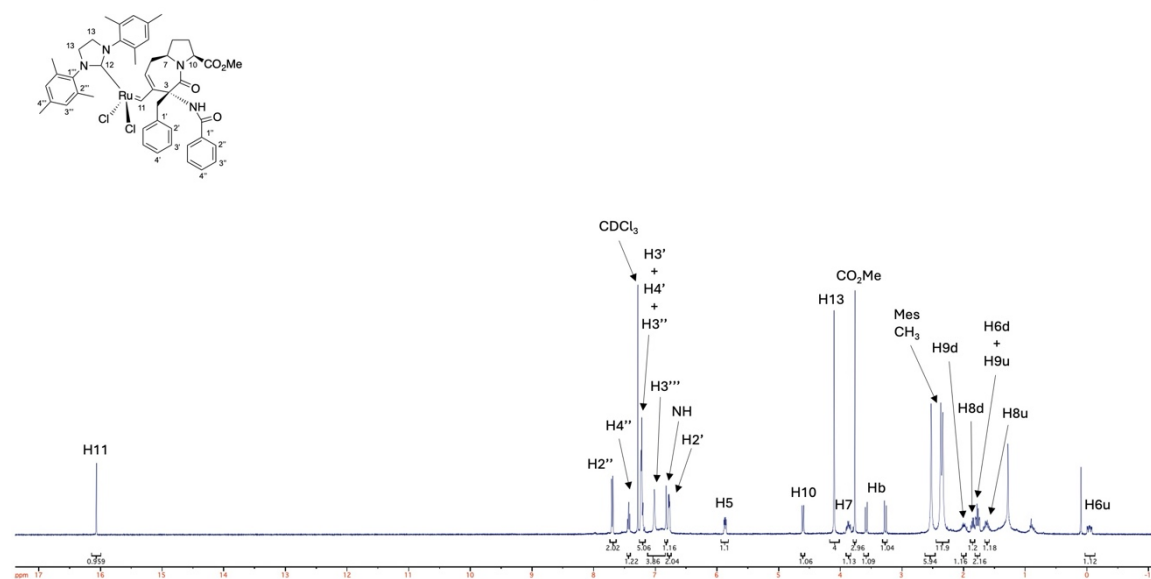


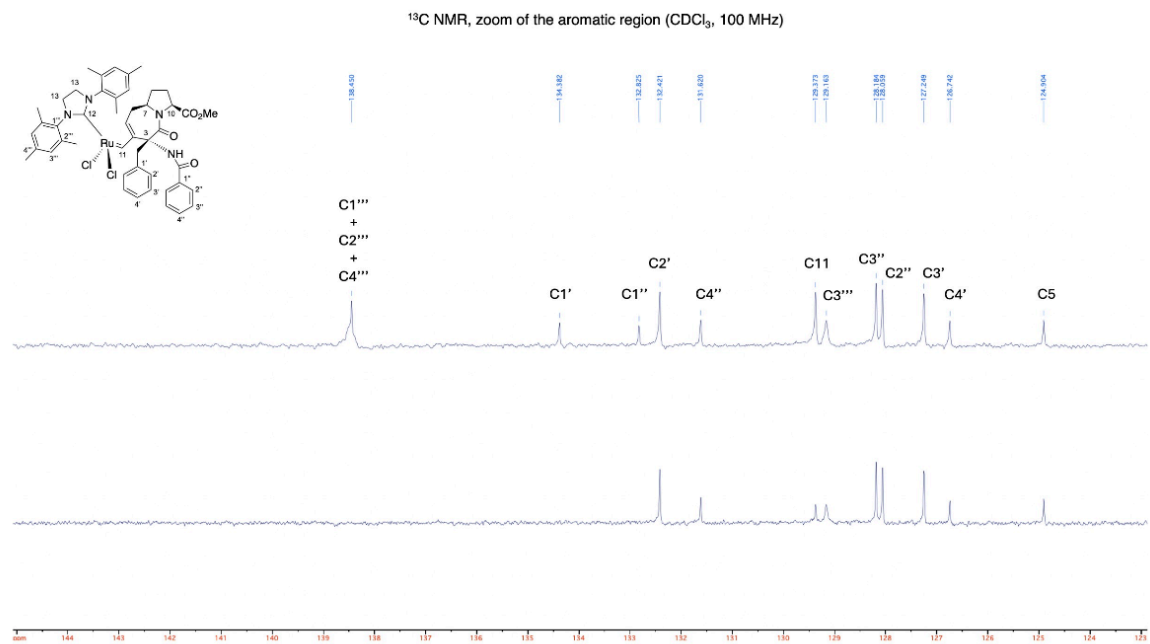


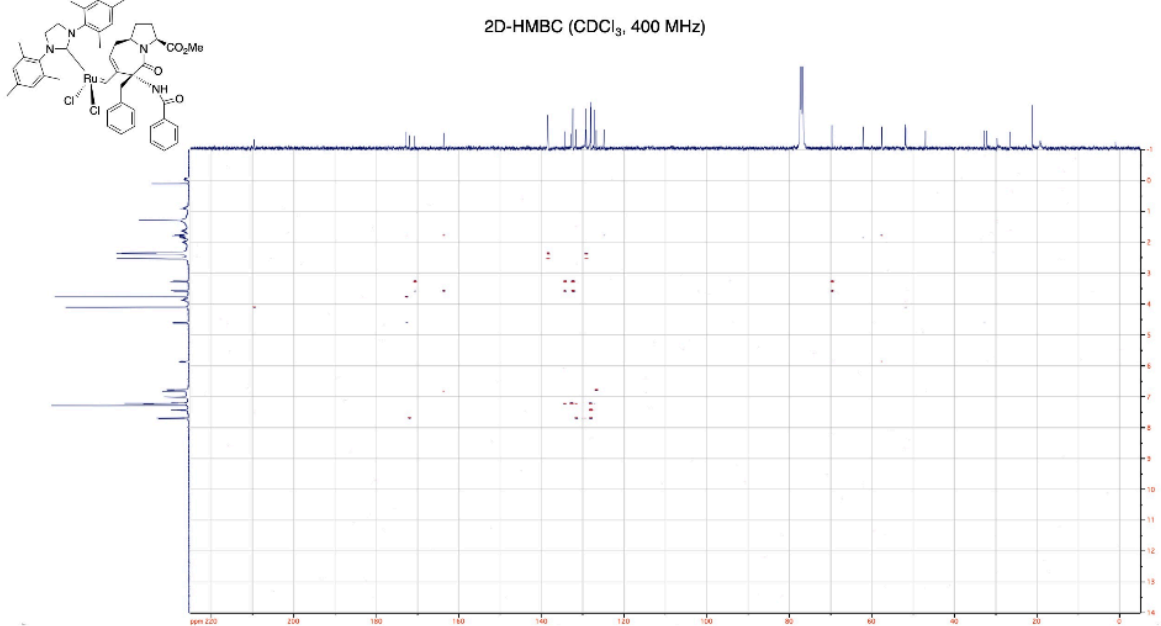
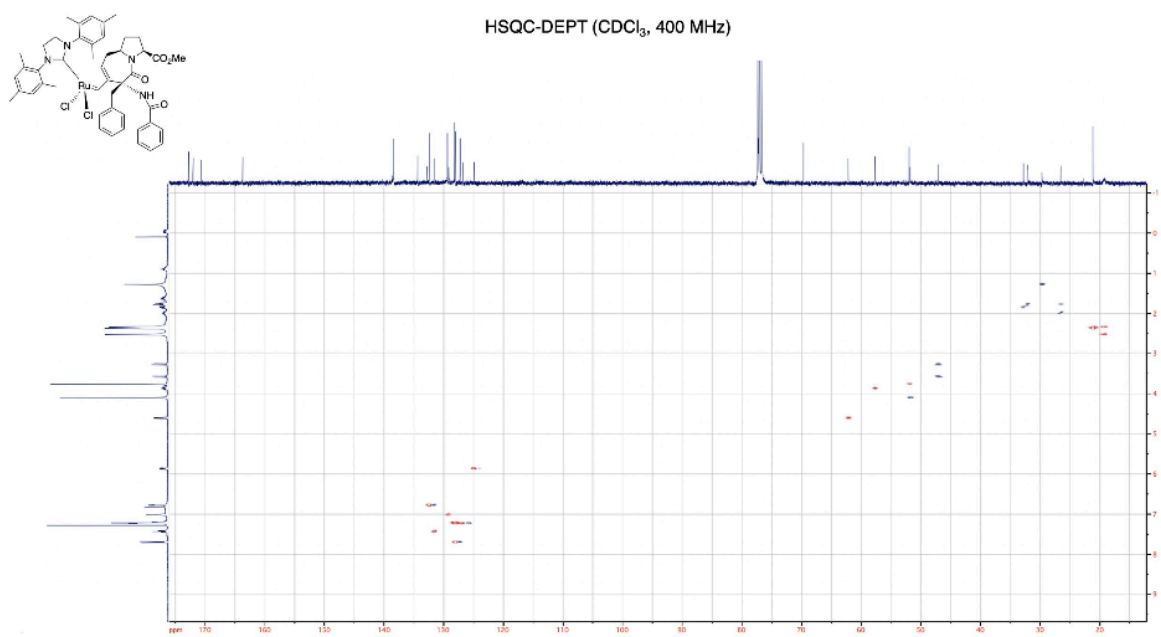


