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Synthesis and development of new molecules for targeted tumor diagnosis and therapy

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Preface

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Introduction

Chemotherapy has been the main modality for medical treatment of cancer for decades. Traditional cytotoxic drugs work primarily through the inhibition of cell division, affecting cancer cells and certain normal tissues. As a result, this treatment cause myelosuppression and have also gastrointestinal, cardiac, hepatic, renal, neurologic and respiratory toxicity. Cancer chemotherapy is limited not only by toxicity to normal cells, but also by intrinsic or acquired multi-drug resistance (MDR) of tumor cells. Because of the rapid elimination and widespread distribution of drugs into non-targeted organs and tissues, the drug needs to be administered in large quantities, which, even non considering the economic issue, complicates the drawback of non-specific toxicity. Consequently, chemotherapy often involves a complex compromise between possible benefits and anticipated side effects. Therefore, the selective targeting of tumor cells is the goal of modern cancer therapy aimed at overcoming this non-specific toxicity. Targeted therapy block the proliferation of cancer cells by interacting with specific molecules which may be present in normal tissues, but they are mutated or over-expressed in tumors. The main advantages of targeted drug delivery systems consists in the more specific delivery of cytotoxic agents to tumor expressing receptors and subsequently a more selective killing of cancer cells. Moreover, the sparing of normal tissues from the harmful side effects of systemic therapy with a reduced peripheral toxicity, allows to avoid the necessity of dose escalation overcoming the intrinsic resistance of some tumors to chemotherapeutic agents.

Antibodies, especially monoclonal human or humanized antibodies (mAbs) against tumor antigens, were the first, and so far the mainly, used as vectors of conjugated moieties for targeted tumor therapy or diagnosis. The recent approval of antibodies such as Herceptin (1998) for breast cancer, Erbitux (2004) and Avastin (2004) for colorectal cancer have revolutionized chemotherapy. Although unmodified mAbs may show some therapeutic potency, their affect tend to be various and ultimately not curative when not used in combination with classical chemotherapy. These antibodies need to be administered to patients in massive doses to be effective, thus leading to serious side effects. As a result, mAbs have been armed with drugs, toxins or radionuclides opening the door to the clinical use of targeted tumor therapy. Among new anticancer drugs approved by U.S. Food and Drug Administration (FDA) since 2000, fifteen have been targeted therapies, compared with only five traditional chemotherapeutic agents.[1] Although traditional cytotoxic drugs remain the treatment of choice for many malignancies, targeted therapies are now a component of treatment for many types of

cancer. Despite all the advancements, most tumor-specific drugs which have undergone registration or are undergoing clinical or preclinical trials (among them almost 20 are immuno-conjugates[2]) are devoid of cytotoxic activity and ability to kill or damage tumor cells.

The effect of these therapies is mainly cytostatic, so not curative, and consist in the retardation of tumor growth or reduction of tumor mass. The main drawbacks on the use of immunoglobulins as tumor targeting agents are the instability of the conjugate and suboptimal pharmacokinetics and biodistribution of the mAb, as their non-specific uptake by the liver and reticuloendotelial system.

Most hormonal approaches to target cytotoxic agents and radioisotopes to cancers are now based on peptides. Peptides became potential vectors for tumor targeting when receptors for different endogenous peptides were observed to be expressed and over-expressed in different human tumours.[3] In particular the finding that somatostatin receptors are expressed on the plasma membrane of several human neuroendocrine tumors opened the way for peptide receptor targeting.[4,5] As tumor targeting agents, peptides have several advantages over antibodies[8], including:

- better organ or tumor penetration and more efficient cellular internalisation, because the antibody molecules are relatively large and might not reach easily the tumor.
- greater stability (long storage at room temperature)
- lower manufacturing costs (recombinant production takes approximately twice as much, in terms of time and costs than chemical synthesis) and more uniform batch-to-batch production parameters, due to the advances in solid-phase peptide synthesis
- fewer and less expensive regulatory requirements (chemical synthesis versus recombinant production) and easier and quicker authority approval.

For these reasons, peptide-derived drugs are now widely used for tumor therapy and diagnosis.[6-8] DOTATOC and OctreoScan®, modified somatostatin analogues conjugated to a metal chelator, are currently used for respectively the therapy and diagnosis of neuroendocrine tumors in humans. Nonetheless, the applications are just limited to neuroendocrine tumors that express sufficient somatostatin receptors. Other regulatory peptides are now candidates as tumor-targeting agents, (such as neurotensin, lutheinizing peptides, gastrin realising peptides, bombesin analogues, substance P, glucagon-like peptide 1). Moreover an increasing number of tumors can be addressed by 'magic-bullet' ('peptide-bullet') strategy, conjugating peptides with functional units

(diagnostic or cytotoxic moieties) which can be specifically delivered into the tumor cell by internalization of the receptor-ligand complex by endocytosis (Figure 1).

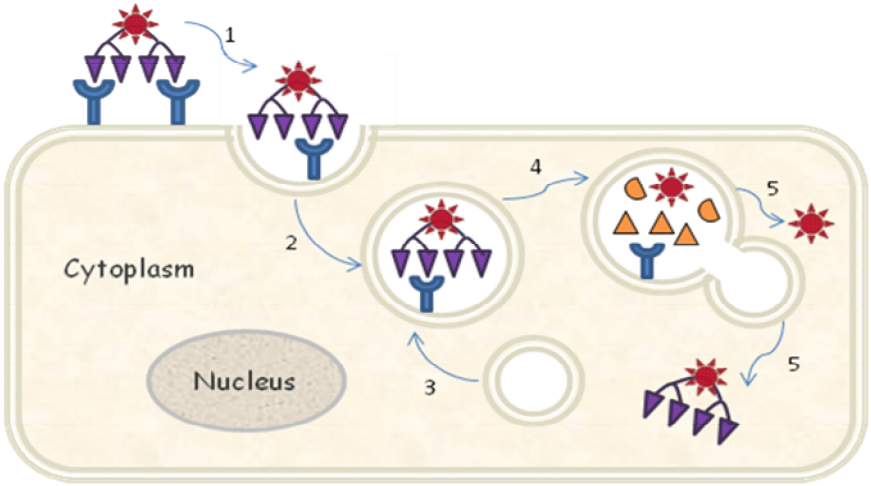


Figure 1. Endocytosis for intracellular drug delivery: 1. Binding of drug delivery unit to a specific ligand; 2. Formation of endosome; 3. Endosome-lysosome fusion; 4. Degradation of the endodomal content by lysosomal enzyme; 5. Endosomal escape, and subsequent delivery of drug to the cytoplasm.

Neurotensin (NT) is a 13-amino acids regulatory peptide, sharing significant similarity in its C-terminal portion of amino acids with several other neuropeptides (Figure 2). This region is responsible for the biological activity as neurotransmitter while the N-terminal portion has a modulatory role. Neurotensin is distributed throughout the central nervous system, with highest levels in the *hypothalamus*, *amygdala* and *nucleus accumbens*. It induces a variety of effects, including: analgesia, hypothermia and increased locomotor activity. It is also involved in regulation of dopamine pathways. In the periphery, neurotensin is found in endocrine cells of the small intestine, where it leads to secretion and smooth muscle contraction.

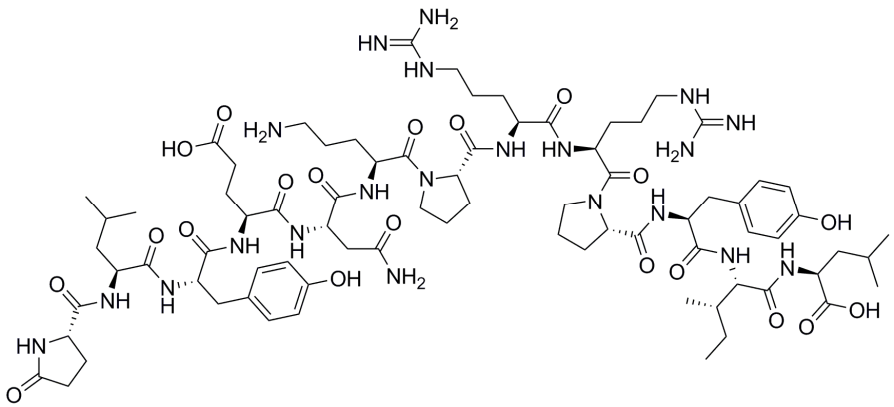


Figure 2. QLYENKPRRPYIL: Neurotensin (NT)

Three different receptors for neurotensin (NTR1, NTR2, and NTR3) have been discovered, cloned and studied so far.[9,10] Neurotensin receptors, in particular the high-affinity NTR1, are expressed in several human tumors, such as small cell lung cancer and colon, pancreatic, and prostate carcinomas (Table 1).[4,8,10,11] Over 75% of all ductal pancreatic carcinomas were reported to overexpress neurotensin receptors, whereas normal pancreas tissue, pancreatitis, and endocrine pancreas do not.[12]

Table 1

TUMOR TYPE	COLON	PANCREAS	PROSTATE	LUNG
NT Expression & Synthesis	*Yes*	*Yes*	*Yes*	*Yes*
NTR1 Expression	*Yes*	*Yes*	*Yes*	*Yes*
NTR2 Expression	*No*	*No*	*No*	_
NTR3 Expression	*Yes*	*Yes*	*Yes*	_
MAPK Activation	*Yes*	*Yes*	_	*Yes*
Proliferative stimulus	*Yes*	*Yes*	*Yes*	*Yes*

Neurotensin is considered the best possible candidate for peptide based therapy of exocrine pancreatic carcinomas,[8] due to the high incidence and density of neurotensin receptors in these tumors, and of whole the gastrointestinal tract cancers in general.
Unfortunately the use of protein and peptides, as Neutotensin, in therapy is hampered by their short half-life, due to physiological degradation by peptidases and proteases.
A truncated COOH-terminal fragment NT(8-13:RRPYIL), fundamental for the interaction peptide-receptor, is slightly more stable than NT while maintaining neurotensin receptor affinity. However, the half-life of NT(8-13) is still too brief for tumor targeting *in vivo*.[13]
To circumvent this problem, various neurotensin analogues have been synthesized, including linear peptides,[13-15] cyclic peptides,[16] and non-peptide molecules,[17] but chemical modification of the native peptide may radically modify receptor affinity and specificity. Prof. Luisa Bracci's group previously reported that synthesis in branched form (Figure 3) increased the biostability of certain peptides, including neurotensin, obtaining promising results *in vitro* and *in vivo* both for diagnostic and therapeutic purposes.[18,19]

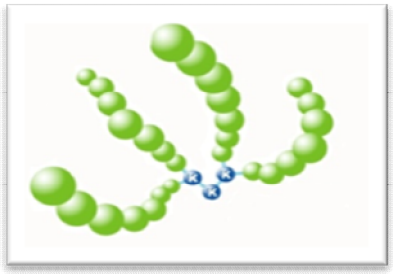


Figure 3 Schematic MAP representation

Synthesis of bioactive peptides in Multiple Antigen Peptide (MAP) dendrimeric form can result in increased half-life, due to acquired resistance to protease and peptidase activity. MAPs, introduced for the first time by Dr. James Tam, have a peptidyl core of radially branched lysine residues onto which peptides can be added using standard solid-phase chemistry (Figure 4).[20] MAPs can be more efficient than monomeric peptides in diagnostic applications and moreover have the possibility to be conjugated to various Functional Units such as fluorophores, photosensitizers, cytotoxic groups or chelators for radioisotopes.

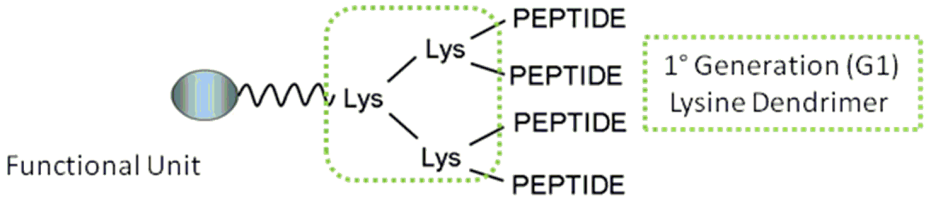


Figure 4 Peptide tetramer with three-lysine branched core

The *in vitro* and *in vivo* efficiency of dendrimeric peptides like MAPs is generally ascribed to their multimeric nature, which enables polyvalent interactions.
In order to test the possible influence of peptide length, number of peptide copies, and steric hindrance on branched peptide stability to peptidases, the monomeric, two-branched and tetra-branched forms of NT and NT(8–13) were synthesized and their stability, after incubation with human plasma and serum was compared. A correlation was made between the number of peptide copies in branched neurotensin and NT(8–13) and stability in plasma and serum. Prof. Bracci found that the tetra-branched form of neurotensin and its short analogue NT(8–13) were stable in both human plasma and serum for 24 h, whereas the two-branched for 5 h and and the monomeric analogues were degraded in roughly 5 h (Tables 2 and 3).[19]

Table 2. *Stability of monomeric, dimeric and tetra-branched NT in plasma and serum*

	PLASMA			SERUM		
PEPTIDE	*2h*	*5h*	*24h*	*2h*	*5h*	*24h*
QLYENKPRRPYIL	+	-	-	+	-	-
$(QLYENKPRRPYIL)_2K$	+	+	-	+	+	-
$(QLYENKPRRPYIL)_4K_2K$	+	+	+	+	+	+

Table 3 *Stability of monomeric, dimeric and tetra-branched NT(8-13) in plasma and serum*

	PLASMA			SERUM		
PEPTIDE	*2h*	*5h*	*24h*	*2h*	*5h*	*24h*
RRPYIL	+	-	-	+	-	-
$(RRPYIL)_2K$	+	+	-	+	+	-
$(RRPYIL)_4K_2K$	+	+	+	+	+	+

The tetrameric form has also receptor affinity, which in the case of tetrabranched NT(8-13), is even higher than that of the monomeric peptide,[18,19] probably due to the polyvalent interactions.

As expected, the native and short peptide have similar resistance to plasma and serum proteases, because most proteolytic enzymes that inactivate NT have their cleavage sites in the short NT(8–13) sequence (Figure 5),[21] the longer sequence of NT does not introduce new sites.

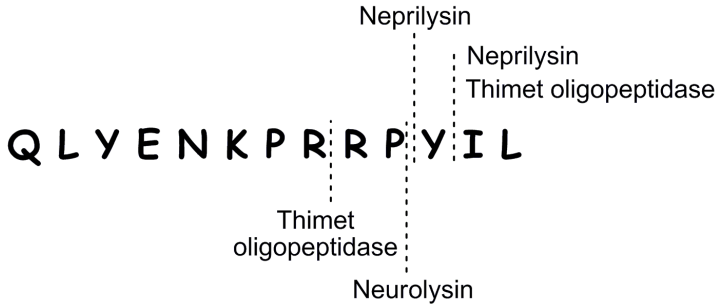


Figure 5. Neurotensin specific cleavage enzymes

In order to determine whether multimericity or branched structure contributes to peptide stability, linear and branched multimeric peptides were compared. Tetrameric peptides showed a dramatic difference in proteolytic resistance. The linear tetramer was very labile being cleaved by blood proteases like the linear monomer (Table 4), whereas the branched tetramer was stable up to 24 h in plasma and serum. This established that, as a general rule, multimeric branched forms are more stable than the corresponding monomeric forms and that multimericity progressively enhances the stability of peptides. Peptidases acting on small endogenous peptides are mainly Zn metallopeptidases like neurolysin, the catalytic centre of which is located in a deep channel, to which only small peptides have access (Figure 6).[22,23] Although binding of the peptide in the catalytic pocket may still be possible, the cleavage site may be geometrically unreachable by catalytic residues, especially in tetrabranched peptides that, unlike two-branched ones, cannot achieve an extended conformation. As a result, the steric hindrance may limit their access to the cleavage site of these peptidases, lengthening the peptide half-life, with obvious advantages for their use as drugs.

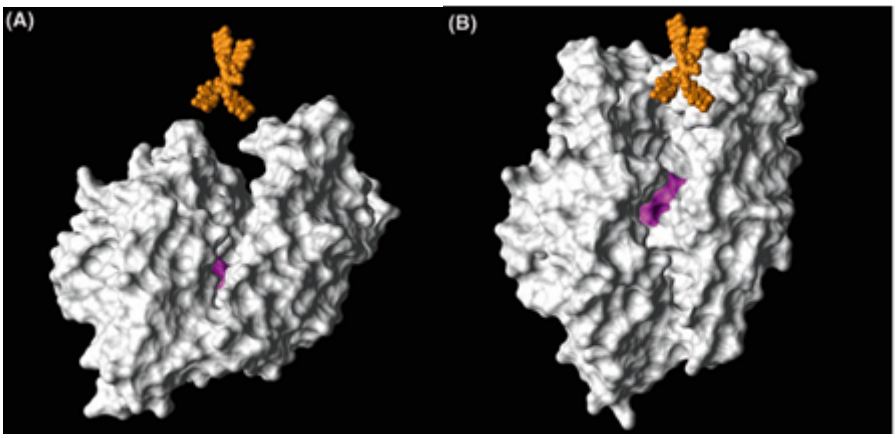


Figure 6 shows Neurolysin (white) and tetrabranched NT (orange) as seen parallel (A) and perpendicular (B) to the major axis of the groove containing the catalytically active zinc site of the enzyme.

The possibility to form supramolecular aggregates is another feature of branched peptides probably accounting for their increased stability.

Tetrabranched peptides may arrange their four arms in parallel structures stabilized by intramolecular H-bonding of the backbone. This structure would resemble that of self-assembling β-sheet peptides. Prof Bracci's group analyzed the presence of aggregates in branched peptide in solution using gel filtration, finding that the tetrabranched NT(8–13) monomer might be in equilibrium with a small aggregate of four or five units.[19]

The formation of small aggregates might decrease the rate of proteolysis and has to be taken into account for the increased half-life of branched peptides.

As already suggested, the finding of new specifically targeted cytotoxic drugs is an important challenge in cancer research, where receptor-specific small peptides may have major potential applications, provided that they are stabilized to overcome their degradation by plasma proteases. Prof. Bracci's results indicate that synthesis in dendrimeric form may be a general method to increase *in vivo* stability of bioactive peptides and so a step forward on this direction.

On the basis of these observations in 2005 "Associazione Italiana per la Ricerca sul Cancro" (AIRC) granted to three groups, including that of my tutor at the University of Florence, a research project titled "*Selection and development of new molecules for tumor therapy and in vivo diagnosis"*. The Florence unit used part of this grant for the present PhD course. The aim of the project was to set up a general method for the design and synthesis of peptide molecules for tumor targeting, both for therapy and for in vivo imaging. These new molecules have been designed as dendrimeric neurotensin derivatives carrying functional units as fluorophores, photosensitizer, cytotoxic drugs and chelators for radionuclides. The project is based on the data reported above, demonstrating that peptides in such a dendrimeric form are particularly suitable for *in vivo* use because they acquire a notable resistance to degradation by proteolytic enzymes, though maintaining or even increasing their natural biological activity. Neurotensin and its functional fragment, synthesised as tetramers, offers even accessible linking units for coupling of functional moieties.

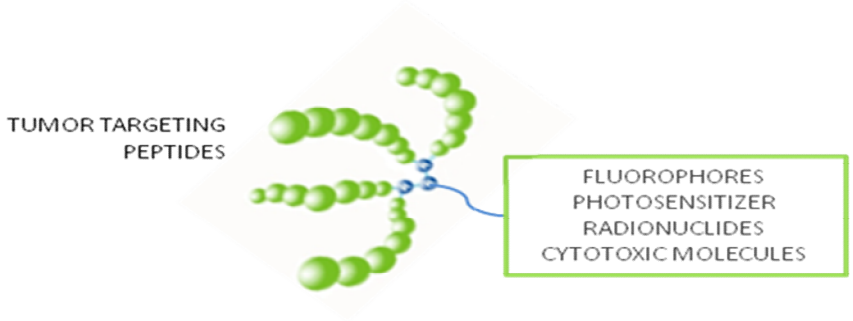


Figure 7. Functional Units conjugated to the dendrimeric carrier

Results obtained with tetra-branched NT(8-13) bound to biotin or fluorescein demonstrate that it fully retains its binding activity on neurotensin specific receptor on HT-29 (colon adenocacinoma) tumor cell line.[24] These outcomes bring new perspectives to the use of dendrimeric NT(8-13) as a carrier, multifunctional targeting

molecule, where cancer detection, screening, diagnosis and therapy are obtained by means of the same molecule, with no modification in target binding, but 'simple exchange' of functional units. Since cancer cells are very different from one another in terms of drug sensibility, not only in different tumors, but in different patients and stages of the disease, this approach prefigures the synthesis of a number of constructs conjugated with different chemotherapeutics. Bracci's group has set up a general method allowing conjugation by standard solid-phase peptide synthesis. The C-terminal lysine's side chain was functionalized with the chemotherapeutics spaced by a PEG (polyethylenglycol) moiety, chosen in consideration of synthetic and biological issues. The hindrance of the branched peptide might impair the access of the activated chemotherapeutic and therefore give poor reaction yields. Moreover the drug moiety, if closely linked to the branched peptide, might impair receptor recognition and, for slow realizing compounds, the closeness of branched peptide might compromise drug-target interaction inside the cell. The pending amino group is then coupled to Functional Units, carrying an accessible COOH group, so that the synthesis can be led in succession, directly on the resin support.

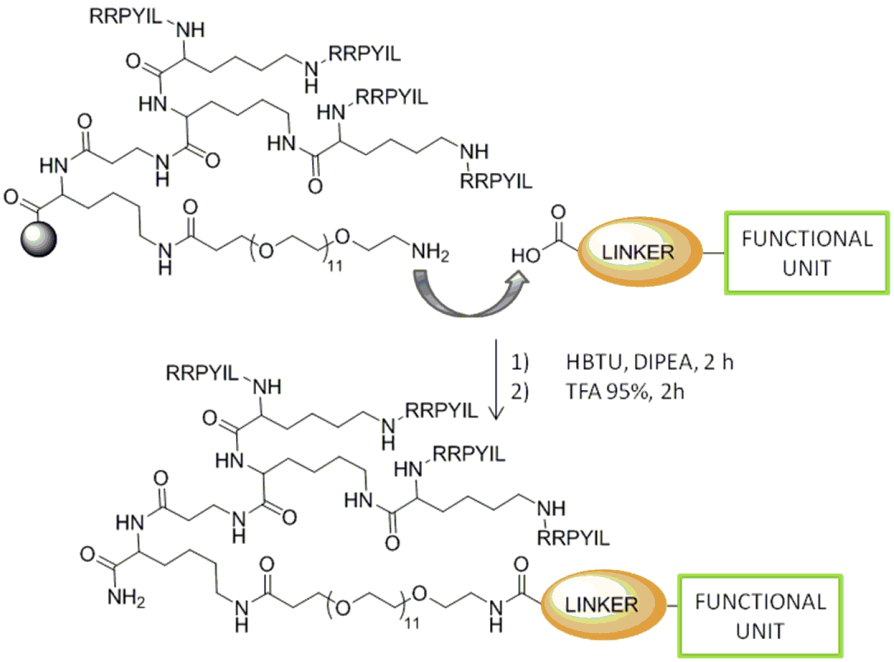


Figure 8. Conjugation procedure

The choice of linker's structure to be inserted between tetrabranched NT and the Functional Unit is fundamental because in the case of cytotoxic molecules the link has

to be broken preferably upon internalization into the tumor cell. In other words the conjugate can be regarded as a 'prodrug', defined as non-toxic form of active drug which is converted in the body into the active species either chemically or metabolically.[25] Non-specific prodrugs are activated systematically (e.g. in plasma) and are normally employed to improve solubility or pharmacokinetic properties. In contrast, tumor-activated prodrugs aim to improve specificity of action of cytotoxins, by providing non-toxic forms that can distribute systemically but which are specifically activated to the toxic form only or preferably in tumor tissue. The activation step makes use of some unique physiological, metabolic or genetic differences between tumour and normal cells, such as different oxygen, ligands and enzymes concentrations.[26] We decide to use disulfide or ester linkage as covalent bonds suitable for safety delivering the drug inside the cell where the releasing of the anticancer agent occurs respectively by reaction with citoplasmatic thiols (for example glutathione, GSH) or by hydrolysis (mediated by ubiquitous esterases). The preparation of selected conjugated pro-drugs, drugs and diagnostic tools have been the main challenge of this PhD project. As it will be described in the following sections, we have synthesized a variety of carboxylic derivatives decorating the most common anticancer drugs (Combretastatin A-4 (CA4), Tirapazamine (TPZ), 6-Mercaptopurine (6-MP), 5-Fluoro-2'-deoxyuridine (5-FdUrd), Gemcitabine, Monastrol) and metal chelators (DOTA) (Figure 9).

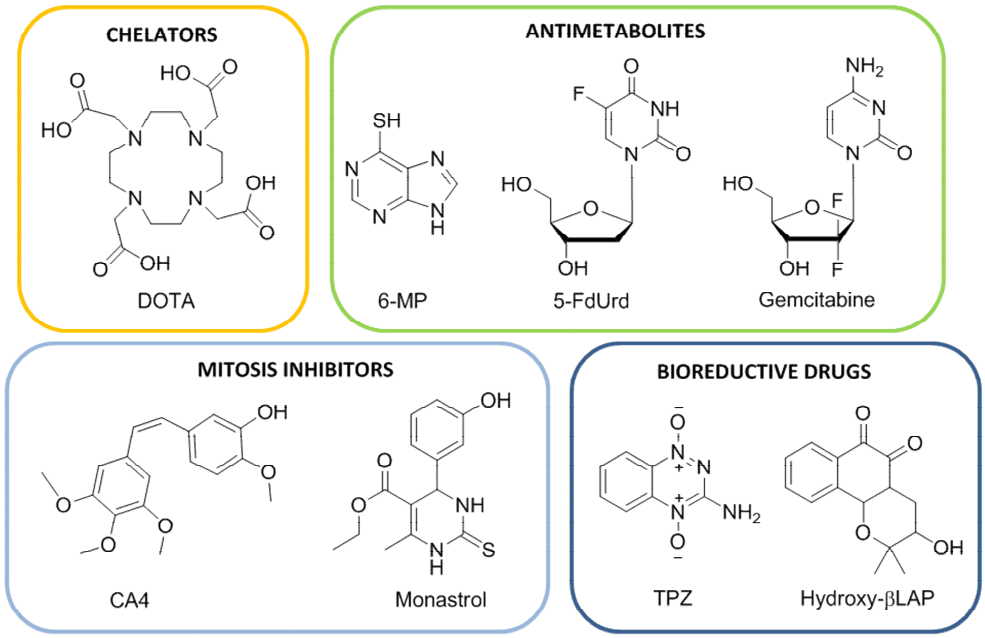


Figure 9. Common anticancer drugs which have been modified during this PhD.

The accessible COOH groups have been then coupled with the NH_2 group, introduced *ad hoc* on the tetrameric peptide, by standard solid phase peptide synthesis.
The synthetic results obtained as well as the anti-tumour activity of these derivatives, once conjugated to the tetrameric peptide NT(8-13), will be reported in this thesis.

1. Synthesis of modified drugs

1.1 Chelating agents (DOTA analogues)

Current interest in the clinical use of chelating agents as vehicles for metals in biological systems has led to the synthesis and study of a large number of new cyclic polioxa or polyaza ligands. One class that has shown great promise for clinical applications is the polyazamacrocyclic polycarboxilate ligands.[27] Particularly, **1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid** (**DOTA**) (Figure 10) and its analogues became a subject of universal interest because they form stable complexes with a wide variety of metal ions. Furthermore metal-DOTA complexes have been shown to be kinetically inert under physiological conditions.[28]

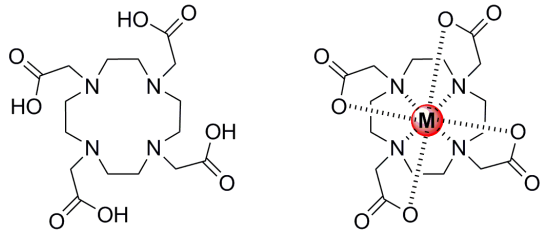


Figure 10 DOTA and octa-coordination around a metal

Among metal ions which can be strongly encapsulated by DOTA, radiometals such as ^{111}In(III), ^{67}Ga(III) and ^{99m}Tc(VII) have physical properties which are well suited for tumor imaging, while ^{90}Y(III), ^{67}Cu(II), ^{186}Re(VII) and ^{177}Lu(III) have cytotoxic properties which can be exploited for therapy.

Paramagnetic ions, as Gd(III), are instead generally exploited for Magnetic Resonance Imaging (MRI), which is a technique able to visualize intact opaque organisms in three dimensions and, therefore, provide an alternative to light microscopy and radiopharmaceutical methods.[29] The image is based upon the NMR signal from the water's proton, where the signal intensity in a given volume element is a function of the water concentration and relaxation times (T_1 and T_2). Gadolinium-based contrast agents for MRI enhance tissue contrast by increasing the relaxation rate ($1/T_1$) of water protons and are widely used in clinical diagnostics.

One of the major problems in conventional contrast agents and radiopharmaceuticals is that they are primarily extracellular with an aspecific biodistribution.

For these reasons, the next generation of contrast agents will include systems able to recognize specific molecules on the cellular surface that act as early reporters of a given pathology. The targeting of over-expressed membrane receptors with specific radiopharmaceuticals is already a well-established diagnostic method in nuclear medicine for several types of tumors. A somatostatin radioligand, DOTA-[D-Phe1-Tyr3]-octreotide (DOTATOC), has been the first synthesized for therapeutic purposes, because of its stable and easy labeling with ^{90}Y. Starting from this successful compound, the number of peptide-metal chelate based molecular sensors, diagnostic and therapeutic agents is rapidly increasing and is becoming one of the most challenging areas of research.[30]

Considering our perspectives in peptide targeted tumor therapy we decided to conjugate a chelating agent to our carrier and exploit its efficacy on detecting or killing of tumors, once complexed with an appropriate metal. For this purpose we selected, as a suitable chelating agent, DOTA because of the promising results obtained and the opportunity of direct coupling between the petide's free amine and its carboxylic functionalities.

In literature is reported that DOTA-modified peptides are generally synthesized either in solution[31] or on solid support[32] attaching the DOTA residue to a free amine of the resin bound peptide. DOTA or a derivative can be both directly attached to the peptide or a linker may be included between the peptide chain and the chelator. The selection of the linker is dependent on the desired pharmacokinetic and chelating properties of the conjugate. Based on this, we designed the synthesis of three DOTA derivatives **1-3** (Figure 11) functionalised with a single pending carboxylic acid moiety to permit their coupling with the free amino group of the dendrimeric peptide using standard solid phase peptide synthesis. The carboxylic acids not to be involved in the coupling, were protected as *t*-Bu esters, to overcome side reactions by polyactivation of the four carboxylic groups of DOTA, and easily deprotected during the final acid cleavage of the modified peptide from the solid support.

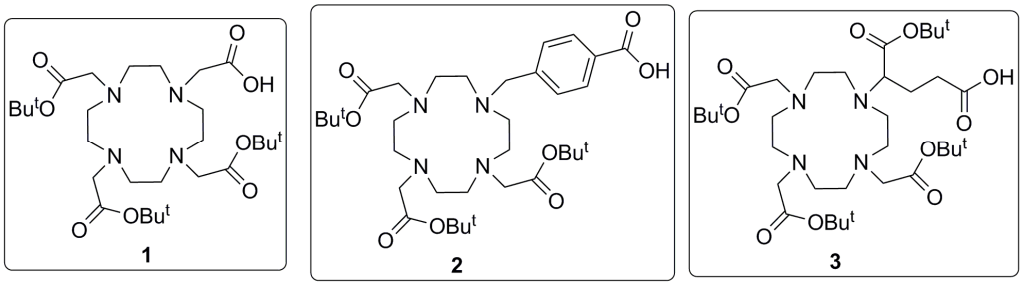
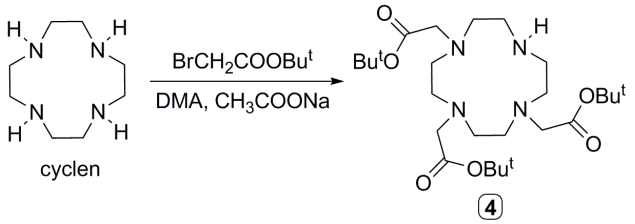


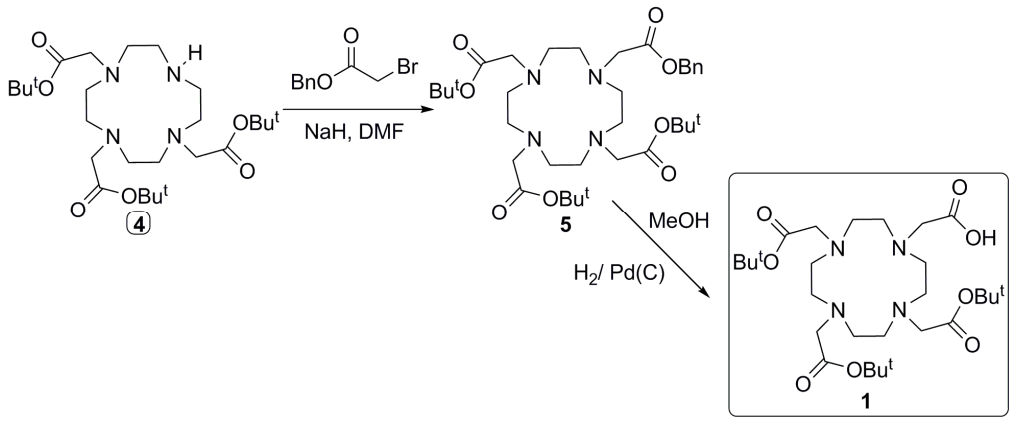
Figure 11 The three DOTA derivatives synthesized

As a suitable common starting material, 1,4,7,10-tetraazacyclododecane-1,4,7-tris(*tert*-butylacetate) **4**, was prepared, as shown in Scheme 1, reacting in dimethylacetamide (DMA) *tert*-butylbromoacetate (3 eq.) with a solution of tetraazacyclododecane (1 eq.), commercially available as cyclen, and Na(CH_3COO) (3 eq.). The *tert*-butyl ester of DO3A (1,4, 7,10-tetraazacyclododecane-1,4,7-triacetic acid) **4** was obtained by precipitation after 20 days under stirring in 55% yield (Scheme 1).



Scheme 1

The derivative **1** was obtained, as previously reported,[31] by adding benzylbromoacetate to compound **4** in DMF in the presence of NaH. The reaction mixture was then stirred for a day at room temperature (r.t.), then a solution of 5% citric acid was added and the mixture extracted with chloroform. The product obtained was purified by flash chromatography to yield *N*-(benzylcarboxymethyl)-1,4,7,10-tetraazacyclododecane-*N*',*N*'',*N*'''-triacetic acid tri-*tert*-butyl ester **5**. A mixture of this compound and 10% Pd/C was hydrogenated in methanol under a positive H_2 atmosphere for 6 hours to yield the modified DOTA macrocycle **1** in 50% yield (meant for two reactions) (Scheme 2).

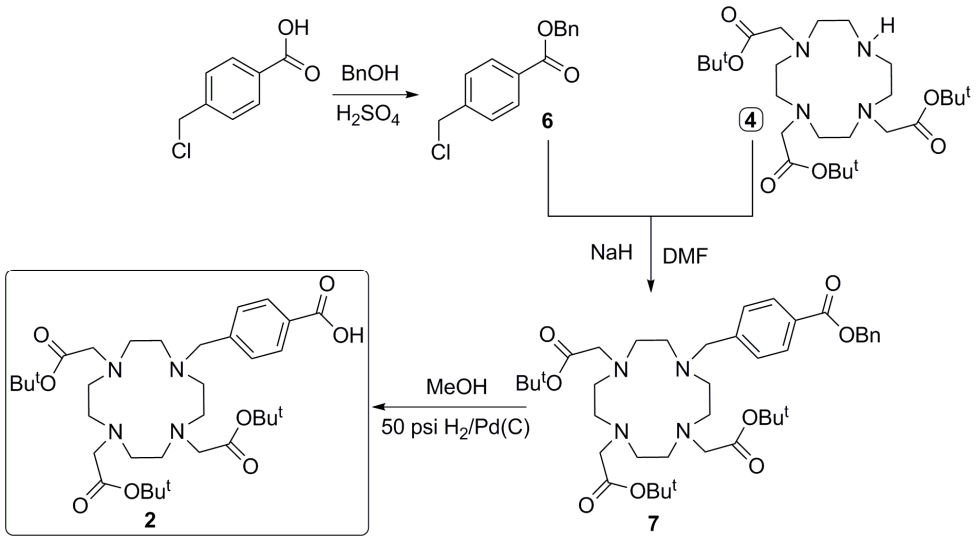


Scheme 2

With the aim to modulate the linker between the chelating moiety and the targeting peptide and so hopefully improve the chelating ability, we designed the synthesis of derivative **2** where a benzyl group is interjected.

Compound **2** was synthesised with a similar procedure to the one reported above, but using another halogenated compound, which has been prepared expressly by heating a suspension of 4-chloromethylbenzoic acid in benzyl alcohol, which works both as solvent and reagent, in acidic conditions. The crude product obtained was purified by column chromatography to give the desired benzyl derivative **6**.

The benzyl ester of 4-chloromethylbenzoic acid **6** (1 eq.) was added to a solution of **4** (1 eq.), and 3 eq. of K_2CO_3, in dry MeCN at 60 °C. After 2 days, the suspension obtained was filtered through celite and concentrated, giving DO3A derivative **7** without further purification. The following hydrogenation was performed at high pressure, 50 psi of H_2, for 60 hours, to obtain benzyl derivative **2** after purification by column chromatography in an overall 50% yield of two reactions (from **4** to **2**) (Scheme 3).



Scheme 3

Structure of compounds **1** and **2** ensure a selective and efficient coupling with the dendrimeric peptide, as it is shown in chapter 3, through the formation of a single amide linkage. However this will cause a lower ability of encapsulating metals due to the lack of a free carboxylic acid group when compared with unmodified DOTA. (Figure 12)

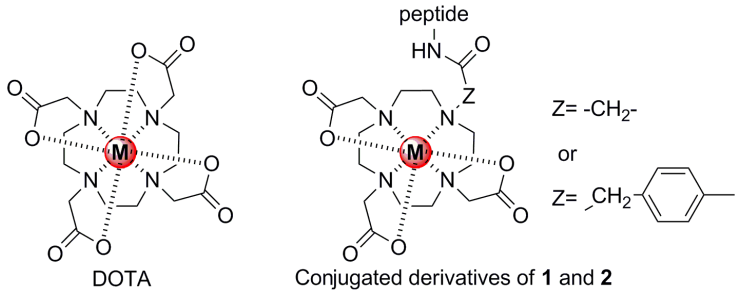


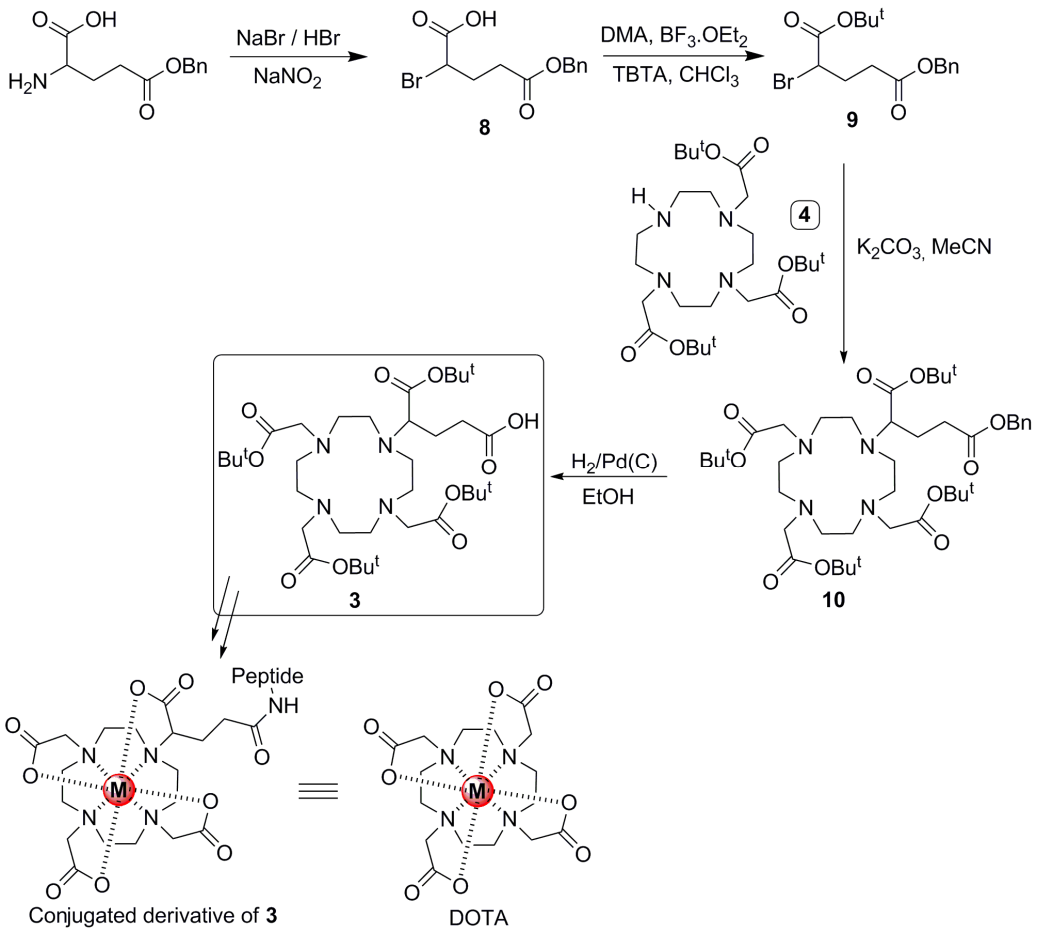
Figure 12 Complexation

Therefore we planned to synthesize the DOTA derivative **3**, by modification of previous methods,[34] which contemporarily ensures the possibility of an active coupling, using the standard solid phase peptide chemistry, and an octa-coordination around the selected metal once the *t*-butyl esters have been hydrolyzed.

Operatively, starting from the commercially available L-glutamic acid-5-benzylester (1 eq.), using a method analogous to Holmberg,[32] a suspension with sodium bromide (3 eq.) in a cooled 1N solution of bromidric acid was made and sodium nitrite (2 eq.) was added portionwise. The yellow solution was stirred for 2 hours and finally 560 µL of 98% sulfuric acid were added, followed by diethylether. The crude product was purified by flash chromatography to obtain bromoacid **8** in 82% yield.

The protection of the free carboxylic group was obtained adding dropwise a solution of *tert*-butyltrichloroacetimidate (TBTA) (2 eq.) in cyclohexane to a solution of **8** (1 eq.) in $CHCl_3$. During the addition a white precipitate formed, which was dissolved by the addition of DMA before adding a catalytic amount of boron trifluoridiethyl etherate (0.1 eq.). The reaction mixture was stirred for three days at r.t.. Flash chromatography was then necessary to isolate bromoester **9** in 50% yield.