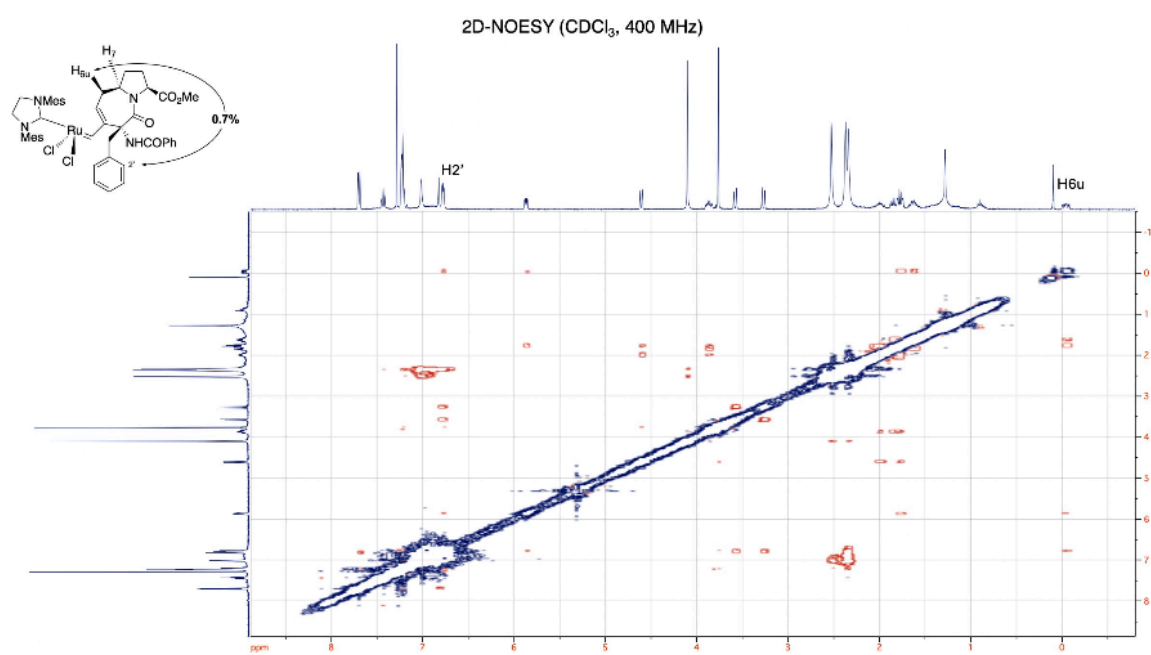
Ruthenium-diene complex (50b)

^1H NMR (CDCl_3 , 400 MHz)

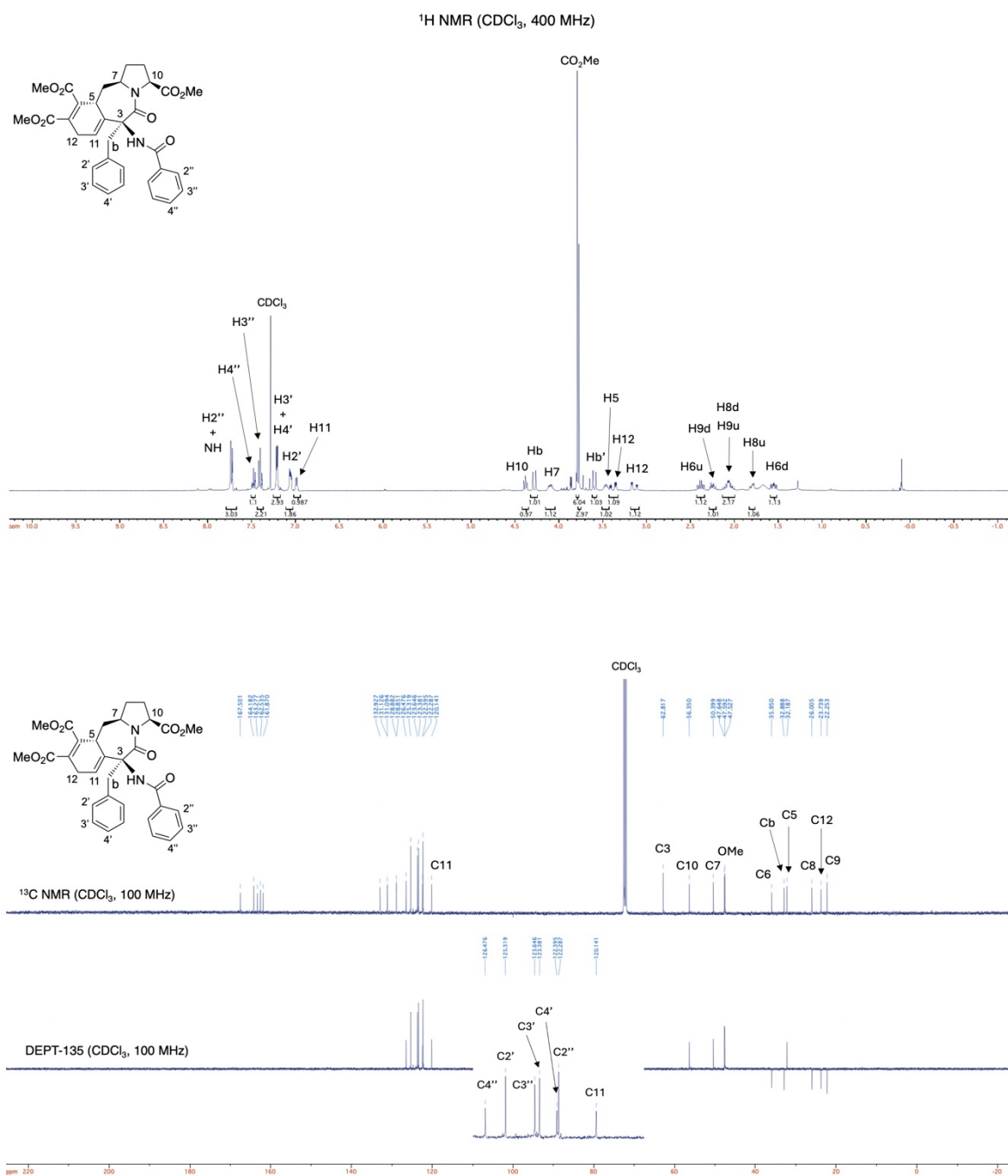




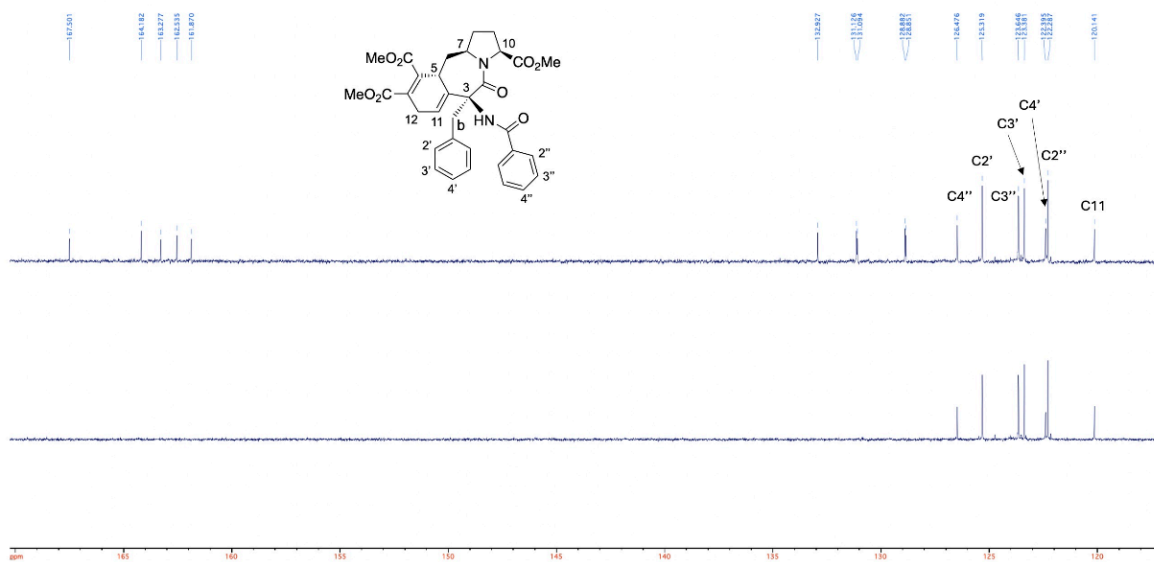




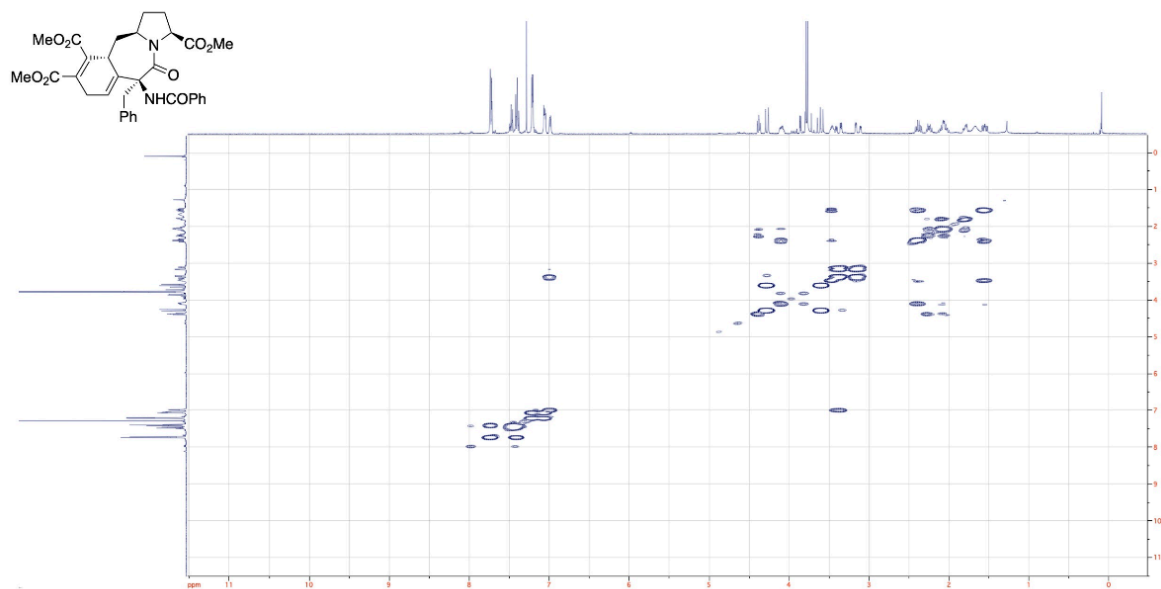
Trimethyl (3S,6S,10aR,11aR)-6-benzamido-6-benzyl-5-oxo-2,3,5,6,8,10a,11,11a-octahydro-1H-benzo[d]pyrrolo[1,2-a]azepine-3,9,10-tricarboxylate (51a)



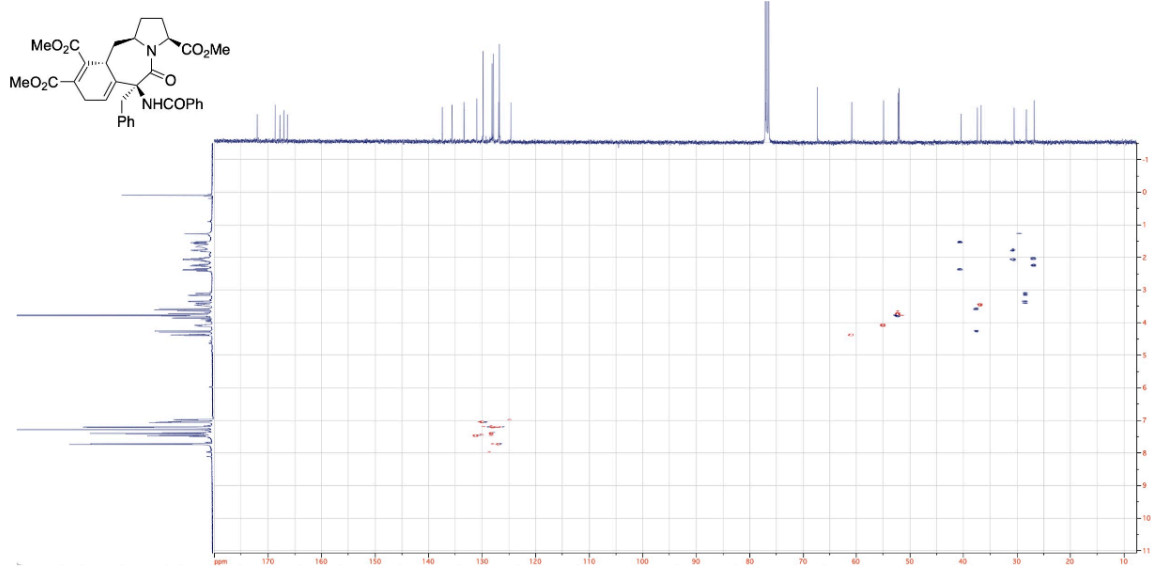
^{13}C NMR, zoom of the aromatic region (CDCl_3 , 100 MHz)



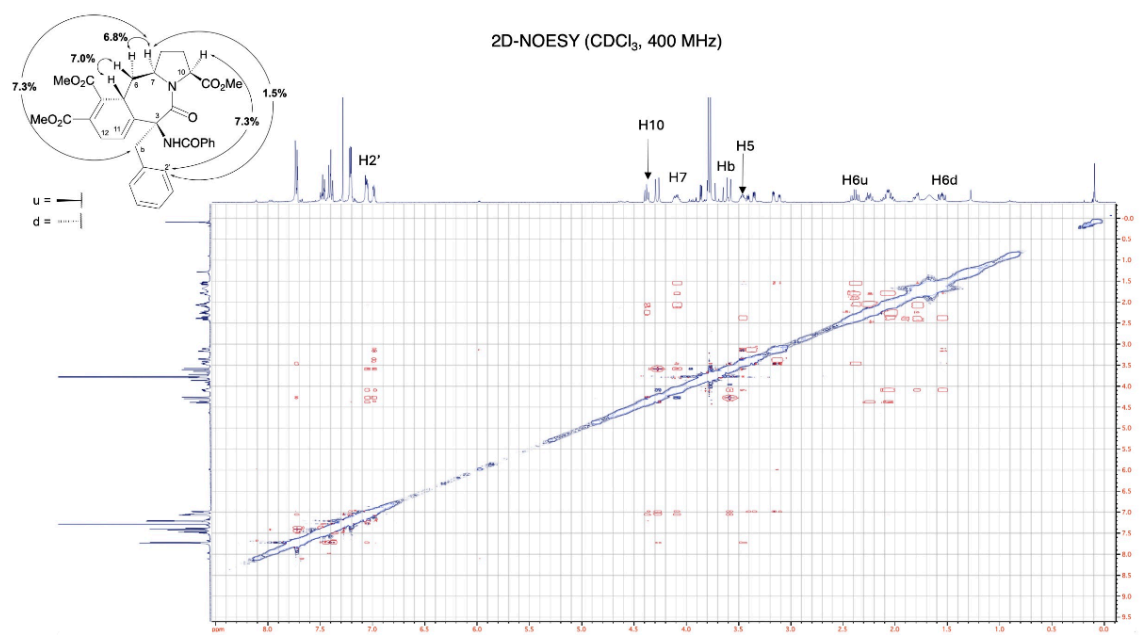
2D-COSY (CDCl_3 , 400 MHz)



HSQC-DEPT (CDCl₃, 400 MHz)