The following coupling with the macrocyclic ring was performed as usual, in mild basic conditions. A mixture of **4** (1 eq.) and K_2CO_3 (3 eq.) was stirred in dry acetonitrile at 60 °C for 10 min before adding dropwise, over a period of 40 min, a solution of **9** (2 eq.) in dry acetonitrile. After 42 hours under stirring at 60° C the yellow solution was filtered through celite and concentrated. The residue was chromatographed to obtain benzyl ester **10** isolated in 59% yield. Eventuallly **10** was dissolved in absolute ethanol and hydrogenated over 10% Pd/C under a positive H_2 atmosphere for 48 hours to give the desired product **3** in 79% yield without the necessity of a further purification (Scheme 4).



Scheme 4

As it is shown in Scheme 4 this derivative (**3**) can be conjugated to the dendrimeric peptide without affecting its complexation ability, because, like for DOTA, an octa-coordination around the metal, resulting in a compact square-antiprism involving four nitrogen atoms in one plane and the four oxygen atoms in a second plane, is perfectly available.

1.2 Cytotoxins

Cytotoxic chemotherapeutic agents are defined as anticancer drugs that kill cells and especially cancer cells. Almost six decades separate us from the first clinical application of an antitumor drug. Nearly all major achievements in cytotoxic therapy were made in early stages, 20-30 years ago, when anticancer drugs such as Cisplatin, Doxorubicin, Cyclophosphamide, 5-Fluorouracil, Tamoxifen, 6-Mercaptopurine, etc., were developed.

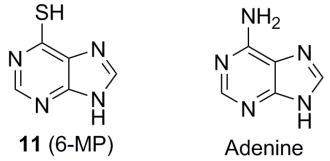
Today a large variety of registered and under clinical trials cytotoxins are used in the treatment of malignant tumors. Among these drugs we selected the most useful, taking in consideration the type of tumors where the neurotensin receptors (NTRs) are over-expressed (Table 1), and at the mean time the most suitable to be synthetically modified in order to be conjugated to the targeting dendrimer.

The selection regarded efficient but non-specific chemotherapeutics from some of the most common anticancer classes, such as *antimetabolites*, *bioreductive drugs* and *mitosis inhibitors*, with the aim to transform them into tumor selective drugs. The simple exchange of Functional Units allowed to test a wide variety of different cytotoxins with different mechanism of action, cytotoxicity and problems, therefore increasing the chances of success.

1.2.1 Antimetabolites

Antimetabolites are substances bearing a close structural resemblance to ones required for normal physiological functioning, and exerting their effect by interfering with the utilization of the essential metabolites. In the cell, antimetabolites are mistaken for the metabolites they resemble, and are processed in the cell in a manner analogous to the normal compounds. The presence of the 'decoy' antimetabolites prevents the cells from carrying out vital functions and the cells are unable to grow and survive. In cancer treatment they can be used, as they interfere with DNA production and, therefore cell division and tumor growth. Because cancer cells spend more time dividing than other cells, inhibiting cell division harms tumor cells more than other cells. Even if the clinical application of these drugs have shown an inadequate selectively which brings serious side effects, antimetabolites can be used for their high cytotoxicity as Functional Units in tumor-targeting systems.[35]

a) The antimetabolite **6-Mercaptopurine** (**6-MP**) **11** is a synthetic analogue of the natural purine, adenine, where the amine group is replaced by a thiol (Figure 13).



11 (6-MP) Adenine

Figure 13

The biological activity of 6-MP is complex and involve cellular metabolism in several ways. Two of 6-MP metabolites, 6-thioguanosine-5'-phosphate (6-thioGMP) and 6-thioinosine (T-IMP), inhibit nucleotide interconversions and *de novo* purine synthesis, thereby blocking the formation of purine nucleotides and inhibiting DNA replication. This agent is also incorporated into DNA in the form of deoxythioguanosine, which results in the disruption of DNA replication. In addition, mercaptopurine is converted to 6-methylmercaptopurine ribonucleoside (MMPR) by 6-thiopurine methyltransferase; MMPRs are also potent inhibitors of *de novo* purine synthesis.

6-MP has been widely used as a chemotherapeutic agent and, to a lesser extent, as an immunosuppressant for almost five decades. The drug is still used in the treatment of a variety of conditions, including leukemias, limphomas, adenocarcinomas,[36] rheumatologic disorders and prevention of rejection following organ transplantation. However, oral absorption of 6-MP is erratic with only 10-50 % of the administered dose reaching the systemic circulation. The limiting factors in the use of mercatopurine are the very short half-life in plasma (0.5-1.5 h) and its very variable bioavailability (about

16 %). Moreover, severe bone marrow toxicity, with a subsequent risk of infections, anaemia and bleeding, and liver toxicity are associated with 6-MP treatment. Strategies to circumvent these problems have included the design of prodrugs that might decrease the systemic toxicity of **11** while ensuring its delivery to the target tissue.

Prodrugs are biologically inert or substantially inactive forms of the parent or active compound, where the release rate of the active drug is influenced especially by the type of bond joining the parent drug to the modifier.

Glutathione (GSH) is an ubiquitous compound that is important in cellular defense mechanism and amino acid transport. Elevated GSH content and levels of glutathione S-transferases (GSTs), GSH peroxidase and GSH reductase (GR) were detected in many tumors compared with the healthy tissues.[37] Moreover, increased levels of GSH were associated to drug resistance. This condition has been useful to design 6-MP prodrugs such as azathioprine (AZA) **12**, that has been shown to be superior to 6-MP as an immunosuppressant, whereas its therapeutic index against leukaemia and adenocarcinoma is similar to that of **11**. AZA has now replaced 6-MP in the treatment of organ transplant patients and other structural analogues have been exploited as anticancer drugs. The biological activity of AZA and its structural analogues (AVTP, **13**, and PTA, **14**) is due to their GSH-mediated metabolism to 6-MP.[36,38] Gunnarsdottir and Elfarra have characterized the metabolism of *cis*-3-(9*H*)-purin-6-ylthio)acrylic acid (PTA, **14**), targeting tumors with up-regulated GSH levels. Structurally, PTA is a propenoic acid conjugate of 6-MP and a Michael acceptor that undergoes addition-elimination reaction with nucleophiles to yield the parent drug.

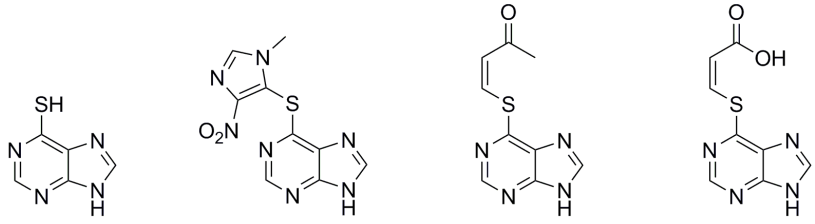
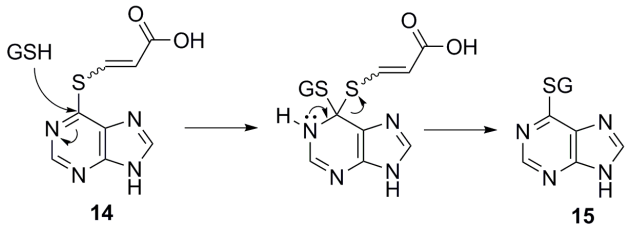


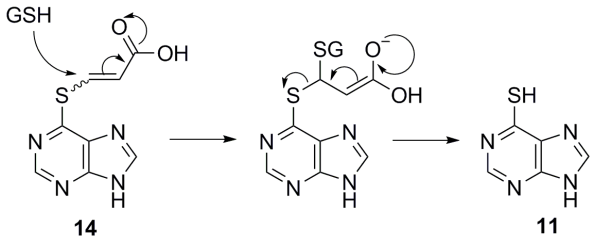
Figure 14

6-MP is formed via two distinct pathways, in the first **11** is obtained indirectly via the formation and further metabolism of *S*-(9*H*-purin-6-yl)glutathione (PG) **15**, the major metabolite formed in the reaction between PTA and GSH after the attack on the *C*-6 of the purinic ring (Scheme 5).



Scheme 5

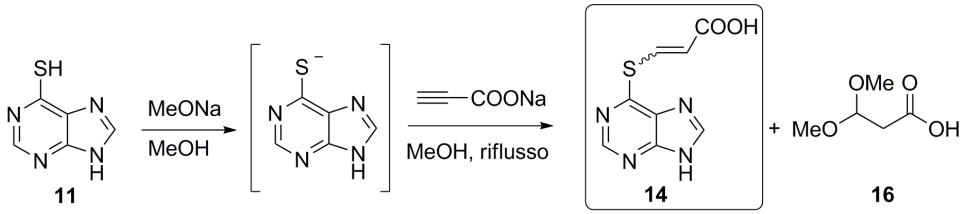
In the second pathway, 6-MP was formed directly from PTA through an addition-elimination reaction with GSH, as is shown in Scheme 6. This mechanism is dependent on the GSH concentration and on pH and it can occur in a non-enzymatic way.



Scheme 6

Therefore, the presence of a carboxylic moiety and the ability to release 6-MP preferably into the cancer cells, due to the high levels of GSH and its enzymes, make PTA a suitable prodrug to be coupled to our targeting peptide.

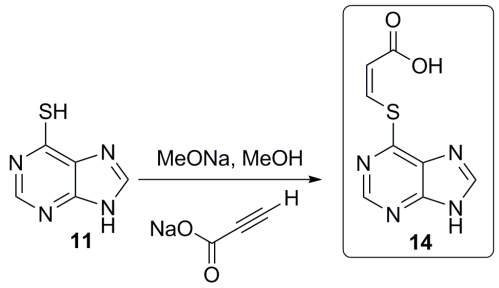
We decided to synthesise compound **14** by modification of previous methods,[38] because with the amount of MeONa reported in literature, 3 eq., we obtained the undesired product **16**, resulting from the bis-addition of the methoxide, onto the propiolic acid, with a subsequent low yield of **14** (Scheme 7).



Scheme 7

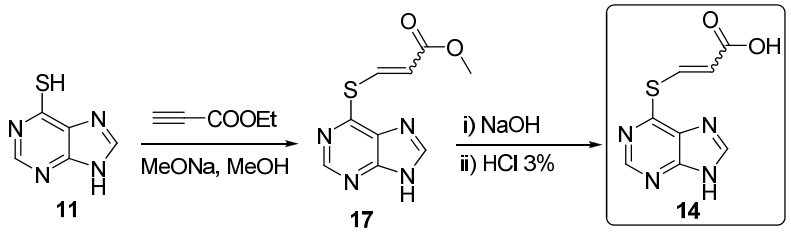
Instead, adding a stoichiometric amount of a freshly prepared solution of MeONa (1 eq.) in dry MeOH and propiolic acid sodium salt (1 eq.) to a suspension of **11** (1 eq.) in dry MeOH, acid **14** precipitated as pure product in 43% yield, after the addition of HCl

1N until pH 2. This procedure allowed the achievement of only the *Z* isomer (Scheme 8).



Scheme 8

We have also verified the formation of PTA from the ethyl propiolate using the same strategy shown in Scheme 7, with stoichiometric amounts of sodium methoxide, led to derivative **17**, generated from a trans-esterification to the corresponding methyl ester, in 14% yield. The alkaline hydrolysis and following acidification gave **14** quantitatively as a mixture of *E* and *Z* isomers (Scheme 9).



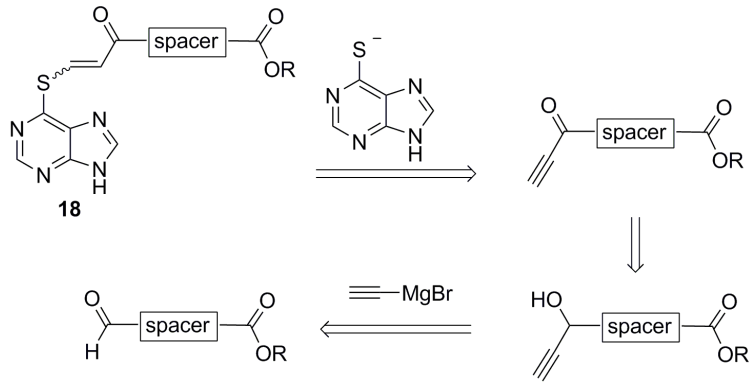
Scheme 9

Since the efficiency of the linker in releasing the drug is crucial for this kind of prodrug and since releasing of 6-MP from PTA is quite slow,[36] possibly because the propenoic acid moiety is ionized at physiological pH slowing the Michael addition, we decided to prepare other types of 6-MP derivatives differently conjugated to the carrier peptide.

For this reason, we studied the properties of a structural analog of PTA, 6-(2-acetylvinylthio)purine AVTP **13** (Figure 4) where the sulfur is conjugated to a butenone moiety. This α,β-unsaturated ketone undergoes a more efficient addition-elimination reaction with GSH than PTA, to yield 6-MP.

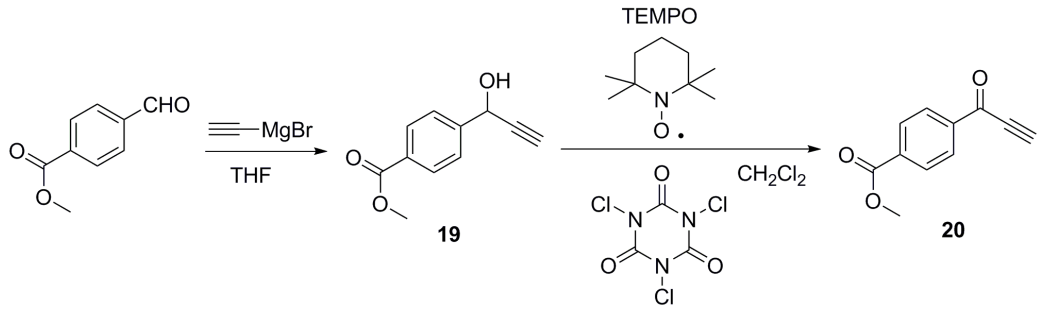
Therefore, we designed the synthesis of an α,β-unsaturated prodrug with general structure **18**, bearing a carboxylic group to allow the conjugation to the branched peptide. We imagined to obtain this type of molecules by the same addition between an

electron-poor alkyne and the thiolate group of 6-MP observed in Scheme 7. The retrosynthetic Scheme 10 shows the designed procedure.



Scheme 10

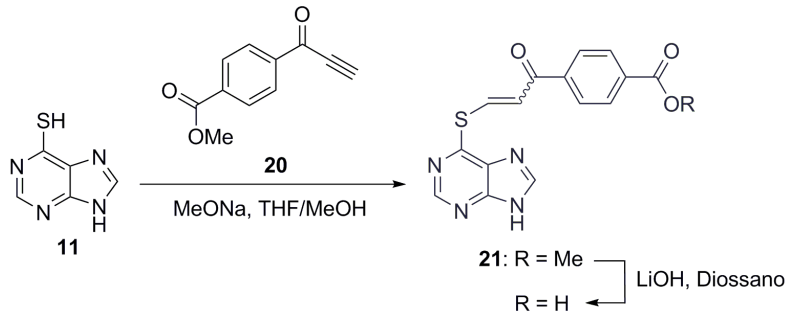
After browsing all the commercial sources of carboxyaldehydes we selected methyl-4-formylbenzoate as the first suitable starting material to verify our retrosynthetic scheme. Ethynyl magnesium bromide (1 eq.) was reacted with methyl-4-formylbenzoate (1 eq.) in THF at -78 °C to give the correspondent secondary alcohol **19** in 69% yield, without affecting the ester function. The oxidation to ketone **20** was carried out in 97% yield, following a mild and chemoselective oxidation strategy,[39] adding catalytic amounts of 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO) to a solution of **19** and trichloroisocianuric acid (TCICA) in CH_2Cl_2. The reaction mixture was stirred for 3 min at r.t. then simply filtered through celite (Scheme 11).



Scheme 11

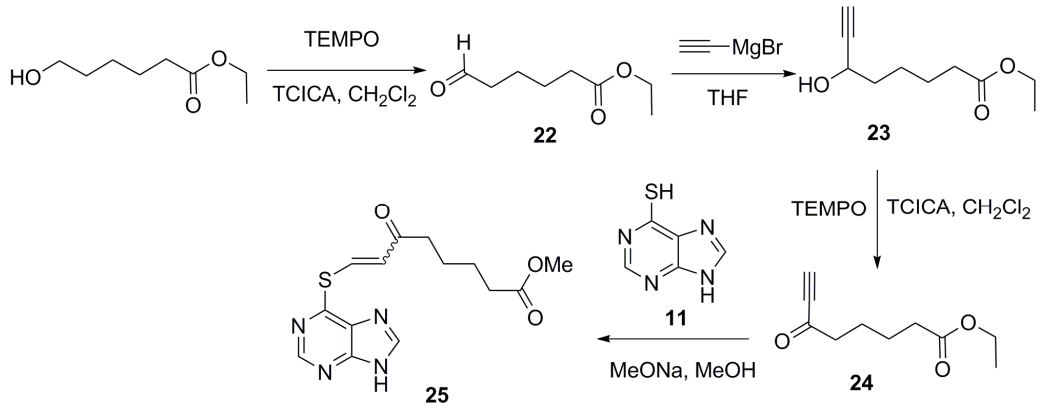
Once obtained the desired alkyne **20** was subjected to the usual reaction with ionized 6-MP, as shown in Scheme 12, but the crude product, where reasonably there was compound **21**, was insoluble in all the solvents tried, even after deprotection of the ester group by alkaline hydrolisys. Probably the benzene ring considerably reduces the low mercaptopurine's solubility and this would have been a big disadvantage especially in

the next peptide coupling. We therefore realized that another suitable α,β-unsaturated modified prodrug had to be exploited.



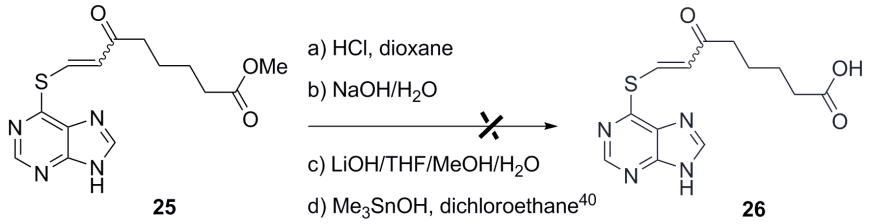
Scheme 12

To circumvent the problem caused from the benzene ring we decided to introduce an alkylic chain on the sulphur atom of 6-MP. Commercially available ethyl-6-hydroxyhexanoate was quantitatively oxidised to the corresponding aldehyde **22** again with trichloroisocianuric acid (TCICA) (1 eq.) and catalytic amount of 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO) in CH_2Cl_2. Reaction of the aldehyde **22** (1 eq.) with ethynyl magnesiumbromide (1 eq.) at -78°C in THF allowed the preparation of propargylic alcohol **23** (43%) which was purified by flash chromatography. The alkyne **24** containing an α,β-unsaturated carbonyl group was eventually obtained using the above described oxidation method adding TEMPO and TCICA to a solution of **23** in CH_2Cl_2 at 0 °C. The reaction mixture was stirred at r.t. for 20 h, diluted with CH_2Cl_2, filtered through celite and after purification by flash chromatography we obtained **24** in quantitative yield. Finally, the addition of the 6-MP thiolate, generated in the presence of MeONa, to the acetylenic group gave the prodrug derivative **25** (47%), which has a good solubility in organic solvents as we expected (Scheme 13).



Scheme 13

Disappointingly in spite of all the efforts done in both acidic or basic conditions, as reported in Scheme 14, we couldn't isolate the desired acid **26**.

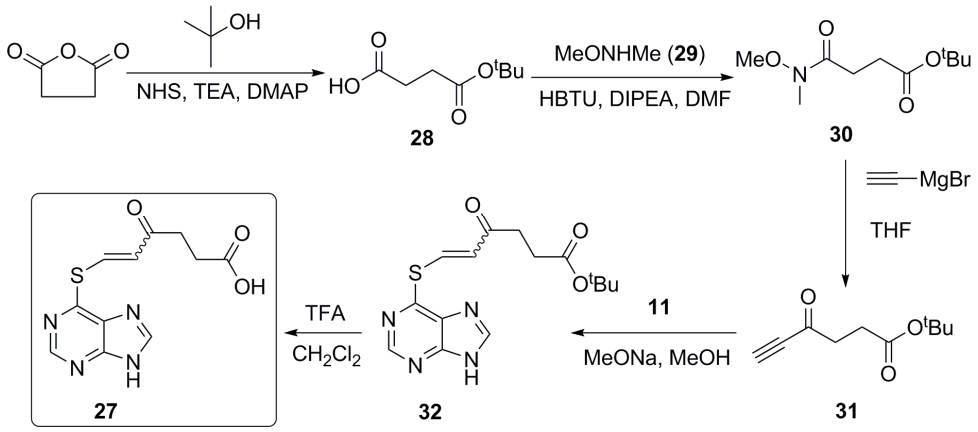


Scheme 14

On the other hand an advanced hydrolysis of derivative **24** to its corresponding acid offered a solution to these deprotecting problems, but the so prepared acid did not react with 6-MP to give **26**.

After all these attempts that in any case led to insoluble or undesired compounds, an α,β-unsaturated prodrug (**27**) was obtained starting from succinic anhydride. In literature is reported the preparation of α,ω-ketoacids by reaction of a Grignard reagent with a cyclic anhydride.[41]

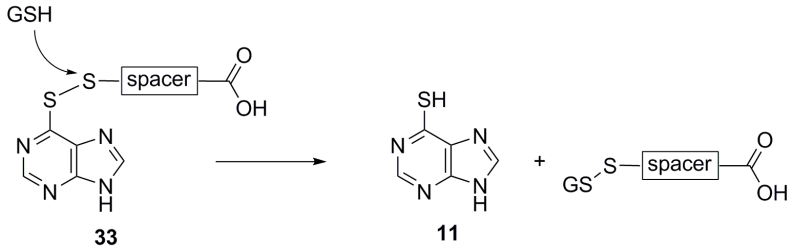
Succinic anhydride (1 eq.) was desymmetrizated as mono-*t*-butyl ester **28** by the addition of N-hydroxy succinimide (NHS) (0.3 eq.), 4-dimethylaminopyridine (DMAP) (0.1 eq.), *tert*-butanol (3 eq.) and triethylamine (TEA) (0.3 eq.).[42] After one day under reflux the crude was purified by flash chromatography to give **28** in 73% yield. This carboxylic acid was activated utilizing the standard peptide coupling strategy, adding O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) (1 eq.) and N,N-diisopropylethylamine (DIPEA) to its solution in dry DMF. Then a solution of N,O-dimethylhydroxylamine **29** was added to the activated acid **28** and stirred for 3 h to obtain the corresponding Wienreb amide **30** in 76% yield without further purification. The reaction of **30** with an excess (5 eq.) of ethynyl magnesium bromide in THF afforded the alkynyl-γ-ketoester **31** in 47% yield. Michael addition of 6-MP anion to the electron poor triple bond of **31** followed by acid hydrolysis of the *t*-butyl ester group of **32** with TFA in CH_2Cl_2, allowed the isolation of compound **27** in 84% yield (Scheme 15).



Scheme 15

The last reaction described in Scheme 15 demonstrated the stability of the ketoacid **27** in TFA, which is a fundamental requirement for all the molecules that we prepared. In fact TFA is the reagent used at the end of the process for the cleavage of the conjugated peptide from the resin support.

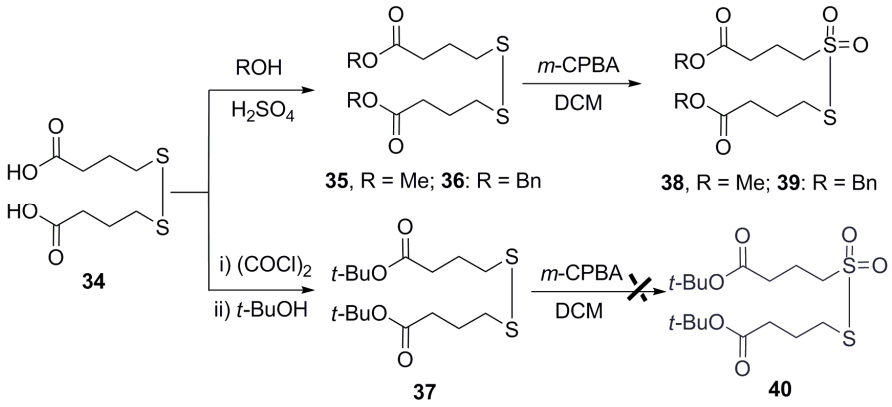
We have imagined another GSH-mediated mechanism for the releasing of 6-MP from a reductively activated prodrug of **11** possible with the introduction of a disulfide linkage (Scheme 16).[43] Along similar lines Vrudhula et al.[44] have synthesized several C2'-disulfide Taxol (paclitaxel) derivatives, based on the similar notion that the disulfide bond could be reduced to free thiol in the hypoxic environment of the tumor site. As shown below we designed a 6-MP derivative with general formula **33** with an alkylic chain separating the disulfide linkage and the carboxylic moiety. The releasing of **11** inside tumor cells should take place through a simple thiol-disulfide exchange with, for example GSH, considering the elevated levels in the tumor tissue of endothiols.



Scheme 16

The desired disulfide was so prepared by two different strategies both starting from dithiobutyrric acid **34** which was protected as methyl (**35**) and benzyl ester (**36**) both by a Fischer direct esterification and as t-butyl ester (**37**) by the formation of the

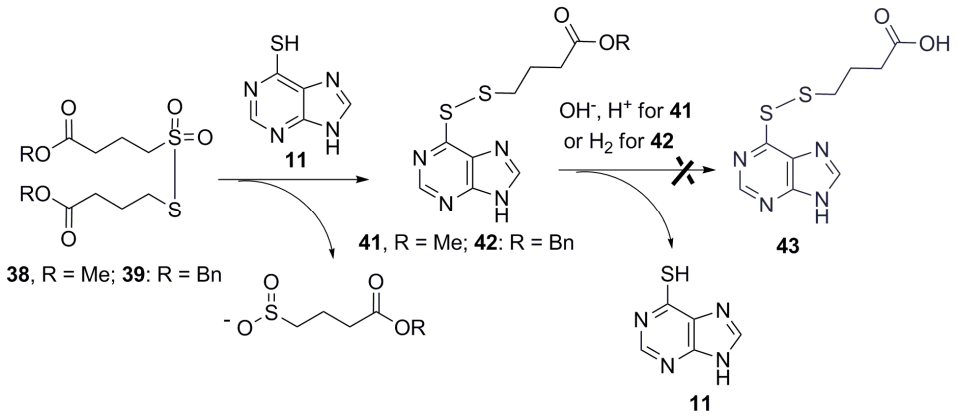
corresponding acyl chloride. Initially we hypothesized a reaction between a thiolsulfonate acting as an electrophile and the sulfur of 6-MP used as the nucleophile. Derivatives **35** and **36** were so oxidized with *m*-CPBA (*meta*-chloroperoxybenzoic acid, 2.2 eq) in CH_2Cl_2 for 8 h. The opaque solution was washed with a 10% solution of sodium thiosulfate and twice with a saturated solution of sodium carbonate. The residue obtained was purified by flash chromatography to give thiolsulfonate **38** and **39**. The same reaction conduced on *t*-butyl ester **37** instead didn't allow the isolation of the desired thiolsulfonate **40** (Scheme 17).



Scheme 17

The derivatives **41** and **42** were prepared reacting for 12 h thiolsulfonates **38** and **39** with 6-MP **11** in dry DMF, at 60°C in the presence of TEA (1 eq.). The crude was diluted with a saturated solution of ammonium chloride, extracted with EtOAc and the organic layer was washed with water. The residue was purified by flash chromatography to yield the mercaptopurine derivatives **41** and **42**.

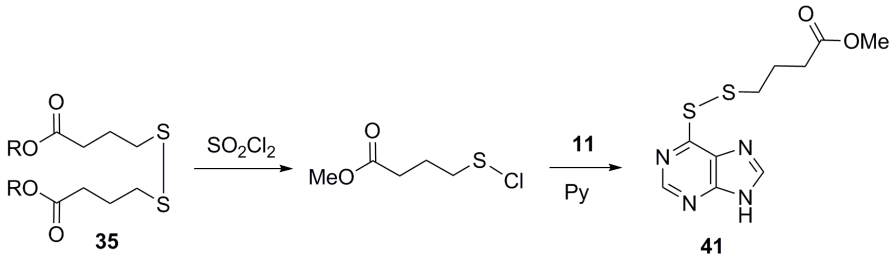
All the various attempts to hydrolyze the methyl ester function, run both in basic and acid conditions as well as in the presence of aluminum chloride ($AlCl_3$) and N,N-dimethylaniline, failed. In the same way the hydrogenation in neutral conditions of **42** gave as the unique product the 6-MP. The reason was clearly the high instability of purinic disulfides regardless of the reaction conditions.



Scheme 18

While trying to obtain compound **41** in high yields, we designed another strategy[45,46] where the same product can be obtained by a two-step sequence as it follows.

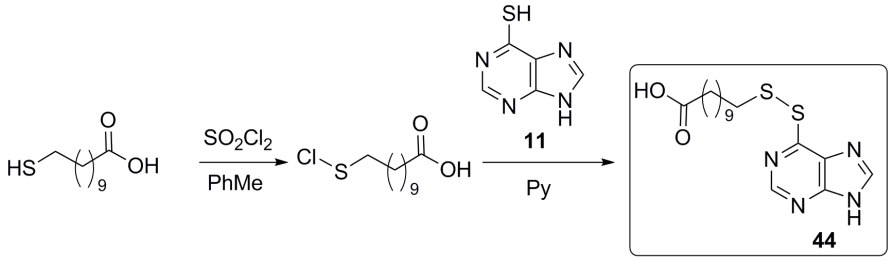
To a solution of ester **35** in dry toluene was added dropwise a solution of SO_2Cl_2 (1eq.) in toluene dry at -15 °C. The reaction mixture was stirred for 20 minutes, the formation of the sulfenyl chloride was verified by titration with cyclohexene. The solution of the sulfenic species obtained (roughly 15 eq.) was added dropwise to a suspension of 6-MP **11** (10 eq.) in pyridine. The reaction mixture was stirred at 84 °C for 2 h and later purified by flash chromatography to give **41** in a whole yield of 27% (Scheme 19).



Scheme 19

As already reported we couldn't deprotect **41** because of the instability of the disulfide linkage under both acid and basic ester-cleavage conditions. So we tried this strategy, just tested on derivative **35**, without protecting carboxylic group, to overcome the above reported problems using an ester as starting material. This synthetic pathway started with the preparation of a sulfenyl chloride which can react with the 6-MP sulfur atom. Operatively, to a cooled solution of 11-mercaptoundecanoic acid (2 eq.) in dry toluene a solution of SO_2Cl_2 (1 eq.) was added, the mixture was stirred for 15 min, then nitrogen was then bubbled inside to eliminate residual traces of SO_2 and HCl. Before undergoing

the next reaction we verified again the effective formation of sulfenyl chloride product by titration with cyclohexene. This allowed us verifying the actual formation of the expected sulfenic species and estimating a 46% yield for the first reaction. The so obtained sulfenyl chloride was heated with a suspension of **11** (1 eq.) in dry pyridine to give disulfide **44** in 30% yield (for two reactions) (Scheme 20).



Scheme 20

b) **5-Fluoro-2'-deoxyuridine (5-FdUrd**, **45**) was synthesized in 1979 by Cook *et al*.[57] It is commonly called doxifluridine or Furtulon® and has a molecular structure consisting of a molecule of 5-Fluorouracil (5-FU) to which a pseudopentose is bonded in position 1 (Figure 15). Doxifluridine represented an important improvement in terms of tumor selectively, efficacy and safety from 5-FU. Compound **22** cannot be phosphorylated and therefore incorporated into nucleic acids without pre metabolic transformation into 5-FU.

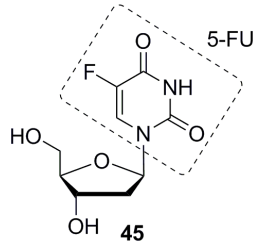


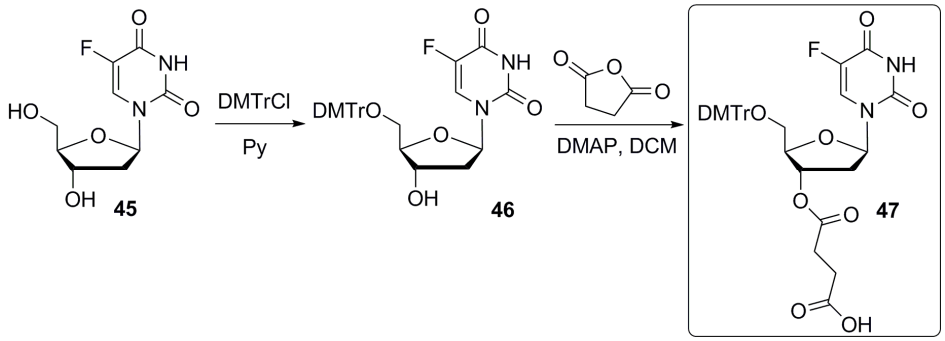
Figure 15 5-FdUrd

The rationale for its development has been the need of the enzyme Thymidine Phosphorylase (TP), a tumor-associated angiogenesis factor,[47] for cleavage into 5-FU, and thus activation. The high level of this enzyme in tumors and in the intestinal tract compared with normal proliferating tissues[48] gave an enhanced selectivity. However, very high activity of TP is found in normal human liver, thus the liver toxicity.

5-FdUrd has a relevant role in the treatment of metastatic cancers, either by exerting an appropriate antitumor activity via blocking thymidylate synthase to inhibit DNA synthesis or by incorporation of its metabolite (5-Fluorouracyl) into DNA or RNA.[49]

Although clinically effective, 5-FdUrd exhibits various side effects as a result of its action on highly mitotic tissues such as the gastrointestinal tract and bone marrow. Further, doxifluridine like most other nucleoside anticancer agents, suffers from low and erratic oral absorption. Strategies that can improve the oral absorption and reduce toxicity can be of a great benefit, therefore the synthesis of a proper linker will be useful for this purpose.[50] Previous studies demonstrated that ester derivatives of 5-FdUrd undergo hydrolysis to effectively release **45** under physiologic conditions and thereby produce a considerable antitumor effect.[51] Considering that esters are the most common prodrugs used,[52] we choose to follow this strategy, inserting a succinate linker, on the 5-FdUrd structure, as a suitable covalent bond able of a safety release of the cytotoxic molecule **45** by both enzymatic and non-enzymatic hydrolysis.

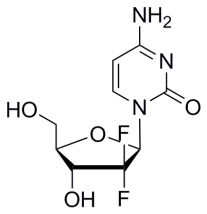
Synthesis, as reported in literature,[53] started by protecting the primary alcohol with 4,4'-dimethoxytriphenylmethylchloride (DMTrCl) (1.5 eq.), to overcome possible side reactions in the next coupling with succinic anhydride. DMAP (1.5 eq.) and succinic anhydride (4.0 eq.) were then added to a solution of **46** in dry CH_2Cl_2. The desired acid **47** was obtained as the unique product in 99% yield, by simple series of basic and acidic extractions to remove impurities (Scheme 21).



Scheme 21

The deprotection of ether function on primary alcohol is not necessary at this stage because the removal of DMTr group will occur contemporary with cleavage of the modified peptide from the solid support at the end of the process.

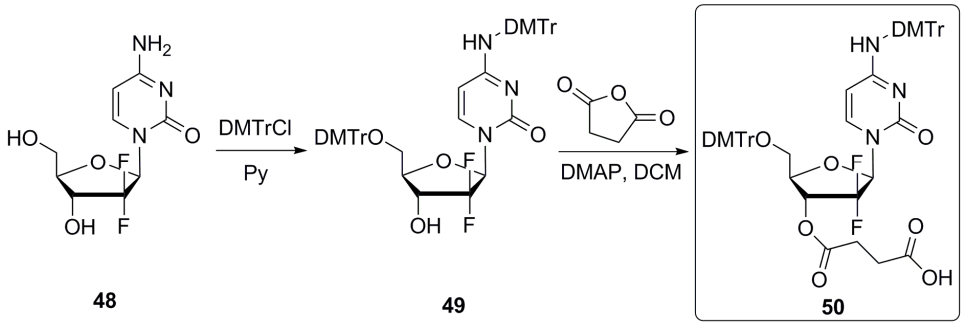
c) **Gemcitabine** is a difluorinated analog of the naturally occurring nucleoside deoxycytidine. 5-Fluorouracil has long been the only available drug that offered some promise in the palliative treatment of advanced pancreatic cancer, but objective responses were rarely achieved. The moderate activity of fluorouracil may therefore explain the unsatisfactory results reported for fluorouracil-containing adjuvant regimens in patients undergoing pancreatectomy.



48

Figure 16 Gemcitabine

The development of gemcitabine is considered a major advance in the treatment of pancreatic cancer. Gemcitabine has a good safety profile with a low incidence of toxicities and has shown significant clinical activity in a variety of solid tumors. In 1997, Burris *et al.* reported[54] the first results of a phase III study that demonstrated significant improvements both in survival and clinical benefit (pain relief, improved performance status, or both) with single agent gemcitabine compared with fluorouracil as first-line chemotherapy for advanced pancreatic cancer.[55] Now gemcitabine is a drug used to treat certain types of breast, pancreatic, ovarian, and lung cancer and is being studied in the treatment of other types of cancer. Gemcitabine is metabolized intracellularly by nucleoside kinases to the active diphosphate (dFdCDP) and triphosphate (dFdCTP) nucleoside metabolites that interfere with DNA replication, arresting tumor growth. The mechanism of action as well as the structure of gemcitabine and 5-fluoro-2'-deoxyuridine are very similar one to each other, so we decided to use the same strategy adopted before. The primary alcohol of **48** was protected with DMTrCl (1.5 eq.), affecting also the amine group and then the secondary alcohol of **49** was coupled with succinic anhydride to obtain the desired acid **50** as the unique product in 15% yield (of two reactions), which is ready to be conjugated to the targeting carrier (Scheme 22).



48 **49** **50**

Scheme 22

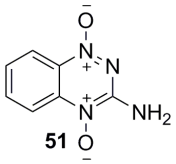
As well as for derivative **47**, the ether deprotection is not necessary for **50** because it will occur while cleaving the conjugated peptide from the solid support at the end of the process.

1.2.2 Bioreductive drugs

Hypoxia specific cytotoxins, also known as bioreductive drugs, are compounds that undergo reductive metabolism under low oxygen conditions to produce toxic products.[56] The rationale for their use in cancer is the hypoxia associated with tumors. Robust tumor growth requires the presence of a local vascular network that supplies oxygen and nutrients. Hypoxia occurs as a result of poor blood supply, and therefore oxygenation, due to the faster development of the highly proliferating mass of tumour cells than the vasculature.[57] It is well established that many solid tumors contain significant regions which are poorly oxygenated (hypoxic) and this is directly correlated with disease progression and patient survival. Furthermore, the hypoxic cells are often resistant to non-surgical treatments of cancer, primarily radiation and anticancer drugs. This hypoxic condition increases the levels of reductive enzymes and nucleophyles, such as GSH and this is a fact to take in consideration while designing new molecules for cancer therapy (as we showed for 6-MP).[58]

There is currently an impressive interest in exploiting tumor hypoxia to produce cytotoxins from prodrugs which selectively attack chemo- and radio-resistant hypoxic tumor cells.[59]

a) The drug **tirapazamine 25** (3-amino-1,2,4-benzotriazine 1,4-dioxide, TPZ) is the most clinically advanced bioreductive, hypoxia selective prodrug.[60]



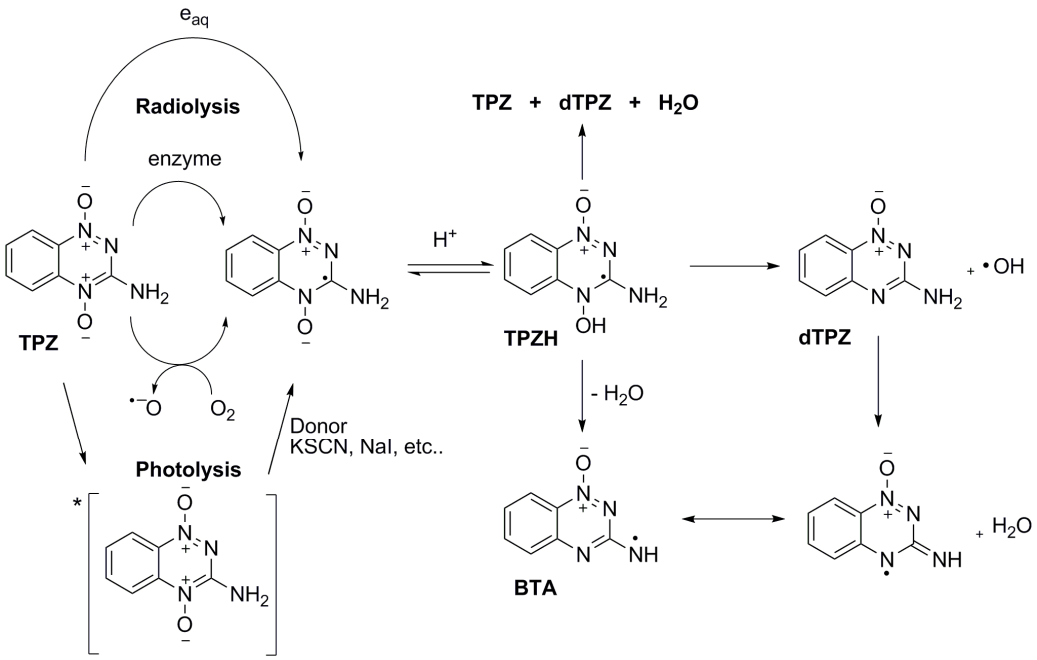
51

Figure 17 Tirapazamine

TPZ is the lead compound in the class of benzotriazine-di-N-oxides, but the real mechanism of action is still unclear.

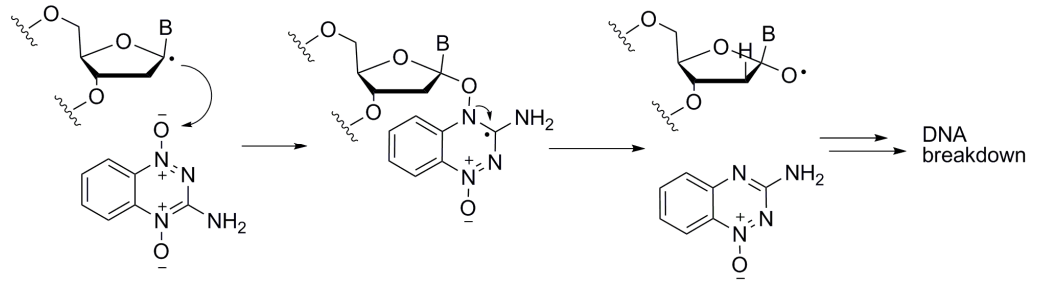
Is something of a paradox that TPZ promotes free radical oxidative cleavage of DNA, yet it is more active in the absence than in the presence of molecular oxygen. On the basis of these observations it has been proposed a dual mechanism of action.[61] First, in

a bioreductive process TPZ generates high oxidative free radicals which break DNA as it is shown in Scheme 23. Molecular oxygen quenches this process by accepting an electron from TPZ radical, hence the greater activity of the drug in hypoxic conditions.



Scheme 23 Generation of free radicals from TPZ

As an alternative mechanism, TPZ reacts directly with DNA sugar radicals followed by oxidation of DNA, thus it is the drug and not oxygen which oxygenates DNA-centered radicals (Scheme 24).



Scheme 24 Reaction between DNA and TPZ

By these ways TPZ is able to kill hypoxic cells 50-200 times more effectively than well oxygenated cells, depending on the cell line.

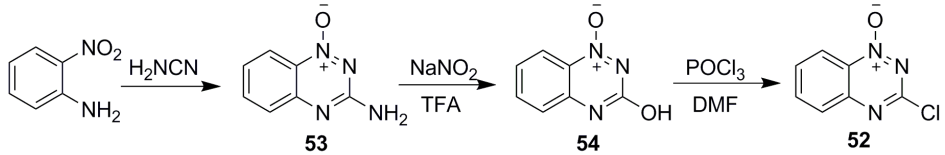
TPZ has a quite low aqueous solubility, and a combination of rapid metabolism and slow extra-cellular diffusion combine to limit its ability to penetrate into hypoxic tissues. Moreover, in contrast to most animal studies, clinical studies report a wide variety of acute toxicities. Even if systematic clinical examinations of long-term toxicity have not yet been performed, is clear that there is necessity for a way to improve bioavaibility and tumor selectivity.

Therefore we designed TPZ derivatives, with similar or improved drug abilities, to be conjugated with the targeting peptide. For this purpose the introduction of a carboxylic acid on the molecule's skeleton by an appropriate linker is necessary, as for all the drugs reported in this thesis.

The choice of a versatile or permanent linker between the peptide and the drug can also be useful to predict the mechanism of action of TPZ. In any case we used the chloride **52**, prepared by modifications of previous methods[62], as the key starting material.

2-Nitroaniline (1 eq.) and cyanamide (5 eq.) were heated together with stirring at 100°C until a deep red melt formed. The reaction was cooled at 50 °C and concentrated HCl was added dropwise. The reaction mixture was warmed at 100 °C, stirred for 4 h, then made strongly alkaline by the careful addiction of a 30% solution of NaOH. After the solution was heated again for 3 h, crystals formed on cooling which were collected and washed with water and diethylether to give 3-amino-1,2,4-benzotriazine-1-oxide **53** as a yellow solid in 72% yield.

A solution of **53** (1 eq.) in trifluoroacetic acid (TFA) was cooled at 5°C. Over a period of 15 min sodium nitrite (3 eq.) was added portionwise. The reaction mixture was stirred at r.t. for 4 h, poured onto ice/water and stirred 30 min. After filtration, the precipitate was washed with water and dried to give 3-hydroxy-1,2,4-benzotriazine-1-oxide **54**. Chloride **52** was finally obtained by adding DMF (3 eq.) and phosphorous oxychloride (7 eq.) directly to **54**. The mixture was refluxed for 1 h, then poured on ice and the precipitate formed filtered. The crude product was recristyllized from methanol to give **52** in 50% yield (for two reactions) (Scheme 25).

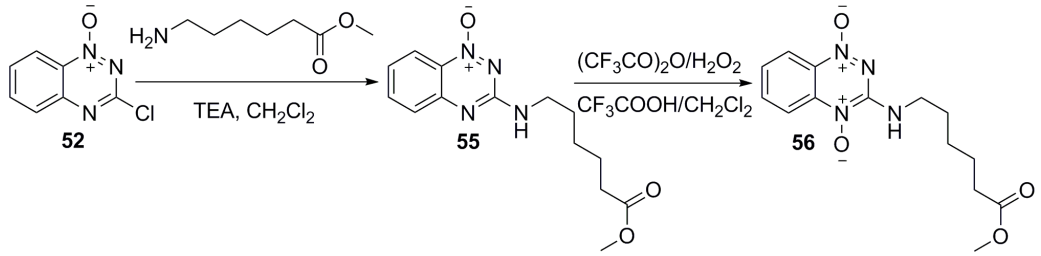


Scheme 25

After obtaining the key intermediate **52**, the chloride atom was substituted by a coupling reaction with an amine under basic conditions. The amine choice permitted synthesis of TPZ-derivatives with different properties and utilizations.

Firstly to a solution of methyl-6-aminohexanoate (3 eq.) in CH_2Cl_2 a solution of **52** (1 eq.) and TEA (2 eq.) in CH_2Cl_2 was added. The reaction mixture was stirred for 116 h at r.t. and distilled to dryness in vacuo. The residue was purified by flash chromatography with 50/1= CH_2Cl_2 /MeOH as eluent to obtain 1-oxide **55** in 75% yield.

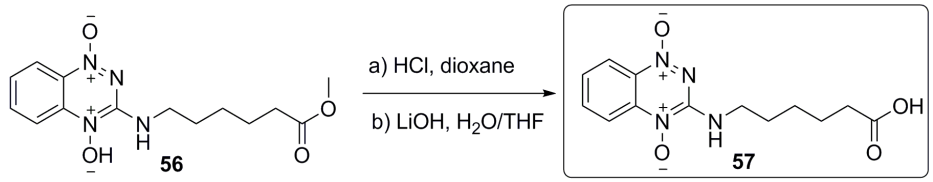
We have optimized the oxidation reaction, after various disappointingly attempts using MCPBA[63] or the commonly sold solutions of H_2O_2 (30 and 50%),[64] as reported in literature. The best result was obtained when we treated a solution of 50% H_2O_2 in MeOH with Na_2SO_4 to increase its oxydizing properties. A solution of the treated H_2O_2 (20 eq.) in MeOH was added to a stirred solution of trifluoroacetic anhydride (20 eq.) in dry CH_2Cl_2 at 5 °C. After 10 min stirring at r.t. the oxidizing agent, tri-fluoroperacetic acid, was formed, then cooled to 5 °C before adding of a solution of **55** (1 eq.) and trifluoroacetic acid (9 eq.) in dry CH_2Cl_2. After 4 days under stirring the red solution was diluted with EtOAc and washed with a saturated solution of $NaHCO_3$. The organic phase was dried and concentrated to give the red coloured 1,4-dioxide **56** in 90% yield (Scheme 26).



Scheme 26

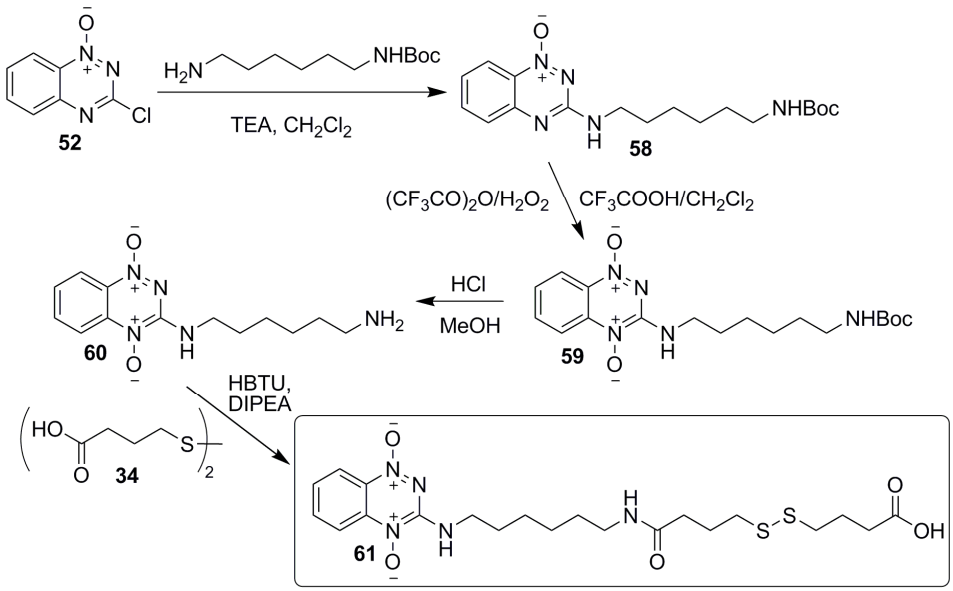
Ester hydrolysis of **56** to free acid **57** was achieved either in acid or basic conditions. In fact adding 37% HCl (35 eq.) to a solution of **56** (1 eq.) in dioxane and stirring at r.t. for 48 h, we obtained **57** in 83% yield, after purification by flash chromatography.

Otherwise, we added 1M LiOH (10 eq.) to a solution of **56** (1 eq.) in 1/1=THF/MeOH. The mixture was stirred for 5min and 1M HCl was added until pH 5. The acid aqueous phase was extracted several times with EtOAc and the combined organic layer was dried and the solvent evaporated to obtain again **57** but this time in 98% yield without further purification (Scheme 27).



Scheme 27

With the aim to have an active linker, between TPZ and the dendrimeric peptide, able to release the drug in hypoxic conditions, we decided to prepare a disulfide containing TPZ-analogue. Unlike 6-MP derivative **44**, here the sulfur-sulfur bond does not directly involve the pharcophore part of the molecule but it is separated by an alkyl chain. Starting again from the chloride **52**, the conjugation was carried out in the presence of N-Boc-1,6-hexanediamine, adopting the same conditions reported before for the synthesis of **55**, to give derivative **58** in 90% yield. The oxidized product **59** was prepared, with a 99% yield, using treated H_2O_2 with the same procedure shown before**,** confirming its efficiency. Once it was obtained, the red compound **59** was dissolved in MeOH and gaseous HCl was bubbled inside for 2 min. The solution instantly became yellow and was stirred for 17 h at r.t., diluted with water and extracted several times. The aqueous phase was basified by adding a saturated solution of $NaHCO_3$ and extracted many times with $CHCl_3$. These final recollected organic layers were dried and concentrated to give the amine **60** in 55% yield. Finally adopting the strategy usually utilized for the standard solid phase conjugation, the carboxylic group of dithiobutirric acid (1 eq.) was activated by adding to its solution in dry DMF, HBTU (1 eq.) and DIPEA (2 eq.). After stirring for 10 min this solution was added to a solution of **60** (1 eq.). The reaction mixture was stirred for 6 h, acidified to pH 5 with 1N HCl and diluted with $CHCl_3$. The two phases were separated and the organic layer was washed with water, dried and concentrated. The crude product was then purified in a TLC plate to obtain **61** in 55% yield (Scheme 28).

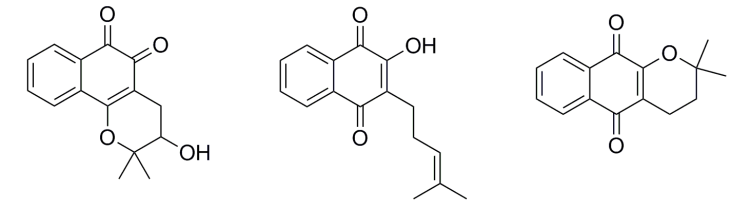


Scheme 28

The high instability of disulfide molecules, already noticed in the case of 6-MP derivatives, forced us to verify the behaviour of this specific molecule **61** in the acid conditions used during the already mentioned peptide cleavage. We therefore demonstrated the stability of **61** in 99% TFA for 18 h.

b) **β-Hydroxy Lapachone (62)**

Mass screening programs of natural products by the National Cancer Institute have identified the quinone moiety as an important pharmacophoric element for cytotoxic activity.[65] Naturally occurring naphthoquinones comprise an important class of natural products with a wide range of biological activity[66] arising from their ability to cause DNA modification via redox cycling of the quinone moiety and the generation of reactive oxygen species. In the structurally diverse naphthoquinone natural products, dihydropyranonaphthoquinones (α- and β-lapachones) have attracted special attention because of their promising antitumor ability,[67] among various other bioactivities.

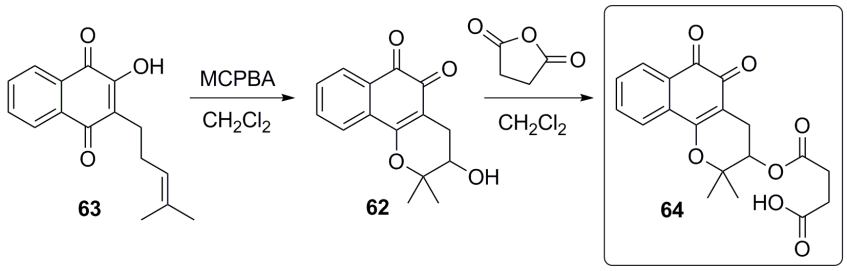


β-hydroxylapachone, **62** lapachol α-lapachone, **63**

Figure 18

Heterocyclic naphthoquinones of the lapachone family are found as minor components in the stem bark of many trees of the *Tabebuia* genus in Central and South America.[68] α-Lapachones have a wider distribution than β-lapachones, and are additionally found in *Ekmanianthe longiflora* in America and in *Capalta ovata* trees in many east Asian countries. Among these pyranonaphthoquinones, β-lapachone derivatives have so far received the most extensive investigations, mainly owing to their stronger antitumor activity. β -lapachone exerts its anti-tumor effect by indirect actions of inducing p53-independent apoptosis and cell cycle arrest mediated through altered activities of cell cycle control regulatory proteins, including down-regulating retinoblastoma protein (pRB), a transcriptional repressor target at transcription factor E2F-1, as well as induces expression of cyclin dependent kinase inhibitor 1A (CDKN1A or p21). Both E2F-1 and p21 are required for G1/S-phase transition during cell cycle. This agent also inhibits DNA topoisomerase I by a mechanism distinct from that of camptothecin, and thereby blocks the formation of a cleavable complex leading to enzyme inhibition and prevent DNA repair. Furthermore, beta-lapachone could induce reactive oxygen species in vivo, and result in cytotoxicity acting as a bioreductive drug.[69]

As already reported for the prodrug synthesis of other cytotoxins, we exploited the presence of a hydroxyl group on the structure of β-hydroxy lapachone **62** by succcinate formation. The synthetic procedure started from the commercially available lapachol (**63**), which was treated with MCPBA (1.2 eq.) in CH_2Cl_2 for a day to yield the drug **62** (57%). The next coupling with succinic anhydride gave prodrug **64** in quantitative yield without further purification (Scheme 29).



Scheme 29

1.2.3 Mitosis Inhibitors

There is a considerable interest in the discovery and development of novel small molecules able to affect tubulin polymerization. Such compounds impair dynamic microtubule elements of the cell cytoskeleton responsible for the formation of the mitotic spindle and required for proper chromosomal separation during cell division.[70] Current drugs that inhibit cell proliferation, such as paclitaxel and docetaxel, are standard treatments for many types of cancers, but these drugs also target other proteins that are essential to important cellular functions and thus, their use is limited by side effects. More recently, it has been established that some tubulin binding agents selectively target the vascular system of tumors. These compounds induce morphological changes in the endothelial cells of the tumor's blood vessels, resulting in their occlusion and interruption of blood flow.[71] These agents has been termed antivascular agents, in contrast with antiangiogenic agents, which can act by inhibiting proliferation of new tumor vasculature. Vascular targeting drugs instead specifically and rapidly act on preexisting tumor blood vessels.

a) **Combretastatin A-4** (**65**), isolated from the bark of the South African tree *Combretum caffrum*, is a potent antimitotic and cytotoxic agent which strongly inhibits the polymerization of tubulin by binding to the colchicine site.[72]

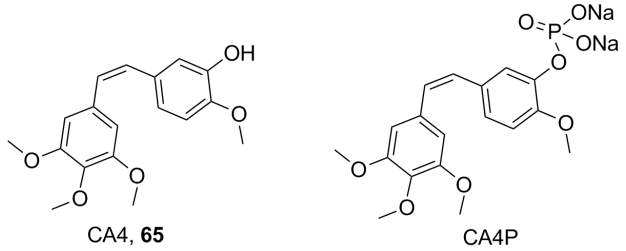


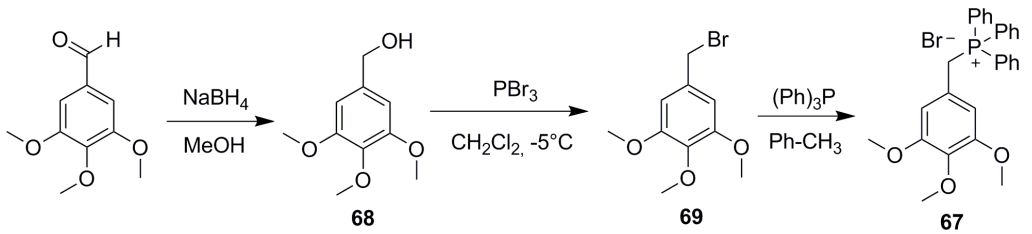
Figure 19 Combretastatin A-4 (CA4) and its prodrug Combretastatin A-4 phosphate (CA4P)

It is also able to elicit irreversible vascular shutdown within solid tumors, leaving normal vascolature intact, behaving as an antivascular agent. Combretastatin A-4 inhibits cell growth even at low nanomolar concentartions and exhibits inhibitory effects on multidrug resistant cancer cell lines.

A prodrug of **65**, its water soluble phosphate derivative (CA4P), is now in phase II of clinical trials[73] and a wide number of its analogues have been synthesized and evaluated.[74] Its structural simplicity, along with its ability to selectively damage tumor neovascolature, makes CA4 of a great interest for medicinal chemistry applied to cancer treatment.

Total synthesis and introduction of the suitable linker will allow the delivery of the drug by the branched-peptide. We imagined to exploit the same releasing mechanism adopted for 5-FdUrd, gemcitabine and lapachone derivatives (**47**, **50** and **64**) transforming the free phenolic OH of **65** in a functionalized ester. Combretastatin was prepared by a modification of previous methods[75] developed by Pettit et al., that exploits a Wittig reaction between aldehyde **66** and corresponding ylide of **67** as the key step.

The synthesis of the phosphonium salt **67** started from the reduction of 3,4,5-trimethoxybenzaldehyde (1.0 eq.) by the addition of $NaBH_4$ (1.2 eq.) in MeOH at 0 °C to yield benzyl alcohol **68** in 88% yield. To a solution of **68** (1.4 eq.) in dry CH_2Cl_2, cooled to -5 °C, a solution of PBr_3 (1.0 eq.) in dry CH_2Cl_2 was added dropwise to give **69** in 96% yield. Finally, a solution of triphenylphosphine (1 eq.) in toluene was added to a solution of bromide **69** (1 eq.). The reaction mixture was stirred for 24 h at r.t. and the precipitate formed filtered and dried to obtain phosphonium bromide **67** in 62% yield (Scheme 30).

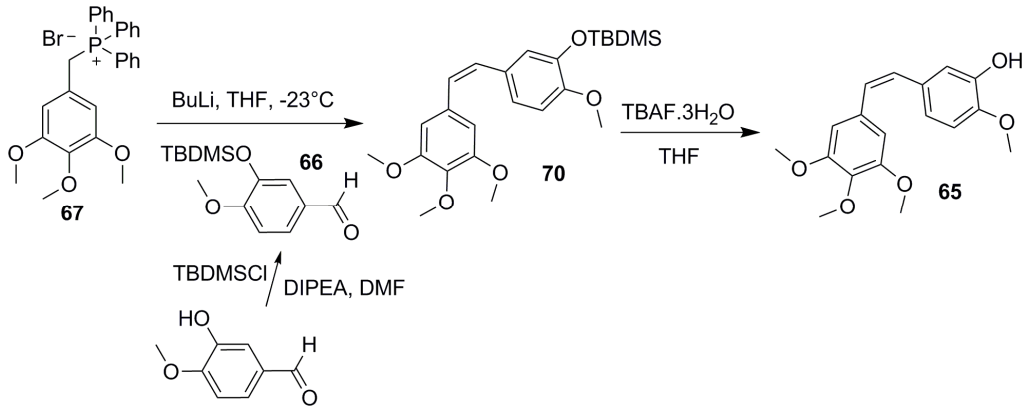


Scheme 30

Aldehyde **66** had to be protected to undergo the Wittig reaction and we choose *tert*-butylsilyl (TBDMS) as protecting group. To a solution of isovanillin (1.0 eq.) in dry DMF, DIPEA (2.0 eq.) and TBDMSCl (1.6 eq.) were added. The crude product, obtained after 4 h of reaction, was purified by flash chromatography to give **66** in 98% yield.

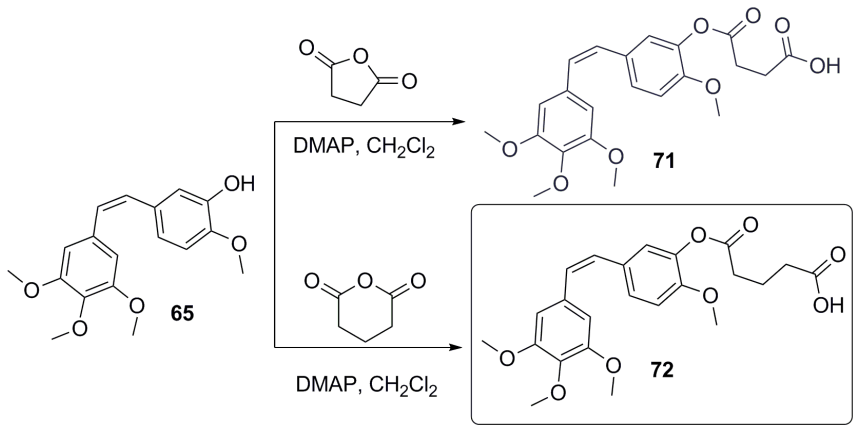
A suspension of phosphonium bromide **67** (1.0 eq.) in dry THF under nitrogen was cooled to -23 °C and retained at that temperature for 2 h. Butyllithium (1.0 eq.) was added dropwise, the resultant orange solution was stirred at the same temperature for 1 h and finally **66** (1.04 eq.) was added. The reaction mixture was stirred at -23 °C for 4 h and at r.t. for 18 h, ice-water was added and two phases separated. The aqueous phase was washed with Et_2O and the etheral solution added to the THF layer. The combined organic phase was washed with water and dried. The crude product was purified by

flash chromatography to give both the desired *Z* product **70** in 61% yield and a 20% of the corresponding *E* product. The careful control of temperature is critically correlated with the obtainment of an excess of *Z*. Deprotection of **70** (1 eq.) occurred in dry THF in the presence of TBAF·$3H_2O$ (1 eq.) after 20 min stirring. The residue was then chromatographed to obtain the natural product CA4 (**65**) in 77% yield (Scheme 31).



Scheme 31

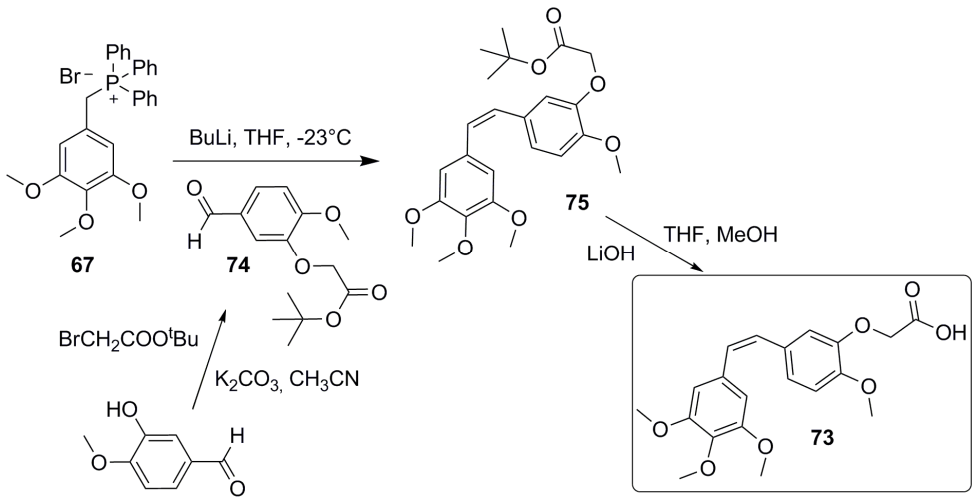
The next reaction with succinic anhydride, led as usual with DMAP in CH_2Cl_2, gave ester **71**, as verified by NMR analysis, but this derivative resulted instable even in silica gel, making impossible not only the purification but also the coupling with the dendrimeric peptide. Since the other succinate derivatives prepared (**47**, **50** and **64**) were stable even in extremely acid conditions, we realised that the aromatic ring promote the ester cleavage because it stabilizes the resulting phenate ion. Therefore, we imagined the formation of a more stable ester, just increasing the chain length. Glutaric anhydride (4 eq.) was added to a solution of combretastatin **65** (1 eq.) in dry CH_2Cl_2. The mixture was stirred for 16 h at r.t., refluxed for 24 h and poured onto ice/water/$NaHCO_3$. The suspension was washed several times to remove other glutaric derivatives and give the desired acid **72** in 50% yield. This product is much more stable than the corresponding compound obtained by the reaction with succinic anhydride, so could be used for the next coupling (Scheme 32).



Scheme 32

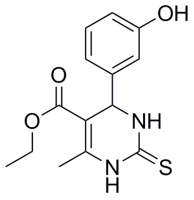
Many of the prodrugs synthesised to be coupled to the targeting unit, count a realising *in vivo* of the corresponding drugs. Since it is difficult to demonstrate the effective release in an animal system, we thought to prepare other derivatives with a non-cleavable ether bond, instead of an 'active' ester function. These molecules can't undergo neither enzymatic nor non-enzymatic hydrolysis and so can be used as a 'control' to effectively demonstrate the importance of the linker in our molecules. For this purpose we synthetised derivative **73** by modifications of the previous method. Isovanillin was functionalized with *tert*-butylbromoacetate and directly utilized for the next Wittig reaction with the same ylide used previously.

Operatively, to a solution of 3-hydroxy-4-methoxybenzaldehyde (1 eq.) in dry CH_3CN, was added K_2CO_3 (3 eq.). The mixture was heated while adding a solution of *t*-butylbromoacetate (1 eq.) and stirred for a day to give *tert*-butyl 2-(5-formyl-2-methoxyphenoxy)acetate **74** with 88% yield after aqueous work up. A homogeneous suspension of phosphonium bromide **67** (1 eq.) in dry THF under nitrogen was cooled to -23 °C and retained at that temperature for 2 h. Butyllithium 1.6 M in hexane (1 eq.) was added dropwise, the resultant orange solution was stirred at the same temperature for 1 h and finally **74** (1.04 eq.) was added dropwise. The reaction mixture was stirred at -23 °C for 4 h and at r.t. for 18 h, After aqueous washings, the crude product was purified by flash chromatography to give both the *Z* (**75**) and the *E* stilbenes in 13 and 15% yield respectively. Finally the hydrolysis of **75** (1 eq.) was carried in 1M LiOH (10 eq.) in a 1/1=THF/MeOH mixture to give acid **73** in 83% yield without further purification.



Scheme 33

b) **Monastrol**, a 4-(3-hydroxyphenyl)-2-thione derivative, is the first specific small molecule inhibitor of the vertebrate kinesin-5 subfamily KSP (human kinesin spindle protein), also known as HsEg5.[76] It is cell-permeable, and its effect on dividing cells is to bring about collapse of pre-existing bipolar spindles and cell cycle arrest with a monopolar spindle. The cocrystal structure of the monastrol-bound KSP motor domain complex revealed that monastrol is an allosteric inhibitor that binds to an induced-fit pocket 12 Å away from the ATP binding pocket. Monastrol is not competitive with ATP but is competitive with microtubules in inhibition of the KSP ATPase.[77] For this mechanism of action is the first small-molecule inhibitor of mitosis that does not target tubulin.

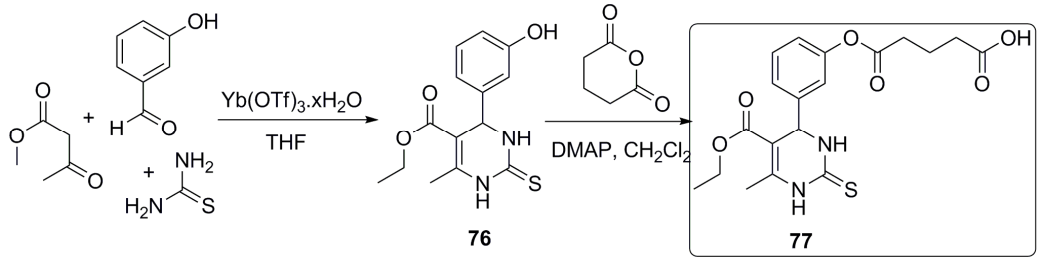


76

Figure 20 Monastrol

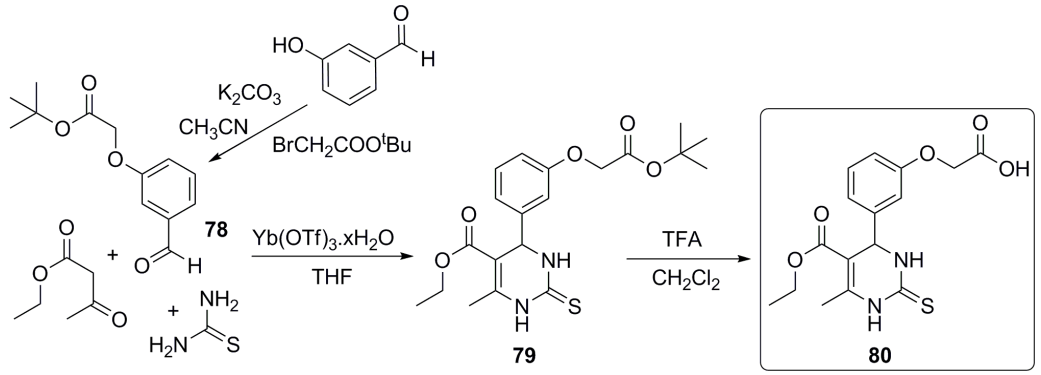
Its analogue ispinesib (Cytokinetics) is currently being studied in multiple phase II clinical trials. Because KSP is not involved in non-mitotic processes, such as neuronal transport, ispinesib may be less likely to cause the peripheral neuropathy often associated with the tubulin-targeting agents. To allow the coupling with branched neurotensin, racemic monastrol **76** was decorated at the phenolic OH with a carboxylic

acid group trough and 'active' ester link in **77** and a permanent ether bond in **80**. Monastrol can be efficiently prepared in high yield (95%) *via* a $Yb(OTf)_3$ catalysed three components Biginelli reaction.[78] The use of the Lewis acid $Yb(OTf)_3$ as the promoter instead of the three component system $CuCl/AcOH/BF_3.Et_2O$, usually adopted in this type of reaction, is due to the incompatibility of this system with the sulfurated version, employing thiourea.[79] Operatively ethyl acetoacetate (1 eq.), 3-hydroxybenzadehyde (1 eq.) and thiourea (3 eq.) were mixed together in THF and Lewis acid (0.1 eq.) was added. The reaction mixture was refluxed for 2 h, to afford monastrol **76** by aqueous work up followed by flash chromatography. The reaction of **76** (1 eq.) with glutaric anhydride (3 eq.) in dry CH_2Cl_2 under basic conditions (DMAP, 3 eq.) allowed the preparation of derivative **77** (56%) with the required pending carboxylic acid, purified by simple acid/basic washings (Scheme 34).



Scheme 34

The reaction of 3-hydroxybenzaldehyde with *t*-butylbromoacetate, used to introduce the ether link, followed by the condensation under the reaction conditions used above, allowed the isolation of the modified Biginelli adduct **79** (15%, non-optimized) which was transformed into the ether linked acid derivative **80** (50%) by hydrolysis with TFA in CH_2Cl_2 (Scheme 35).



Scheme 35

In summary the synthetic efforts made allowed the prepation and isolation of fifteen different carboxylic acid derivatives, shown in Figure 21, with chelating or anti-tumor activity to be adopted as Functional Units in our targeting system.

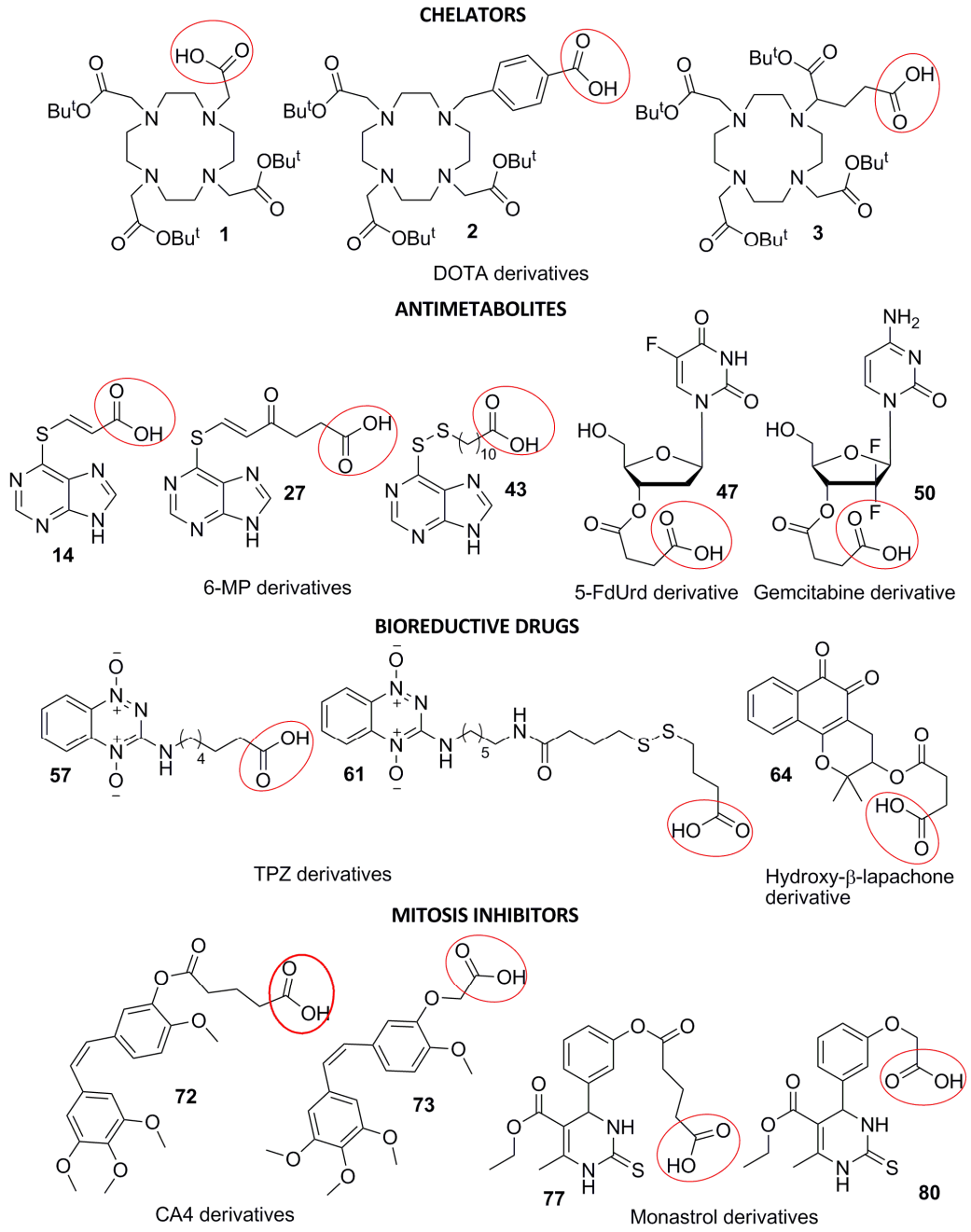


Figure 21 Carboxylic acid derivatives prepared during these three years.

2. 6-MP release from prodrugs

We synthesized three 6-MP derivatives **14**, **27**, **43** which are supposed to be prodrugs, biologically inert or inactive molecules which are able to release the active drug *in vivo*. Solid tumors are often in an hypoxic environment where GSH is normally abundant.[42,43] Pro-drugs as **14** and **27**, in the presence of nucleophiles like GSH that promote an addition-elimination reaction on the double bond, release 6-MP. Derivative **43** instead releases 6-MP by a thiol-disulfide exchange.

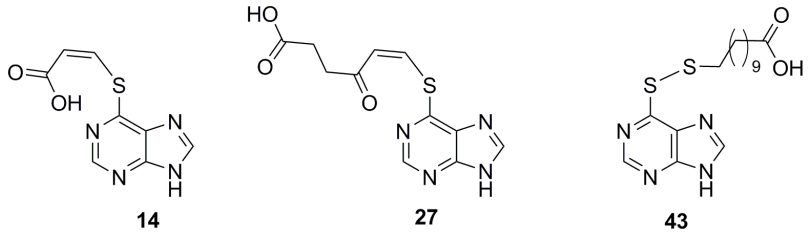


Figure 22 6-MP derivatives

In order to corroborate these findings, compounds **14**, **27** and **43** were incubated at 37°C in a phosphate buffer solution (pH = 7.4) in the presence of 1, 5 and 15 equivalents of GSH. These three concentrations had been chosen because they represent respectively the average concentration of GSH in normal cells, tumor cells and high hypoxic cells. The release of 6-MP, in all the three solutions incubated at 37 °C, was measured by HPLC at different time intervals.

Disulfide **43** was extremely reactive with nucleophiles and after 5 min, 77% of 6- MP was already released. Moreover the release of 6-MP was observed even in absence of GSH. In fact after 3 h of incubation of derivative **43** in buffered solution the 6-MP release was 50%. The instability of the compound was incompatible even with the conditions required for the resin cleavage (i.e. 95% TFA). We therefore gave up with the development of **43** and of any disulfide analogue.

Compound **14**, on the other hand, came out to be far too stable, since in the presence of 15 equivalents of GSH, after 18 h it only released 10% of 6-MP.

Satisfactorily, the most interesting releasing profile was shown by **27,** in fact the derivative **27** released 86% of 6-MP after 135 min in the presence of 1 equivalent of GSH (Figure 23, squares) and complete release after 30 min with 5 equivalents of GSH (Figure 23, rhombus). The possibility to modulate the release of the drug as function of

cellular endothiols concentration is an important feature of the pro-drug that was then carried forward for further characterization.

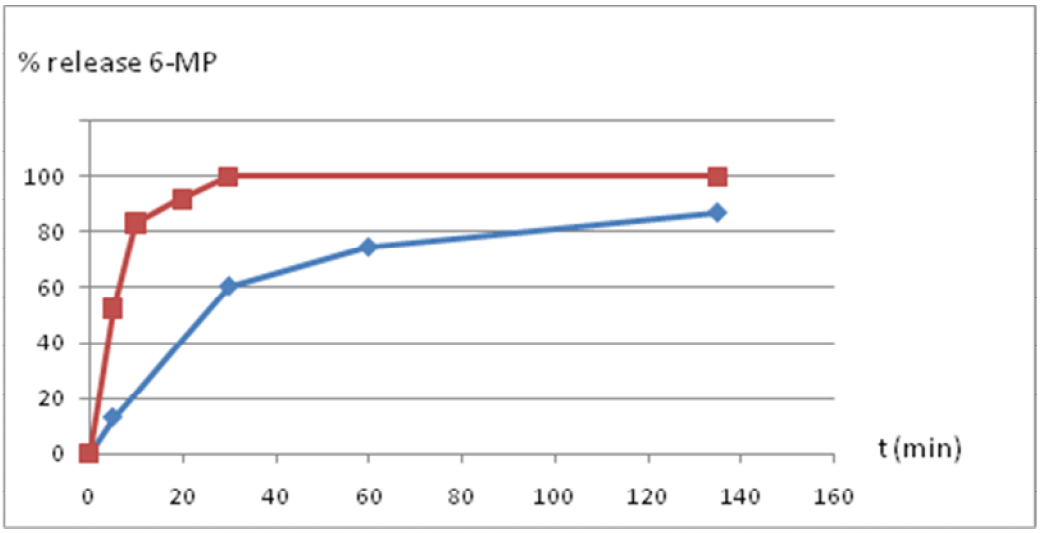


Figure 23 Release (%) of 6-MP at 37 °C with concentrations of **27** 1 mM and in presence of GSH at 1 mM (blue rhombus) ad 5 mM (red squares) concentrations

3. Conjugation to branched peptides (NT4)

We have synthesized, as we described in chapter 1, a variety of carboxylic derivatives decorating the most common anticancer drugs [Combretastatin A-4 (CBTST), Tirapazamine (TPZ), 6-Mercaptopurine (6-MP), 5-Fluoro-2'-deoxyuridine (5-FdUrd), Gemcitabine, Monastrol (MON)] and metal chelators (DOTA). The accessible COOH groups of these Functional Units should be then coupled with the tetrameric NT peptide in order to provide a tumor targeting system for diagnostic or therapeutic applications.

The conjugation with the NH_2 group, introduced *ad hoc* on the dendrimer, was carried out by standard solid phase synthesis for most of the derivatives prepared.

Operatively, peptide synthesis was performed on a MultiSynTech Syro automated multiple peptide synthesizer (Witten Germany), employing 9-fluorenylmethoxycarbonyl (Fmoc) chemistry with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) / N,N-diisopropylethylamine (DIPEA) activation. Side chain protecting groups were trityl for His, 2,2,4,6,7-pentamethyldihydro-benzofuran-5-sulfonyl for Arg, tert-butyl ether for Ser and Tyr, tert-butyl ester for Asp and Glu, and tert-butyloxycarbonyl for Trp. Considering the large variety of molecules to be conjugated with the dendrimeric peptide, prof. Bracci's group tested different approaches to find the most simple and versatile way to prepare tetramers. Tetra-branched analogues were synthesized on resin NovaSyn TGR. A mixture of 5 eq. of Fmoc protected aminoacid, 5 eq. of HBTU dissolved in DMF and 10 eq. of DIPEA were added to the resin beads. The reaction was automatically performed at r.t. for 40 minutes and the coupling step repeated twice. After each aminoacid addition the Fmoc protecting group was removed with a 40% solution of piperidine in DMF and extensively washed with DMF before adding the next one. All tetrameric peptides were built employing two consecutive Fmoc-Lys(Fmoc)-OH coupling steps to form the branched core.

In particular, tetrabranched NT(8-13)-PEG derivatives of DOTA, [NT4(8-13)-**1**] **81** and [NT4(8-13)-**3**] **82**; 6-Mercaptopurine, [NT4(8-13)-**14**] **83** and [NT4(8-13)-**27**] **84**; 5-Fluorodeoxyuridine, [NT4(8-13)-**47**] **85**; Gemcitabine, [NT4(8-13)-**50**] **86**; Tirapazamine, [NT4(8-13)-**57**] **87** and [NT4(8-13)-**61**] **88**; Combretastatin, [NT4(8-13)-**72**] **89** and [NT4(8-13)-**73**] **90**; and Monastrol, [NT4(8-13)-**77**] **91** and [NT4(8-13)-**80**] **92** (Scheme 36), were synthesized with Fmoc-Lys(Dde)-OH as first coupling step and Fmoc-β-Ala-OH as second coupling.

Fmoc-Lys(Fmoc)-OH was used to build the three-lysine branched core. C-terminal stepwise automated elongation [(Arginine (R), Arginine (R), Proline (P), Tyrosine (Y), Isoleucine (I), Leucine (L)] was then carried out by the above described HBTU/DIPEA method. Boc-Arg(Pbf)-OH was used as last amino acid of the neurotensin sequence, so that the last step occurred selectively on the side chain arm. In fact, Dde protective group was removed with 2% hydrazine for 10 min at room temperature and the free amino group was coupled with all the new functional units, which are provided with a carboxylic acid function. Moreover, the side chain amine group to be used for the functional unit coupling was rendered more accessible stretching it with Fmoc-PEG-OH. After deprotection of the Fmoc group on PEG, the functionalized peptide was finished by activation and coupling of the carboxylic acid of the Functional Unit (R'-OH).

Peptides were cleaved from the resin and deprotected by treatment with trifluoroacetic acid (TFA) containing water and triisopropylsilane (95/2.5/2.5), and precipitated with diethyl ether (Scheme 36).