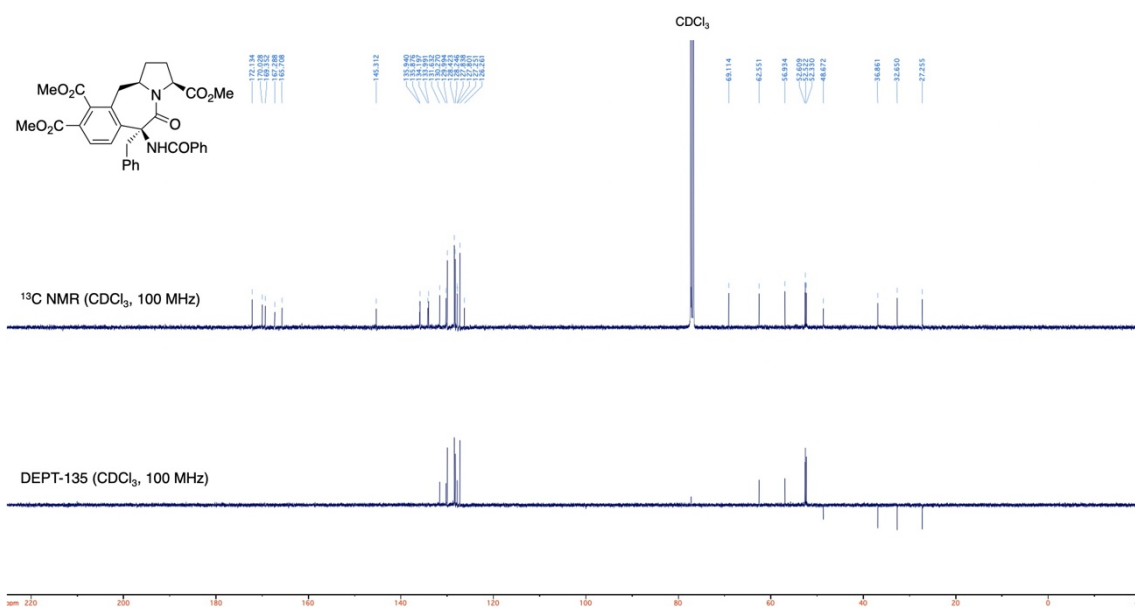
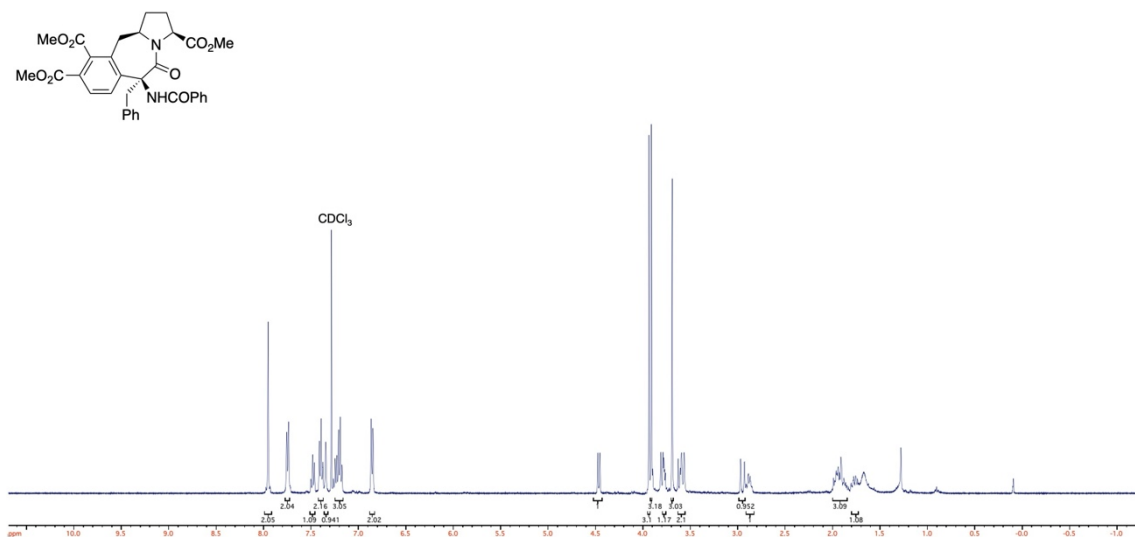


2D-NOESY (CDCl₃, 400 MHz)



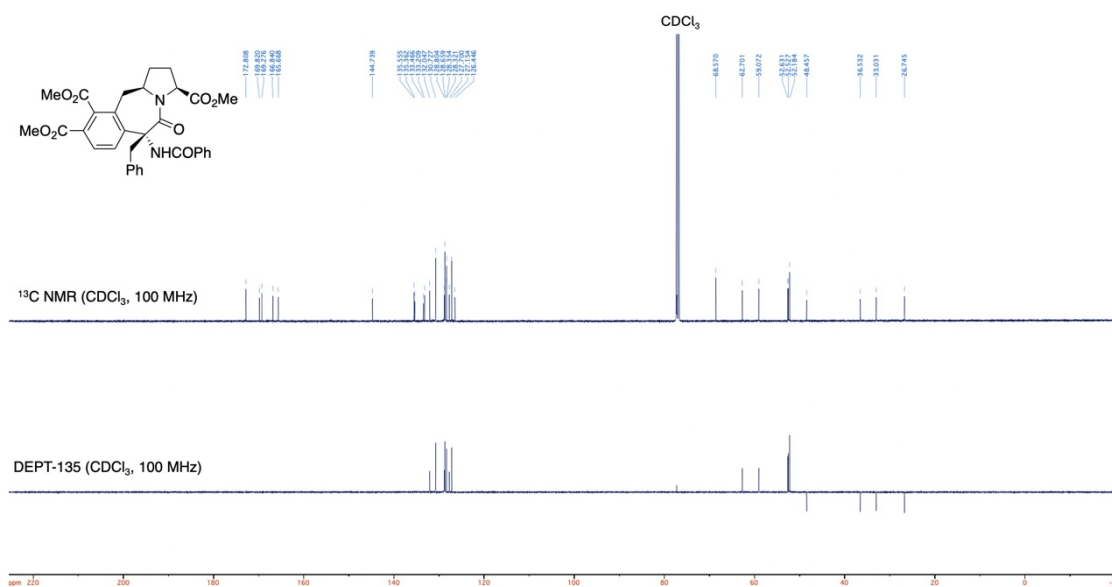
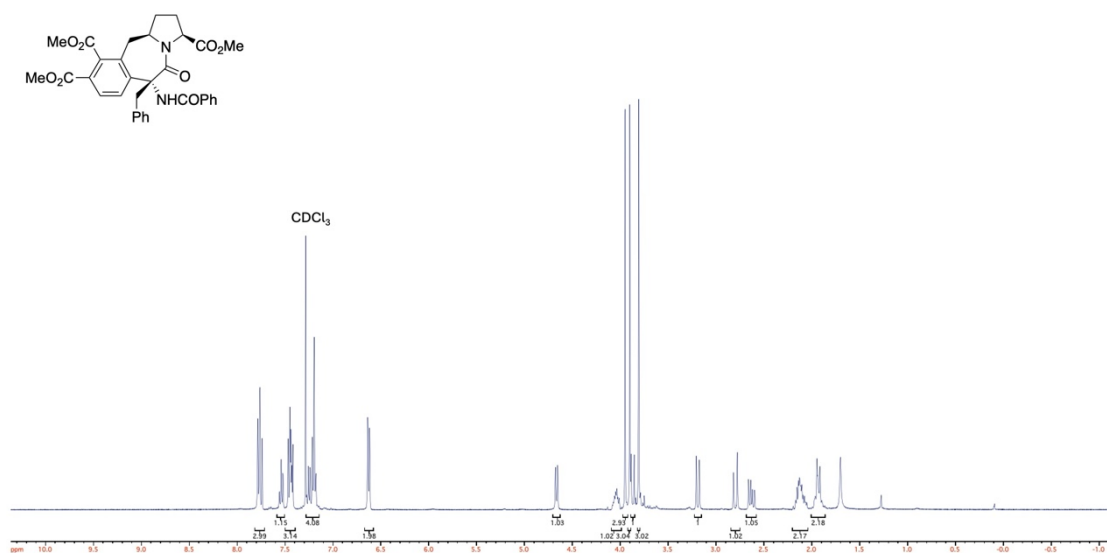
Trimethyl (3S,6S,11aR)-6-benzamido-6-benzyl-5-oxo-2,3,5,6,11,11a-hexahydro-1H-benzo[d]pyrrolo[1,2-a]azepine-3,9,10-tricarboxylate (52a)

^1H NMR (CDCl_3 , 400 MHz)

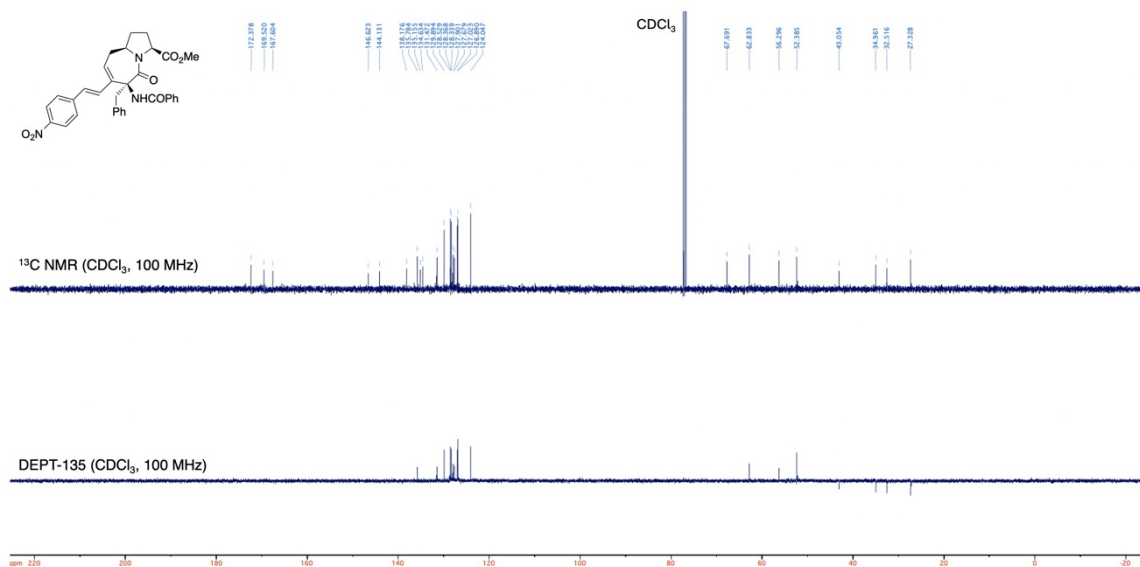
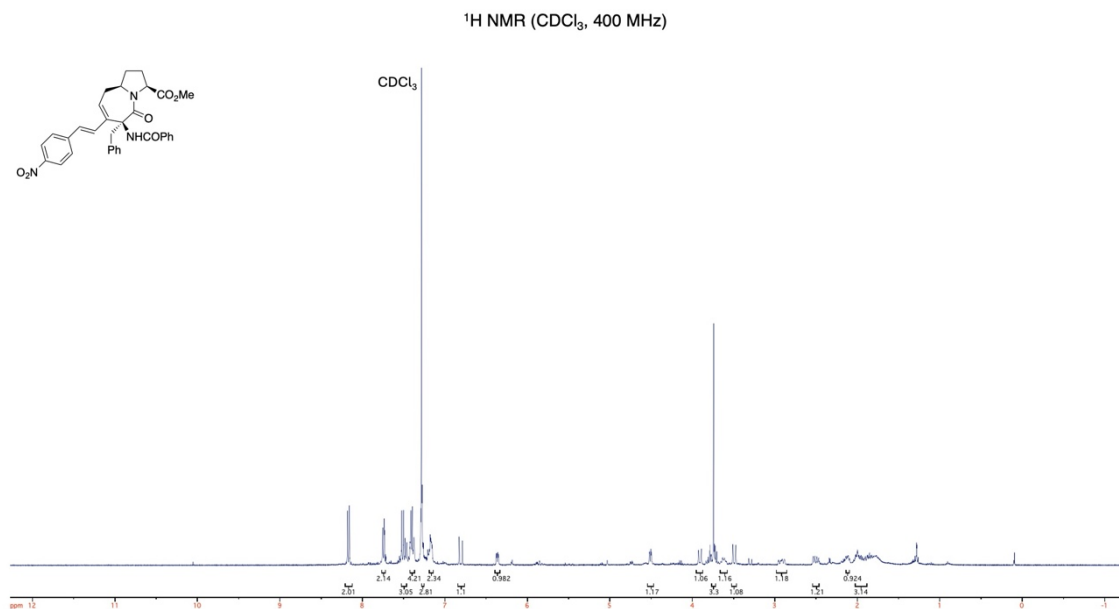


Trimethyl (3S,6R,11aR)-6-benzamido-6-benzyl-5-oxo-2,3,5,6,11,11a-hexahydro-1H-benzo[d]pyrrolo[1,2-a]azepine-3,9,10-tricarboxylate (52b)

^1H NMR (CDCl_3 , 400 MHz)

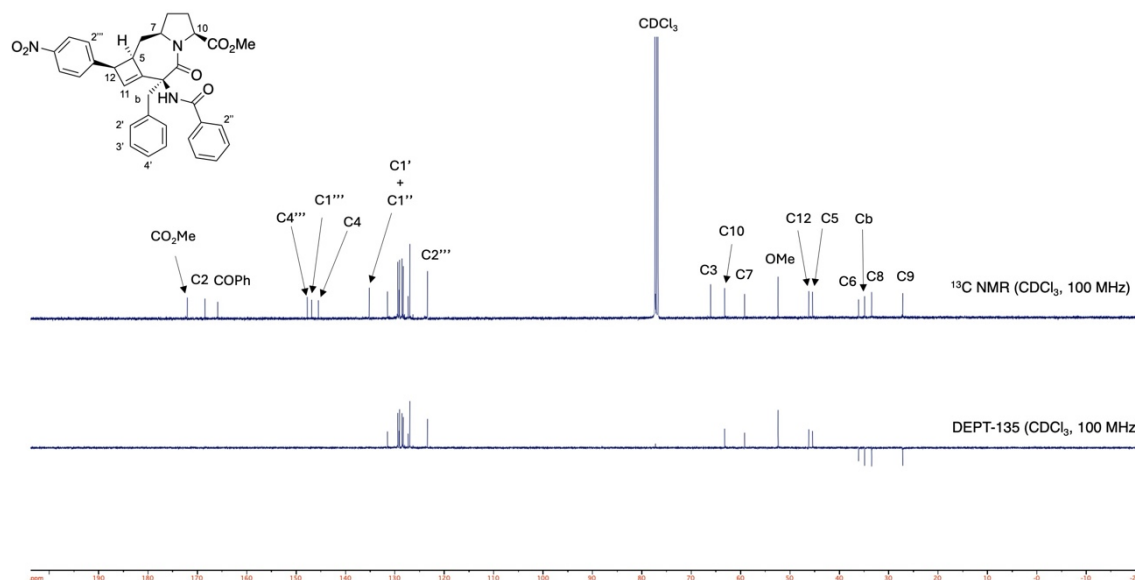
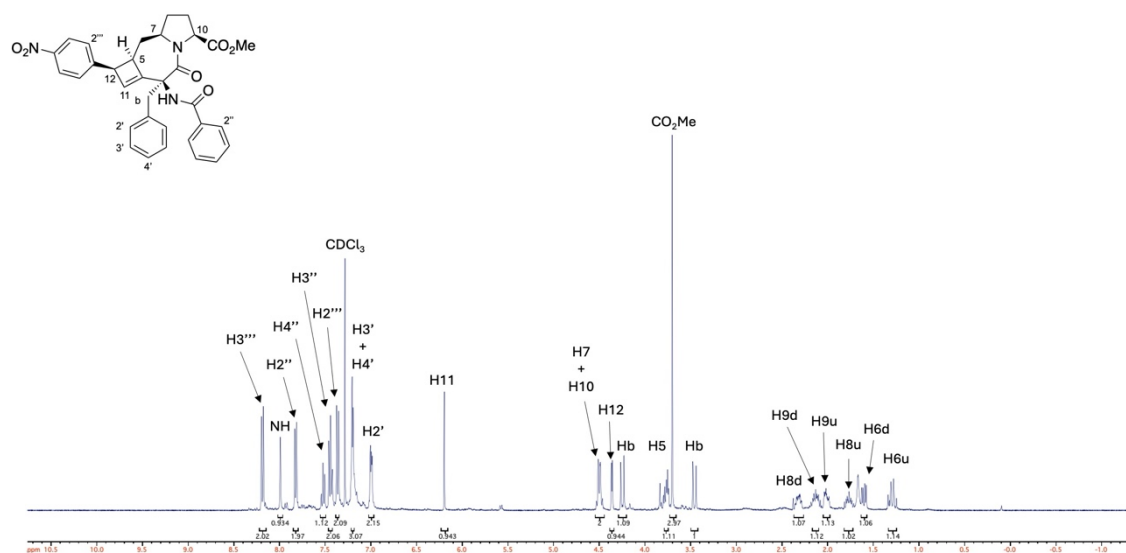


Methyl (3*S*,6*S*,9*a**R*)-6-benzamido-6-benzyl-7-(4-nitrostyryl)-5-oxo-2,3,5,6,9,9*a*-hexahydro-1*H*-pyrrolo[1,2-*a*]azepine-3-carboxylate (53a)