The same procedure was also used to synthesized NT4(8-13)-Bio (Biotin) (Figure 24), starting with Fmoc-Lys-Bio-OH as the first group attached to the solid support and the spacer Fmoc-PEG-OH as second step.

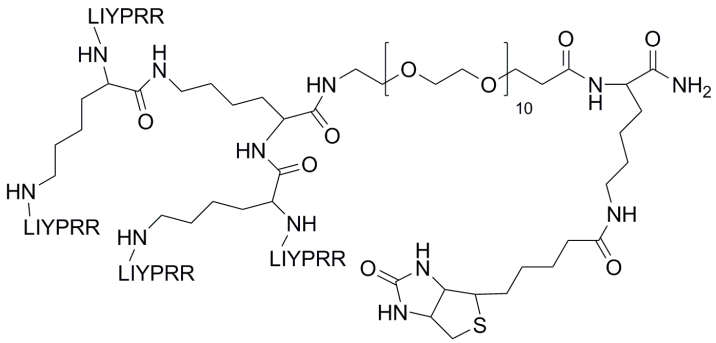
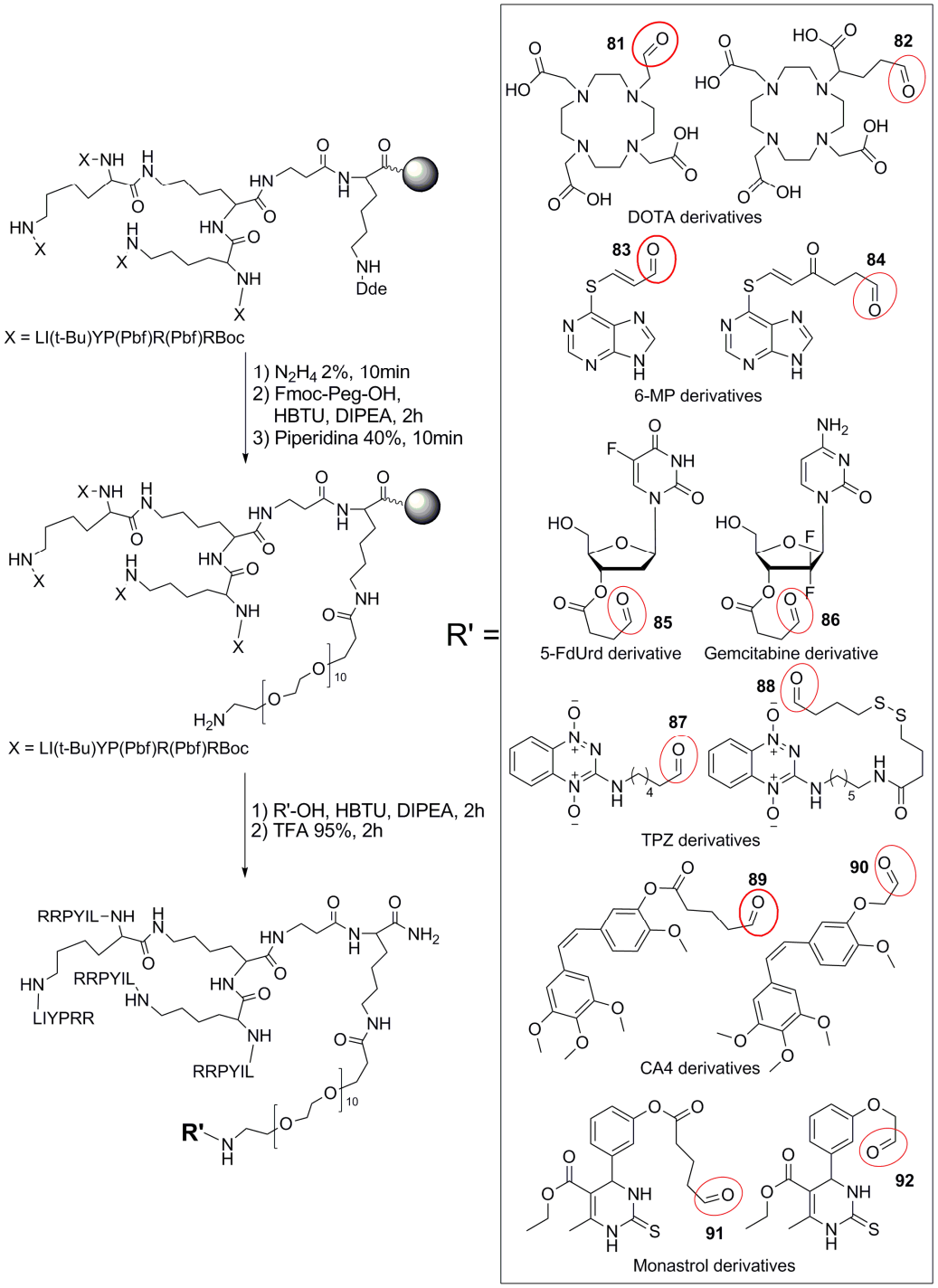


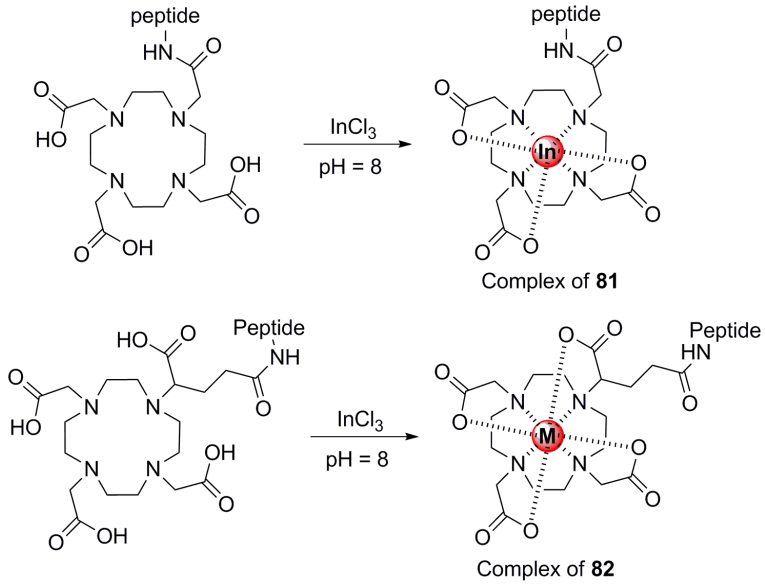
Figure 24 NT4(8-13)-Bio


X = LI(t-Bu)YP(Pbf)R(Pbf)RBoc
1) N_2H_4 2%, 10min
2) Fmoc-Peg-OH, HBTU, DIPEA, 2h
3) Piperidina 40%, 10min
X = LI(t-Bu)YP(Pbf)R(Pbf)RBoc
1) R'-OH, HBTU, DIPEA, 2h
2) TFA 95%, 2h
LIYPRR
RRPYIL
R' =
81
82
DOTA derivatives
83
84
6-MP derivatives
85
86
5-FdUrd derivative
Gemcitabine derivative
87
88
TPZ derivatives
89
90
CA4 derivatives
91
92
Monastrol derivatives

Scheme 36

All compounds were characterized by MS on a Ettan MALDI-TOF mass spectrometer and purified by HPLC on a C18 Vydac column. [NT4(8-13)-**57**] **87** characterization has been initially complicated, because the compound undergoes reduction of the tirapazamine moiety by single or double oxygen extraction during MS analysis.

NT(8-13)4-PEG-DOTA **81** and **82** were then used to chelate $^{115}In^{3+}$ in a typical chelation reaction: the peptide and $InCl_3$ were dissolved in acetate buffer pH 8 and stirred for four hours at room temperature (Scheme 37).



Scheme 37

The reaction and purification procedures will be reproduced with ^{111}In for biodistribution experiments in mice.

4. Cytotoxicity of drug-conjugated tetra-branched NT peptides

All the experiments have been carried out in three different cell lines to represent the diverse tumors that over-express NT receptors (Table 1): HT-29 (colon adenocacinoma), PANC-1 (pancreas epitheloid carcinoma) and PC-3 (prostate carcinoma).

Our tumor targeting system is based on the specific delivery of the functional unit into the tunor cell by peptide binding and subsequent internalization of the receptor-ligand complex by endocytosis, as shown in Figure 1. In order to validate NT branched peptides as possible targeting agents for therapy of either colon or pancreas or prostate adenocarcinoma, binding of tetra-branched NT peptides to human tumor cell lines, was analysed and their rate of internalization was quantified.

Peptide binding and internalization of tetra-branched NT(8-13) conjugated to Biotin (NT(8-13)4-Biotin) was analysed by confocal microscopy in HT-29, PANC-1 and PC-3 cell lines**.** It was found that NT4 conjugated to different fluorophores or to biotin, followed by fluorophore-conjugated streptavidin, specifically bind to the three cell lines, which express the NT receptors.[24] Peptide binding on membrane receptors was observed after 30 minutes incubation at room temperature. Cells were then washed and further incubated for 1, 2 or 4 hours to follow peptide internalization by confocal microscopy as it is shown in Figure 25. Peptides are degraded inside the cells within 18 hours. Binding of tetra-branched NT(1-13) and NT(8-13) was clear in all the cell lines, whereas that of corresponding monomeric peptides was scarcely evident, which demonstrated the higher binding efficiency of branched peptides. NT(1-13) and NT(8-13) branched peptides were completely internalized by the three cell lines, despite different internalization rates among the cell lines. Internalization of both tetra-branched NT(1-13) and NT(8-13) was quicker in PANC-1 and PC-3 than in HT-29 (Fig. 2). As a matter of fact HT-29 still showed a considerable amount of peptide on the cell membrane after a 2-hour incubation, whereas in PANC-1 and PC-3, peptides were completely internalized after a 1-hour incubation. No binding was observed either with an analogously conjugated branched peptide containing an unrelated sequence (DDHSVA) or with equivalent concentrations, in terms of peptide units, of the conjugated monomeric NT(1-13) or NT(8-13) sequences.[80]

Drug-conjugated tetra-branched NT peptides' internalization rate

PANC-1 cells were incubated with tetra-branched NT conjugated to Functional Units and with the unrelated peptide (DDHSVA). Cells were centrifuged, washed and then

lysed. The presence of uncleaved peptides was assessed by MALDI spectrometry both in the cell supernate and in cell lysate. Functional Units were detected as uncleaved molecules in cell supernate and cell lysate even after 48 h. Unrelated peptides were found intact after 48 hours in the supernate and never detected in the lysed cells. Linear NT(1-13) (Sigma) was detectable in cell supernate after 2 hours but was undetectable in the lysed cells.

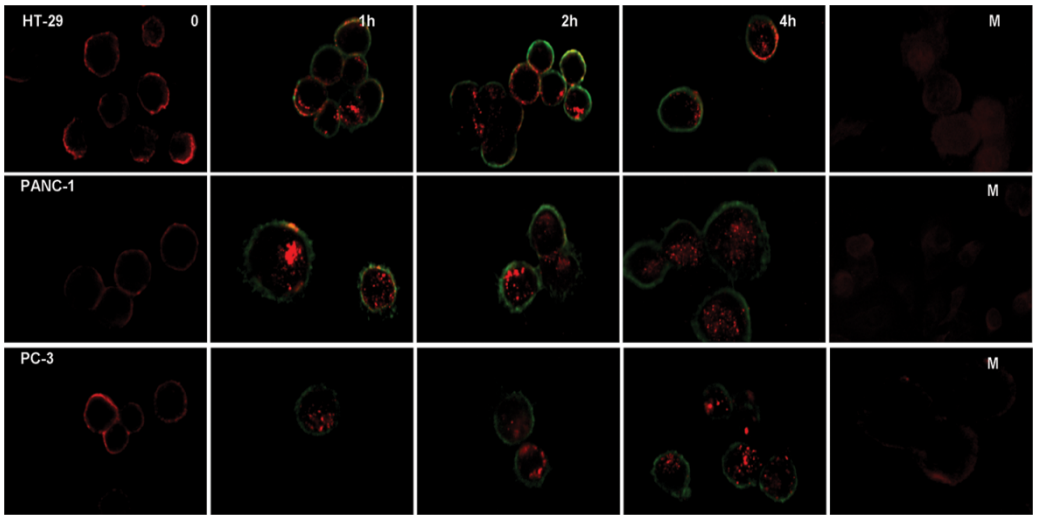


Figure 25 Binding and internalization in tumor cell lines. HT-29, PC-3 and PANC-1 were exposed for 30 minutes (time 0) to NT(8-13)4-Biotin or monomeric NT(8-13)-Biotin (M) followed by Streptavidin-Cy3. Internalization of NT(8-13)4-Biotin was observed after 1, 2 and 4 hours of further incubation in medium at 37°C. Cell membrane was stained with Lectin-FITC.

The ability of tetrabranched peptides conjugated to a functional unit to bind cancer cell lines through NT receptors, to be rapidly internalized in cells and still detectable after 4 hours, is an important feature for possible therapeutic applications of these molecules.[81]

Finally we tested the possible application of these new NT-based molecular tools, conjugated to different drugs, for personalized therapy by determining their cytotoxicity in different human cancers.

Cytotoxicity of drug-conjugated NT4 in different tumor cell lines

It is well known that classical chemotherapeutics used in the clinical practice have different activity on different tumors. This is due to natural resistance of cancer cells to the drugs, caused by different mechanisms, including a decreased uptake or increased export of drugs by the cell, increased inactivation of drugs inside the cell or enhanced repair of the DNA damage produced by DNA-alkylating agents.

Previously reported cytotoxicity experiments, performed by the Prof. Bracci's research group on HT-29,[24] demonstrated that conjugation of methotrexate (MTX) or of the photosensitizer, chlorine-e6, to tetra-branched NT(8-13) produces pro-drugs like molecules. Such molecules can no longer be transported across plasma membranes by the mechanism of the corresponding free drug and can only be 'activated' via peptide-receptor binding, thus profoundly decreasing non-specific drug toxicity.

Introduction of a novel, peptide receptor-mediated, mechanism of cell internalization of the drug might allow by-passing natural mechanism of cell resistance. As already shown in chapter 1, we designed and prepared several derivatives of different drugs, commonly used in classical tumor chemotherapy. Almost all these molecules were tested either as free drugs or conjugated to the tetra-branched NT(8-13) on HT-29, PANC-1 and PC-3 tumor cell lines.

In details, HT-29, PANC-1 or PC-3 cells were plated at a density of 2.5 x 10^4 per well in 96-well microplates. Different concentrations of free or NT(8-13)-conjugated drugs, from 0.15 to 30 μmol/L, were added 24 h after plating. Cells were grown without changing the medium for 6 days. Growth inhibition was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. EC50 values were thereby calculated by non-linear regression analysis using GraphPaD prism 3.02 software.[2]

The cellular toxicity of all the drug-conjugated NT4 was tested on the three cell lines and compared with the cytotoxicity of corresponding free drugs and also with that of an unrelated tetra-branched peptide (U4), identically conjugated to the same drug.

We must say first that 5-Fluorodeoxyuridine, monastrol and combretastatin have never been proved to be inactivated by the conjugation, so at the state of the art it is not known if **47**, **72** and **77** are prodrugs or not. However it was necessary to know their rate of drug release to identify the true cytotoxic species (free or conjugated drugs). For this reason derivatives **72** and **77** were conjugated to NT4 via a bifunctional linker that bears an ester bond, which should be released *in vivo*, at one end and an amide at the other end, whereas **73** and **80** bifunctional linker bears a non-hydrolyzable ether bond at one end and an amide at the other end. Stability of the conjugated molecule was then assessed by incubation with HT-29 for different time intervals and the supernate and cell lysate analysed by HPLC and MS resulting in a complete release of the drug moiety after 2 hours (data not shown). The ester bond allows a faster release of the drug and the branched derivatives were then classified as *fast releasing* **84**, **85**, **89** and **91** - and *slow releasing* **83**, **90** and **92** and their cytotoxicities compared.

When cells were incubated with the fast releasing **85** and **89** peptide drugs in experimental protocol of six days of incubation without washing (Figure 19 and 22) part of the chemotherapeutic moiety was released outside the cell and internalized via a different mechanism, probably by membrane diffusion. This was proved since unrelated conjugated peptides had a cytotoxic effect that could only be ascribed to the release of the 5-FdUrd **45**, Combretastatin **65** or Monastrol **76**, since we demonstrated that the unrelated peptide cannot be internalized.[24]

The washing procedure was then essential to follow the activity of fast releasing adducts so to exclude the additive effect of the released free drug.

Cytotoxicity of fast releasing drug-conjugated tetra-branched peptides was then tested[80,81] in HT-29, PANC-1 and PC-3 in experiments where cells were exposed to a 1 hour pulse of free or NT-conjugated drug, washed and incubated for 6 days or, alternatively incubated for 6 days with the peptides, without additional washing. The additional washing was performed in order to avoid diffusion of free drug inside the cells during the following six days (Figure 20, 23, 25).

*Cytotoxicity of **83** and **84**, derivatives of 6-Mercaptopurine.* (Figure 26 and 27)

Mercaptopurine **11** itself is a chemotherapy drug that is most commonly used to treat acute leukaemias but is also given as a treatment for some other types of cancer.

The cytotoxicity test for derivative **83**, where its analog, PTA, is conjugated to the dendrimeric neurotensin was evaluate as described above, in two cell lines HT-29 and PANC-1. As expected, 6-MP derivative **83,** a very slow releasing adduct**,** was completely inactive in this in vitro model, as well as U4-6MP.

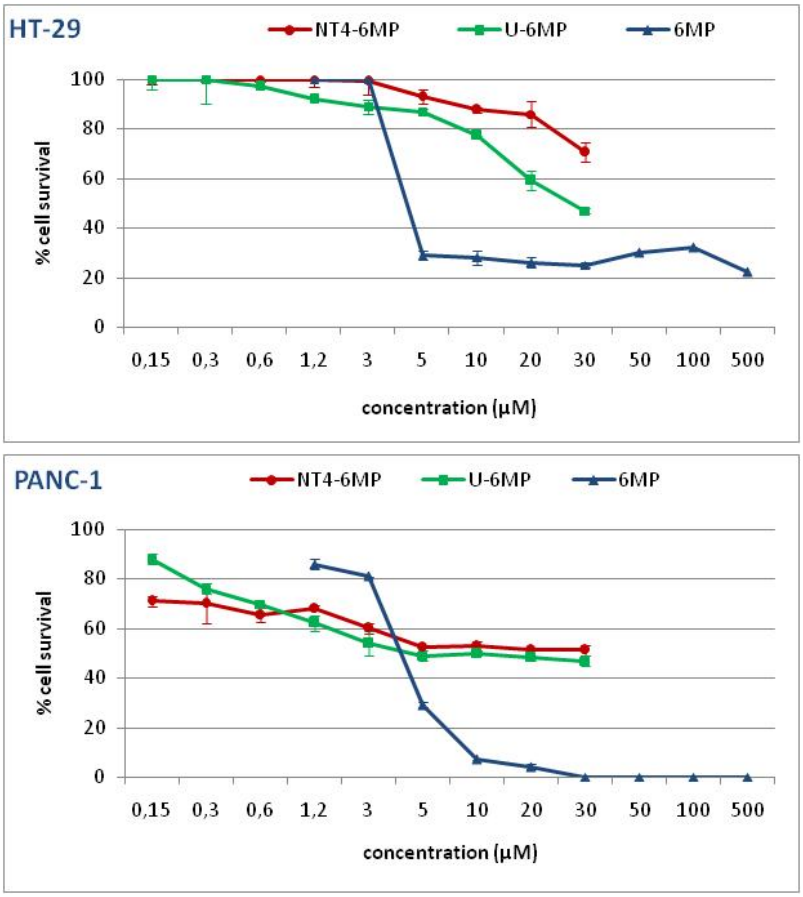


Figure 26 Cytotoxicity of conjugate [NT4-**14**] **83** (indicated like NT4-6MP in the figure) compared to the unrelated conjugate U4-**14** (indicated like U-6MP) and free 6MP

Regardless the promising releasing behaviour, **27** didn't give the expected results, in fact, the conjugated derivative (**84**) showed a cytotoxic activity comparable to 6-MP itself. (Figure 27)

On the other hand, this behavior means that this derivative is effectively a pro-drug and it is able to release 6-MP in this experimental model. Probably there are enzymatic or

GSH-mediated mechanisms which permit the drug releasing both by neurotensin peptide and its *unrelated* analog.

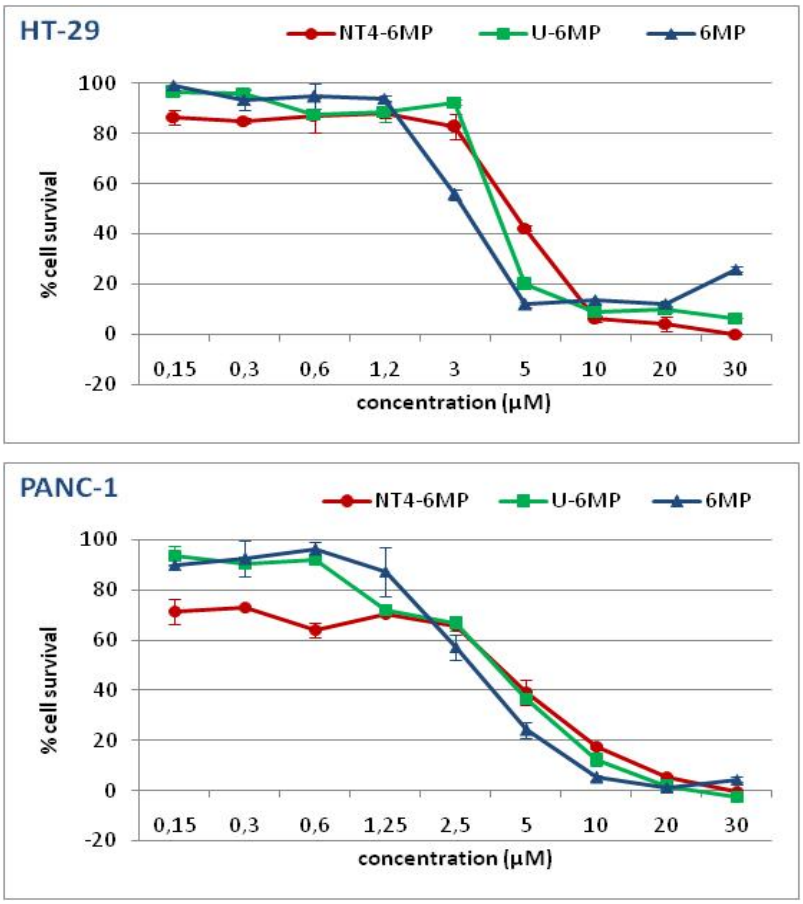


Figure 27 Cytotoxicity of conjugate [NT4-**27**] **84** (indicated like NT4-6MP in the figure) compared to the unrelated conjugate U4-**27** (indicated like U-6MP) and free 6MP

*Cytotoxicity of **85**, NT4(8-13) derivative of 5-Fluorodeoxyuridine.* (Figure 28 and 29)

5-FdUrd (5-fluorodeoxyuridine) **45** as already said is a thymidylate synthase inhibitor, it is transformed into different cytotoxic metabolites which are then incorporated into DNA and RNA, finally inducing cell cycle arrest and apoptosis. Its principal use is in colorectal and pancreatic cancers.

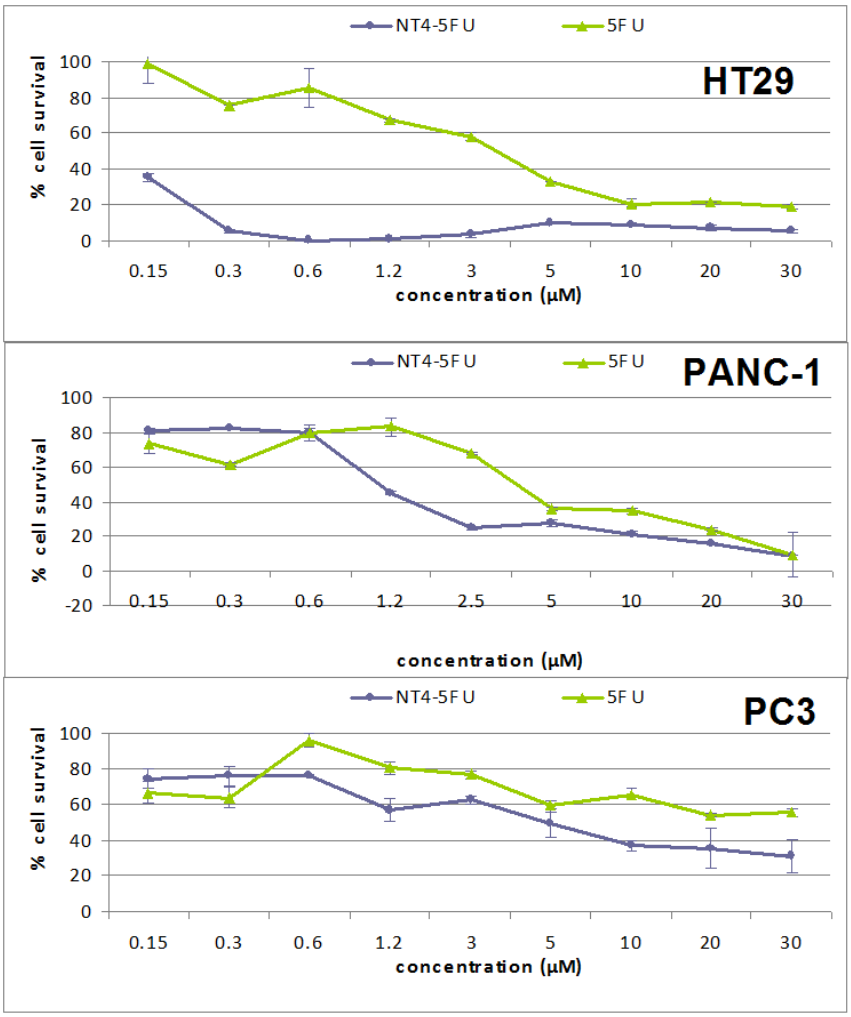


Figure 28 Cytotoxicity of conjugate [NT4-**47**] **85** (indicated like NT4-5FU) compared to free 5FU

5-Fluorouracile was used as a control in the experiment of cytotoxicity and shows analogue activity on PC-3, PANC-1 and HT-29 cell lines. The conjugated molecule NT4-5FU showed increased activity with respect to the free drug especially on HT-29 where the IC50 drops to 1.0 nM, whereas in PC-3 and PANC-1, IC50 it is in the micromolar range, respectively 3.8 µM and 1.6 µM.

An additional washing was performed in order to avoid the diffusion of free 5-FdU released by hydrolysis of the ester bond of **85** during the 6 day incubation period and the data obtained with this procedure are reported below (Figure 29).

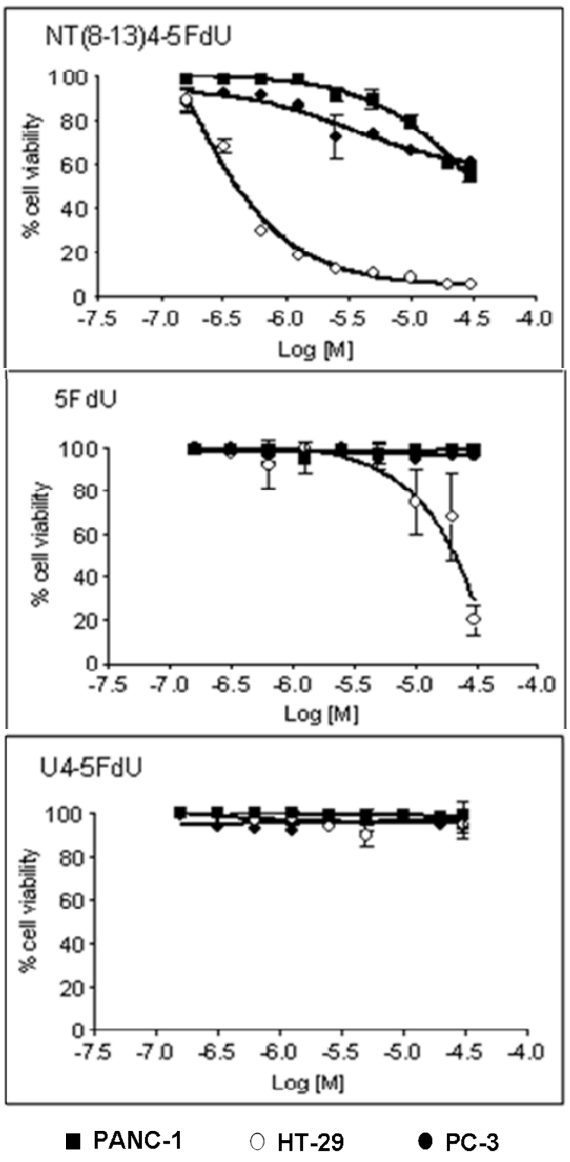


Figure 29 Cytotoxicity of conjugate [NT4-**47**] **85** (indicated like NT4-5-FU) compared to free 5FU and the unrelated conjugate U4-**47** (indicated like U4-5FU) after washing

Free 5-FdU was completely ineffective in PANC-1 and PC-3 cell lines at the doses used and slightly effective in HT-29. When conjugated to branched NT, it became effective not only in HT-29, where the EC50 became 1.1e-007, with nearly two logs improvement, but also in PANC-1 and PC-3 (Figure 29).

*Cytotoxicity of **87**, NT4(8-13) derivative of Tirapazamine.* (Figure 30)

Tirapazamine **51** is selectively activated by multiple reductases to form free radicals in hypoxic cells, thereby inducing single-and double-strand breaks in DNA, base damage, and cell death. This agent also sensitizes hypoxic cells to ionizing radiation and inhibits the repair of radiation-induced DNA strand breaks via inhibition of topoisomerase II. Curreently, there are numerous clinical trials using this agent, alone or in combination, in cervical cancer, non-small cell lung cancer, head and neck cancer, glioblastoma multiforme, and ovarian carcinoma.

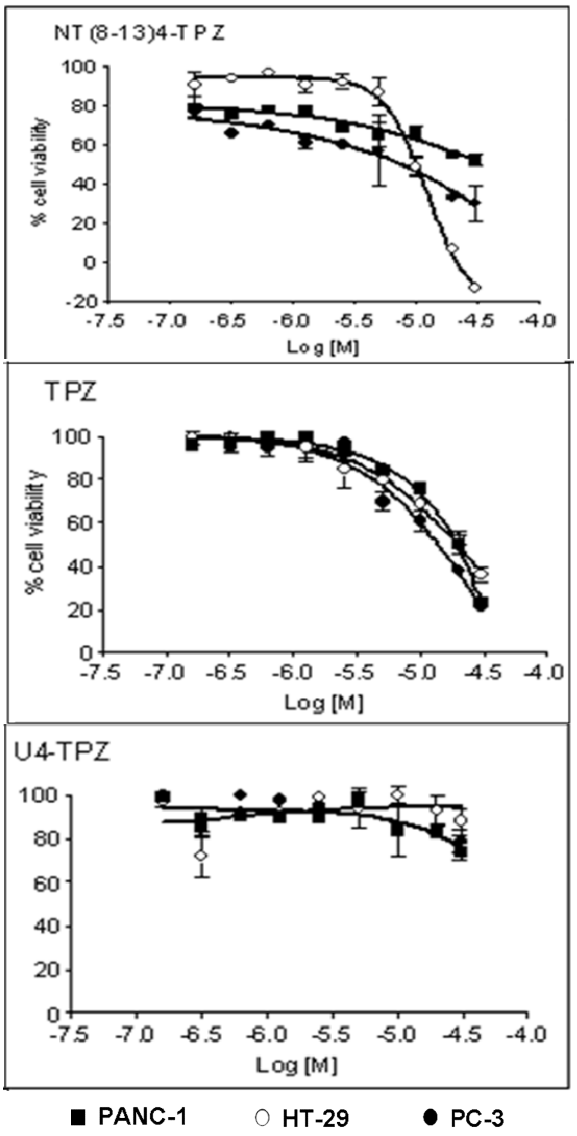


Figure 30 Cytotoxicity of conjugate [NT4-**57**] **87** (indicated like NT4-TPZ) compared to the free TPZ and unrelated conjugate (U-TPZ).

As we can see from Figure 30 free TPZ was more active than when it is conjugated to the branched peptide, which therefore didn't bring any advantage in PANC-1 and PC-3 cell lines. Interestingly conjugation to branched NT increased drug efficiency in HT-29. We have to consider the bioreductive nature of TPZ, which is able to kill hypoxic cells 50-200 times more effectively than well oxygenated cells. Therefore, this study has to be repeated under hypoxic conditions to have a real evidence of the TPZ activity. Moreover the cytotoxicity of the conjugated disulfide derivative of TPZ **88** has still to be evaluated as well as its ability to effectively release the TPZ and consequently increase the activity of the conjugate.

*Cytotoxicity of **89**, NT4(8-13) derivative of Combretastatine.* (Figure 31 and 32)
Combretastatin A-4 inhibits cell growth even at low nanomolar concentrations and exhibits inhibitory effects on multidrug resistant cancer cell lines.

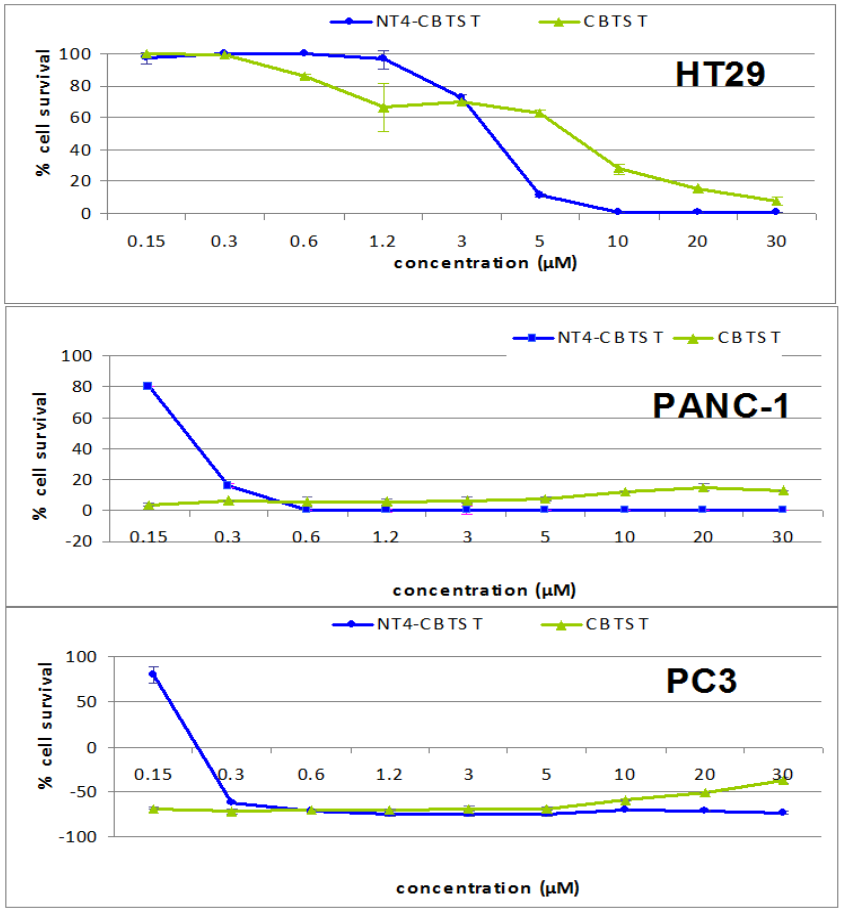


Figure 31 Cytotoxicity of conjugate [NT4-**72**] **89** (indicated like NT4-CBST) compared to the free CBST.

The strong toxic activity of the free drug is evident in these human tumors cell lines and especially against PANC-1 and PC-3. The conjugated derivative NT4-**72** preserves its activity in the three cell lines and reflects the same trend, in facts, the IC50 is 3.5 µM on HT-29 and drops to 0.5 and 0.4 nM respectively on PANC-1 and PC-3.
As for [NT4-**47**] **85** the additional washing was performed and the results compared.

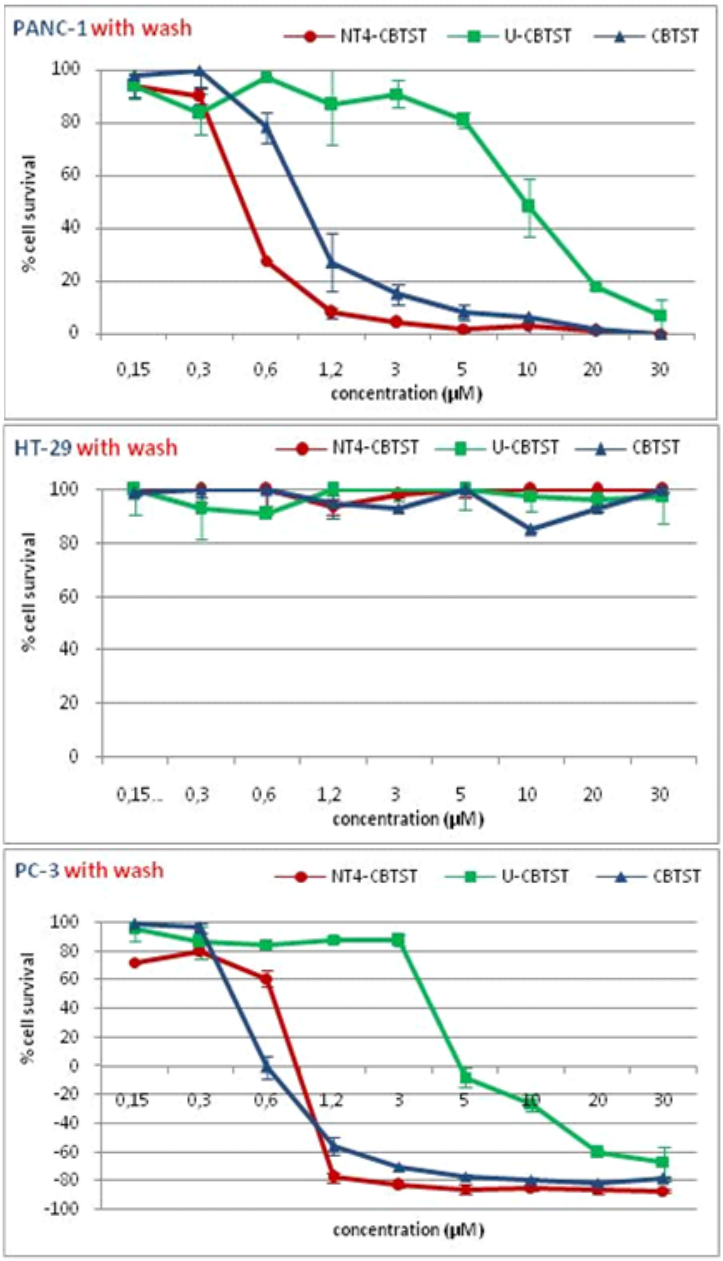


Figure 32 Cytotoxicity of conjugate [NT4-**72**] **89** (indicated like NT4-CBST) compared to the unrelated conjugate (indiacted like U-CBST) and free CBST after washing

Compound **89** deserves major interest, since the activity of the drug is clearly increased by conjugation to branched NT4 through a fast releasing linker (Figure 31) and this is evident though the unfavourable conditions of the additional washing.

*Cytotoxicity of **90**, NT4(8-13) derivative of Combretastatin.* (Figure 33)

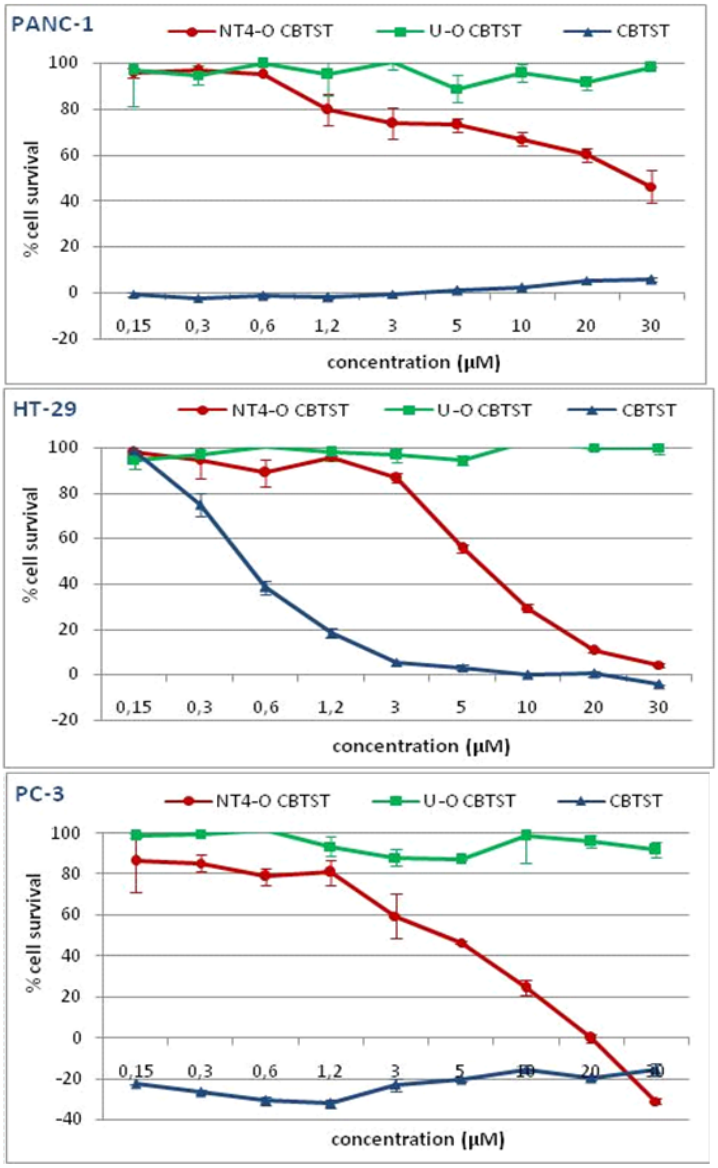


Figure 33 Cytotoxicity of conjugate [NT4-**73**] **90** (NT4-O-CBST) compared to the unrelated conjugate (U4-O-CBST) and free CBST

Comparison of results obtained with Combretastatin derivatives **89** and **90**, indicates that this system works better if Combretastatin can be released inside the cell than when it remains bound to the carrier peptide.

*Cytotoxicity of **91**, NT4(8-13) derivative of Monastrol.* (Figure 34)

Monastrol and its analog Ispinesib selectively inhibits the mitotic motor protein, kinesin spindle protein (KSP), resulting in inhibition of mitotic spindle assembly, induction of cell cycle arrest during the mitotic phase, and cell death in tumor cells that are actively dividing. They are undergoing multiple clinical trials for breast, prostate, ovarian and other solid tumors or lymphomas.

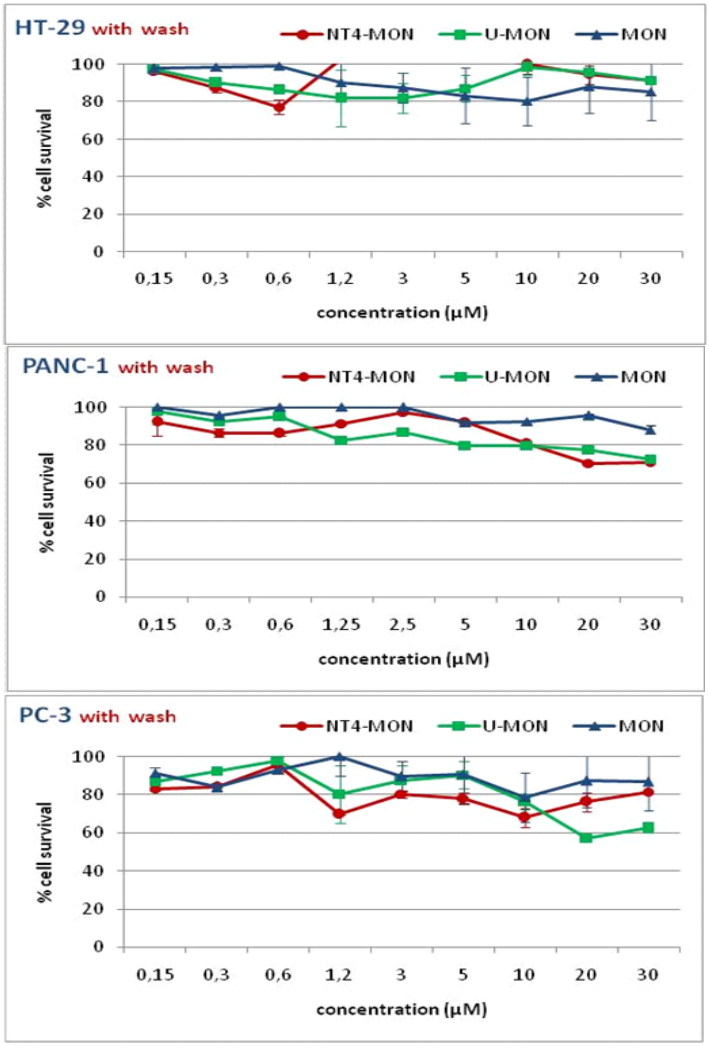


Figure 34 Cytotoxicity of conjugate [NT4-**77**] **91** (indicated like NT4-MON) compared to the unrelated conjugate (indicated like U4-MON) and free MON after washing

Monastrol has a very poor cytotocity as a free drug for the three cell lines tested and its activity is not increased by the conjugation to the peptide.

*Cytotoxicity of **92**, NT4(8-13) derivative of Monastrol*. (Figure 35)

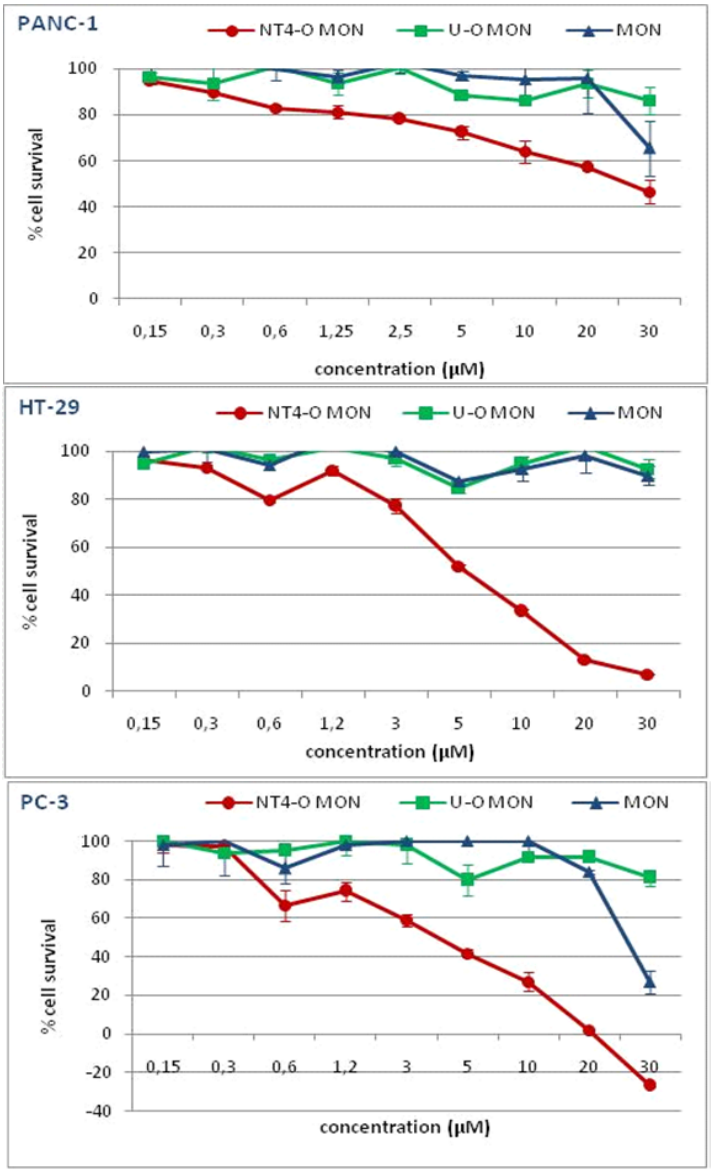


Figure 35 Cytotoxicity of conjugate [NT4-**80**] **92** (indicated like NT4-OMON) compared to the unrelated conjugate (indicated like U4-OMON) and free MON

In the case of Monastrol, very poor in citotoxicity as a free drug, conjugation to NT4 by an ether linkage, converts free drug inefficacy to moderate cytotoxicity of the

conjugated drug. As we can see from Figure 34 and 35 the effect of the washing on the activity of Monastrol is pronounced. We are planning to repeat the experiments in order to find the best incubation interval before washing. Nevertheless *in vivo* studies will be the most exhaustive to appreciate the releasing pattern of the drug.

In conclusion, as expected, the washing procedure, in [NT4-**47**] **85**, [NT4-**72**] **89** and [NT4-**77**] **91** experiments, lowers sensibly the resulting EC50 and this has to be taken into account when considering the overall goodness of the tumor targeting peptide. To have an idea of the loss of initial drug concentration, by means of the additional washing, the behaviour of free CA-4 can be followed in Figure 31 and 32: free CA-4 for PANC-1 and PC-3 give 100% cell death at all tested concentrations, but when cells are washed after 2 hours and re-incubated EC50 falls to 12 μM and 0.4 μM respectively for PANC-1 and PC-3 .

All the experiments assessed that the conjugation to tetrabranched NT(8-13) profoundly modified drug activity, which might result from the combination of both cell and drug features, including: i) cell sensitivity to the drug; ii) drug mechanism of action; iii) mechanisms of cell resistance to the drug; iv) efficiency of membrane transport of the conjugated-drug. As expected, activities of the free drugs are very different from one another and from cell line to cell line. In principle, the conjugation to tetrabranched NT may produce as a result:

-an increase in drug specificity,

-an increase in drug activity,

-an increase in both specificity and activity or no improvement of the free drug.

When the free drug is very efficient, slow release conjugation to NT4 apparently decreases drug efficiency (like in the case of Combretastatin derivative **90** in PANC-1 and PC-3). Interestingly, in other cases conjugation to branched NT can increase drug efficiency (as for Monastrol derivative **92** in the three cell lines, Tirapazamine derivative **87** in HT-29). Two negative examples of this type of conjugation are represented by **83** [tetrabranched NT(8-13)-**14**] (slow release) and **91** [tetrabranched NT(8-13)-**77**] (fast release) these two compounds gained no improvement compared to the parent free drugs. Results with fast releasing tetra-branched NT are very interesting, since in the case of both 5-Fluorodeoxyuridine and Combretastatin derivative, respectively **47** and **72**, the activity of the drug is clearly increased by conjugation to branched NT4 (Figure 28-33). Comparison of results obtained with slow and fast releasing molecules, indicate that fast releasing molecules can be even more active than

slow-releasing compounds. A clear advantage of the branched peptide carrier is its target specificity, demonstrated by the lack of activity on the three cell lines of any drug, when coupled to an unrelated branched peptide.

Interestingly, cells that are not affected by a drug, such as PC-3 by 5-Fluorouracil itself, can become sensitive to it, when conjugated to branched NT (Figure 28 and 29).

Changing the mechanism of membrane transport, by switching to a peptide receptor-mediated mechanism, can deeply modify drug transport from outside to inside the cells. Moreover, conjugation to branched peptides might as well impair mechanisms of drug export from inside to outside the cell, entrapping the conjugated drug into the target cell. This is extremely important for the therapy of tumors that over-express NT receptors and do not respond to classical chemotherapy.

In conclusion, almost all our derivatives show very interesting profiles, in any case we can see an increased selectivity and/or an increased activity.

Especially [NT4-**47**] and [NT4-**72**] have both increased their activity, and seem to be very efficient cytotoxic agents, while the branched scramble peptide conjugated to **47** and **72** are inactive.

These two new promising molecules **85** and **89** will be so enrolled for the *in vivo* studies, to determine the inhibition of tumor growth in nude mice bearing HT-29 tumor xenografts.

5. Nanoparticle therapeutics

During my PhD I had the opportunity to spend six months in the group of Professor Vincent M. Rotello at University of Massachusetts, working on a project directed towards the synthesis of nanoparticles to be used in therapeutic applications.

Nanomaterials are currently receiving considerable attention because of their potential applications in biology and medicine. In particular, nanoparticles (NPs) are artificially created structures in which all three dimensions are between 1 and 100 nm and which are stable dispersed in solvent.[82,84] Depending on material, size and shape, NPs possess different functional properties. Some of these properties make them interesting as contrast markers for imaging cancerous tissue (MRI), diagnostic tools,[82] radiotherapy enhancers, as well as drug and gene deliverers.[83,84]

Several methods have been proposed to target a tumor efficiently, exploiting some useful characteristics of the newly formed vasculature. For example the enhanced permeability and retention (EPR) phenomenon in tumors, where microvasculature allows permeation of molecules and the absence of lymphatic drainage leads to retention and accumulation of the delivered compounds in a tumor[83] (Figure 36). In EPR targeting, also called passive targeting, the therapeutic agent is subsequently released locally and taken up by tumor cells to achieve its effect. In contrast, the tumor receptor or antigen targeting, utilizing respectively peptides (like NT) and mAbs as targeting agents, specifically aims the therapeutic agent at tumor cells (active targeting).

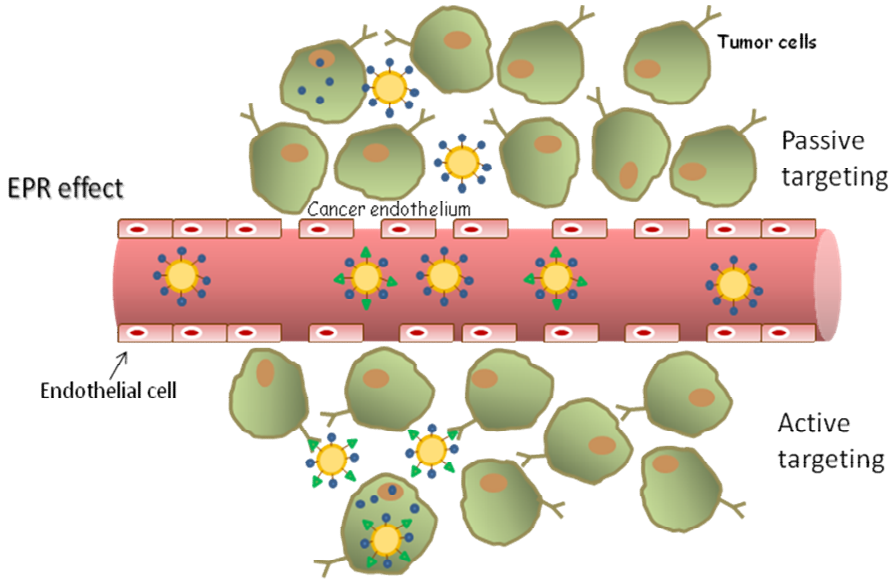


Figure 36 Passive and active targeting

NPs exploiting either passive or active targeting are emerging as attractive candidates for delivery of various payloads, which can be small drugs or large biomolecules, like proteins, DNA or RNA.

Drug delivery

Many of the pharmacological properties of conventional ("free") drugs can be improved through the use of drug delivery systems (DDS) composed primarily of lipids and or polymers.[86] DDS are designed to alter the pharmacokinetics (PK) and biodistribution (BD) of their associated drugs, or to function as drug reservoirs. Alterations in the BD of DDS can occur through EPR effect (passive targeting).

Multifunctionality is the fundamental advantage of nanovectors[86] for the cancer-specific delivery of therapeutic and imaging agents. Primary functionalities include the avoidance of biobarriers and biomarker-based targeting, and the reporting of therapeutic efficacy. A huge number of nanovectors are now under study and the first examples, liposome-encapsulated formulations of doxorubicin[86] were approved more than ten years ago for the treatment of Kaposi's sarcoma, and are now used against breast cancer and refractory ovarian cancer. Liposome carriers are to date the most effective, but they do not provide control for the time of drug release and usually do not achieve effective intracellular delivery of the drugs[87], therefore limiting their potential, especially against multidrug-resistant cancers. In contrast, inorganic nanoparticles generally possess versatile properties suitable for cellular delivery, including wide availability, rich functionality, good biocompatibility (low toxicity), potential capability of targeted delivery and controlled release of carried drugs, thus offering a new appealing alternative.[83] Thus in the last decade an increasing efforts in research and development have been devoted worldwide to various inorganic materials as novel carriers in the last decade.

Gold nanoparticles (AuNPs) have been recently emerged as an attractive candidate for drug and gene delivery applications for their functional versatility,[88] biocompatibility,[89] and low toxicity.[90] Efficient release of these therapeutic agents is a prerequisite for effective therapy. Both covalent and non-covalent approaches can be applied to these DDSs. Recent studies have demonstrated release of payload, shown in Figure 37, by intracellular thiols, as GSH.[84,91] However controlled dissociation of drugs from covalent conjugates is still a challenge for therapeutic applications.

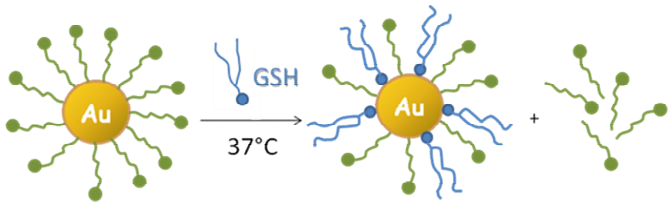
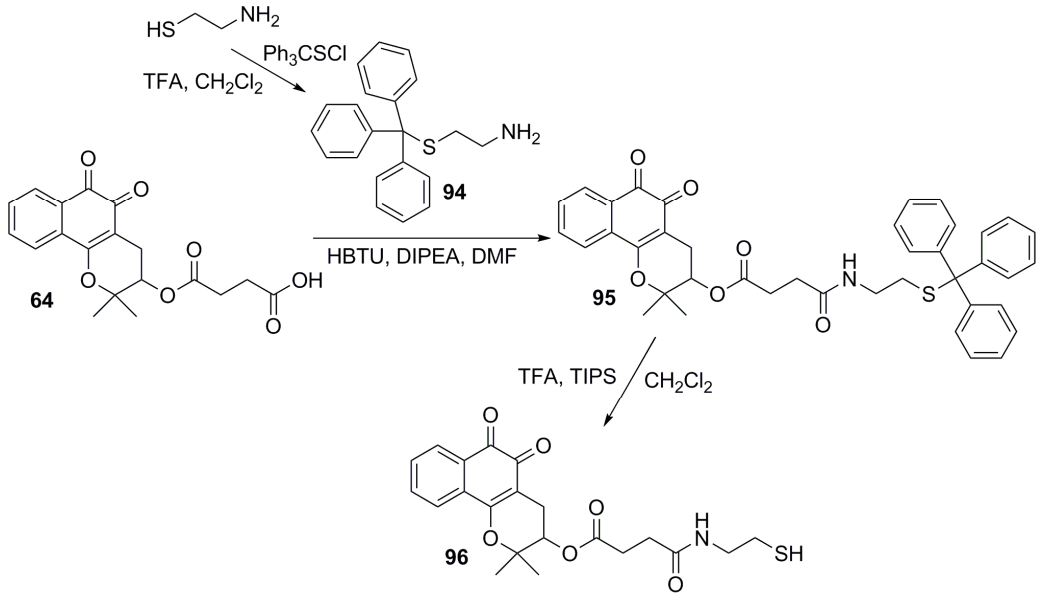


Figure 37 GSH-mediated payload release

During my period in the U.S.A. I have been involved in the synthesis of a thiol-derivative of the ester prodrug of β-hydroxylapachone (LAP) **64** which was also conjugated to the NT targeting peptide, see Chapter 1.2.2. We imagined the same release of the cytotoxic molecule exploited for this type of conjugates, by both enzymatic and non-enzymatic hydrolysis. In this case the release will occur after thiol-exchange on the Au NPs by GSH.

β-hydroxylapachone (LAP) derivative **64** was functionalized with the previously prepared[1] protected thiol **94** affording the amide **95** which was then deprotected with TFA to obtain the desired thiol **96** (Scheme 38).

This thiol will be used in the future to functionalize AuNPs and subsequently release the covalently linked cytotoxic drug *in vivo* by a combined action of GSH and esterases.



Scheme 38

Non-covalent incorporation of drugs onto carriers can be achieved either by encapsulation of hydrophobic drug molecules into non-polar cavities or by

complexation between functional groups on the surface of NPs and drugs with opposite charges. These non-covalent inclusions or complexes offer a variety of promising advantages over the free drug molecules, such as enhanced water solubility and drug stability, programmed release of drugs from the matrixes, and improved PD and PK behaviors. This approach provides an alternative delivery strategy with the potential for avoiding drug release and prodrug processing issues. Encapsulation methods, however, are not without intrinsic limitations as inefficient drug loading and clearance by the reticuloendothelial system (RES) remain a challenge. Interestingly, it has been shown that smaller structures can evade RES capture and exhibit the ability to accumulate in a broader range of tumors creating a necessity for nano-sized delivery vehicles.

We hypothesized that hydrophobic drugs would partition, as organic solutes do, into the "hydrophobic pockets" inside monolayer of water-soluble AuNPs.

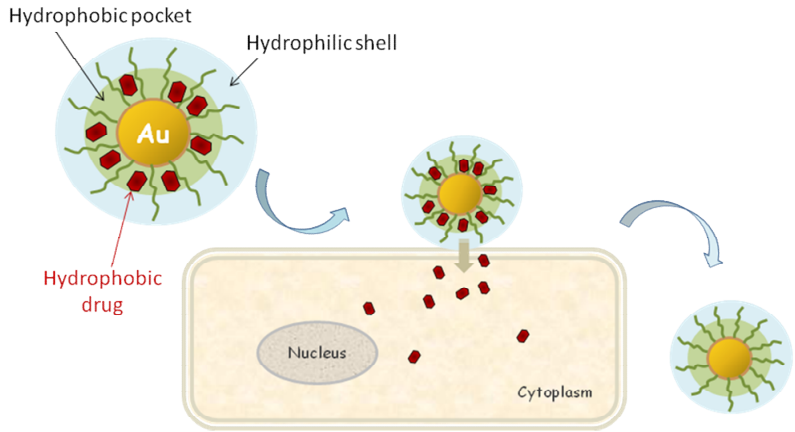


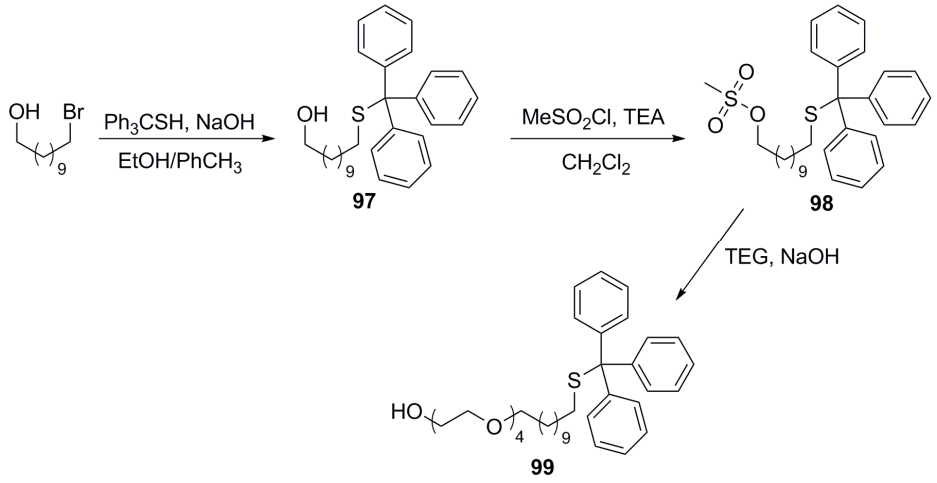
Figure 38

For this purpose we synthesized a variety of thiols, to functionalize AuNPs, featuring two functional domains: a hydrophobic alkanethiol interior and a hydrophilic shell.

Initially we decided to prepare biocompatible AuNPs with a zwitterionic headgroup, which minimize non-specific binding with biomolecules.[92] The principle of stabilizing small nanoparticles of less than 10 nm by zwitterionic ligands has been proved by various studies.[93]

For the synthesis of the zwitterionic thiol ligands we started from 11-bromo-1-undecanol (1 eq.) which was reacted, in the presence of 12 M NaOH, with triphenylmethanethiol (1.2 eq.) in a ethanol/toluene mixture. The thioether **97** was obtained in 99% yield after purification by column chromatography. Methanesulfonyl chloride (1.5 eq.) was then added to a solution of **97** (1 eq.) and TEA (2 eq.) in CH_2Cl_2 to give sulfonyl derivative **98** in 80% yield**.** The tetraethylenglycol (TEG) functionality

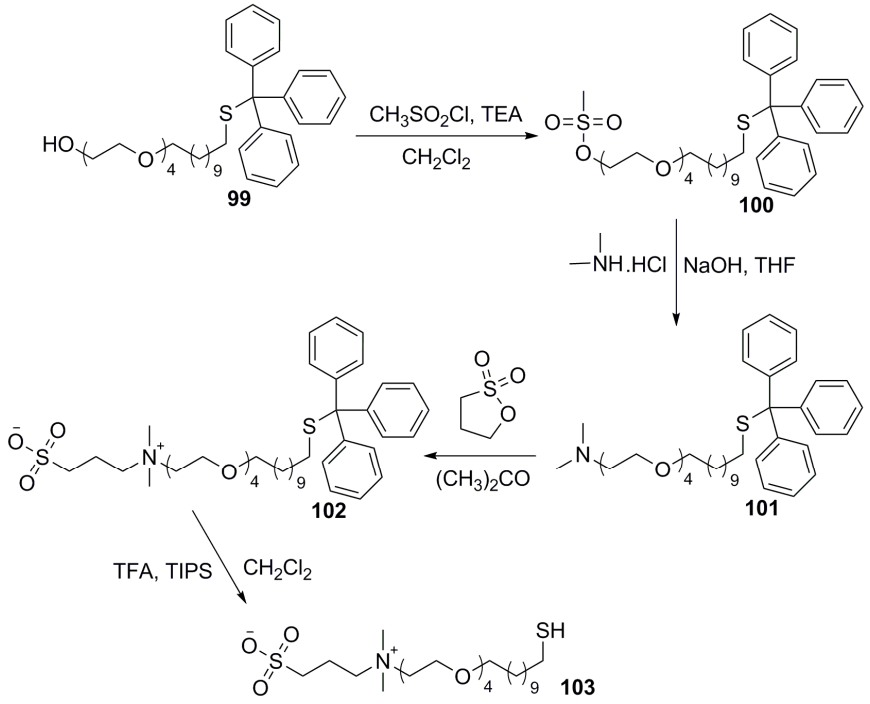
was then introduced heating **98** with TEG-OH in basic conditions, flash chromatography was then necessary to isolate **99** (58%) (Scheme 39).



Scheme 39

TrS-C_{11}-TEG-OH (**99**) was the key intermediate for the synthesis of various functionalized thiols used in this study.

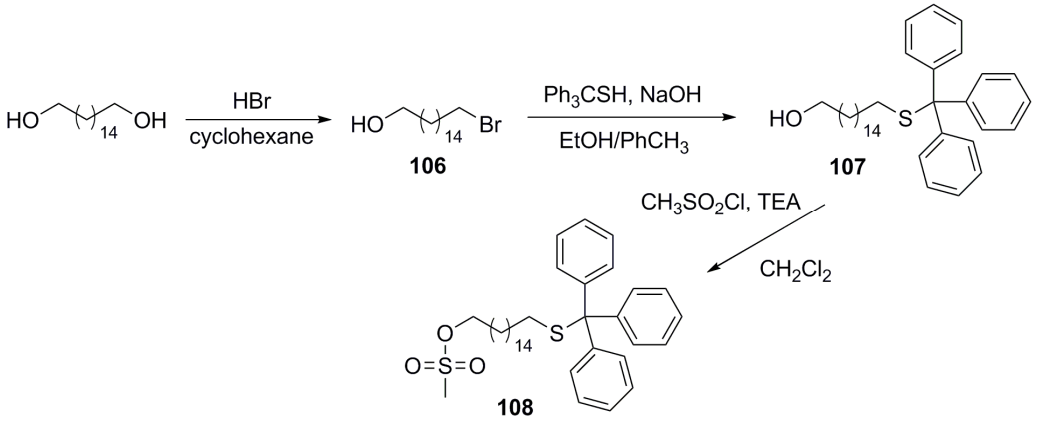
Methanesulfonyl chloride (1.5 eq.) was added to a solution of compound **99** (1 eq.) and TEA (2 eq.) in CH_2Cl_2 to obtain sulfonyl derivative **100** in 89% yield without further purification. The dimethylamine was then bubbled inside a solution of **100** in THF for 5 h, the reaction mixture was stirred overnight, washed with water to give tertiary amine **101** (85%). Finally the zwitteronic functionality was introduced by reaction with 1,3-propanesulton in acetone. Purification by flash chromatography allowed to obtain zwitterion **102** in 85% yield. Deprotection of the trityl group was carried out in CH_2Cl_2 with TFA/triisopropylsilane (TIPS) giving thiol **103** which is ready for the coupling with the gold nanoparticles (Scheme 40).



Scheme 40

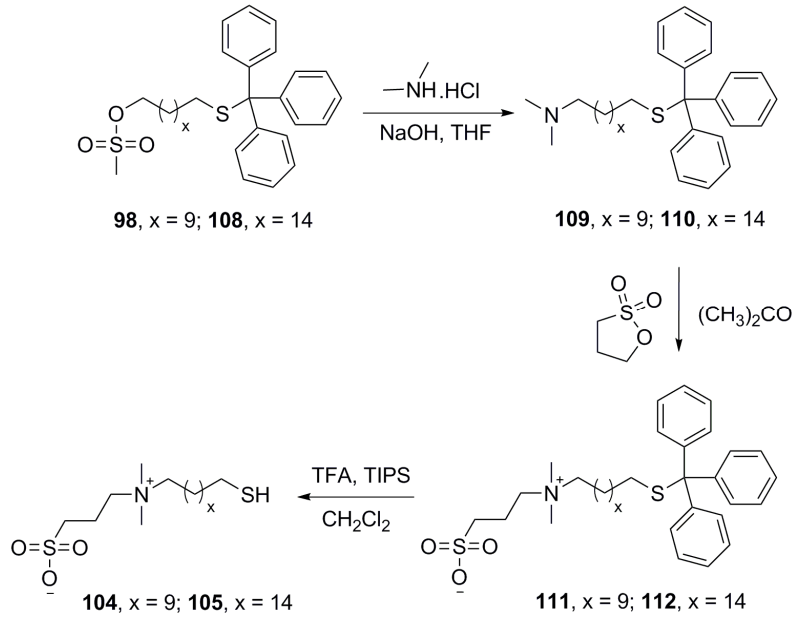
We prepared other zwitterionic thiols with a different hydrophobic/hydrophilic ratio in order to evaluate the changing of encapsulation's ability of the resulting AuNPs. Two ligands without TEG functionality were synthesized either with a C_{11} (**104**) or C_{16} (**105**) alkyl chain.

In the case of **104** we prepared also the starting bromide **106** that is not commercially available. We started from hexadecane-1,16-diol which was reacted with HBr in cyclohexane to give bromide **106** quantitatively. The thio ether functionality was then introduced by reaction with triphenylmethanethiol obtaining **107**, which underwent the next sulfonylation with methanesulfonyl chloride to give the activated derivative **108** (Scheme 41).



Scheme 41

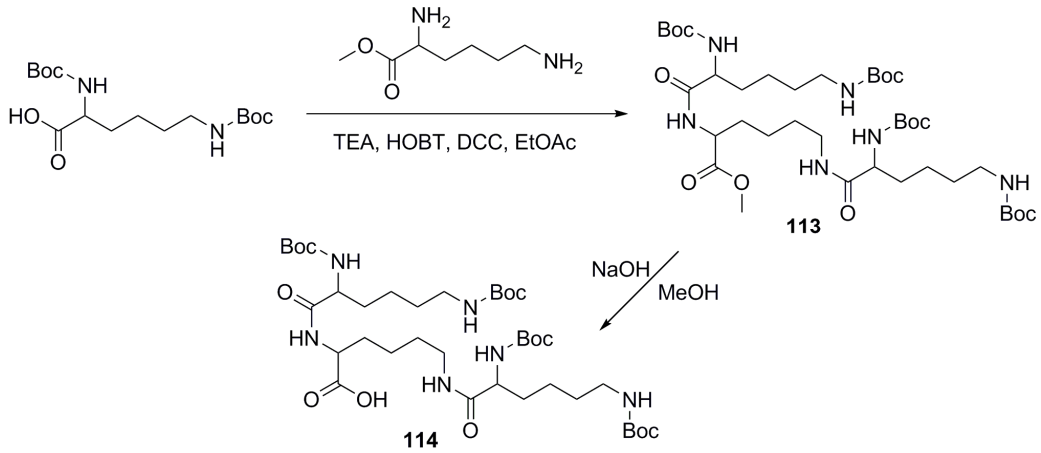
The two sulfonyl derivatives **98** and **108** were reacted with dimethylamine to obtain **109** and **110** which were transformed in the zwitterionic species **111** and **112** using 1,3-propanesultone. Deprotection of the trityl group of **109** and **110** was carried out in CH_2Cl_2 with TFA/triisopropylsilane (TIPS) giving the desired thiols **101** and **102**, ready for the coupling with the gold nanoparticles (Scheme 42).



Scheme 42

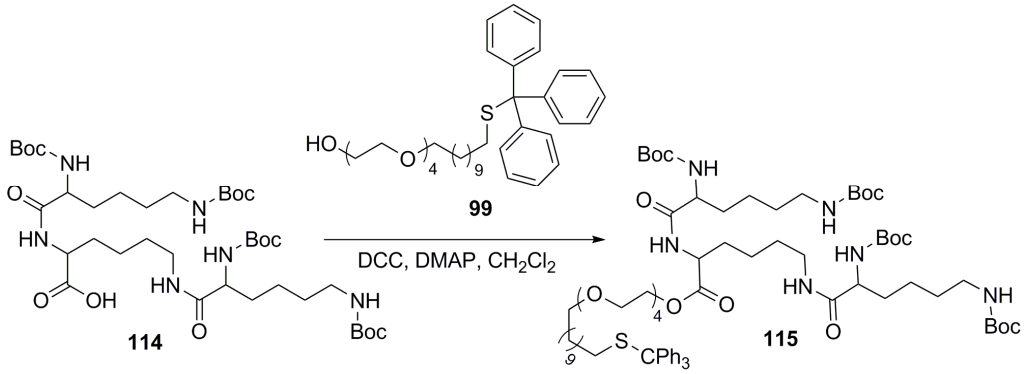
In order to obtain a wide hydrophobic pocket able to encapsulate big molecules we decided to synthesized a thiol with a dendrimeric chain. We chose the same lysine dendrimer adopted for the tetramerization of NT peptides for its versatility and

biocompatibility. In this case the G1-lysine dendrimer was prepared in solution starting from L-lysine methyl ester dihydrochloride (1 eq.) and Boc-Lys(Boc)-OH (2 eq.). These two compounds were suspended in EtOAc in the presence of TEA (2 eq.), then N-hydroxybenzotriazole (HOBT, 2 eq.) and dicyclohexylcarbodiimide (DCC, 2 eq.) were added to activate the carboxylic acid of Lysine. Three lysine molecules were so connected together in the G1-dendrimer **113**, which was then deprotected on the methyl ester in basic conditions (NaOH) to afford acid **114** (Scheme 43).



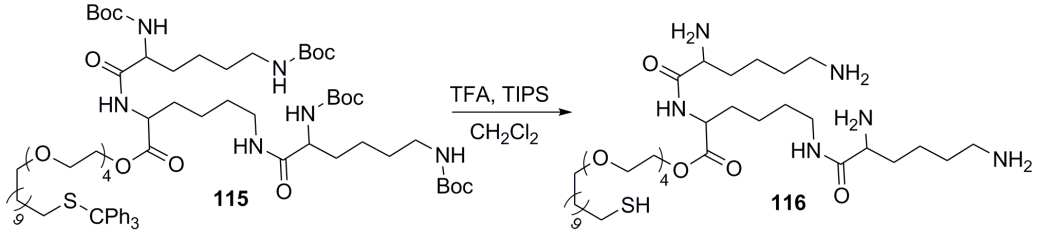
Scheme 43

Esterification of the key intermediate **99** (1 eq.) with the acid **114** (1.4 eq.) previously activated with DCC (2 eq.) and DMAP (0.5 eq.), allowed the isolation of G1-conjugated **115** in 99% yield, after the purification by flash chromatography (Scheme 44).



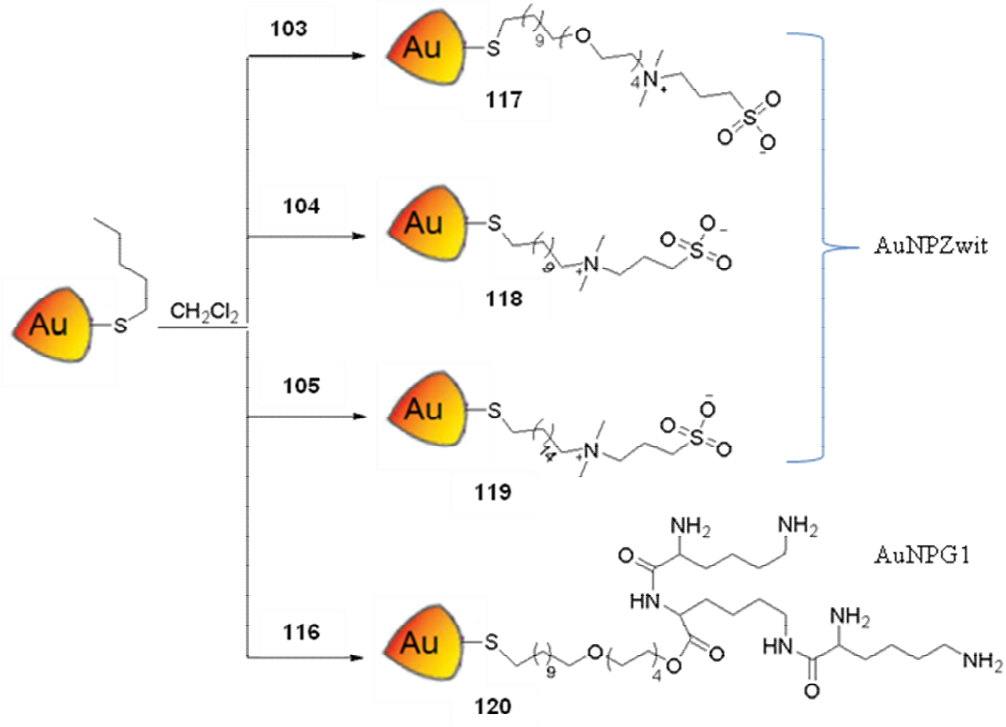
Scheme 44

Eventually, deprotection of derivative **115** on the Boc functionalities and trityl group was carried out directly with TFA/TIPS in CH_2Cl_2, allowing the isolation of thiol **116** in quantitative yield (Scheme 45).



Scheme 45

Once obtained the desired thiols, these were used to conveniently functionalize AuNPs. Gold nanoparticles, C_5NP (~ 2 nm) were prepared by following the Brust-Schiffrin two phase method using 1-pentanethiol as capping ligand. Ligand place exchange reaction was then carried out, mixing 10 mg of C5 NP with 30 mg of thiol (1:3 in weight) in CH_2Cl_2. After washing with diethylether the water-soluble functionalized gold nanoparticles were dialysed in MilliQ water (Scheme 46). The effectiveness of the place exchange was verified by the reversal solubility in water and ^{1}H NMR analysis.



Scheme 46

All the AuNPs synthesised resulted water soluble and stable for long time in solution.
In order to prepare AuNPs complexes with a guest compound, solvent displacement method was carried out using water and acetone. An acetone solution of 10 mg of the guest compound and aqueous solution of AuNP were mixed together. Then, acetone was evaporated slowly. During the evaporation of the solvent, some guest compounds were entrapped inside AuNP due to hydrophobic interactions while the residue precipitated out. The particles were purified by multiple filtrations, through a molecular weight cutoff filter until no absorbance of guest molecules in filtrate was detected by spectrophotometer. The complexes were characterized by ^{1}H NMR using the signal of the broad peak of the guest molecule as indication of the entrapment into the hydrophobic pocket of AuNP. In the case of AuNPZwit the entrapment of the guest occurred only for NPs **117**, but not for **118** and **119**. Therefore the presence of a TEG moiety is probably fundamental for the formation of the complexes.
Moreover AuNPs **117** didn't form any complex with the guest compounds tried, probably the hydrophobic pocket is too wide to retain the guest once entered.
AuNPZwit **117** were the only able to efficiently entrap guest compounds were, therefore other studies were performed in order to test the stability and cytotoxicity of these complexes.
We chose three different hydrophobic guest compounds: 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (Bodipy) as a fluorescent probe, and the highly hydrophobic therapeutics tamoxifen (TAF) and β-lapachone (LAP) as drugs.
The number of entrapped guest compounds per particle was calculated by measuring the integrals from ^{1}H NMR spectrum and NaCN-induced decomposition experiments. The AuNPZwit-dye/drug complexes were stable in buffer for more than 1 month and to dialysis, indicating a level of kinetic entrapment greater than the one observed with dendrimers.[94]
Prof. Rotello's group explored also the ability of the delivery systems to release the payload *in vitro*. Initially a two-phase CH_2Cl_2-water system was adopted and AuNPZwit-Bodipy released the dye into the organic phase probably via a particle-interface process, since no AuNPZwit particle was observed in CH_2Cl_2. Afterwards the delivery of the dye/drug to cells using human breast cancer cells (MC-7) was determined by laser scanning microscopy (CLSM), transmission electron microscopy TEM and inductively coupled plasma mass spectrometry (ICP-MS) (data not shown[93]).
Little or no cellular uptake of AuNPZwit was noticed while efficient delivery of the dye to the cytosol was observed and an efficient cytotoxicity of the encapsulated drugs was

demonstrated (Alamar blue assay). Notably AuNPZwit **115** itself was non-toxic but once complexed with TAF and LAP acquired a comparable toxicity to the free drugs making these systems suitable for targeting strategies.

Gene delivery

Gene therapy provides an ideal therapeutic for treatment of a variety of diseases, like cancer and genetic disorders of both innate and acquired origin.[95] Although viral vectors are very effective, they have raised many safety concerns such as unpredictable cytotoxicity and immune responses.[96] Synthetic vectors, which include polymers, lipids, nanomaterials and combinations thereof,[97] although highly effective *in vitro* have a low efficiency *in vivo* also due to their cell toxicity and immunogenicity. The effective delivery vehicles should provide efficient protection of nucleic acids from degradation, efficient cell entry and release of the nucleic acid in functional form. Gold nanoparticles are particularly attractive scaffolds for gene delivery because they provide an high surface-to-volume ratio and a controlled surface functionality, which allow tuning of the charge and hydrophobicity.

Prof. Rotello's group showed in earlier studies that quaternary ammonium-functionalized cationic GNPs bind plasmid DNA through electrostatic interactions.[98-99] The releasing of the bound DNA from these systems is regulated by manipulation of intracellular GSH levels (like in Figure 37). These studies demonstrated that DNA delivery efficiency and therefore their ability to condense DNA, strongly depends on the structure of the head-groups. Amino-acid functionalized gold nanoparticles provide a versatile scaffold for recognition and delivery of DNA and in particular the GNP-LysG1 (Lysine Dendron) **121** was the most potent vector (Figure 39).[99]

In order to increase the transfection efficiency of these vectors we modified the thiol ligand used to functionalize the GNPs. In particular we introduced a TEG functionality into the alkyl chain trying to provide a better permeation.

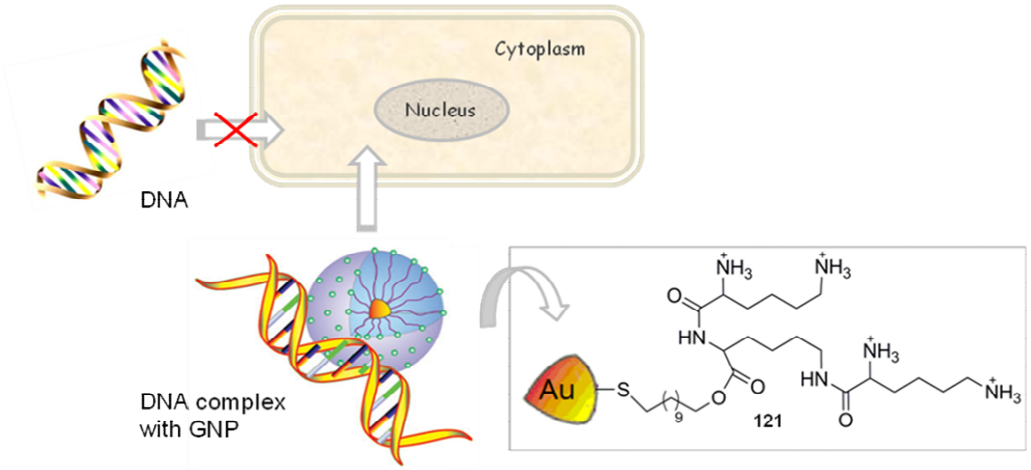
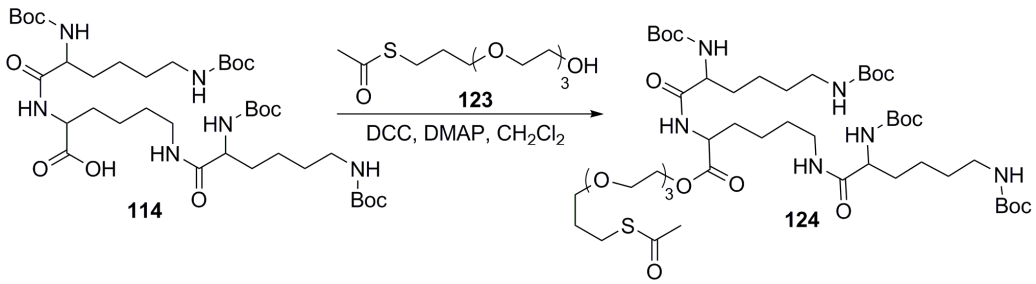


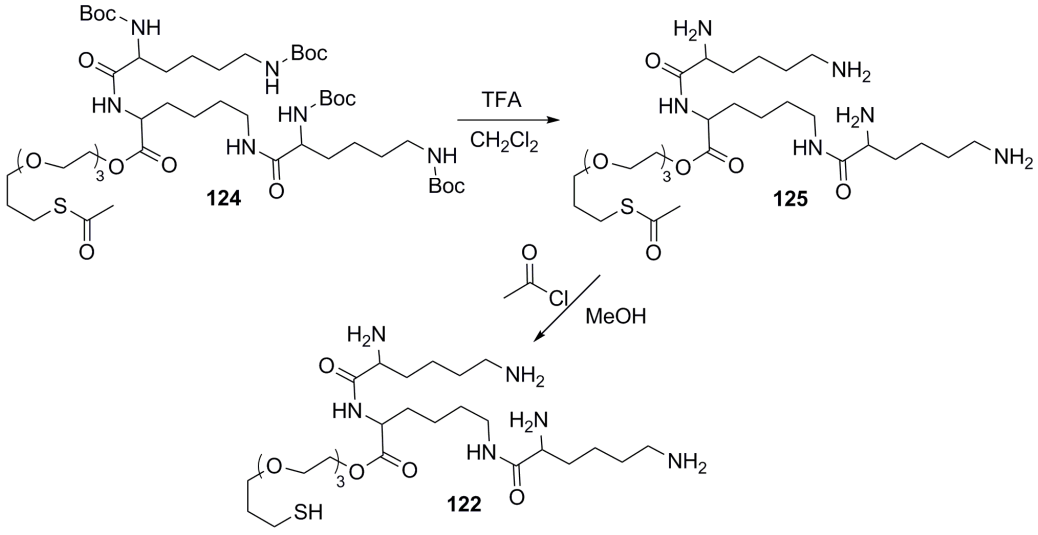
Figure 39

Synthesis of derivative **122** started from the G1-Lysine dendron **114** and protected thiol **123**. The activation of the carboxylic group with DCC allowed the conjugation with the alcohol **123** and formation of the desired ester **124** in quantitative yield (Scheme 47).