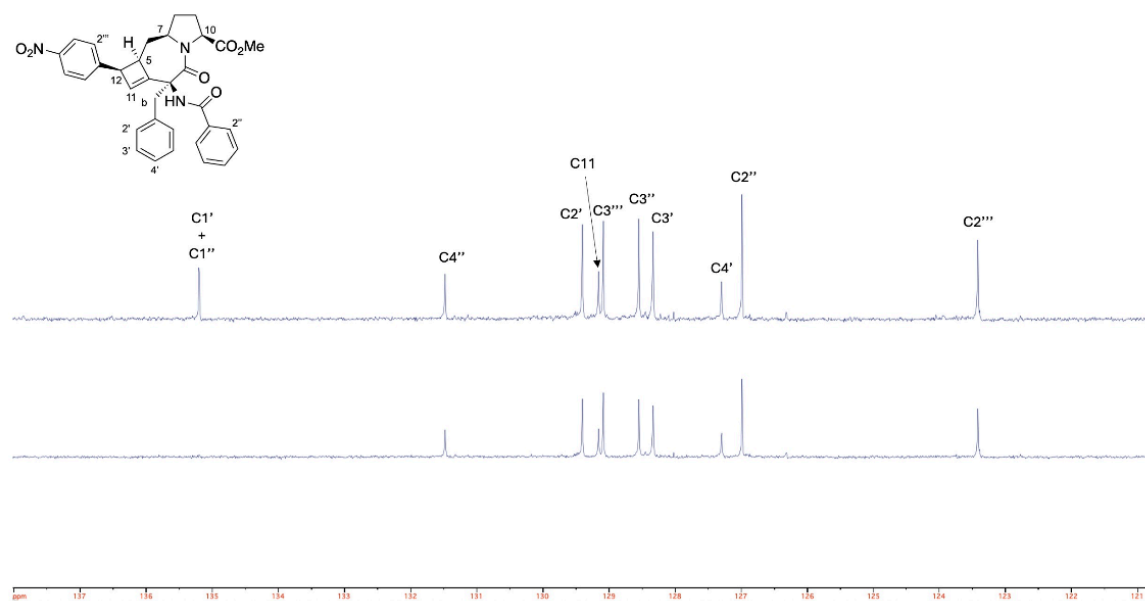


Methyl (3S,6S,8R,8aR,9aR)-6-benzamido-6-benzyl-8-(4-nitrophenyl)-5-oxo-
2,3,5,6,8,8a,9,9a-octahydro-1H-cyclobuta[d]pyrrolo[1,2-a]azepine-3-carboxylate (54a)

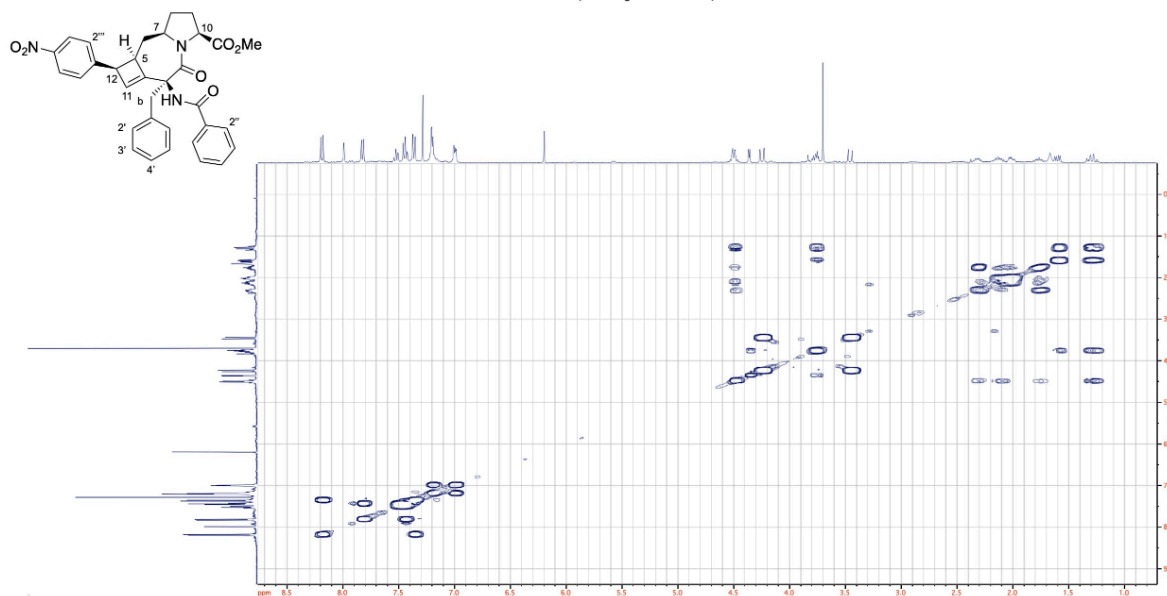
^1H NMR (CDCl_3 , 400 MHz)

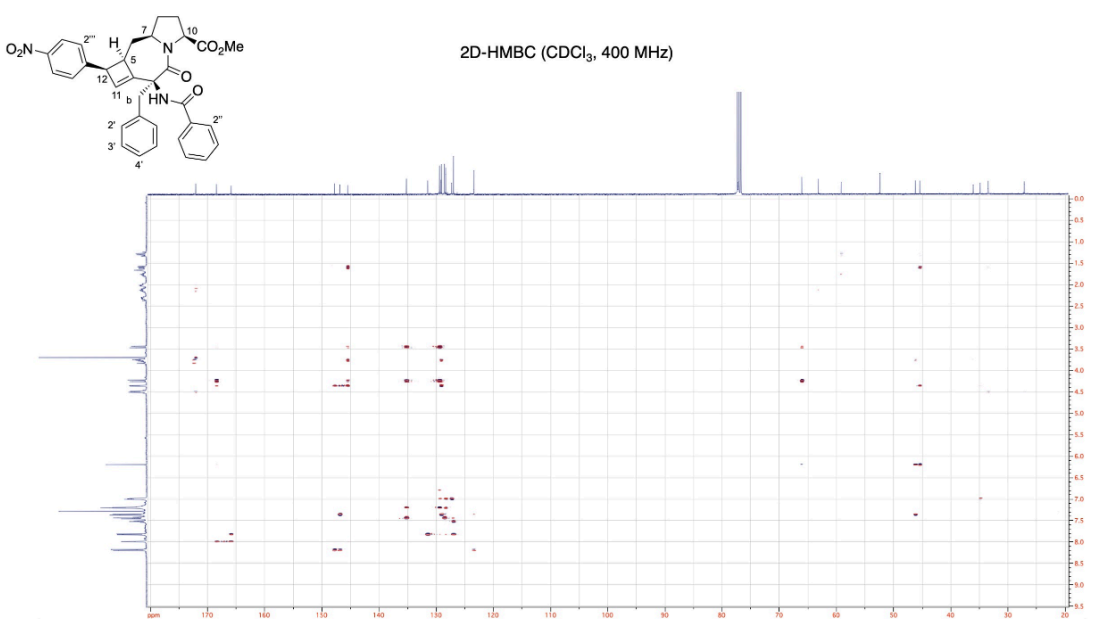


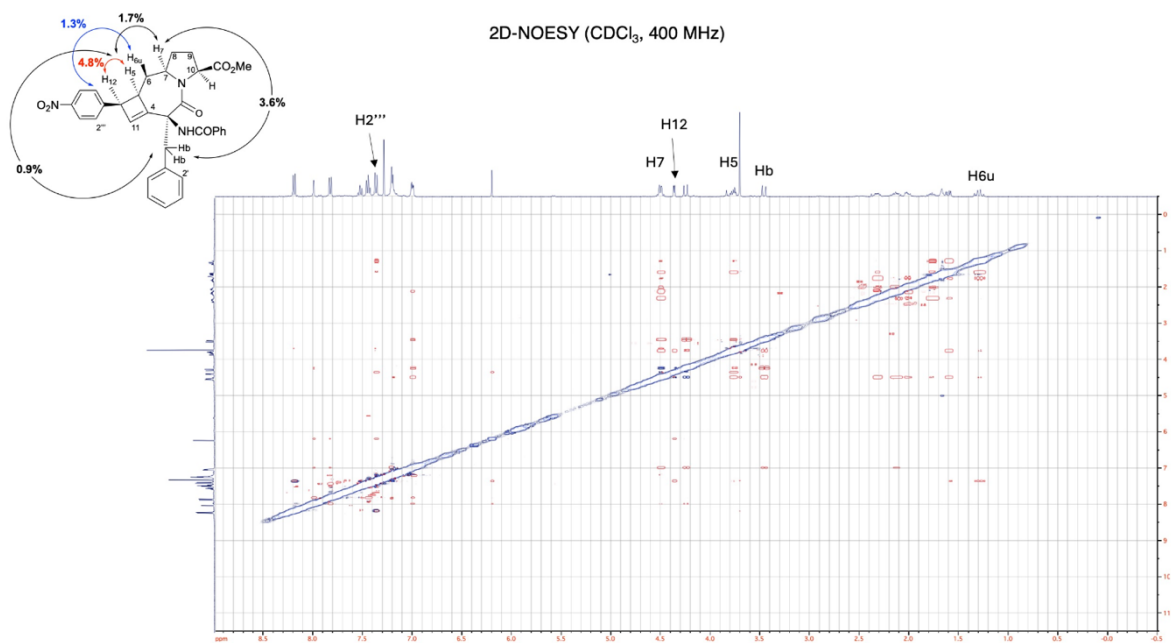
^{13}C NMR, zoom of the aromatic region (CDCl_3 , 100 MHz)