Scheme 47

The deprotection was performed in two steps. Initially Boc protecting groups were cleaved by the addition of TFA to a solution of **124** in CH_2Cl_2 to give free amine **125**. Then acetyl chloride was added to remove thioacetyl group, allowing the isolation of thiol **122** in 86% yield without further purification (Scheme 48).



Scheme 48

Once obtained the desired thiol, ligand place exchange reaction was carried out, mixing 10 mg of C5 NP with 30 mg of **122** (1:3 in weight) in CH_2Cl_2. After washing with diethylether the water-soluble functionalized gold nanoparticles were dialysed in MilliQ water (Scheme 46). The effectiveness of the place exchange was verified by the reversal solubility in water and ^{1}H NMR analyses.

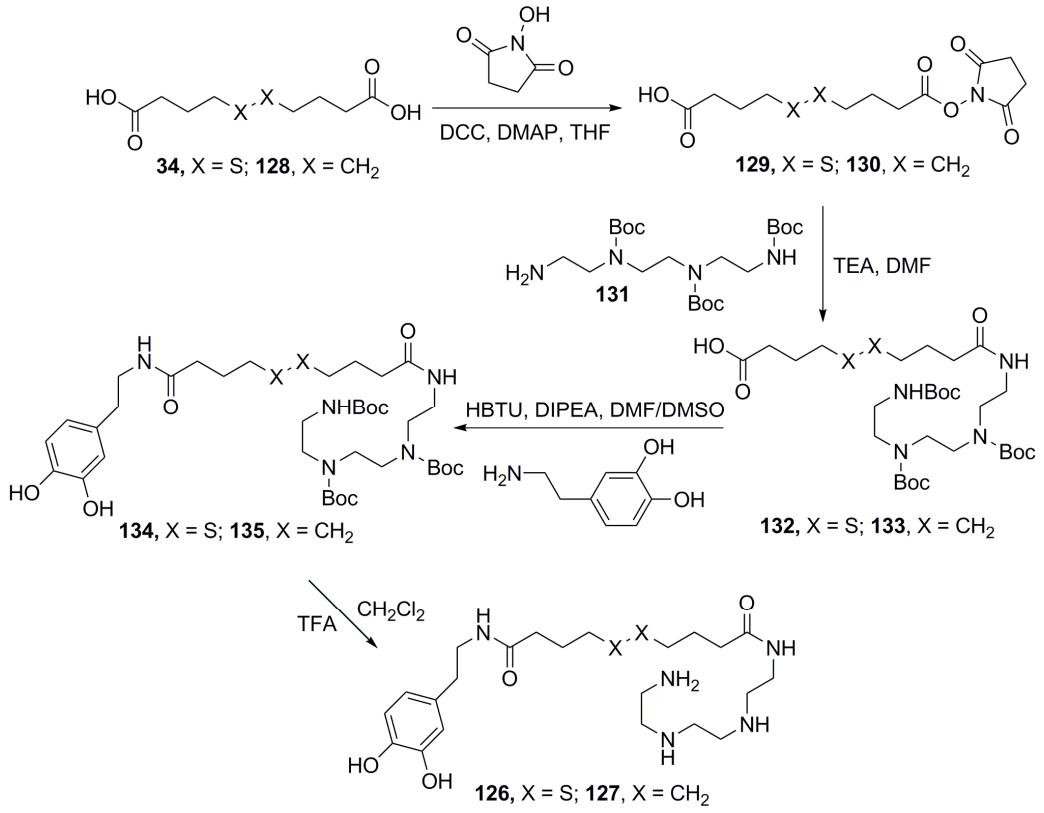
FePt nanooparticles have never been exploited as gene delivery agents.

Their easy functionalization with different ligands on iron and platinum allows a better tuning of the charge and hydrophobicity, making them interesting tools in DNA delivery applications. Since PEI (polyethylenimines) has shown high transfection efficiency both *in vitro* and *in vivo* and attracted great attention, we designed the synthesis of PEI functionalized FePt NPs.

FePt NPs (~ 4 nm) were prepared by following the Shouheng Sun et al. method using oleic acid and oleyl amine as capping ligands respectively for Fe and Pt.[100]

The functionalization of Fe can be achieved using either carboxylic acid or dopamine groups while Pt can attach thiols or amines. We chose to prepare two dopamine derivatives: **126**, carrying a disulfide linkage, which should be able to release the DNA by thiol-disulfide exchange with endogenous thiols like GSH, and **127**, with a C_8 alkyl chain. The synthetic procedure started in both cases from a commercially available dicarboxylic acids **34** or **128**. One acid group was activated with N-hydroxysuccinimide to give **129** and **130**. The reaction with Boc-protected oligoethylamine **131**, previously synthesized as reported in the experimental part,[101] in basic conditions afforded the

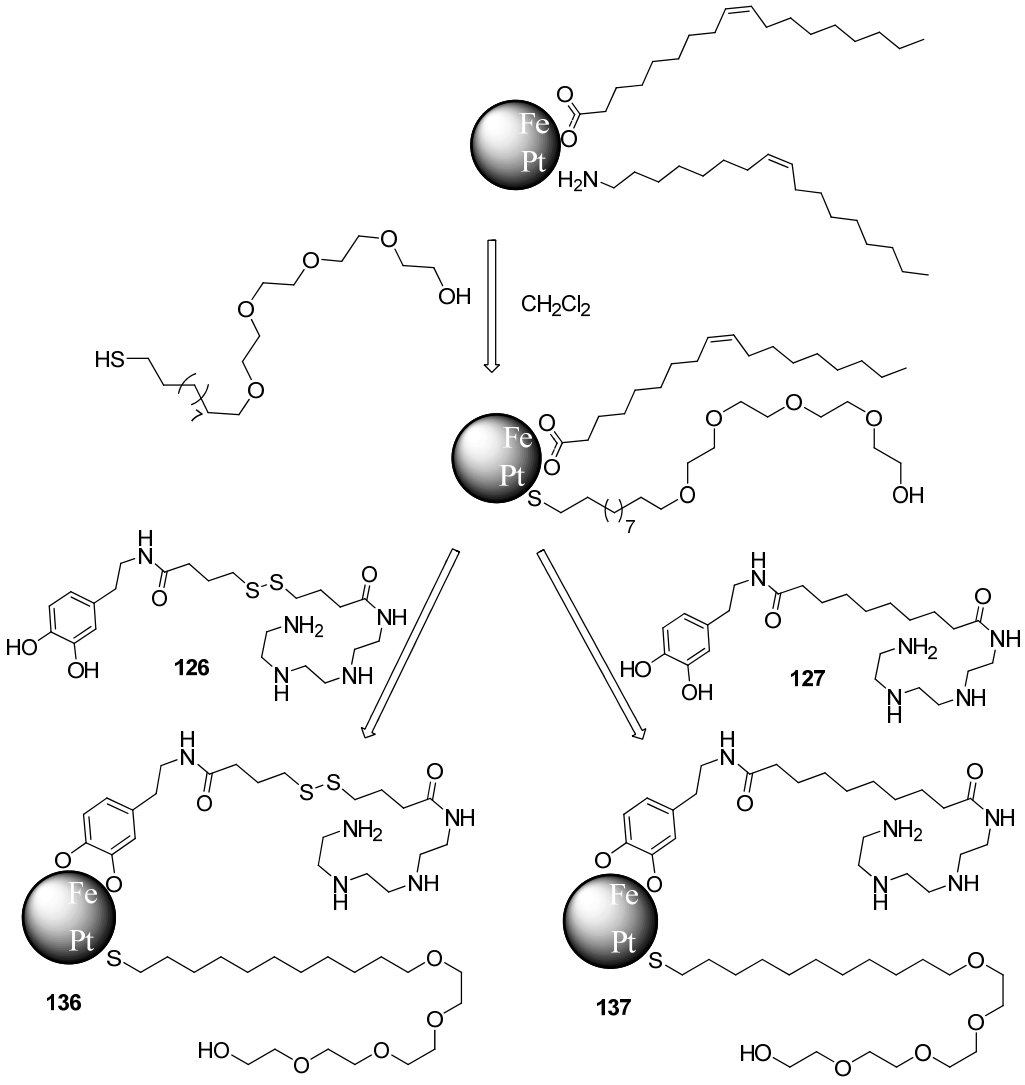
corresponding amides **132** and **133** in quantitative yields without further purification. The conjugation with dopamine of both the free acids and the deprotection of the Boc protecting groups with TFA allowed the isolation of the PEI-Dopamine derivatives **126** and **127** (Scheme 49).



Scheme 49

In a typical experiment the FePt functionalize NPs were prepared by mixing 5 mg of oleic acid and oleylamine stabilized FePt NPs with 10 mg of deprotected derivative of **99** in CH_2Cl_2. The mixture was stirred overnight then to the resulting SH-C_{11}-TEG-OH functionalized FePt NPs, 7.5 mg of DOP-PEI (**126** or **127**) were added and the reaction mixture stirred for 2 days at 35 °C. The functionalized NPs **136** and **137** were then purified by multiple filtrations through a molecular weight cut-off filter and dissolved in water.

The introduction of SH-C_{11}-TEG-OH provided a better water solubility (Scheme 50).



Scheme 50

The transfection efficiency of mammalian cells by these FePtNPs is now under investigation.

General

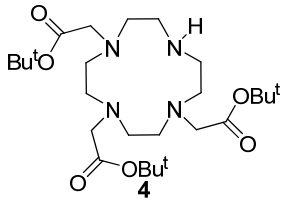
All the reactions were monitored by TLC on commercially available precoated plates (silica gel 60 F 254) and the products were visualized with acidic vanillin solution. Silica gel 60, 230–400 mesh, was used for column chromatography, unless otherwise stated. EtP refers to light petroleum, bp 40–60 °C and EtOAc to ethylacetate.. ^{1}H and ^{13}C NMR spectra were recorded at 400, 200 and 100, 50 MHz, respectively. Melting points were measured on a microscopic apparatus and are uncorrected. FTIR spectra were recorded in KBr pellets or in $CDCl_3$ solutions. Mass spectra were measured with a Shimadzu QP5050 or by FAB (*m*-nitrobenzyl alcohol as matrix) or by ESI using JEOL MStation JMS700.

THF was distilled from sodium in the presence of the blue colour of benzophenone kethyl, toluene was distilled from sodium, CH_2Cl_2 was distilled from CaH_2 and MeOH from Mg.

Commercial available reagents, catalysts and ligands were used as obtained, unless otherwise stated, from freshly opened container without further purifications.

Synthesis of 2-(4,7,10-tris(2-tert-butoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)acetic acid (1)

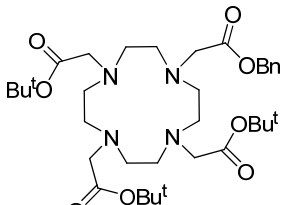
*1,4,7,10-tetraazacyclododecane-1,4,7-tris(tert-butylacetate) (**4**)*



4

To a solution of tetraazacyclododecane (1.50 g, 8.72 mmol) and Na(CH_3COO) (2.14 g, 26.13 mmol) in dimethylacetamide (31 mL), *tert*-butylbromoacetate (5.10 g, 26.13 mmol) was added. The white suspension was stirred for 20 days, then the precipitate was filtered and dried to give **4** as a white solid in 55% yield. ^{1}H NMR, 200 MHz , $CDCl_3$, δ: 1.44 (s, 9H, tBu); 1.45 (s, 18H, tBu); 2.82-2.96 (m, 12H, CH_2); 3.03-3.12 (m, 4H, CH_2); 3.28 (s, 2H, CH_2); 3.36 (s, 4H, CH_2); 10.00 (s, 1H, NH). ^{13}C NMR, 50 MHz, $CDCl_3$, δ: 28.1 (6C, CH_3); 28.2 (3C, CH_3); 47.5, 49.2, 51.3, 58.2 (2C, CH_2); 81.6 (1C, N-CH_2); 81.8 (2C, N-CH_2); 169.6 (1C, CO); 170.5 (2C, CO).

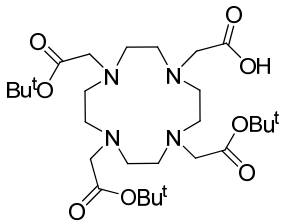
*N-(benzylcarboxymethyl)-1,4,7,10-tetraazacyclododecane-N',N'',N'''-triacetic acid tri-tert-butyl ester (**5**)*



5

Compound **4** (2.38 g, 7.44 mmol) was added to a suspension of 60% NaH in DMF, after 2 hours stirring, benzylbromoacetate (2 g, 8.7 mmol) was added dropwise below 0 °C. The reaction mixture was stirred for 27 h at room temperature (r.t.), then a solution of 5% citric acid was added and the mixture extracted with chloroform. The product obtained was purified by flash chromatography with 8/1= CH_2Cl_2/MeOH as eluent to give **5** in 67% yield. ^{1}H NMR, 200 MHz , DMSO-*d6*, δ: 1.40 (bs, 18H); 1.44 (s, 9H); 2.43-1.94 (bs, 6H); 3.29-2.71 (bs, 7H); 3.31 (s, 2H); 3.46–3.39 (m, 2H); 5.13 (s, 2H); 7.35 (s, 5H). ^{13}C NMR (DMSO-d6) (d, ppm): 31.2; 50.5; 52.3; 52.6; 53.8; 57.1; 58.3; 75.3; 85.7; 85.7; 128.7; 129.8; 144.7; 172.9; 174.3.

2-(4,7,10-tris(2-tert-butoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)acetic acid ***(1)***

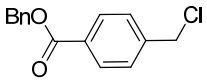


1

A mixture of **5** (0.83 g, 1.25 mmol) and 10% Pd/C (0.11 g) was hydrogenated in methanol under a positive H_2 atmosphere for 6 h. The catalyst was removed by filtration, and the filtrate was evaporated to yield the modified DOTA macrocycle **1** in 50% yield without further purification. ^{1}H NMR, 200 MHz , $CDCl_3$, δ: 1.40 (s, 9H); 1.41 (s, 18H); 2.64 (bs, 4H); 2.72 (bs, 4H); 2.87 (bs, 4H); 3.33 (bs, 4H); 3.36 (s, 6H); 3.46 (m, 2H); ^{13}C NMR, 200 MHz, DMSO, δ: 36.83, 36.88, 56.09; 58.59; 60.78; 61.88; 64.61; 65.63; 89.53; 179.30. MALDI-MS (m/z) for $[M+H]^+$: 572.6. ESI-MS (m/z) for $[M+H]^+$: 573.4.

Synthesis of 4-((4,7,10-tris(2-tert-butoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)methyl)benzoic acid (2)

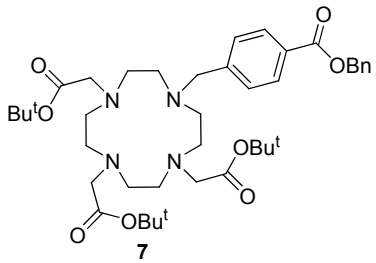
*Benzyl 4-(chloromethyl)benzoate (****6****)*



6

4-chloromethylbenzoic acid (500 mg, 2.93 mmol) was suspended in 3.8 mL of benzylic alcohol and three drops of concentrated sulfuric acid. The reaction mixture was stirred at 64°C for 3h, then diluted with CH_2Cl_2 and a saturated solution of $NaHCO_3$ was added until neutral pH. The two phases where separated and the organic layer was washed with water (3x50mL), dried over Na_2SO_4, filtered and the solvent removed under vacuum. The oil yielded was purified by flash chromatography with 10/1=EtP/EtOAc as eluent to give benzyl derivative **6** in 34% yield. ^{1}H NMR, 200 MHz , $CDCl_3$, δ: 4.61 (s, 2H, CH_2); 5.37 (s, 2H, CH_2); 7.36-7.48 (m, 7H, H_{arom}); 8.07 (d, 2H, J = 8.4 Hz, H_{arom}).

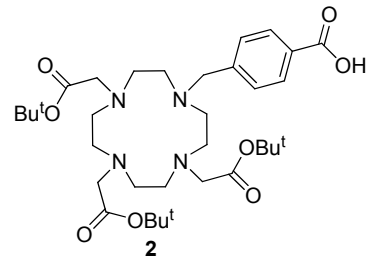
*Tert-butyl-2,2',2''-(10-(4-(benzyloxycarbonyl)benzyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (**7**)*



7

1,4,7,10-tetraazacyclododecane-1,4,7-tris(*tert*-butylacetate) **4** (100 mg, 0.19 mmol) and K_2CO_3 (68 mg, 0.49 mmol) were suspended in 4 mL of CH_3CN and the mixture was stirred at 60°C for 15 min. At this temperature the benzyl ester of 4-chloromethylbenzoic acid **6** (55 mg, 0.21 mmol) was added dropwise over 1 h. After 48 h, the yellow suspension obtained was filtered through celite and concentrated to obtain an oil which was used in the subsequent reaction without further purification, **7**. ^{1}H NMR, 200 MHz, C_6D_6, δ: 1.30 (s, 18H, *t*Bu); 1.34 (s, 9H, *t*Bu); 2.46-2.50 (m, 4H, CH_2); 2.76-2.83 (m, 10H, CH_2); 3.14 (s, 4H, CH_2); 3.28 (s, 2H, CH_2); 3.30 (s, 2H, CH_2); 5.16 (s, 2H, CH_2); 7.03-7.18 (m, 5H, H_{arom}); 7.36 (d, 2H, J = 8.0 Hz, H_{arom}); 8.15 (d, 2H, J = 8.0 Hz, H_{arom}).

*4-((4,7,10-tris(2-tert-butoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)methyl)benzoic acid (**2**)*



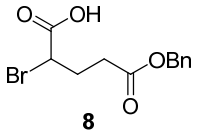
2

7 (67 mg, 0.09 mmol) was dissolved in 1.9 mL of absolute ethanol, added with 10% Pd/C (26 mg) and hydrogenated at 50 psi of H_2 for 60 h. The catalyst was removed by filtration, the filtrate evaporated, and the crude purified by flash chromatography with 8/1→5/1=CH_2Cl_2/MeOH as eluent to obtain benzyl derivative **2** in 50% yield (from **4** to **2**). ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.42 (s, 9H, *t*Bu); 1.45 (s, 18H, *t*Bu); 2.00-3.22 (m, 12H, CH_2); 3.22-3.40 (m, 4H, CH_2); 3.47(s, 2H, CH_2); 4.43 (s, 1H, OH); 7.45 (d, 2H, J = 7.8 Hz, H_{arom}); 8.05 (d, 2H, J = 7.8 Hz, H_{arom}). ^{13}C NMR, 50 MHz, $CDCl_3$, δ: 27.8, 27.9 (3C, CH_3); 28.1 (6C, CH_3); 49.2, 49.7, 50.5 (2C, CH_2); 51.2 (4C, CH_2); 51.9, 55.9,

59.3 (2C, CH_2); 81.8, 82.1, 82.4, 83.1 (1C, N-CH_2); 88.8 (3C, $C(CH_3)_3$); 129.5 (2C, CH_{arom}); 130.2 (3C, CH_{arom} and C_{arom}); 140.9 (1C, C_{arom}); 169.3, 169.9, 172.2, 173.2 (1C, CO). IR: 3426, 2971, 2930, 2837, 1731, 1715, 1369, 1230, 1163 cm^{-1}. MS: (MALDI-ToF) $M^{+\cdot}$= 673.105 (100%).

Synthesis of 5-*tert*-butoxy-5-oxo-4-(4,7,10-tris(2-tert-butoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)pentanoic acid (3)

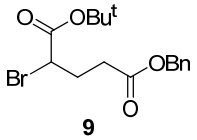
*5-(benzyloxy)-2-bromo-5-oxopentanoic acid (**8**)*



8

To a suspension of L-glutamic acid-5-benzylester (1.50 g, 6.48 mmol) and sodium bromide (2.27 g, 22.08 mmol) in a 1N solution of bromidric acid, cooled at 0°C, sodium nitrite (0.79 g, 11.45 mmol) was added portionwise. The yellow solution was stirred at this temperature for 2 h and finally 560 µL of 98% sulfuric acid was added, followed by diethylether. The water phase was extracted several times with Et_2O and the combined organic layers washed with brine. The crude product was purified by flash chromatography with 1/1=EtOAc/EtP, to obtain bromo-derivative **8** in 82% yield. ^{1}H NMR, 200 MHz, $(CD_3)_2SO$, δ: 2.00-2.40 (m, 4H, CH_2); 4.38-4.50 (m, 1H, CH-Br); 5.08 (s, 2H, CH_2); 5.74 (s, 1H, OH); 7.35 (m, 5H, H_{arom}). ^{13}C NMR, 50 MHz, $CDCl_3$, δ: 29.3 (1C, CH_2); 31.4 (1C, CH_2); 44.3 (1C, C-Br); 66.6 (1C, CH_2); 128.0 (1C, C_{arom}); 128.2, 128.4 (2C, CH_{arom}); 135.3 (1C, C_{arom}); 172.0, 173.3 (1C, CO).

*5-benzyl 1-tert-butyl 2-bromopentanedioate (**9**)*

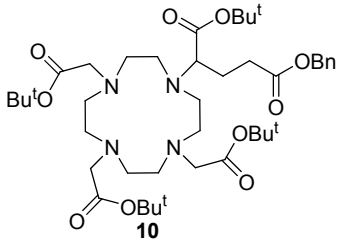


9

A solution of *tert*-butyltrichloroacetimidate (TBTA) (2.20 mL, 12.05 mmol) in 8 mL of cyclohexane was added dropwise to another solution of **8** (1.70 g, 5.63 mmol) in 7 mL of $CHCl_3$. During the addition a white precipitate formed, which was dissolved by the addition of DMA followed by boron trifluoridiethyl etherate (120 µL, 0.56 mmol) as a catalyst. The reaction mixture was stirred for three days at r.t. then concentrated and the remaining DMA phase was extracted with hexane (3x10mL). The hexane phase was evaporated and the crude product product was purified by flash chromatography with 20/1=EtOAc/EtP followed by 10/1, to give Bu^t ester **9** in 50% yield. ^{1}H NMR, 200

MHz, $(CD_3)_2SO$), δ: 1.47 (s, 9H, CH_3); 2.20-2.50 (m, 2H, CH_2); 2.52-2.60 (m, 2H, CH_2); 4.24 (dd, 1H, J_1 = 6.0 Hz, J_2 = 8.2 Hz, CH-Br); 5.13 (s, 2H, CH_2); 7.36 (m, 5H, H_{arom}).

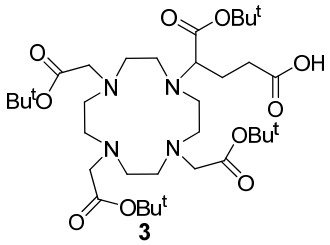
*5-benzyl 1-tert-butyl 2-(4,7,10-tris(2-tert-butoxy-2-oxoethyl)-1,4,7,10-tetraaza cyclododecan-1-yl)pentanedioate (**10**)*



10

To a solution of **4** (918 mg, 1.79 mmol) in 31 mL of dry CH_3CN, K_2CO_3 (740 mg, 5.36 mmol) was added. The reaction mixture was stirred at 60 °C for 10 min before adding dropwise, over a period of 40 min, a solution of **9** (1.14 g, 3.58 mmol) in 15 mL of dry CH_3CN. After 42 h under stirring at 60° C the yellow solution was filtered throw celite and concentrated. The residue was chromatographed with 20/1=CH_2Cl_2/MeOH followed by 8/1 to obtain **10** in 59% yield. Melting point: 145°C. ^{1}H NMR, 200 MHz, $CDCl_3$, δ: 1.45 (s, 36H, CH_3); 1.90-3.60 (m, 27H); 5.07 (s, 2H, CH_2); 7.33 (m, 5H, H_{arom}).

*5-tert-butoxy-5-oxo-4-(4,7,10-tris(2-tert-butoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)pentanoic acid (**3**)*

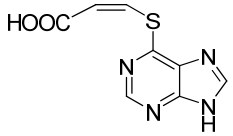


3

10 (100 mg, 0.127 mmol) was dissolved in 2.8 mL of absolute ethanol and to this solution 37 mg of 10% Pd (C) were added. The reaction mixture was hydrogenated for two days, filtered over cotton wool pad and the filtrate concentrated to give **3** with 79% yield without further purification. Melting Point = 180-182°C. ^{1}H NMR, 400 MHz, CD_3OD, δ: 1.49 (s, 9H, CH_3); 1.50 (s, 18H, CH_3); 1.53 (s, 9H, CH_3); 1.98-2.30 (m, 8H); 2.46-2.88 (m, 11H); 2.90-3.20 (m, 4H); 3.42-3.6 (m, 4H). ^{13}C NMR, 100 MHz, CD_3OD, δ: 28.3 (6C, CH_3); 28.1 (6C, CH_3); 33.3 (1C, CH_2); 45.2, 53.9, 54.0, 56.6, 56.9

(12C, CH_2); 61.3 (1C, CH); 82.9 (2C, $C(CH_3)_3$), 83.0, 83.6 (1C, $C(CH_3)_3$); 174.6, 174.7, 176.5, 176.7 (1C, CO). MS IR: 3422, 2974, 2929, 2840, 1726, 1362, 1223, 1166 cm^{-1}. : ESI(MS) $M^{+\cdot}$=701, 723 (100%); CI, 56(100%), 157, 600.

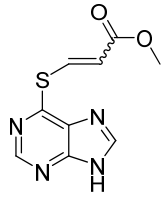
Synthesis of 3-(9*H*-purin-6-ylthio)acrylic acid, PTA (14)



14

To a suspension of 6-mercaptopurine (**11**) (1.00 g, 5.88 mmol) in 35 mL of anhydrous methanol, a freshly prepared 1.17 M solution of MeONa in MeOH (5.03 mL) was added with continuous stirring until complete solution formed. Propiolic acid (0.36 mL, 5.88 mmol) was dissolved in a 5.00 mL portion of the 1.17 M solution of MeONa in MeOH, and added to the solution of 6-MP. The reaction mixture was refluxed overnight under a nitrogen atmosphere. Addition of 3% HCl to the mixture, until pH 2, caused the formation of a precipitate which was recollected by filtration, washed with water and dried. NMR control of the crude product reveal apart from the desired product, the presence of some residual **11**. A saturated solution of $NaHCO_3$ was added to the residue under stirring to give a suspension. After filtration, HCl 1N was added to the solution causing the precipitation of pure PTA **14** which was isolated by filtration as single *Z* isomer in 43% yield. Mp 235°C, dec. ^{1}H NMR, 400 MHz, DMSO-*d6*, δ: 6.26 (d, 1H, *J*=10.0 Hz, C=CH-S); 8.53 (s, 1H, CH_{arom}); 8.74 (d, 1H, *J*=10.0Hz, C=CH-CO); 13.69 (s, 1H, NH); 8.79 (s, 1H, CH_{arom}). ^{13}C NMR, 100 MHz, DMSO-*d6*, δ: 117.2 (1C, C=C), 137.5 (1C, C_{arom});, 144.7 (1C, CH_{arom}); 151.8 (1C, C=C); 156.3 (1C, C_{arom}), 161.9 (1C, CH_{arom}); 167.8 (1C, CO). IR, KBr: 1569; 1597; 1681; 3462 cm^{-1}.

*Methyl 3-(9H-purin-6-ylthio)acrylate (**17**)*

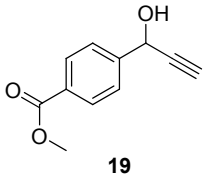


17

To a suspension of 6-MP (**11**) (1.00 g, 5.88 mmol) in 45 mL of dry MeOH, 3.5 mL of a 1.67 M solution of MeONa (5.88 mmol) were added under stirring, until a complete dissolution of **11**. 1.8 mL of ethylpropiolate (17.6 mmol) were then added and the reaction mixture was refluxed for 15 h, diluted with 50 mL of a saturated solution of NH_4Cl and extracted with EtOAc. The combined organic layers were washed with water, dried over Na_2SO_4, filtered and evaporated to give adduct **17** in 14% yield as a mixture 77/23 of isomers *cis* and *trans*. ^{1}H NMR, 200 MHz, DMSO-*d6*, δ: 3.75 (s, 6H, O-$CH_{3(cis+trans)}$); 6.39 (d, 1H, *J*= 10.2 Hz, S-CH_{cis}); 6.54 (d, 1H, *J* = 16.0 Hz, S-CH_{trans}); 8.59 (s, 2H, $CH_{arom(cis+trans)}$); 8.84 (s, 2H, $CH_{arom(cis+trans)}$); 8.86 (d, 1H, *J* = 8.8 Hz, CH_{cis}-CO); 8.89 (d, 1H, *J* =14.6 Hz, CH_{trans}-CO); IR (KBr), cm^{-1}: 3366 (stretch. N-H), 1701 (stretch. C=O), 1567 (bend. N-H).

Synthesis of methyl 4-propioloylbenzoate (20)

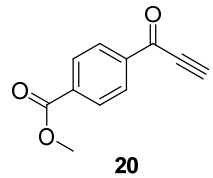
*Methyl 4-(1-hydroxyprop-2-ynyl)benzoate (**19**)*



19

To a solution of methyl-4-formylbenzoate (1.00 g, 6.10 mmol) in 25mL of dry THF, a 0.5 M solution of ethynyl magnesium bromide in THF (12.2 mL, 6.10 mmol) at -78°C in 20 min. After the addition the reaction was stirred at -78°C for 50min, at r.t. for 2 h, and finally neutralized with a saturated solution of NH_4Cl, extracted with Et_2O (3x40mL) and dried over Na_2SO_4. The crude product was purified by flash chromatography with 3/1=EtP/EtOAc as eluent, to give **19** in 69% yield. ^{1}H NMR, 200 MHz, $CDCl_3$, δ: 2.70 (d, 1H, *J* = 2.2 Hz, C≡C-H); 3.92 (3H, CH_3);. 5.53 (dd, 1H, *J* = 2.2 e 6.2 Hz, CH-OH); 7.63 (d, 2H, *J* =8.8 Hz, H_{arom}); 8.06 (d, 2H, *J* = 8.8 Hz, H_{arom}).

*Methyl 4-propioloylbenzoate (**20**)*

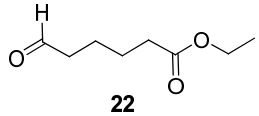


20

To a solution of derivative **19** (0.80 g, 4.21 mmol) in 15 mL of CH_2Cl_2 at 0°C, TEMPO (0.01 g, 0.84mmol) followed by trichloroisocianuric acid (TCICA) (1.07 g, 4.63 mmol) were added. The resulting suspension was maintained at 0°C for 5 min and at r.t. for 40 min. The reaction mixture was diluted with CH_2Cl_2 and then filtered through celite. The filtrate was washed with a saturated solution of Na_2CO_3 (2x25 mL), a 1N solution of HCl (25 mL) and finally with water (25 mL). The solution was dried over Na_2SO_4 and the solvent removed under vacuum to obtain derivative **20** in 97% yield. ^{1}H NMR, 200 MHz, $CDCl_3$, δ: 3.51 (s, 1H, C≡C-H); 3.96 (s, 3H, CH_3); 8.15-8.25 (m, 4H, CH_{arom}). IR ($CHCl_3$), cm^{-1}: 3215 (stretch.C≡C-H), 2095 (stretch. C≡C), 1717 (stretch. O-C=O), 1633 (stretch. C=O).

Synthesis of methyl 8-(9H-purin-6-ylthio)-6-oxooct-7-enoate (25)

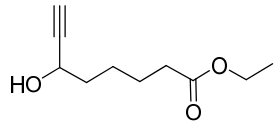
*Ethyl 6-oxohexanoate (**22**)*



22

To a solution of ethyl-6-hydroxy-hexanoate (2.00 mL, 12.30 mmol) in 32 mL of CH_2Cl_2, trichloroisocianuric acid (2.90 g, 12.30 mmol) was added. After 10 min under stirring, TEMPO (0.020 g, 0.12 mmol) was added and the reaction stirred for 3 min. After dilution with CH_2Cl_2, the mixture was filterd through celite and the filtrate washed with a saturated solution of Na_2CO_3 (3x40 mL), 40mL of 1N HCl and finally with water. After drying over Na_2SO_4, filtration and evaporation aldehyde **22** was obtained in 98% yield without further purification. ^{1}H NMR, 200 MHz, $CDCl_3$, δ: 1.37 (t, 3H, *J* = 7.0Hz, CH_2CH_3); 1.42-1.68 (m, 4H); 2.19-2.23 (m, 2H, CH_2-COO); 2.25-2.42 (m, 2H, CH_2-CHO); 4.04 (q, 2H, *J* =7.4 Hz, CH_2CH_3); 9.70 (t, 1H, *J* = 1.6 Hz, CHO). IR ($CDCl_3$), cm^{-1}: 2827, 2724 (stretch. H-C=O), 1720 (stretch. C=O), 1390 (bend. H-C=O).

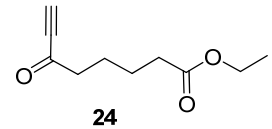
*Ethyl 6-hydroxyoct-7-ynoate (**23**)*



23

To a solution of **22** (1.90 g, 12.00 mmol) in 25 mL of anhydrous THF at -78°C and under nitrogen atmosphere, a 0.5 M solution of etynyl magnesium bromide in THF (24.00 mL, 12.00 mmol). The reaction mixture was stirred at -78°C for 50 min, and at r.t. for 1 h, then was neutralized by the addition of a saturated solution of NH_4Cl and extracted with diethyl ether (3x40 mL). The recollected organic layers were dried over Na_2SO_4, filtered and the solvent removed. The crude product was purified by flash chromatography with 3/1=EtP/EtOAc as eluent, to give alcohol **23** in 43% yield. ^{1}H NMR, 200 MHz, $CDCl_3$, δ: 1.25 (t, 3H, *J* = 7.1, CH_2CH_3); 1.40-1.90 (m, 6H); 2.32 (at, 2H, *J* = 7.4 Hz, CH_2-COO); 2.45 (d, 1H, *J* = 2.2 Hz, C≡C-H); 4.13 (q, 2H, *J* = 7.0 Hz, CH_2CH_3); 4.38 (dt, 1H, *J* = 1.80 Hz, *J* = 6.0 Hz, CH-OH).

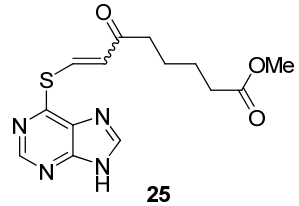
*Ethyl 6-oxooct-7-ynoate (**24**)*



24

To a solution of **23** (0.60 g, 3.30 mmol) in 10 mL of CH_2Cl_2 at 0°C, TEMPO (0.01 g, 0.84 mmol) followed by trichloroisocianuric acid (TCICA) (0.84 g, 3.63 mmol) were added. The resulting suspension was maintained at 0°C for 40 min and at r.t. for 16 h. The reaction mixture was diluted with CH_2Cl_2 and then filtered through celite. The filtrate was washed with a saturated solution of Na_2CO_3 (2x35 mL), a 1N solution of HCl (35 mL) and finally with water (35 mL). The solution was dried over Na_2SO_4 and the solvent removed under vacuum to obtain derivative **24** in 99% yield without further purification. ^{1}H NMR, 200 MHz, $CDCl_3$, δ: 1.25 (t, 3H, *J* = 7.1, CH_2CH_3); 1.55-1.80 (m, 4H); 2.32 (at, 2H, CH_2-C(O)O); 2.62 (at, 2H, CH_2-C=O); 3.13 (s, 1H, C≡C-H); 4.13 (q, 2H, *J* = 7.0 Hz, CH_2CH_3). IR ($CDCl_3$), cm^{-1}: 3297 (stretch.C≡C-H), 2097 (stretc. C≡C), 1727 (stretch. C=O estereo), 1683 (stretch, C=O aldeidico).

*Methyl 8-(9H-purin-6-ylthio)-6-oxooct-7-enoate (**25**)*

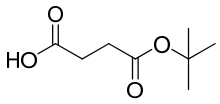


25

To a suspension of 6-mercaptopurine (**11**) (0.22 g, 1.28 mmol) in 10 mL of anhydrous methanol, a freshly prepared 5.1 M solution of MeONa in dry MeOH (0.25 mL, 1.28 mmol) was added with continuous stirring until complete solution formed. **24** (0.70 g, 3.85 mmol) was dissolved in a 2.00 mL of MeOH and added.and added to the solution of 6-MP. The reaction mixture was stirred at r.t. overnight under a nitrogen atmosphere. A saturated solution of NH_4Cl was added to the mixture, which was then extracted with EtOAc, washed with water and dried. The crude product was purified by flash chromatography with 13/1=CHCl3/MeOH to obtain derivative **25** in 47% yield as a mixture 80/20 of *cis* and *trans* isomers. ^{1}H NMR, 200 MHz, DMSO-*d6*, δ: 1.14 (t, 6H, *J* = 7.0 Hz, $CH_2CH_{3(cis+trans)}$); 1.23-1.65 (m, 8H, C-CH_2CH_2-$C_{(cis+trans)}$); 2.05-2.40 (m, 4H, CH_2-$COO_{(cis+trans)}$); 2.42-2.70 (m, 4H, CH_2-$CO_{(cis+trans)}$), 4.01 (q, 4H, *J* = 7.0 Hz, $CH_2CH_{3(cis+trans)}$); 6.75 (d, 1H, *J* = 19.0 Hz, S-CH_{trans}); 6.79 (d, 1H, *J* = 10.0 Hz, S-CH_{cis}); 8.56 (s, 2H, $CH_{arom(cis+trans)}$); 8.70 (d, 1H, *J* = 10.0 Hz, CH_{cis}-CO); 8.80 (s, 2H, $CH_{arom(cis+trans)}$); 8.85 (d, 1H, *J* = 19.0 Hz, CH_{trans}-CO). ^{13}C NMR, 200 MHz, DMSO-*d6*, δ: 14.1, 23.0, 24.0, 33.3, 42.0 (10C, $C_{alif(cis+trans)}$); 59.6 (2C, $OCH_{2(cis+trans)}$); 121.9 (2C, $C=C_{(cis+trans)}$); 144.5 (2C, $C=C_{(cis+trans)}$); 121.9, 135.0, 135.7, 151.2, 151.4 (10 $C_{arom(cis+trans)}$); 172.5 (2C, $COO_{(cis+trans)}$); 196.4 (1C, C=C-$CO_{(trans)}$); 199.2 (1C, C=C-$CO_{(cis)}$). IR (KBr), cm^{-1}: 3462 (stretch. N-H), 1731 (stretch. O-C=O), 1669 (stretch. C=O), 1594 (stretch. C=C), 1569 (bend. N-H). ESI-MS, *m/z*: 357 ($M+Na^+$); 335 ($M+H^+$). ESI-MS, *m/z*: 333 ($M-H^-$)

Synthesis of 6-(9*H*-purin-6-ylthio)-4-oxohex-5-enoic acid (27)

*4-Tert-butoxy-4-oxobutanoic acid (**28**)*

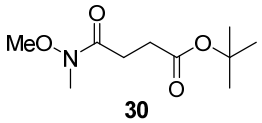


28

A suspension of succinic anhydride (5.00 g, 50.00 mmol) in 30 mL of dry toluene was manteined under a nitrogen atmosphere and reacted in sequence with *N*-hydroxysuccinimide (1.73 g, 15.00 mmol), 4-dimethylaminopyridine (DMAP, 0.61 g,

5.00 mmol), dry *tert*-butanol (14.00 mL, 150.00 mmol) and triethylamine (TEA, 2.09 mL, 15.00 mmol). The mixture was stirred for 30 min at r.t., and heated at reflux for 24 h. After cooling, ethyl acetate (80 mL) was added, the organic phase washed with a 10% citric acid solution (3x80 mL) and brine (2x60 mL), dried on Na_2SO_4 and concentrate under reduced pressure. The crude product (7.00 g), was purified by flash cromathography with 2/5=ethyl acetate/dichloromethane, to give acid **28** in 73% yield, as a pale yellow solid. Mp 50°C. ^{1}H NMR, 200 MHz, $CDCl_3$, δ: 1.44 (s, 9H, *t*Bu); 2.69-2.48 (m, 4H, CH_2). IR, $CDCl_3$: 1716; 1746; 3450-2550 cm^{-1}.

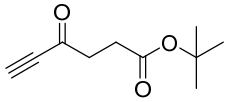
*Tert-butyl 4-(methoxy(methyl)amino)-4-oxobutanoate (**30**)*



30

To a suspension of **28** (1.00 g, 5.74 mmol) in dry DMF, HBTU (2.18 g, 5.74 mmol) and DIPEA (2.00 mL, 11.50 mmol) were added, and the mixture stirred at r.t. for 10 min. Finally, a solution Wienreb's amine **29** (0.56 mg, 5.74 mmol) in dry DMF (15 mL) was added. After stirring at r.t. for 3 h, the mixture was diluted with H_2O (50 mL) and extracted with $CHCl_3$ (3x40 mL). The combined organic phases were dried on Na_2SO_4 and concentrated under reduced pressure, to give **30** in 76% yield, used without further purification in the next step . ^{1}H NMR, 200 MHz, $CDCl_3$, δ: 1.45 (s, 9H, *t*Bu); 2.50-2.75 (m, 4H, CH_2); 3.71 (s, 3H, OCH_3); 3.18 (s, 3H, NCH_3). IR, $CDCl_3$: 1658; 1718 cm^{-1}.

*tert-butyl 4-oxohex-5-ynoate(**31**)*

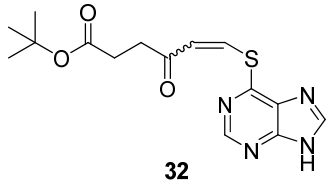


31

A solution of **30** (0.15 g, 0.70 mmol) in dry THF (25 mL) was kept under a nitrogen atmosphere, cooled at -78 °C and, after adding of a 0.5 M solution of ethynyl magnesium bromide in THF (7.00 mL, 3.50 mmol), stirred at -78 °C for 30 min and at r.t. for 3 h. Then, the mixture was quenched adding Et_2O (60mL), ice and a solution of 1M KH_2PO_4 (150mL), under vigorous stirring. After extraction with Et_2O (3x50 mL), the combined organic phases were washed subsequently with 1M KH_2PO_4 (1x150 mL), satured Na_2CO_3 (2x150mL) and brine (3x150 mL), dried over Na_2SO_4 and concentrated under reduced pressure to give ynoate **31** in 47% yield as a pure solid. ^{1}H NMR, 200

MHz, $CDCl_3$, δ: 1.44 (s, 9H, *t*Bu); 2.56 (t, 2H, *J*=6.4 Hz, CH_2); 2.87 (t, 2H, *J*=6.4 Hz, CH_2); 3.24 (s, 1H, C≡CH). ^{13}C NMR, 50 MHz, $CDCl_3$, δ: 28.1 (3C, CH_3); 30.4 (1C, CH_2); 40.2 (1C, CH_2); 78.9 (2C, C≡C); 81.0 (1C, $C(CH_3)_3$); 170.7, 184.9 (1C, CO).

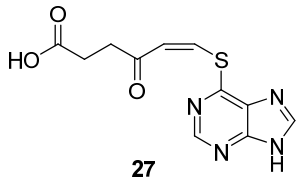
*tert-butyl 6-(9H-purin-6-ylthio)-4-oxohex-5-enoate (**32**)*



32

To a suspension of **11** (0.19 g, 1.10 mmol) in 10 mL of anhydrous MeOH, maintained under a nitrogen atmosphere, a freshly prepared 0.65 M solution of MeONa in dry MeOH (1.70 mL, 1.10 mmol) was added with continuous stirring until complete solution. To this solution, **31** (0.20 g, 1.10 mmol) in dry MeOH (4 mL), was added and the mixture stirred at r.t. overnight. The mixture was diluted with saturated NH_4Cl (30 mL) and extracted with AcOEt (3x30 mL). The combined organic phases were dried over Na_2SO_4 and concentrate under reduced pressure. The crude product (0.72 g) was washed with EtP and purified by flash chromatography with 15/1=$CHCl_3$/MeOH, to give **32** in 84% yield as a 75/25 mixture of *E/Z* isomers. ^1H NMR, 200 MHz, DMSO, δ: 1.37 (s, 9H, *t*Bu); 2.5 (at, 4H, J=6.6 Hz, $CH_{2(E+Z)}$); 2.84 (at, 4H, J=6.4 Hz, $CH_{2(E+Z)}$); 6.82 (d, 1H, J=16.0 Hz, S-CH_E); 6.86 (d, 1H, J=9.8 Hz, S-CH_Z); 8.57 (s, 2H, $CH_{arom(E+Z)}$); 8.73 (d, 1H, J=9.8 Hz, CH_Z-CO); 8.83 (s, 2H, $CH_{arom(E+Z)}$); 8.91 (d, 1H, J=16.6 Hz, CH_E-CO). ^{13}C NMR, 50 MHz, DMSO, *Z* + *E*, δ: 27.7, 28.8 (3C, $C(CH_3)_3$); 35.2, 37.4 (2C, CH_2); 79.6 (2C, $C(CH_3)_3$); 121.8 (2C, C=C); 126.5 (2C, C_{arom}); 134.8, 135.7 (2C, C C_{arom}); 144.4 (2C, C=C); 151.0, 151.3 (2C, CH_{arom}); 171.0 (2C, COO); 194.9 (1C, CO); 197.4 (1C CO). Elementary analysis for $C_{15}H_{18}N_4O_3S$: Calc.: C, 53.88%; H, 5.43%; N, 16.75%; Found: C, 53.67%; H, 5.39%; N, 16.38%.

*6-(9H-purin-6-ylthio)-4-oxohex-5-enoic acid (**27**)*

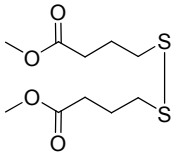


27

A solution of *tert*-butyl 6-(9H-purin-6-ylthio)-4-oxohex-5-enoate **32** (0.05 g, 0.19 mmol) in TFA (2.5 mL) was stirred at r.t. for 2 h. Compound **27** was obtained as pure product as unique *Z* isomer in 84% yield by simple evaporation of the excess of TFA. ^{1}H NMR, 200 MHz, DMSO-*d6*, δ: 2.52 (at, 2H, *J*=7.0 Hz, CH_2), 2.86 (at, 2H, *J*=6.2 Hz, CH_2), 6.88 (d, 1H, *J*=9.8 Hz, S-CH_Z=C), 8.59 (s, 1H, CH_{arom}), 8.74 (d, 1H, *J*=10.4 Hz, C=CH-CO), 8.83 (s, 1H, CH_{arom}). ^{13}C NMR, 50 MHz, DMSO-*d6*, δ: 27.8, 37.4 (1C, CH_2); 121.9 (1C, C=C); 134.8 (1C, C_{arom}); 144.4 (1C, C=C); 151.2 (2C, CH_{arom}); 157.3 (1C, C_{arom}); 173.3 (1C, COOH); 197.6 (1C, COOH). IR, KBr: 1546; 1664; 1709; 3546; 2470-3550 cm^{-1}.

Synthesis of Methyl 4-((9H-purin-6-yl)disulfanyl)butanoate (41)

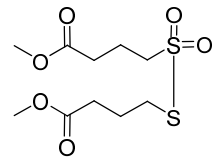
*Dimethyl 4,4'-disulfanediyldibutanoate (**35**)*



35

To a suspension of dithiobutirric acid **34** (1.50 g, 6.29 mmol) in 35 mL of MeOH, 0.5 mL of H_2SO_4(96%) were added and the mixture was refluxed for 1 h. The reaction was then neutralized with a saturated solution of $NaHCO_3$ and extracted with CH_2Cl_2 (3x40 mL). After anhydrification over Na_2SO_4 the solvent was removed under vacuum to give disulfide **35** in 96% yield. ^{1}H NMR, 200 MHz, $CDCl_3$, δ: 2.01 (aq, 4H, *J* = 6.90 Hz, C-CH_2-C); 2.42 (at, 4H, *J* = 7.30 Hz, CH_2-CO); 2,68 (at, 4H, *J* = 7.2 Hz, CH_2-S); 3.65 (s, 6H, CH_3).

*Dimethyl 4,4'-thiolsolfonyldibutanoate (**38**)*

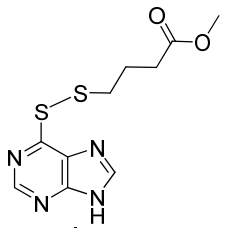


38

To a solution of **35** (1.55 g, 5.83 mmol) in 30 mL of CH_2Cl_2, *m*-chloroperbenzoic acid (70%) (1.58 g, 6.41 mmol) in 27 mL of CH_2Cl_2 was added at 0°C. The reaction mixture was stirred for 10 min at 0°C then at r.t. for 90 min.

Another portion of *m*-chloroperbenzoic acid (70%) (2.15 g, 8.70 mmol) in 35 mL of CH_2Cl_2 was added at 0°C, and the suspension stirred for 10 min at 0°C, for 2 h at 30°C and for 15h at r.t. The reaction mixture was diluted with 40 mL of CH_2Cl_2, washed with a saturated solution of $Na_2S_2O_3$ (3x60 mL), and Na_2CO_3 (3x60 mL).The organic phase was dried over Na_2SO_4, filtered and evaporated. The crude product was purified by column chromatography utilizing Florisil and 12/1=$CHCl_3$/MeOH as eluent to give **38** in 65 % yield. ^{1}H NMR (200 MHz, $CDCl_3$), δ: 1.96-2.30 (m, 4H, C-CH_2-C); 2.38-2.60 (m, 4H, CH_2-CO); 3.19 (at, 2H, *J* = 7.20 Hz, CH_2-S); 3.42 (at, 2H, *J* = 7.50 Hz, CH_2-SO_2); 3.68 (s, 3H, CH_3); 3.69 (s, 3H, CH_3).

*Methyl 4-((9H-purin-6-yl)disulfanyl)butanoate (**41**)*



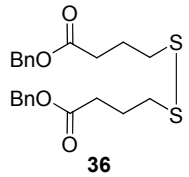
41

To a solution of thiolsulfonate **38** (0.70 g, 2.35 mmol) in 17 mL of dry DMF a solution of 6-MP (**11**) (0.40 g, 2.35 mmol) in 28 mL of dry DMF was added dropwise followed by 0.64 mL of dry TEA. The reaction mixture was heated at 60°C for 18 h, neutralized with NH_4Cl and extracted with EtOAc (6x40 mL). The recombined organic layers were washed with H_2O (5x30 mL), dried over Na_2SO_4, filtered and evaporated. The crude product was purified by column chromatography with 15/1= $CHCl_3$/MeOH to give disulfide **41** in 27% yield. ^{1}H NMR, 200 MHz, CD_3OD, δ: 2.05 (aq, 2H, *J* = 7.1 Hz, C-CH_2-C); 2.53 (at, 2H, *J* = 7.2 Hz, CH_2-CO); 2.97 (at, 2H, *J* = 7.0 Hz, CH_2-S); 3.65 (s, 3H, CH_3); 8.37 (s, 1H, CH_{arom}); 8.92 (s, 1H, CH_{arom}), ^{1}H NMR, 200 MHz, $CDCl_3$, δ: 2.05 (aq, 2H, *J* = 7.2 Hz, C-CH_2-C); 2.50 (at, 2H, *J* = 7.3 Hz, CH_2-CO); 2.95 (at, 2H, *J*

= 7.0 Hz, CH_2-S); 3.62 (s, 3H, CH_3); 8.41 (s, 1H, CH_{arom}); 8.90 (s, 1H, CH_{arom}). ^{13}C NMR, 200 MHz, CD_3OD, δ: 24.1 (1C, C-CH_2-C); 32.5 (1C, CH_2-CO); 38.4 (1C, CH_2-CS); 51.9 (1C, CH_3); 130.4 (1C, C_{arom}); 142.7 (1C, CH_{arom}); 150.5 (1C, C_{arom}); 152.1 (1C, CH_{arom}); 159.5 (1C, C_{arom}); 173.46 (1C, CO). IR (KBr), cm^{-1}: 3452 (stretch. N-H), 1737 (stretch. C=O), 1566 (bend. N-H). MS: *m/z*: 284 ($M^{+\cdot}$, 12), 252 (M- CH_3OH, 8), 152 (6-MP, 100), 119 (6-MP- $SH^{\cdot}$, 30).

Synthesis of benzyl 4-((9H-purin-6-yl)disulfanyl)butanoate (42)

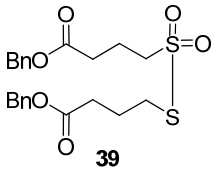
*Benzyl 4,4'-disulfanediyldibutanoate (**36**)*



36

To a suspension of dithiobutirric acid **34** (1.50 g, 6.29 mmol) in 7.81 mL of benzyl alcohol, 0.5 mL of H_2SO_4(96%) were added and the mixture was stirred at 64°C for 1 h. The reaction was then neutralized with a saturated solution of $NaHCO_3$ and extracted with CH_2Cl_2 (3x40 mL). After anhydrification over Na_2SO_4 the solvent was removed under vacuum and distillation to remove the excess of BnOH. The crude product was purified by column chromatography with 2/3=EtP/CH2Cl2 followed by 1/2 and finally 1/3 to give disulfide **36** in 73% yield. ^1H NMR, 200MHz, $CDCl_3$, δ: 2.03 (aq, *J* = 6.8Hz, 4H, C-CH_2-C); 2.48 (at, 4H, *J* = 7.2Hz, CH_2-CO); 2.69 (at, *J* = 7.2Hz, 4H, CH_2-S); 5.12 (s, 4H, Ar-CH_2),7.33-7.39 (m, 10H, H_{arom}).

*Dibenzyl 4,4'-tiolsolfonyldibutanoate (**39**)*

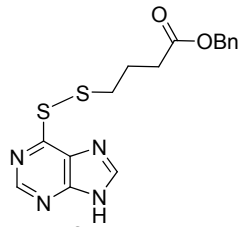


39

To a solution of **36** (1.00 g, 2.34 mmol) in 25 mL of CH_2Cl_2, *m*-chloroperbenzoic acid (70%) (0.63 g, 2.57 mmol) in 27 mL of CH_2Cl_2 was added at 0°C. The reaction mixture was stirred for 10 min at 0°C then at r.t. for 90 min. Another portion of *m*-chloroperbenzoic acid (70%) (0.63 g, 2.57 mmol) in 7 mL of CH_2Cl_2 was added at 0°C, and the suspension stirred for 10 min at 0°C and for 5 h at r.t. The reaction mixture was diluted with 50 mL of CH_2Cl_2, washed with a saturated solution of $Na_2S_2O_3$ (3x60 mL), and Na_2CO_3 (3x60 mL).The organic phase was dried over Na_2SO_4, filtered and

evaporated. The crude product was purified by column chromatography with 3/1=EtP/EtOAc as eluent to give **39** in 48% yield. ^{1}H NMR, 200MHz, $CDCl_3$, δ: 2.20-2.30 (m, 4H, C-CH_2-C); 2.47-2.60 (m, 4H, CH_2-CO); 3.15 (at, 2H, *J* = 7.1Hz, CH_2-S); 3.38 (at, 2H, *J* = 7.60Hz, CH_2-SO_2); 5.13 (s, 4H, Ar-CH_2); 7.28-7.39 (m, 10H, Ar). ^{13}C NMR, 200 MHz, $CDCl_3$, δ: 19.1 (1C, C-CH_2-C); 24.9 (1C, C-CH_2-C); 31.7 (1C, CH_2-C-S); 32.3 (1C, CH_2-CO); 35.3 (1C, CH_2-CO); 61.0 (1C, CH_2-SO_2); 66.4 (2C, CH_2-OCO); 128.1, 128.4 (10C, CH_{arom}); 135.3 (1C, C_{arom}); 135.4 (1C, C_{arom}); 171.4 (1C, CO); 171.8 (1C, CO).

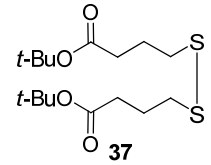
*Benzyl 4-((9H-purin-6-yl)disulfanyl)butanoate (**42**)*



42

To a solution of thiolsulfonate **39** (0.50 g, 1.11 mmol) in 12 mL of dry DMF a solution of 6-MP (**11**)) (0.19 g, 1.11mmol) in 20 mL of dry DMF was added dropwise followed by K_2CO_3 (0.36 g, 1.11 mmol). The reaction mixture was heated at 60°C for 20 h, neutralized with NH_4Cl and extracted with EtOAc (6x40 mL). The recombined organic layers were washed with H_2O (5x30 mL), dried over Na_2SO_4, filtered and evaporated. The crude product was purified by column chromatography with 5/2=EtOAc/CH_2Cl_2 to give disulfide **42** in 26% yield. ^{1}H NMR, 200 MHz, $CDCl_3$, δ: 2.08 (aq, 2H, *J* = 7.20Hz, C-CH_2-C); 2.59 (at, 2H, *J* = 7.30Hz, CH_2-CO); 8.31 (s, 1H, CH_{arom});8.89 (s, 1H, CH_{arom}). IR (KBr), cm^{-1}: 3434 (stretch. NH), 1731 (stretch. C=O), 1560 (bend. NH).

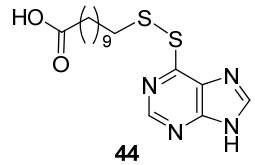
Synthesis of *Tert*-butyl 4,4'-disulfanediyldibutanoate (37)



37

To a solution of dithiobutirric acid **34** (0.12 g, 0.50 mmol) in 8 mL of toluene dry, 0.36 mL of oxalyl chloride (4.20 mmol) were added dropwise at 8°C. The reaction mixture was stirrred at 8°C for 1 h, the solvent was removed and the crude product dissolved in CH_2Cl_2 dry. A solution of tert-butyl alcohol (0.6 mL, 6.25 mmol) and TEA (0.121 mL, 0.88 mmol) in dry CH_2Cl_2 was added to the solution and stirred at 10°C for 4 h. The mixture was then diluted with CH_2Cl_2 and washed with a saturated solution of NH_4Cl (3x15 mL), dried over Na_2SO_4 and evaporated. The crude product was purified by column chromatography with 30/1=EtP/EtOAc as eluent to obtain derivative **37** in 18% yield. ^{1}H NMR, 400 MHz, CD_3OD, δ: 1.44 (s, 18H, $C(CH_3)_3$); 1.97 (aq, 4H, J =7.2 Hz, C-CH_2-C); 2.34 (at, 4H, J =7.2 Hz, CH_2-CO); 2.70 (at, 4H, J = 7.2 Hz, CH_2-S).

Synthesis of 3-((9*H*-purin-6-yl)disulfanyl)undecanoic acid (44)



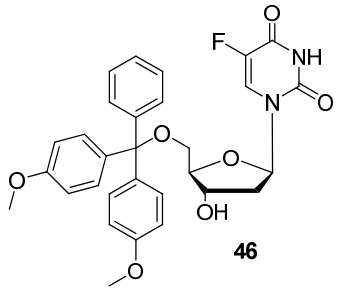
44

To solution of 11-mercaptoundecanoic acid (0.79 g, 3.61 mmol) in dry toluene (5 mL), cooled to -15 °C, a solution of SO_2Cl_2 (0.28 mL, 1.81mmol) in dry toluene (1.5 mL) was added. This mixture was stirred at r.t. for 15 min and nitrogen was bubbled inside to eliminate residual traces of SO_2 and HCl. Before undergoing the next reaction we verified the effective formation of sulfenyl chloride product by titration with cyclohexene. This allowed us verifying the actual formation of the expected sulfenic species and estimating a 46% yield for the first reaction. The so obtained sulfenyl chloride was reacted with a suspension of 6-MP **11** in dry pyridine at 84 °C, for 2 h. The mixture was cooled at r.t., diluted with water and acidified to pH 5 with 3% HCl. The acid aqueous layer was extracted several times with EtOAc and the combined organic phase dried and concentrated. The resulting crude product was purified by flash cromathography with 25/1→15/1=CH_2Cl_2/MeOH to obtain acid **44** in 64% yield. ^{1}H NMR, 200 MHz, CD_3OD, δ: 1.73-1.80 (m, 16H, CH_2); 2.27 (at, 2H, J = 7.2 Hz, CH_2); 6.18 (t, 2H, J = 7.2 Hz, CH_2); 8.44 (s, 1H, CH_{arom}); 8.78 (s, 1H, CH_{arom}). ^{13}C NMR, 50

MHz, DMSO-*d6*, δ: 24.5, 27.7, 28.1, 28.5, 28.7, 28.8, 33.6, 38.1 (CH_2); 142.0, 151.7 (1C, CH_{arom}); 174.5 (1C, COOH).

Synthesis of 2'-Deoxy-5'-*O*-[bis(4-methoxyphenyl)phenylmethyl]-5-fluoro-3'-*O*-(3-carboxypropanoyl)uridine (47)

*2'-deoxy-5'-O-[bis(4-methoxyphenyl)phenylmethyl]-5-fluorouridine (**46**)*

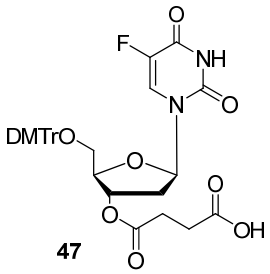


46

2'-Deoxy-5-fluorouridine **45** (0.50 g, 2.03 mmol) was evaporated three times with 3 mL of pyridine dry and then redissolved in 7 mL of anhydrous pyridine. 4,4'-Dimethoxytriphenylmethyl chloride (1.03 g, 3.05 mmol) in pyridine dry (7 mL) was added dropwise at 0 °C under stirring. The reaction mixture was stirred at room temperature for 16 h. The solution was poured onto 50 mL of ice containing 5% $NaHCO_3$, extracted with dichloromethane, dried over anhydrous Na_2SO_4, and evaporated. The crude product was purified by column chromatography using 30/1=CH_2Cl_2/MeOH as eluent obtaining **46** in 62% yield.

^{1}H NMR, 200MHz, $CDCl_3$, δ: 2.60-2.20 (m, 2H, 2'-H_2); 3.41-3.32 (m, 2H, 5'-H_2), 3.76 (s, 6H, OCH_3), 4.20-4.12 (m, 1H, 4'-H), 4.60-4.45 (m, 1H, 3'-H), 6.31 (at, 1H, *J*=6.2, 1'-H), 6.90-6.78 (m, 4H, CH_{arom}), 7.5-7.15 (m, 9H, H_{arom}),7.84 (d, 1H, J=4.4, 6-H,F); ^{13}C NMR, 200MHz, $CDCl_3$, δ: 41.1 (1C, 2'-C), 55.3 (2C, CH_3), 63.4 (1C, 5'-C), 72.0 (1C, 3'-C), 85.6 (1C, 1'-C), 86.5 (1C, C_{quat}), 87.1 (1C, 4'-C), 113.2 (4H, CH_{arom}), 123.7, 124.4, 126.9, 127.8, 129.8, (10C, C_{arom}+ 6-C), 135.2, 135.0 (2C, C_{quat}), 140.4 (d, 1C, *J* = 236.1Hz, 1-CF), 144.0 (1C, C_{quat}), 148.9 (1C, 4-CO), 157.1, 156.6 (2C, C_{quat}-OCH_3), 158.5 (1C, 2-CO).

*2'-Deoxy-5'-O-[bis(4-methoxyphenyl)phenylmethyl]-5-fluoro-3'-O-(3-carboxypropanoyl)uridine (**47**)*

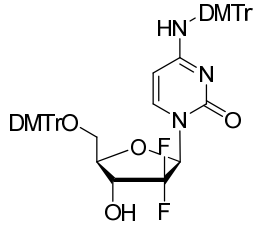


47

To a solution of **46** (0.25 g, 0.46 mmol) in 6 mL of CH_2Cl_2 dry, DMAP (0.08 g, 0.68 mmol) followed by succinic anhydride (0.18 g, 1.83 mmol) were added under nitrogen atmosphere. The reaction mixture was stirred for 18 h at r.t., poured onto a solution of 5% $NaHCO_3$ (50 mL), acidified with 1M HCl to pH 3, and then extracted with CH_2Cl_2. The combined organic layers were washed with water, dried over Na_2SO_4 and the solvent removed to give ester **47** in 90% yield without further purification. ^{1}H NMR, 400MHz, $(CD_3)_2CO$, δ: 2.60-2.40 (m, 6H, 2'-H_2 + CH_2CH_2); 3.58-3.33 (m, 2H, 5'-H_2), 3.78 (s, 6H, OCH_3), 4.22-4.19 (m, 1H, 4'-H), 5.50-5.43 (m, 1H, 3'-H), 6.28 (at, 1H, J = 6.2 Hz, 1'-H), 6.91-6.89 (m, 4H, CH_{arom}), 7.50-7.24 (m, 9H, H_{arom}), 7.88 (d, 1H, J = 6.8 Hz, 6-H,F). ^{13}C NMR, 100 MHz, $(CD_3)_2CO$, δ : 29.4, 30.2 (2C, Succ-CH_2), 38.2 (C-2), 55.4 (2C, OCH_3), 64.5 (C-5), 75.4 (C-3), 84.6 (C-4), 85.7 (C-1), 87.6 (C-Ph_3), 113.9 (2C, C_{arom}), 124.6 (C-6), 127.6 (1C, C_{arom}), 128.6 (1C, C_{arom}), 128.7 (1C, C_{arom}), 130.8 (2C, C_{arom}), 136.1, 136.3 (2C, C_{arom}), 141.3 (C-5), 145.6 (1C, C_{arom}), 150.3 (C-2), 157.3 (C-4), 159.5 (2C, C_{arom}), 172.4, 173.4 (2C, Succ-CO). ESI-MS: m/z = 648 $[M]^+$.

Synthesis of 2'-deoxy-5'-O-[bis(4-methoxyphenyl)phenylmethyl]-4-(bis(4-methoxyphenyl)(phenyl)methylamino)-2',2'-difluoro-3'-O-(3-carboxypropanoyl)cytidine (50)

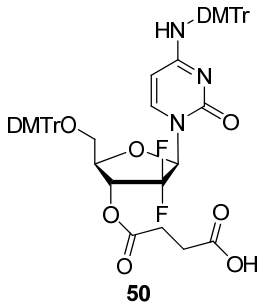
*2'-deoxy-5'-O-[bis(4-methoxyphenyl)phenylmethyl]-4-(bis(4-methoxyphenyl)(phenyl)methylamino)-2', 2'-difluorocytidine(**49**)*



49

Gemcitabine **48** (0.37 g, 1.43 mmol) was evaporated three times with 2 mL of pyridine dry and then redissolved in 20 mL of anhydrous pyridine. 4,4'-dimethoxytriphenylmethyl chloride (1.52 g, 4.49 mmol) in pyridine dry (20 mL) was added dropwise at 0 °C under stirring. The reaction mixture was stirred at room temperature for 16 h. The solution was poured onto 50 mL of ice containing 5% $NaHCO_3$, extracted with dichloromethane, dried over anhydrous Na_2SO_4, and evaporated. The crude product was purified by column chromatography using 15/1=CH_2Cl_2/MeOH as eluent obtaining **49** in 62% yield. ^{1}H NMR, 200MHz, $CDCl_3$, δ: 3.38-3.52 (m, 2H, 5'-H_2), 3.69-3.73 (m, 12H, OCH_3), 4.02-4.06 (m, 1H, 4'-H), 4.40-4.45 (m, 1H, 3'-H), 4.88 (d, 1H, *J*=7.6, H_{vinyl}), 6.30-6.42 (m, 1H, 1'-H), 6.70-6.81 (m, 8H, H_{arom}); 7.01-7.32 (m, 20H, H_{arom}), 7.57 (d, 1H, *J*=7.6, H_{vinyl}); ^{13}C NMR, 200MHz, $CDCl_3$, δ: 55.3 (4C, CH_3), 61.2 (1C, 5'-C), 70.4 (1C, 3'-C), 80.4 (2C, C_{quat}), 84.5 (1C, 1'-C), 86.7 (1C, 4'-C), 95.5 (1C, C_{vinyl}); 113.2, 113.6, 127.4, 127.8, 128.3, 128.4, 129.9, 130.2 (14C, C_{arom}+ 6-C), 135.0, 135.1, 135.7, 136.1 (4C, C_{quat}), 141.0 (1C, C_{vinyl}), 144.2, 144.8 (2C, C_{quat}), 155.1 (1C, CO); 158.4, 158.5 (4C, C_{quat}-OCH_3), 165.3 (1C, 2-CO).

2'-deoxy-5'-O-[bis(4-methoxyphenyl)phenylmethyl]-4-(bis(4-methoxyphenyl)(phenyl)methylamino)-2',2'-difluoro-3'-O-(3-carboxypropanoyl)cytidine ***(50)***

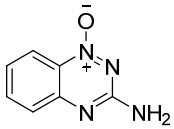


50

To a solution of **49** (0.18 g, 0.20 mmol) in 4 mL of CH_2Cl_2 dry, DMAP (0.04 g, 0.30 mmol) followed by succinic anhydride (0.08 g, 0.81 mmol) were added under nitrogen atmosphere. The reaction mixture was stirred for 24 h at r.t., poured onto a solution of 5% $NaHCO_3$ (50 mL), acidified with 1M HCl to pH 3, and then extracted with CH_2Cl_2. The combined organic layers were washed with water, dried over Na_2SO_4 and the solvent removed to give ester **50** in 90% yield without further purification. ^{1}H NMR, 400MHz, $(CD_3)_2CO$, δ: 2.60-2.70 (m, 4H, CH_2CH_2); 3.28-3.41 (m, 2H, 5'-H_2), 3.73, 3.74, 3.75, 3.76 (s, 12H, OCH_3), 4.06-4.09 (m, 1H, 4'-H), 4.92 (d, 1H, *J*=7.6, H_{vinyl}), 5.45-5.51 (m, 1H, 3'-H), 6.39 (at, 1H, *J* = 6.2 Hz, 1'-H), 6.73-6.82 (m, 8H, CH_{arom}), 7.50-7.24 (m, 18H, H_{arom}), 7.40 (d, 1H, *J*=7.6, H_{vinyl}),. ^{13}C NMR, 100 MHz, $(CD_3)_2CO$, δ : 28.7 (2C, Succ-CH_2), 55.1, 55.2 (4C, OCH_3), 60.9 (C-5), 69.7-69.8 (1C, 3'-C), 70.4 (C_{quat}) 78.2 (C-4), 83.5-83.7 (C-1), 86.6 (C-Ph_3), 95.6 (1C, C_{vinyl}); 113.2, 113.5 (4C, C_{arom}), 123.5 (C-2), 126.8, 127.4, 127.7,127.9, 128.3, 128.5, 129.1, 129.9, 130.1 (18C, C_{arom}), 134.9, 135.7, 140.0 (4C, C_{arom}), 140.8 (CH), 144.1, 144.4 (2C, C_{arom}), 154.6 (C_{arom}), 158.5, 158.6 (4C, C_{arom}), 165.4 (1C, C-NH); 170.6, 175.3 (2C, Succ-CO). ESI-MS: *m/z* = 967$[M]^+$.

Synthesis of 3-(5-carboxypentylamino)benzo[e][1,2,4]triazine 1,4-dioxide (57)

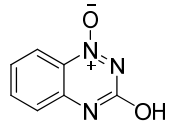
*3-aminobenzo[e][1,2,4]triazine 1-oxide (**53**)*



53

2-Nitroaniline (6.00 g, 43.50 mmol) and cyanamide (7.40 g, 176.20 mmol) were heated with stirring at 100°C until a deep red melt formed. The reaction was cooled at 50 °C and 22 mL of concentrated HCl was added dropwise. The reaction mixture was warmed at 100 °C, stirred for 4h, then made strongly alkaline by the addiction of 70 mL 30% solution of NaOH. After the solution was heated again for 3h, cooling crystals formed. These were collected and washed with water (120 mL) and diethylether (100 mL) to give 3-amino-1,2,4-benzotriazine-1-oxide **53** in 78% yield. ^{1}H NMR, 200 MHz , C_6D_6SO, δ: 7.28-7.38 (m, 3H, H_{arom} e NH_2); 7.53 (d, J = 8.2 Hz, 1H, H_{arom}); 7.78 (m, 1H, H_{arom}); 8.13 (d, J = 8.8 Hz, 1H, H_{arom}).

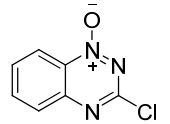
*3-hydroxybenzo[e][1,2,4]triazine 1-oxide (**54**)*



54

A solution of **53** (5.35 g, 33.02 mmol) in 62 mL of trifluoroacetic acid (TFA) was cooled at 5°C. Over a period of 15' sodium nitrite (2.51 g, 36.33 mmol) was added portionwise. The reaction mixture was stirred at r.t. for 4h, poured onto ice/water, stirred 30 min, filtered, washed with water and dried to give 3-hydroxy-1,2,4-benzotriazine-1-oxide **54**. ^{1}H NMR, 200 MHz, $CDCl_3$, δ: 7.72-7.81 (m, 1H, H_{arom}); 7.98-8.01 (m, 2H, H_{arom}); 8.40 (d, J = 8.8 Hz, 1H, H_{arom}).

*3-chlorobenzo[e][1,2,4]triazine 1-oxide (**52**)*

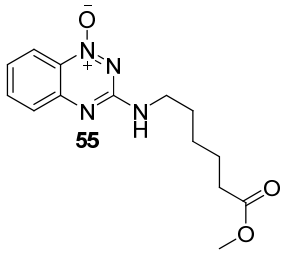


52

Chloride **52** was obtained by adding 0.4 mL of DMF and 82.5 mL of phosphorous oxychloride (7 eq.) directly to **54**. The reaction mixture was refluxed for 1h, then poured on ice and filtered. The crude product was recristyllized from methanol to give

chloride **52** in 60% yield. ^{1}H NMR, 200 MHz, $CDCl_3$, δ: 7.72-7.81 (m, 1H, H_{arom}); 7.98-8.01 (m, 2H, H_{arom}); 8.40 (d, J = 8.8 Hz, 1H, H_{arom}). ^{1}H NMR, 200 MHz, C_6D_6SO, δ: 7.90 (t, J = 7.0 Hz, 1H, H_{arom}); 8.04-8.20 (m, 2H, H_{arom}); 8.37 (d, J = 8.8 Hz, 1H, H_{arom}).

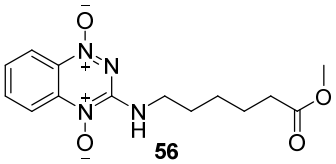
*3-(6-methoxy-6-oxohexylamino)benzo[e][1,2,4]triazine 1-oxide (**55**)*



55

To a solution of methyl-6-aminohexanoate (10.08 g, 55.09 mmol) in 250 mL of CH_2Cl_2 was added a solution of **52** (3.36 g, 18.56 mmol) and TEA (5.20 mL, 37.12 mmol) in 100 mL CH_2Cl_2. The reaction mixture was stirred for 116h at r.t. and distilled to dryness in vacuo. The residue was purified by flash chromatography with 50/1= CH_2Cl_2 /MeOH as eluent to yield 1-oxide **55** in 75% yield. ^{1}H NMR, 200 MHz, $CDCl_3$, δ: 1.38-1.51 (m, 2H, CH_2); 1.61-1.76 (m, 4H, CH_2); 2.33 (t, J = 7.2 Hz, 2H, CH_2); 3.51 (m, 2H, CH_2); 3.65 (s, 3H, CH_3); 5.39 (m, 1H, NH); 7.27 (dt, J_1 = 8.5 Hz, J_2 = 1.5 Hz, 1H, H_{arom}); 7.55-7.72 (m, 2H, H_{arom}); 8.24 (dd, J_1 = 8.6 Hz, J_2 = 1.2 Hz, 1H, H_{arom}). ^{13}C NMR, 50 MHz, $CDCl_3$, δ: 24.5, 26.3, 28.9, 33.8, 41.0 (1C, CH_2); 51.4 (1C, CH3); 120.6, 124.6, 126.3 (1C, CH_{arom}); 130.6 (1C, $C_{arom.}$); 135.3 (1C, $CH_{arom.}$); 148.6, 158.8 (1C, $C_{arom.}$); 173.8 (1C, CO).

*3-(6-methoxy-6-oxohexylamino)benzo[e][1,2,4]triazine 1,4-dioxide (**56**)*

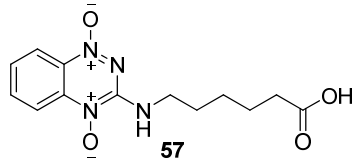


56

50% H_2O_2 (7.5 mL) was diluted with 37.5 mL MeOH and dried on Na_2SO_4 overnight to increase its oxidizing properties. A solution of dried H_2O_2 in MeOH (1.25 mL) was added to a stirred solution of trifluoroacetic anhydride (2.6 mL) in 7.2 mL of dry CH_2Cl_2 at 5°C. The mixture was stirred for 10 min at r.t. then cooled to 5°C before adding a solution of **55** (300 mg, 1.03 mmol) and trifluoroacetic acid (720 µL) in 7.2 mL dry CH_2Cl_2. After 4 days under stirring at r.t. the red solution was diluted with EtOAc and washed with a saturated solution of $NaHCO_3$. The organic phase was dried,

filtered and concentrated to give 1,4-dioxide **56** with 90% yield. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.43-1.50 (m, 2H, CH_2); 1.66-1.78 (m, 4H, CH_2); 3.33 (t, *J* = 7.6 Hz, 2H, CH_2); 3.59-3.66 (m, 2H, CH_2); 3.67 (s, 3H, CH_3); 7.14 (s, 1H, NH); 7.51 (t, *J* = 8.4 Hz, 1H, $H_{arom.}$); 7.88 (t, *J* = 7.6 Hz, 1H, $H_{arom.}$); 8.29 (d, *J* = 8.0 Hz, 1H, $H_{arom.}$); 8.33 (t, *J* = 8.8 Hz, 1H, $H_{arom.}$). ^{13}C NMR, 50 MHz, $CDCl_3$, δ: 24.6, 26.3, 29.3, 33.9, 41.4 (1C, CH_2); 51.7 (1C, CH3); 117.2, 121.6, 127.0, 130.4 (1C, C $H_{arom.}$); 136.0, 138.1, 149.7 (1C, C_{arom}); 173.8 (1C, CO). IR: 3401, 3244, 2952, 1737, 1620, 1597, 1412, 1384, 1357, 1177, 1088, 719 cm^{-1}.MS: (CI) $M^{+\cdot}$= 306; 55 (100%); 65, 90 (70%); 289 (30%); 175 (50%) (**51**); 90 (70%); .

*3-(5-carboxypentylamino)benzo[e][1,2,4]triazine 1,4-dioxide (**57**)*



57

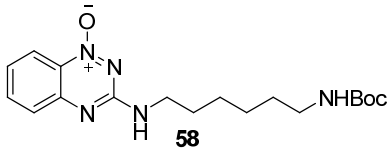
a) 37% HCl (1.4 mL, 13.65 mmol) was added to a solution of **56** (120 mg, 0.39 mmol) in 2 mL of dioxane. The reaction mixture was stirred at r.t. for 48h, concentrated and purified by flash chromatography with 12/1 CH_2Cl_2/MeOH to obtain **57** in 83% yield.

b) 1M LiOH (3.9 mL, 3.90 mmol) was added to a solution of **56** (120 mg, 0.39 mmol) in 1/1=THF/MeOH (4 mL/4 mL). The reaction mixture was stirred for 5', then 1M HCl was added until pH 5 and the aqueous phase was extracted with EtOAc (8x30 mL). Combined organic layers were dried, filtered and evaporated to give acid **57** in 98% yield without further purification.

^{1}H NMR, 400 MHz, $(CD_3)_2SO$), δ: 1.31-1.33 (m, 2H, CH_2); 1.51-1.55 (m, 2H, CH_2); 1.59-1.63 (m, 2H, CH_2); 2.19-2.23 (m, 2H, CH_2); 3.35-3.40 (m, 2H, CH_2); 7.52-7.57 (m, 1H, $H_{arom.}$); 7.90-7.94 (m, 1H, $H_{arom.}$); 8.12 (d, *J* = 8.4 Hz, 1H, $H_{arom.}$); 8.19 (d, *J* = 8.4 Hz, 1H, $H_{arom.}$); 8.27-8.30 (m, 1H, $H_{arom.}$). ^{13}C NMR, 50 MHz, $(CD_3)_2SO$), δ: 24.3, 25.8, 28.5, 33.7, 41.4 (1C, CH_2); 116.8, 121.1, 126.8 (1C, C $H_{arom.}$); 129.8 (1C, $C_{arom.}$); 135.4 (1C, $CH_{arom.}$); 138.1, 149.7 (1C, $C_{arom.}$); 174.4 (1C, CO). IR: 3439, 3249, 2924, 1617, 1594, 1410, 1365, 1315, 1119, 1080, 721 cm^{-1}. MS: (ESI-MS) $M^{+\cdot}$= 292 (CI): 55 (100%); 57 (55%), 99 (45%); 175 (35%); 276 (20%).

Synthesis of 3-(6-(4-((3-carboxypropyl)disulfanyl)butanamido)hexylamino)benzo[e][1,2,4]triazine 1,4-dioxide (61)

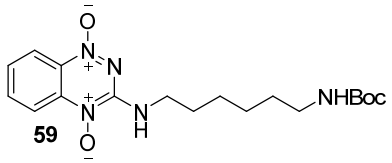
*3-(6-(tert-butoxycarbonylamino)hexylamino)benzo[e][1,2,4]triazine 1-oxide (**58**)*



58

A solution of 6-*t*-butyloxycarbamoylhexylamine (0.13 g, 6.11 mmol) in CH_2Cl_2 was added to a stirred solution of **52** (0.37 g, 2.04 mmol) and TEA (0.57 mL, 4.08 mmol) in CH_2Cl_2 (100 mL), and the solution was stirred at r.t. for 96 h. The solvent was evaporated and the residue chromatographed, eluting with 1/1=EtOAc/EtP, to give 1-oxide **58** in 65% yield. ^{1}H NMR, 400 MHz, $(CD_3)_2SO$), δ: 1.38–1.43 (m, 4 H, CH_2); 1.44 [s, 9 H, $C(CH_3)_3$]; 1.48–1.54 (m, 2 H, CH_2); 1.64–1.72 (m, 2H, CH_2); 3.10–3.13 (m, 2H, CH_2N),); 3.51 (dd, J ¼ 6.8, 6.6 Hz, 2 H, CH2N), 4.55 (br s, 1 H, OCONH); 5.34 (br s, 1 H, NH); 7.27 (dd, *J* ¼ 8:0, 7.5 Hz, 1 H,H 7); 7.59 (d, *J* = 8.5 Hz, 1 H, H5); 7.70 (dd, *J* = 8:2, 7.2 Hz, 1 H, H6);8.26 (d, J ¼ 8:6 Hz, 1 H, H 8). Anal. calc. for C18H27N5O3: C, 59.8;H, 7.5; N, 19.4; found: C, 59.6; H, 7.7; N, 19.2%.

*3-(6-(tert-butoxycarbonylamino)hexylamino)benzo[e][1,2,4]triazine 1,4-dioxide (**59**)*

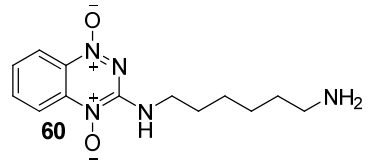


59

50% H_2O_2 (7.5 mL) was diluted with 37.5 mL MeOH and dried on Na_2SO_4 overnight to increase its oxidizing properties. A solution of dried H_2O_2 in MeOH (1.25 mL) was added to a stirred solution of trifluoroacetic anhydride (2.6 mL) in 7.2 mL of dry CH_2Cl_2 at 5°C. The mixture was stirred for 10 min at r.t. then cooled to 5°C before adding a solution of **58** (300 mg, 1.03 mmol) and trifluoroacetic acid (720 μL) in 7.2 mL dry CH_2Cl_2. After 4 days under stirring at r.t. the red solution was diluted with EtOAc and washed with a saturated solution of $NaHCO_3$. The organic phase was dried, filtered and concentrated to give 1,4-dioxide **59** with 99% yield. ^{1}H NMR, 400 MHz, $(CD_3)_2SO$), δ: 1.25-1.31 (m, 2H, CH_2); 1.32-1.40 (m, 13H, CH_2, $C(CH_3)_3$); 1.56–1.61 (m, 2H, CH_2), 2.87-2.92 (m, 2H, CH_2N), 3.32-3.39 (m, 2H, CH_2N), 6.76 (br s, 1H, NH), 7.53-7.57 (m, 1 H, H7), 7.91-7.95 (m, 1H, H6), 8.12 (d, *J* = 8.5 Hz, 1H, H5), 8.19 (d, *J*

= 8.5 Hz, 1 H, H8), 8.30 (dd, *J* = 6.3, 6.1 Hz, 1H, OCONH). Anal. calc. for C18H27N5O4.1/4H_2O: C, 56.6; H, 7.3; N, 18.3; found: C, 56.8; H, 7.3; N, 16.8%.