2D-COSY (CDCl_3 , 400 MHz)

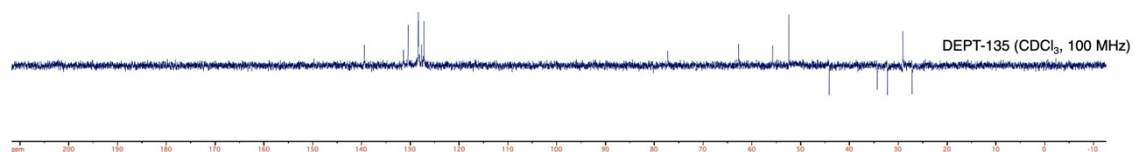
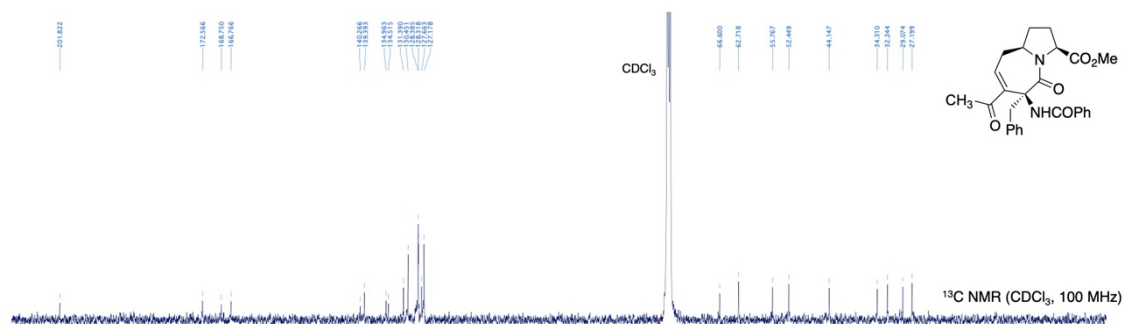
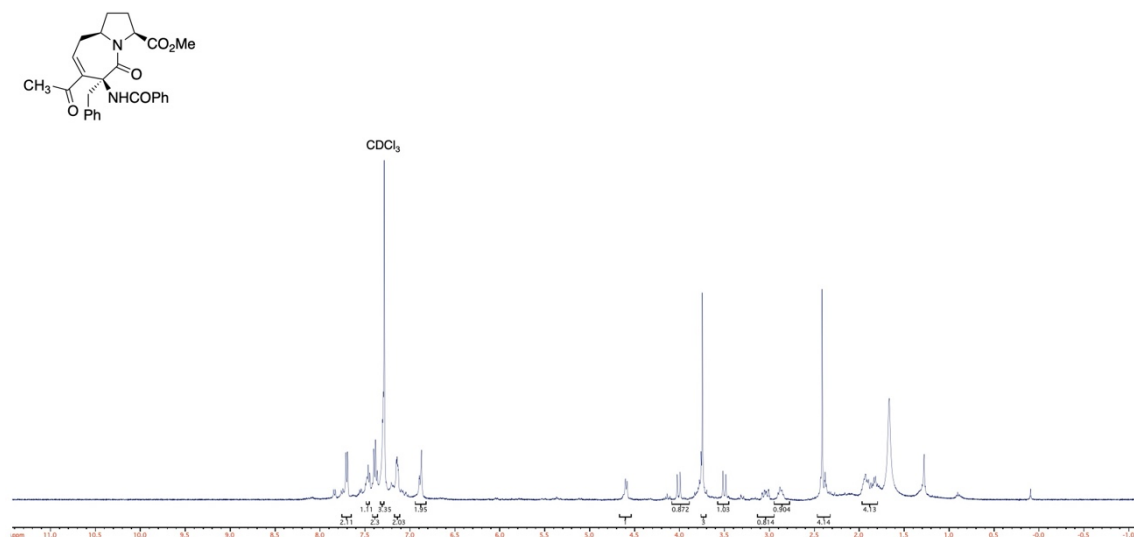






Methyl (3S,6S,9aR)-7-acetyl-6-benzamido-6-benzyl-5-oxo-2,3,5,6,9a-hexahydro-1H-pyrrolo[1,2-a]azepine-3-carboxylate (57a)

^1H NMR (CDCl_3 , 400 MHz)

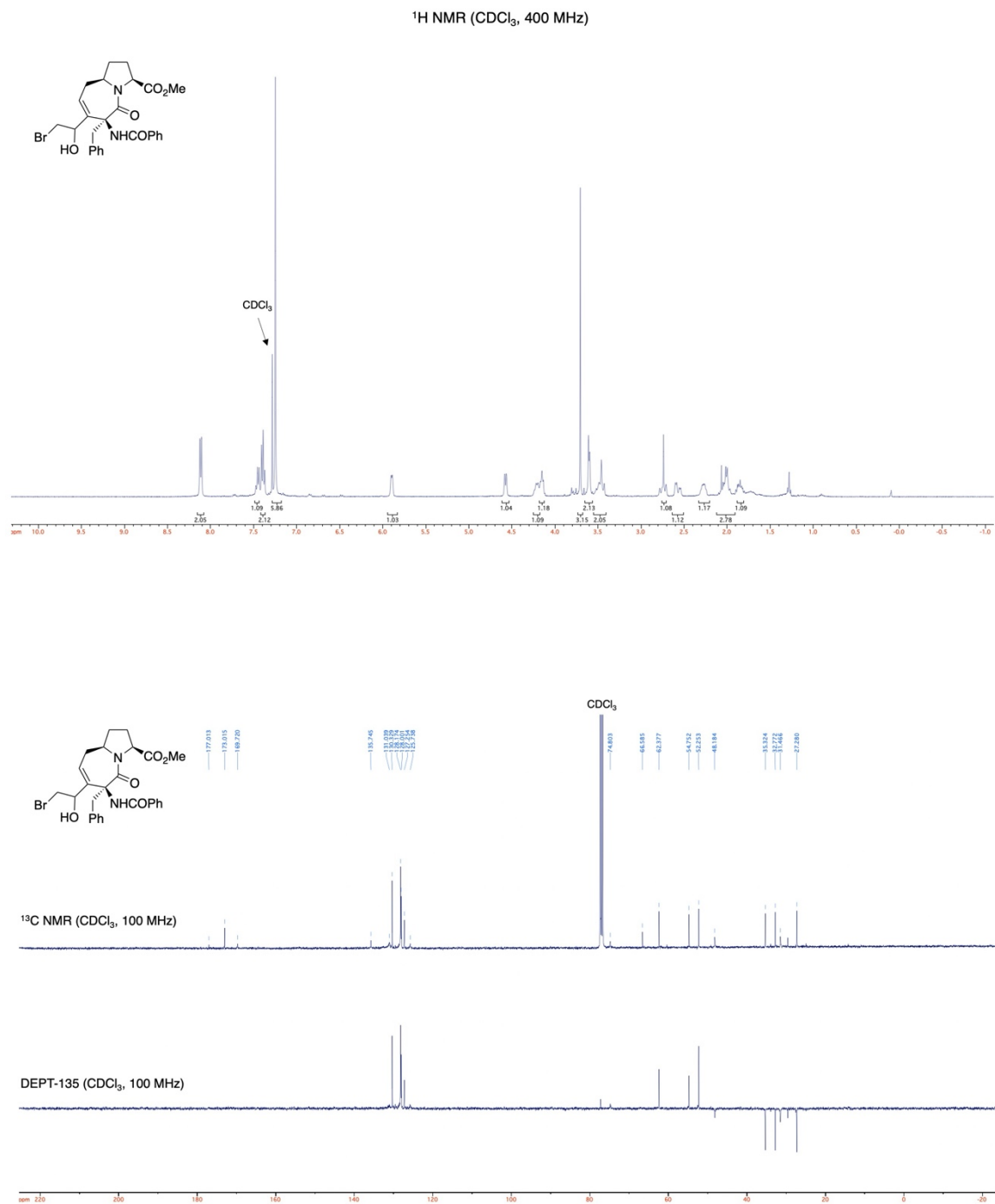


COC(=O)[C@H]1CC[C@@H]1/C=C/[C@H](C(=O)Nc2ccccc2)C(=O)c3ccccc3

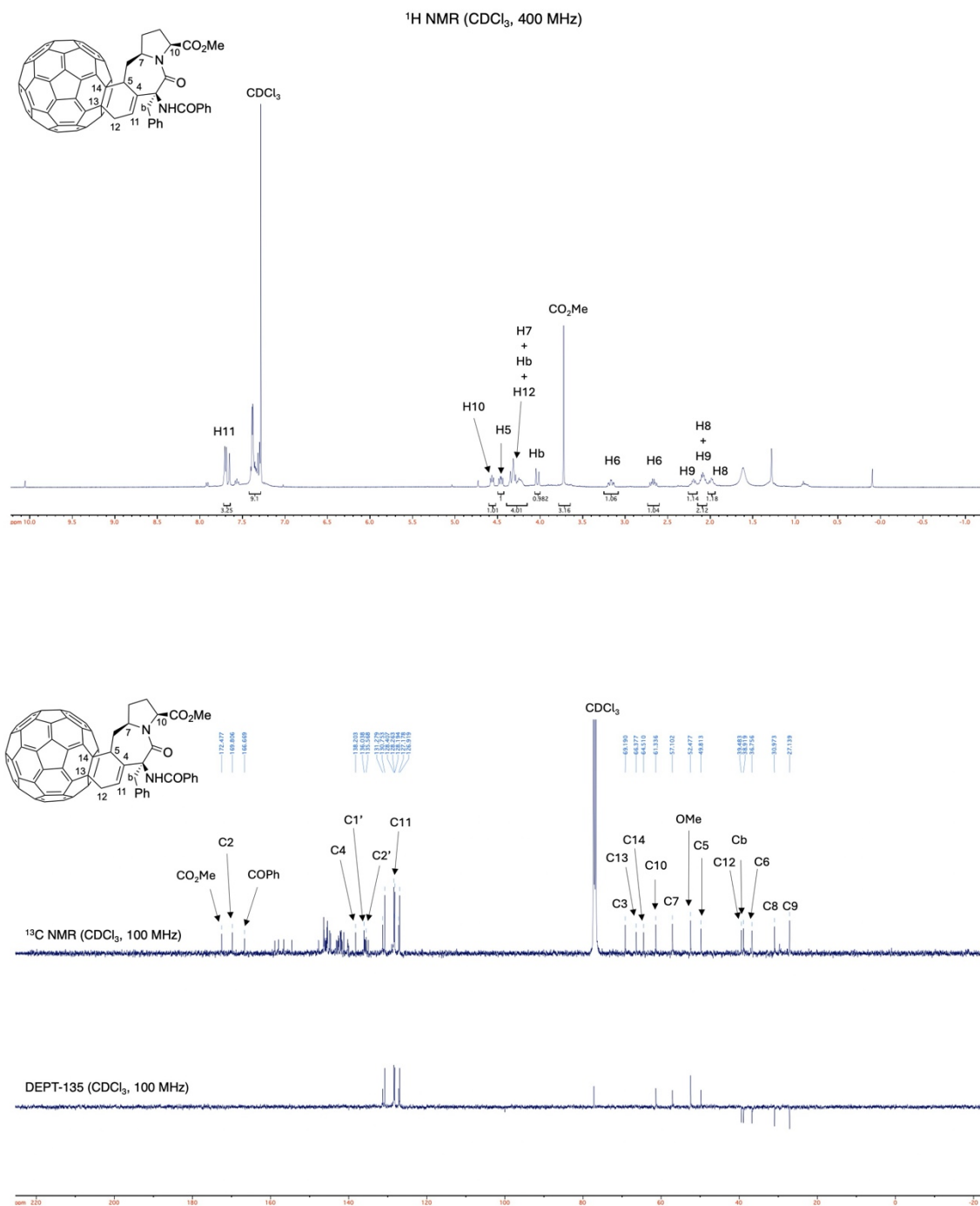
COC(=O)N1C(=O)C(=C(C(=O)O)C(=O)N1Cc2ccccc2)C(=O)c3ccccc3

^1H NMR spectrum (CDCl₃) of compound 10. The spectrum shows peaks corresponding to the structure, with integration values indicated below the baseline. The x-axis represents chemical shift in ppm, ranging from 0.0 to 10.0.

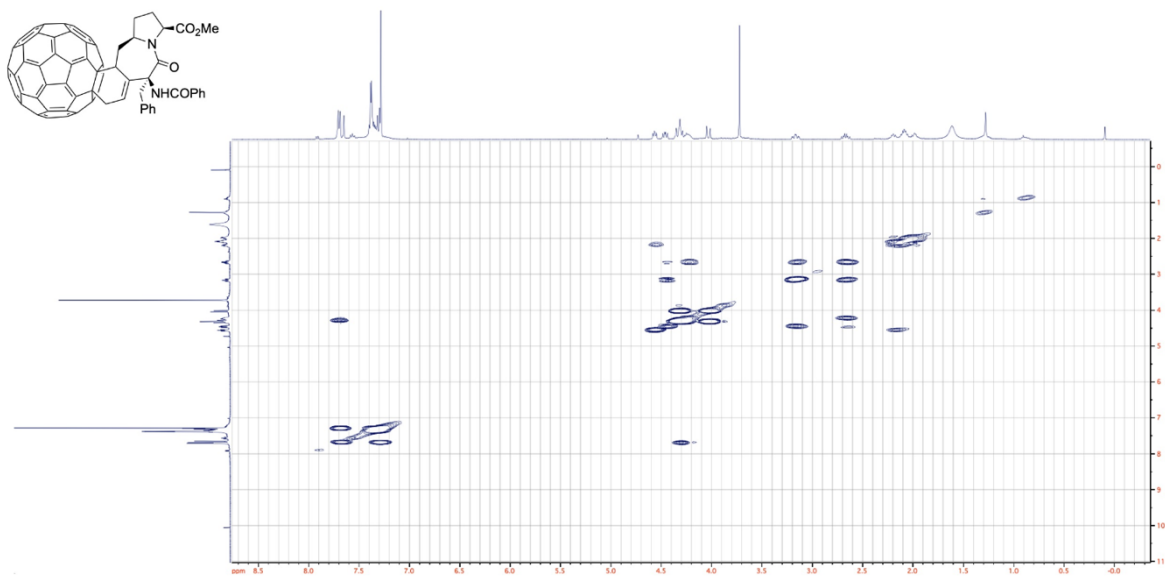
Methyl (3S,6S,9aR)-6-benzamido-6-benzyl-7-(2-bromo-1-hydroxyethyl)-5-oxo-2,3,5,6,9,9a-hexahydro-1H-pyrrolo[1,2-a]azepine-3-carboxylate (60a)



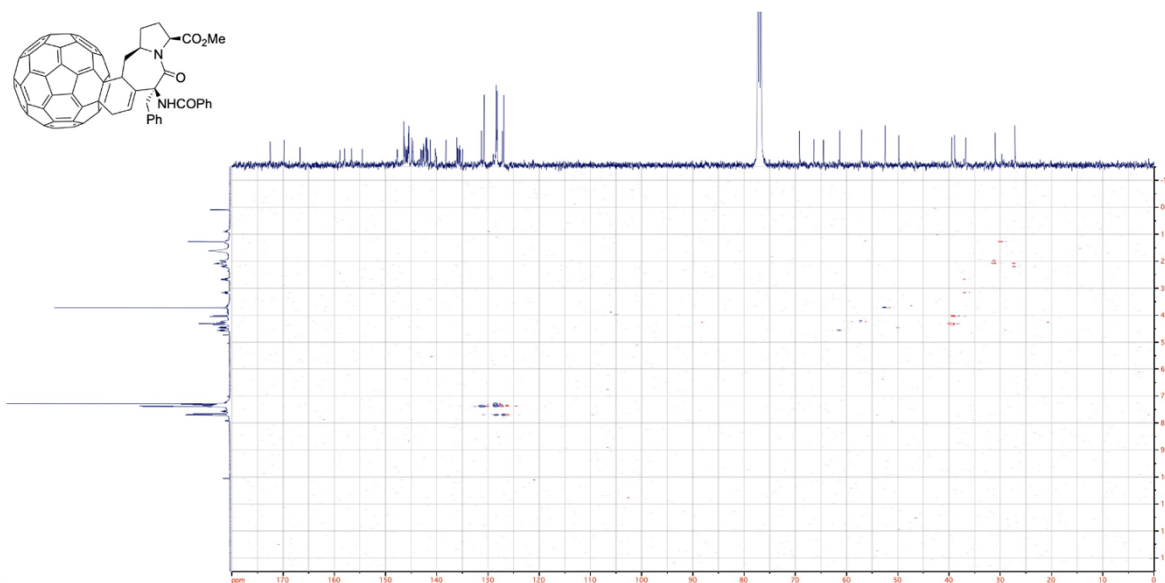
Aba-decorated C60 fullerene (61a)



2D-COSY (CDCl₃, 400 MHz)



HSQC-DEPT (CDCl₃, 400 MHz)



2D-HMBC (CDCl₃, 400 MHz)



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