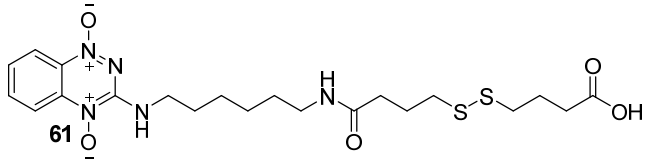
*3-(6-aminohexylamino)benzo[e][1,2,4]triazine 1,4-dioxide (**60**)*



60

59 was then dissolved in MeOH and gaseous HCl was bubbled inside for 2'. The yellow solution was stirred for 17 h at r.t., diluted with water and extracted several times with $CHCl_3$. The aqueous phase was basified by adding of a saturated solution of $NaHCO_3$ and extracted many times with $CHCl_3$. These final recollected organic layers, once dried and concentrated, gave the amine **60** in 55% yield. ^{1}H NMR, 400 MHz, $(CD_3)_2SO$), δ: 1.35-1.50 (m, 6H, CH_2), 1.70-1.76 (m, 2 H, CH_2), 2.70 (t, *J* = 6:8 Hz, 2 H, CH_2N), 3.60 (t, *J* = 7.1 Hz, 2 H, CH_2N), 7.13 (s, 1 H, NH), 7.48-7.52 (m, 1 H, H 7'), 7.87-7.90 (m, 1 H, H 6'), 8.29 (d, *J* = 8.6 Hz, 1 H, H 5'), 8.34 (d, *J* = 8.5 Hz, 1 H, H 8'). Anal. calc. for C13H19N5O2: C, 56.3; H, 6.9; N, 25.3; found: C, 56.3;H, 6.8; N, 22.2%.

*3-(6-(4-((3-carboxypropyl)disulfanyl)butanamido)hexylamino)benzo[e][1,2,4]triazine 1,4-dioxide (**61**)*

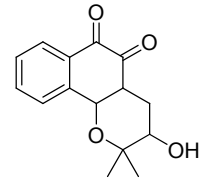


61

Dithiobutirric acid **34** (1 eq.) was activated by adding to its solution in dry DMF, HBTU (1 eq.) and DIPEA (2 eq.). After stirring for 10' this solution was added to another of **60** (1 eq.) in dry DMF. The reaction mixture was stirred for 6h, acidified with 1N HCl to pH 5 and diluted with $CHCl_3$. The organic phase was washed with water and the crude product purified in a TLC plate with 15/1=CH_2Cl_2 /MeOH to obtain **61** (55%). ^{1}H NMR, 400 MHz, $(CD_3)_2SO$), δ: 1.25-1.53 (m, 8H, CH_2), 1.71-1.74 (m, 2H, CH_2), 1.98-2.05 (m, 4H, CH_2), 2.30-2.43 (m, 2H, CH_2), 2.43 (t, *J* = 6:8 Hz, 2 H, CH_2), 2.70-2.74 (m, 4H, CH_2), 3.24-3.27 (m, 2 H, CH_2), 3.59-3.61 (m, 2H, CH_2), 5.93 (br s, 1H, NH), 6.05 (br s, 1H, NH); 7.48-7.52 (m, 1 H, H 7'), 7.87-7.90 (m, 1 H, H 6'), 8.26-8.29 (m, 2 H, H_{arom}).

Synthesis of 4-(2,2-dimethyl-5,6-dioxo-3,4,4a,5,6,10b-hexahydro-2H-benzo[h]chromen-3-yloxy)-4-oxobutanoic acid (64)

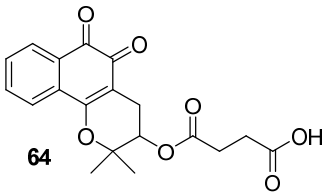
*3-hydroxy-2,2-dimethyl-4,4a-dihydro-2H-benzo[h]chromene-5,6(3H,10bH)-dione (**62**)*



62

Lapachol **63** (0.35 g, 1.47 mmol) was treated with MCPBA (ca. 65%) (0.43 g, 1.76 mmol) in 10 mL of dry CH_2Cl_2 at r.t. for 24 h. The reaction mixture was diluted with a saturated solution of $NaHCO_3$, the organic layer was separated and the aqueous phase extracted with CH_2Cl_2. The combined organic layers were washed with water, dried over Na_2SO_4, filtered and the solvent evaporated. The crude was purified by column chromatography with 4/1=hexane/EtOAc to give **62** in 57% yield. mp 203–205°C. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.45 (s, 3H, CH_3), 1.53 (s, 3H, CH_3), 1.90 (s, 1H, OH), 2.64 (dd, 1H, *J* = 26 and 5.7 Hz, H-4), 2.82 (dd, 1H, *J* = 2.6 and 5.3 Hz, H-4), 3.91 (dd, 1H, *J* = 5.7 and 5.3 Hz, H-3), 7.60-7.66 (m, 3H, H_{arom}), 8.02 (d, 1H, *J* = 6.2 Hz, H_{arom}). ^{13}C NMR, 400 MHz, $CDCl_3$, δ: 21.0, 22.6, 23.2, 69.0, 79.7, 110.0, 124.3, 128.8, 130.1, 131.0, 132.0, 135.0, 161.2, 178.6, 179.4. IR: 1700, 1630 (CO) cm^{-1}.

*4-(2,2-dimethyl-5,6-dioxo-3,4,4a,5,6,10b-hexahydro-2H-benzo[h]chromen-3-yloxy)-4-oxobutanoic acid (**64**)*



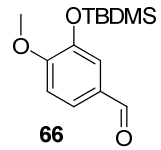
64

To a solution of **62** (0.07 mg, 0.27 mmol) in 7 mL of dry CH_2Cl_2, succinic anhydride (0.08 g, 0.81 mmol) and DMAP (0.10 g, 0.81 mmol) were added and the resulting mixture stirred at r.t. for 18 h and poured onto ice/water/$NaHCO_3$. The suspension was acidified to pH 5 and extracted with CH_2Cl_2 (3x30mL). The recollected organic phases were washed with a saturated solution of $NaHCO_3$ (1x50mL) and water (1x50mL), then dried to give **64** in 92% yield without further purification. ^{1}H NMR, 200 MHz, $CDCl_3$, δ: 1.43 (s, 3H, CH_3), 1.48 (s, 3H, CH_3), 2.62-2.74 (m, 6H, H-4 and CH_2), 5.17 (t, 1H, *J* = 4.8 Hz, H-3), 7.53 (dt, *J* = 1.0 and 6.4 Hz 1H, H_{arom}), 7.64 (dt, *J* = 1.6 and 6.0 Hz 1H,

H_{arom}), 7.84 (dd, *J* = 1.0 and 6.0 Hz 1H, H_{arom}), 7.64 (dt, *J* = 1.4 and 6.6 Hz 1H, H_{arom}). ^{13}C NMR, 400 MHz, $CDCl_3$, δ: 22.5, 23.2, 24.9, 29.0, 29.7, 69.4, 79.7, 109.9, 124.3, 128.8, 130.0, 131.0, 134.9, 137.4, 171.2, 179.2.

Synthesis of (Z)-5-(2-methoxy-5-(3,4,5-trimethoxystyryl)phenoxy)-5-oxopentanoic acid (72)

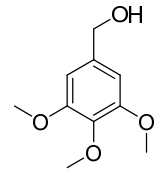
*3-(tert-butyldimethylsilyloxy)-4-methoxybenzaldehyde (**66**)*



66

To a solution of isovanillin (4.00 g, 26.32 mmol) in 30 mL of dry DMF, DIPEA (9.10 mL, 52.61 mmol) and TBDMSCl (6.35 g, 42.13 mmol) were added. The reaction mixture was stirred at r.t. for 4 h, diluted with water and EtP. The phases were separated and the water layer was extracted twice with EtP (2x100mL). The combined organic phases were washed with a saturated solution of $NaHCO_3$ (1x100mL) and water (6x100mL), then dried over Na_2SO_4, filtered and concentrated under vacuum. The crude product was purified by flash chromatography with 80/1=CH_2Cl_2/EtP to give **66** in 99% yield. ^1H NMR, 200 MHz , $CDCl_3$, δ: 0.17 (s, 6H, CH_3); 1.00 (s, 9H, tBu); 3.89 (s, 3H, OCH_3); 6.95 (d, *J*=8.3 Hz, 1H, H_{arom}); 7.37 (d, *J*=1.8 Hz, 1H, H_{arom}); 7 .48 (dd, J_1=8.3 Hz, J_2=1.8 Hz, 1H, H_{arom}); 9.82 (s, 1H, CHO).

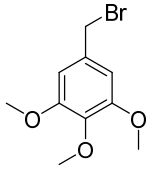
*(3,4,5-trimethoxyphenyl)methanol (**68**)*



68

A solution of 3,4,5-trimethoxybenzaldehyde (4.00 g, 20.41 mmol) in 40 mL of dry MeOH was chilled to 0 °C and $NaBH_4$ (0.94 g, 24.70 mmol) was added in small portions over 1 h. The resulting mixture was stirred overnight at r.t. and concentrated. The crude product was partitioned between Et_2O and water. The aqueous layer was extracted with Et_2O (3x100 mL) and combined organic phases dried and evaporated to obtain **68** in 88% yield. ^1H NMR, 200 MHz , $CDCl_3$, δ: 3.81 (s, 3H, OCH_3); 3.83 (s, 6H, OCH_3); 4.60 (s, 2H, CH_2); 6.57 (s, 2H, H_{arom}).

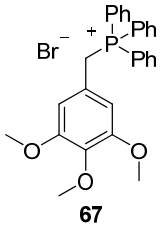
*5-(bromomethyl)-1,2,3-trimethoxybenzene(**69**)*



69

To a solution of **68** (3.02 g, 15.25 mmol) in 20 mL of dry CH_2Cl_2, cooled to -5 °C, a solution of PBr_3 (3.05 g, 11.29 mmol) in 10 mL of dry CH_2Cl_2 was added dropwise. The reaction mixture was stirred at the same temperature for 45 min, poured onto ice, neutralized with $NaHCO_3$ and stirred for 1h. The water phase was extracted with CH_2Cl_2 (5x100mL) and the recollected organic layers were washed with brine (2x150mL) and dried to obtain **69** in 96% yield. ^{1}H NMR, 200 MHz , $CDCl_3$, δ: 3.81 (s, 3H, OCH_3); 3.83 (s, 6H, OCH_3); 4.60 (s, 2H, CH_2); 6.57 (s, 2H, H_{arom}).

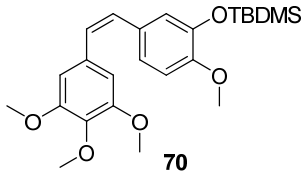
*Triphenyl(3,4,5-trimethoxybenzyl)phsphonium bromide(**67**)*



67

A solution of triphenylphosphine (3.94 g, 14.17 mmol) in 11 mL of toluene was added to a solution of **69** (3.75 g, 14.42 mmol) in 16 mL of toluene. The reaction mixture was stirred for 24 h at r.t. and the white precipitate formed filtered and dried to give the phosphonium salt **67** in 66% yield. ^{1}H NMR, 200 MHz , D_2O, δ: 3.27 (s, 6H, OCH_3); 3.54 (s, 3H, OCH_3); 4.46 (d, J = 14.2 Hz, 2H, P-CH_2); 6.03 (d, J = 2.6 Hz 2H, H_{arom}); 7.38-7.50 (m, 12H, H_{arom}); 7.60-7.76 (m, 3H, H_{arom}).

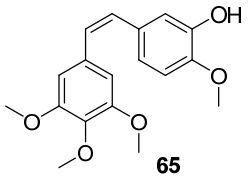
*(Z)-tert-butyl(2-methoxy-5-(3,4,5-trimethoxystyryl)phenoxy)dimethylsilane (**70**)*



70

A homogeneous suspension of triphenylphsphonium bromide **67** (1.00 g, 1.91 mmol) in 60 mL of dry THF under nitrogen was cooled to -23 °C and retained at that temperature for 2 h. Butyllithium 1.6 M in hexane (1.23 mL, 1.91 mmol) was added dropwise, the resultant orange solution was stirred at the same temperature for 1 h and finally aldehyde **66** (0.53 g, 1.99 mmol) was added dropwise. The reaction mixture was stirred at -23 °C for 4 h and at r.t. for 18 h, ice-water was added and the two phases separated. The aqueous phase was washed with Et_2O (3x75mL) and the ethereal solution added to the THF layer. The combined organic phase was washed with water (3x75mL), dried over Na_2SO_4, filtered and the solvent eliminated. The crude product was purified by flash chromatography with 15/1=EtP/EtOAc to isolate both the *Z*-**70** and the *E*-**70** stilbenes in 61 and 20% yield respectively. ^{1}H NMR, 200 MHz , $CDCl_3$, *Z*, δ: 0.05 (s, 6H, CH_3); 0.94 (s, 9H, CH_3); 3.70 (s, 6H, OCH_3); 3.77 (s, 3H, OCH_3); 3.83 (s, 3H, OCH_3); 6.44 (d, *J*=2.2 Hz, 2H, H_{arom}); 6.49 (s, 2H, H_{arom}); 6.71-6.90 (m, 3H, H_{arom}).

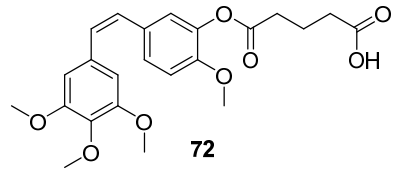
*(Z)-2-methoxy-5-(3,4,5-trimethoxystyryl)phenol (**65**)*



65

To a solution of *Z*-**70** (0.45 g, 1.03 mmol) in 11 mL of dry THF, cooled to 0 °C, TBAF·$3H_2O$ (0.34 g, 1.06 mmol) dissolved in 4 mL of dry THF was added. The reaction mixture was stirred at r.t. for 20 min, then ice was added followed by ether and the organic layer was washed with water (3x30 mL). The residue was chromatographed with 3/2=EtP/EtOAc to give Combretastatin A-4 **65** in 77% yield. ^{1}H NMR, 200 MHz , $CDCl_3$, δ: 3.69 (s, 6H, OCH_3); 3.83 (s, 3H, OCH_3); 3.86 (s, 3H, OCH_3); 5.56 (s, 1H, OH); 6.43 (AB system, *J*=12.3 Hz, 2H, H_{arom}); 6.52 (s, 2H, H_{arom}); 6.70-6.82 (m, 2H, H_{arom}); 6.92 (d, *J*=1.8 Hz, 1H, H_{arom}).

*(Z)-5-(2-methoxy-5-(3,4,5-trimethoxystyryl)phenol)-5-oxopentanoic acid (**72**)*

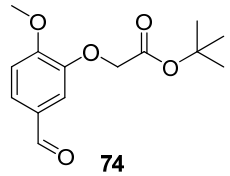


72

To a solution of **65** (0.12 g, 0.38 mmol) in 3 mL of dry CH_2Cl_2, glutaric anhydride (0.70 g, 0.57 mmol) was added and the resulting mixture stirred at r.t. for 16 h then refluxed for 24 h and poured onto ice/water/$NaHCO_3$. The suspension was acidified to pH 5 and extracted with CH_2Cl_2 (3x30mL). The recollected organic phases were washed with a saturated solution of $NaHCO_3$ (1x50mL) and water (1x50mL), then dried to give **72** in 50% yield without further purification. ^{1}H NMR**,** 200 MHz, $CDCl_3$, δ: 2.05 (q, 2H, *J* =7.1 Hz, CH_2); 2.51 (t, 2H, *J*=7.1 H, CH_2); 2.59 (t, 2H, *J*=7.1 Hz, CH_2) (3.70 (s, 6H, OCH_3); 3.80 (s, 3H, OCH_3); 3.83 (s, 3H, OCH_3); 6.45 (s, 2H, H_{arom}); 6.50 (s, 2H, H_{arom}); 6.76 (d, *J*=8.4 Hz, 1H, H_{arom}); 6.99 (d, *J*=1.8 Hz, 1H, H_{arom}); 7.12 (dd, J_1=8.4 Hz e J_2=1.8 Hz, 1H, H_{arom}). ^{13}C NMR, 50 MHz , $CDCl_3$, δ: 20.0, 32.7, 32.9 (1C, CH_2); 55.9 (1C, OCH_3), 56.0 (2C, OCH_3); 60.9 (1C, OCH_3); 105.7 (2C, CH_{arom}); 111.8 (1C, C_{arom}); 122.9, 127.7 (1C, CH_{arom}); 128.4, 129.3 (1C, CH_{vinil}); 129.9 (1C, CH_{arom}); 132.4, 136.9, 139.1, 150.0 (1C, C_{arom}); 152.8 (2C, C_{arom}); 170.6, 177.8 (1C, CO).

Synthesis of (Z)-2-(2-methoxy-5-(3,4,5-trimethoxystyryl)phenoxy)-acetic acid (73)

*Tert-butyl 2-(5-formyl-2-methoxyphenoxy)acetate (**74**)*

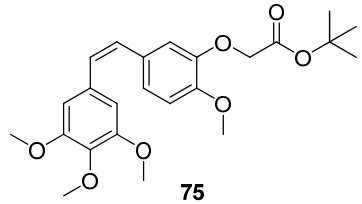


74

To a solution of 3-hydroxy-4-methoxybenzaldehyde (1.50 g, 9.89 mmol) in 55 mL of dry CH_3CN, was added K_2CO_3 (3.40 g, 24.67 mmol). The mixture was stirred at 58 °C for 7 min under nitrogen, then a solution of *t*-butylbromoacetate (2.31 g, 9.84 mmol) in 36 mL of dry CH_3CN was added dropwise in 15 min. The reaction mixture was stirred for 2 h at 60 °C and overnight at r.t. before adding a saturated solution of NH_4Cl and extracting with Et_2O. The organic phase was washed with water, dried, filtered and concentrated to give **74** with 88% yield. ^{1}H NMR, 200 MHz , $CDCl_3$, δ: 1.46 (s, 9H, *t*Bu); 3.95 (s, 3H, OCH_3); 4.63 (s, 2H, OCH_2); 6.98 (d, 1H, *J*=8.1 Hz, H_{arom}); 7.47 (dd, J_1=8.1 Hz, J_2=1.8 Hz, 1H, H_{arom}); 9.85 (s, 1H, CHO); ^{13}C NMR**,** 50 MHz , $CDCl_3$, δ: 28.1 (3C, CH_3); 56.2 (1C, OCH_3); 66.0 (1C, OCH_2); 82.6 (1C, $C(CH_3)_3$); 110.9 (2C,

CH_{arom}); 127.1 (1C, CH_{arom}); 129.7, 147.7, 154.5 (1C, C_{arom}); 167.0 (1C, COO); 190.2 (1C, CHO).

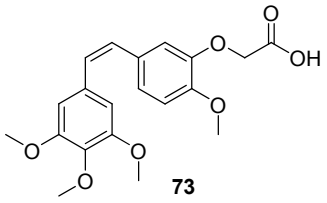
*(Z)-tert-butyl 2-(2-methoxy-5-(3,4,5-trimethoxystyryl)phenoxy)acetate (***75***)*



75

A homogeneous suspension of **67** (0.10 g, 1.91 mmol) in 60 mL of dry THF under nitrogen was cooled to -23 °C and retained at that temperature for 2 h. Butyllithium 1.6 M in hexane (1.20 mL, 1.91 mmol) was added dropwise, the resultant orange solution was stirred at the same temperature for 1 h and finally aldehyde **74** (0.53 g, 1.99 mmol) was added dropwise. The reaction mixture was stirred at -23 °C for 4 h and at r.t. for 18 h, ice-water was added and the two phases separated. The aqueous phase was washed with Et_2O (3x75mL) and the ethereal solution added to the THF layer. The combined organic phase was washed with water (3x75mL), dried over Na_2SO_4, filtered and the solvent eliminated. The crude product was purified by flash chromatography with 3/1=EtP/EtOAc to give both the *Z*-**75** and the *E*-**75** stilbenes in 13 and 15% yield respectively. ^{1}H NMR, 200 MHz , $CDCl_3$, *Z*, δ: 1.41 (s, 9H, *t*Bu); 3.68 (s, 6H, OCH_3); 3.82 (s, 3H, OCH_3); 3.84 (s, 3H, OCH_3); 4.39 (s, 2H, OCH_2); 6.45 (AB system, J = 12.2 Hz, 2H, H_{vinyl}); 6.47 (s, 2H, H_{arom}); 6.70-6.95 (m, 3H, H_{arom}).

*(Z)-2-(2-methoxy-5-(3,4,5-trimethoxystyryl)phenoxy)acetic acid (***73***)*



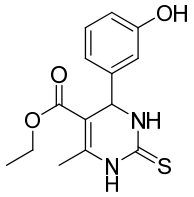
73

1M LiOH (2.40 mL, 2.42 mmol) was added to a solution of *Z*-**75** (0.10 g, 0.24 mmol) in a 1/1=THF/MeOH mixture (8 mL). The reaction mixture was stirred for 2 h, then 1M HCl was added until pH 5 and the aqueous phase was extracted with EtOAc (8x30 mL). Combined organic layers were dried, filtered and evaporated to give acid **73** in 83% yield without further purification. ^{1}H NMR, 400 MHz , CD_3OD, δ: 3.66 (s, 6H, OCH_3); 3.71 (s, 3H, OCH_3); 3.81 (s, 3H, OCH_3); 3.81 (s, 2H, OCH_2); 6.48 (AB system, *J* = 12.0

Hz, 2H, H_{arom}); 6.55 (s, 2H, H_{arom}); 6.86-6.88 (m, 1H, H_{arom}); 6.91 (d, *J* = 1.2 Hz, 1H, H_{arom}). ^{13}C NMR, 100 MHz , $CDCl_3$, δ: 57.0 (3C, OCH_3), 61.3 (1C, OCH_3); 67.2 (1C, OCH_2); 108.0 (2C, CH_{arom}); 114.0 (1C, C_{arom}); 116.8, 124.5, (1C, CH_{arom}); 130.7, 130.8 (1C, CH_{vinil}); 131.7 (1C, CH_{arom}); 134.3, 139.3, 149.1, 151.0 (1C, C_{arom}); 154.9 (2C, C_{arom}); 171.0 (1C, CO). IR: 1124; 1236; 1510; 1577; 1739; 3439 cm^{-1}.

Synthesis of Pentanedioicacid mono-[3-(5-ethoxycarbonyl-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidn-4-yl)-phenyl] ester (77)

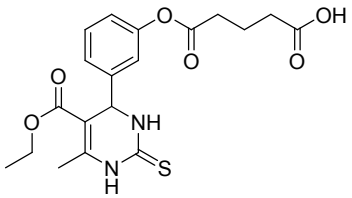
*(3-Hydroxy-phenyl)-6-methyl-2-thioxo-1,2,3,4,-tetrahydropyrimidine-5-carboxylic acid ethyl ester (**76**)*



76

To a mixture of 3-hydroxybenzaldehyde (0.85 g, 7.00 mmol) and thiourea (1.60 g, 21.00 mmol), a solution of ethyl acetoacetate (0.89 mL, 7.00 mmol) in 45 mL of THF dry and $Yb(OTf)_3.xH_2O$ (0.50 g) were added under nitrogen. The suspension was refluxed for 7 h, then diluted with EtOAc and washed with water for 5 times. The organic phase was dried, filtered and evaporated and the crude product was purified by column chromatography with 2/1=cyclohexane/EtOAc as eluent to give **76** in 44% yield. Mp 184–186°C. ^1H NMR,: 1.14 (t, *J*=7.5 Hz, 3H, OCH_2CH_3); 2.30 (s, 3H, CH_3); 4.03 (q, *J*=7.5 Hz, 2H, OCH_2CH_3); 5.11 (d, *J*=3.5 Hz, 1H, CH); 6.61–6.69 (m, 3H, H_{arom}); 7.06–7.17 (m, 1H, H_{arom}); 9.45, 9.62, 10.31 (3 brs, 3H, NH and OH). ^{13}C NMR ,200 MHz , C_6D_6SO, δ: 14.0, 17.2 (1C, CH_3); 54.0 (1C, CH); 59.6 (1C, CH_2); 100.8 (1C, C=C); 113.3, 114.6, 117.0, 129.5 (1C, CH_{arom}); 144.8, 144.9 (1C, C_{arom}); 157.5 (1C, C=C); 165.2 (1C, CO); 174.2 (1C, C=S). IR (KBr) 3300; 3180; 2900–2600; 1670; 1655; 1620; 1575 cm^{-1}.

Pentanedioicacid mono-[3-(5-ethoxycarbonyl-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidn-4-yl)-phenyl] ester (77)

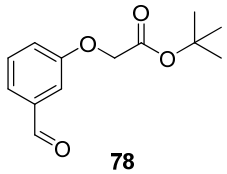


77

Glutaric anhydride (0.18 g, 1.56 mmol) was added to a suspension of **76** (0.11 g, 0.39 mmol) in 10 mL of dry CH_2Cl_2. The resulting solution was stirred for 24 h at r.t. under nitrogen, then refluxed for 27 h, poured onto a saturated solution of $NaHCO_3$ and acidified to pH 5. The aqueous mixture was extracted with $CHCl_3$ and then the organic layer extracted with sat. $NaHCO_3$ solution and water. The recollected aqueous phase was extracted with EtOAc and $CHCl_3$ to give acid **77** in 56% yield, without further purification. ^{1}H NMR, 400 MHz , CD_3OD, δ: 1.14 (t, *J*=7.5 Hz, 3H, OCH_2CH_3); 2.01 (qq, *J*=4.4 Hz, 2H, CH_2); 2.30 (s, 3H, CH_3); 2.43 (t, *J*=4.4 Hz, 2H, CH_2); 2.64 (t, *J*=4.4 Hz, 2H, CH_2); 4.05 (q, *J*=7.5 Hz, 2H, OCH_2CH_3); 5.28 (s, 1H, CH); 7.01–7.07 (m, 2H, H_{arom}); 7.20–7.24 (m, 1H, H_{arom}); 7.28 (t, *J*=7.8 Hz, 1H, H_{arom}); 9.45, 9.62, 10.31 (3 brs, 3H, NH and OH). ^{13}C NMR, 400 MHz , CD_3OD, δ: ^{13}C NMR ,200 MHz , C_6D_6SO, δ: 14.5, 17.7 (1C, CH_3); 21.2, 33.7, 34.0 (1C, CH_2); 56.0 (1C, CH); 61.4 (1C, CH_2); 102.8 (1C, C=C); 121.1, 122.3, 125.1, 130.7 (1C, CH_{arom}); 146.1, 146.4 (1C, C_{arom}); 152.3 (1C, C=C); 167.1 (1C, COO); 173.2 (1C, C=S); 176.3 (1C, COO); 176.6 (1C, COOH).

Synthesis of 4-(3-carboxymethoxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid ethyl ester (80)

*(3-formyl-phenox)-acetic acid tert-butyl ester(**78**)*

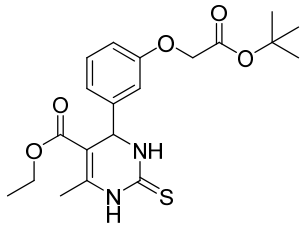


78

To a solution of 3-hydroxybenzaldehyde (1.00 g, 8.20 mmol) in 45 mL of dry CH_3CN, K_2CO_3 was added (2.83 g, 20.49 mmol). The mixture was stirred at 58 °C for 10 min under nitrogen, then a solution of *t*-butylbromoacetate (1.92 g, 9.84 mmol) in 30 mL of dry CH_3CN was added dropwise in 10 min. The reaction mixture was stirred for 2 h at 60 °C before adding a saturated solution of NH_4Cl and extracting with Et_2O. The organic phase was washed with water, dried, filtered and concentrated to give **78** with

90% yield. ^{1}H NMR, 200 MHz , $CDCl_3$, δ: 1.49 (s, 9H, tBu); 4.59 (s, 2H, OCH_2); 7.18–7.55 (m, 4H, H_{arom}); 9.85 (s, 1H, CHO).

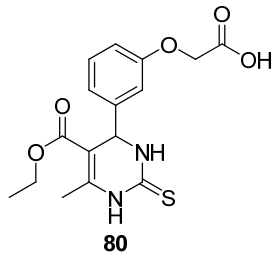
*4-(3-tert-Butoxycarbonylmethoxy-phenyl)-2-thioxo-1,2,3,4,-tetrahydro-pyrimidine-5-carboxylic acid ethyl ester (**79**)*



79

To a mixture of **78** (1.28 g, 5.42 mmol) and thiourea (1.24 g, 16.27 mmol), a solution of ethyl acetoacetate (0.69 mL, 5.42 mmol) in 30 mL of THF dry and $Yb(OTf)_3.xH_2O$ (0.50 g) were added under nitrogen. The suspension was refluxed for 12 h, then diluted with EtOAc and washed with water for 5 times. The organic phase was dried, filtered and evaporated and the crude product was purified by column chromatography with 10/1=cyclohexane/EtOAc as eluent to give **79** in 15% yield (recovering 23% of aldehyde). ^{1}H NMR, 200 MHz, $CDCl_3$, δ: 1.16 (t, *J*=7.4 Hz, 3H, OCH_2CH_3); 1.48 (s, 9H, tBu); 2.35 (s, 3H, CH_3); 4.08 (q, *J*=7.4 Hz, 2H, OCH_2CH_3); 4.45 (s, 2H, OCH_2); 5.17 (d, *J*=3.5 Hz, 1H, CH); 6.65–6.95 (m, 3H, H_{arom}); 7.08–7.22 (m, 1H, H_{arom}); 7.56, 8.08 (2 brs, 2H, NH). ^{13}C NMR, 100 MHz, CD_3OD, δ: 13.7, 17.5 (1C, CH_3); 27.6 (3C, CH_3); 59.7 (1C, CH); 59.9 (1C, CH_2); 65.1 (1C, CH_2); 81.7 (1C, C_{quat}); 101.5 (1C, C=C); 112.9, 113.2, 119.4, 128.9 (1C, CH_{arom}); 143.3, 143.7 (1C, C_{arom}); 157.4 (1C, C=C); 164.6 (1C, COO); 167.3 (1C, COO); 173.1 (1C, C=S).

4-(3-carboxymethoxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid ethyl ester ***(80)***



80

TFA (5.0 mL) was added to a solution of **79** (0.14 g, 0.34 mmol) in 5 mL of CH_2Cl_2. The reaction mixture was stirred at r.t. for 1 h, concentrated and purified by flash chromatography with 7/1=hexane/EtOAc to obtain **80** in 50% yield. ^{1}H NMR, 400 MHz , CD_3OD, δ: 1.17 (t, *J*=7.2 Hz, 3H, OCH_2CH_3); 2.34 (s, 3H, CH_3); 4.09 (q, *J*=7.2 Hz, 2H, OCH_2CH_3); 4.63 (s, 2H, OCH_2); 5.30 (s, 1H, CH); 6.81–6.89 (m, 2H, H_{arom}); 6.92 (d, *J*=7.8 Hz, 1H, H_{arom}); 7.24 (t, *J*=7.8 Hz, 1H, H_{arom}). ^{13}C NMR, 100 MHz, CD_3OD, δ: 14.5, 17.7 (1C, CH_3); 56.2 (1C, CH); 61.3 (1C, CH_2); 65.9 (1C, CH_2); 103.0 (1C, C=C); 114.3, 114.8, 120.7, 130.8 (1C, CH_{arom}); 146.0, 146.3 (1C, C_{arom}); 159.6 (1C, C=C); 167.2 (1C, COO); 172.6 (1C, COOH); 176.2 (1C, C=S).

6-MP release from 14, 27, 43

Quantification of 6-MP release from prodrugs was evaluate via HPLC on a C18 Vydac column with a gradient concentration from 95% A to 5% A in 30 min.

A = H_2O+TFA 0.1%, pH=2-3

B = MeOH

Compounds were revealed at 323nm.

Peptide Synthesis

Peptide synthesis was performed with a MultiSynTech Syro automated multiple peptide synthesizer (Witten, Germany). Protected L-amino acid, coupling reagents (DIPEA and HBTU), NovaSyn TGR resin (crosslinked polystyrene and polyethylene glycol carrying a Rink linker) was purchased from Novabiochem. Side chain protecting groups were trityl for His, 2,2,4,6,7-pentamethyldihydro-benzofuran-5-sulfonyl for Arg, tert-butyl ether for Ser and Tyr, tert-butyl ester for Asp and Glu, and tert-butyloxycarbonyl for Trp. All tetra-branched peptides were built using two consecutive Fmoc-Lys(Fmoc)-OH coupling steps to form the branched core. Peptides were synthesized using Fmoc-Lys(Dde)-OH as the first amino acid on Novasyn TGR resin, then the second aminoacid was Fmoc–PEG–OH. Two coupling steps with Fmoc-Lys(Fmoc)-OH were used to build the core and the tetramer was synthesized using Boc-Arg(Pbf)-OH as the final amino acid of the neurotensin sequence.

Operatively, a mixture of 5 eq. of Fmoc protected aminoacid, 5 eq. of HBTU dissolved in DMF and 10 eq. of DIPEA were added to the resin beads. The reaction was automatically performed at r.t. for 40 minutes and the coupling step repeated twice. After each aminoacid addition the Fmoc protecting group was removed with a 40% solution of piperidine in DMF and extensively washed with DMF before adding the next one. All tetrameric peptides were built employing two consecutive Fmoc-Lys(Fmoc)-OH coupling steps to form the branched core.

The Dde protective group was removed using 2% hydrazine in DMF (v/v) for 10 min at room temperature and the free amino group was coupled with Fmoc–PEG–OH. The Fmoc group was then removed to enable coupling with Functional Unit.

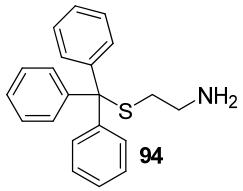
Peptides were cleaved from the resin and deprotected with 95% TFA, 2.5% TIS and 2.5 % H_2O (v/v). The mixture was stirred for 1h at r.t. and the solution removed by filtration. This sequence was repeated teice. After the addition of cold diethylether the peptide precipitated. The crude was centrifuged and the solution removed to eliminate

the impurities. After three washings, dendrimer was dissolved in 0.5 % of acetic acid, freezed and liophylized.

Unrelated analogues were synthesized analogously and the N-terminus was acetylated before the conjugation steps. HPLC purification was performed on a C18 Vydac column and revealed at 280 nm. Water (A) containing 0.1% TFA and methanol (B) were used as eluents, gradients of B in 30 min were run at flow rates of 0.8 ml/min and 4 ml/min for analytical and preparatory procedures, respectively. The gradient used is: from 80% to 1% of A. All compounds were also characterized on an Ettan MALDI-TOF mass spectrometer (Amersham Biosciences, Buckinghamshire, UK). As an example: [NT(8-13)4**-47] 85**. HPLC: t_R = 28.42 min (1-80% B); t_R = 22.21 min (1-70% B). MALDI-ToF *m/z*: $[M]^+$ 4723.93 (calcd 4725.64).

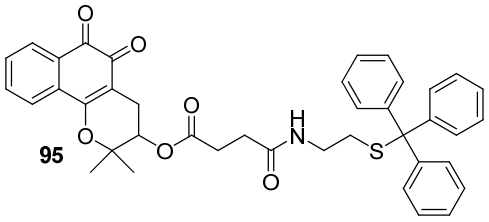
Synthesi of 2,2-dimethyl-5,6-dioxo-3,4,5,6-tetrahydro-2H-benzo[h]chromen-3-yl 4-(2-mercaptoethylamino)-4-oxobutanoate (96)

*2-(tritylthio)ethanamine (**94**)*



Triphenylmethanethio chloride (0.56 g, 2.00 mmol) was dissolved in 1 mL of dry CH_2Cl_2. To this solution cysteamine hydrochloride (0.26 g, 2.30 mmol) and TFA (0.40 mL) were added to form a dark red brown solution. The reaction mixture was stirred at r.t. for 2 h under argon, then 1N NaOH (3mL) was added. The oil obtained was diluted with CH_2Cl_2 (150 mL) and a saturated solution of $NaHCO_3$ (150 mL). The two phases were separated and the aqueous layer was extracted with CH_2Cl_2 (3x50mL). The combined organic layers were washed with water (3x100mL), dried over Na_2SO_4, filtered and evaporated. The crude product was purified by flash chromatography with 6/1=Hexane/Ethylacetate as eluent to give thioether **94** in 99% yield. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 2.36 (t, *J*=6.5 Hz, 2H), 2.63 (t, *J*=6.5 Hz, 2H), 7.23-7.25 (m, 3H), 7.26-7.34 (m, 6H), 7.48-7.50 (m, 6H). ESI-MS: $[M+Na]^+$: 342.5.

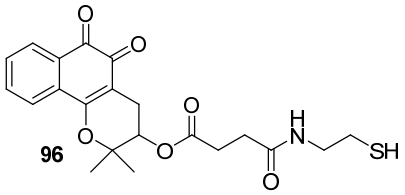
*2,2-dimethyl-5,6-dioxo-3,4,5,6-tetrahydro-2H-benzo[h]chromen-3-yl 4-oxo-4-(2-(tritylthio)ethylamino)butanoate (**95**)*



Compound **94** (0.28 g, 0.44 mmol) and **64** (0.08 g, 0.49 mmol) were dissolved in 8 mL of DMSO. The solution was degassed with argon and cooled to 0 °C before adding HBTU (0.21 g, 0.56 mmol) and DIPEA (0.24 g, 1.79 mmol). The reaction mixture was then stirred at r.t. for 64 h, concentrated and, after the addition of EtOAc, washed with HCl 1M and water. The resulting solution was dried over Na_2SO_4, filtered and concentrated to give the desired derivative **95** in 99% yield without further purification. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.43 (s, 3H, CH_3), 1.48 (s, 3H, CH_3), 2.36 (t, *J*=6.5 Hz,

2H), 2.62-2.74 (m, 8H, H-4 and CH_2), 5.17 (t, 1H, *J* = 4.8 Hz, H-3), 7.23-7.25 (m, 3H), 7.26-7.34 (m, 6H), 7.48-7.50 (m, 6H), 7.53 (dt, *J* = 1.0 and 6.4 Hz 1H, H_{arom}), 7.64 (dt, *J* = 1.6 and 6.0 Hz 1H, H_{arom}), 7.84 (dd, *J* = 1.0 and 6.0 Hz 1H, H_{arom}), 7.64 (dt, *J* = 1.4 and 6.6 Hz 1H, H_{arom}).

*2,2-dimethyl-5,6-dioxo-3,4,5,6-tetrahydro-2H-benzo[h]chromen-3-yl 4-(2-mercaptoethylamino)-4-oxobutanoate (**96**)*

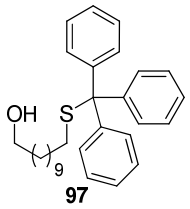


96

Compound **95** (0.45 g, 0.68 mmol) was dissolved in 7 mL of CH_2Cl_2 and TFA (1.80 mL, 15.00 mmol) was added, followed by TIPS (240 µL, 1.13 mmol). The mixture was stirred under argon for 32h. The solvent was removed under vacuum and the residue was purified by washing with hexane (3 times) and Et_2O (4 times) to obtain thiol **96** with 98% yield. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.43 (s, 3H, CH_3), 1.48 (s, 3H, CH_3), 2.36 (t, *J*=6.5 Hz, 2H), 2.62-2.74 (m, 8H, H-4 and CH_2), 5.17 (t, 1H, *J* = 4.8 Hz, H-3), 7.53 (dt, *J* = 1.0 and 6.4 Hz 1H, H_{arom}), 7.64 (dt, *J* = 1.6 and 6.0 Hz 1H, H_{arom}), 7.84 (dd, *J* = 1.0 and 6.0 Hz 1H, H_{arom}), 7.64 (dt, *J* = 1.4 and 6.6 Hz 1H, H_{arom}). ESI(MS): 682.2.

Synthesis of 26,26,26-triphenyl-2,5,8,12-tetraoxa-25-thiahexacosan-1-ol (99)

*12-(tritylthio)dodecan-1-ol (**97**)*

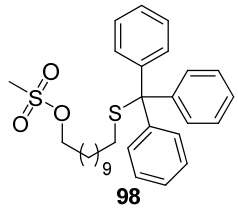


97

Triphenylmethanethiol (26.41 g, 95.54 mmol) was dissolved in EtOH/toluene (67 mL/67 mL). To this solution 10 mL of NaOH 12M (4.78 g, 119.43 mmol) and a solution of 11-Bromo-1-undecanol (20.00 g, 79.62 mmol) in EtOH/toluene (33 mL/33 mL) were added. The red mixture was stirred at 60°C for 4h, then the solvent was removed. The oil obtained was diluted with CH_2Cl_2 (150 mL) and a saturated solution of $NaHCO_3$ (150 mL). The two phases were separated and the aqueous layer was extracted with CH_2Cl_2 (3x50mL). The combined organic layers were washed with water

(3x100mL), dried over Na_2SO_4, filtered and evaporated. The crude product was purified by flash chromatography with 6/1=hexane/EtOAc as eluent to give thioether **97** in 99% yield. PM=446.6 ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.30-1.42 (m, 18H, CH_2); 1.52-1.59 (m, 2H, CH_2); 2.13 (t, J=7.2 Hz, 2H, CH_2); 3.64 (t, J=6.4 Hz, 2H, CH_2); 7.18-7.25 (m, 3H, H_{arom}); 7.26-7.46 (m, 12H, H_{arom}).

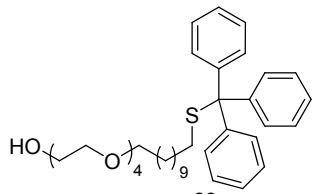
*12-(tritylthio)dodecyl methanesulfonate (**98**)*



98

TEA (22.00 mL, 159.20 mmol) was added to a solution of **97** (35.60 g, 79.60 mmol) in 140 mL CH_2Cl_2 at 0°C. At this temperature Methanesulfonyl chloride (9.2 mL, 119.4 mmol) was added dropwise by a syringe. The reaction mixture was allowed to go to room temperature, stirred for 3h and then diluted with CH_2Cl_2. The solution was washed with a saturated solution of $NaHCO_3$ (4x100 mL) and water (2x100mL). The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated. The crude product was purified by flash chromatography starting with 80/1 and gradually going to 5/1 =Hexane/Ethylacetate as eluent to give sulfonyl derivative **98** in 80% yield. PM= 524.24 ^{1}H NMR, 400 MHz, CD_3OD , δ: 1.23-1.35 (m, 16H, CH_2); 1.70-1.80 (m, 2H, CH_2); 2.16 (t, *J*=7.6 Hz, 2H, CH_2); 3.08 (s, 3H, CH_3); 4.26 (t, *J*=6.4 Hz, 2H, CH_2); 7.24-7.26 (m, 3H, H_{arom}); 7.30-7.33 (m, 6H, H_{arom}). 7.41-7.44 (m, 6H, H_{arom}).

*26,26,26-triphenyl-2,5,8,12-tetraoxa-25-thiahexacosan-1-ol (**99**)*



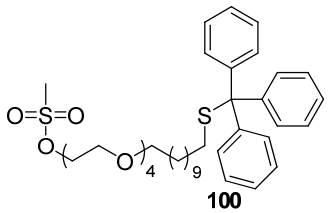
99

To tetraethyleneglycol (TEG) (160.00 mL, 932.80 mmol), 50% aq. NaOH (2.45 g, 62.20 mmol) was added. The resulting mixture was heated at 90°C for 1h under argon, then allowed to return to r.t. and poured onto **98** (32.60 g, 62.20 mmol). The reaction was stirred under argon at 90°C for 24, extracted with 5/1=Hexane/EtOAc (6x100 mL) and concentrated. The residue was purified by flash chromatography with 5/1=

Hexane/EtOAc → EtOAc as eluent to yield alcohol **99** in 58% yield. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.10-1.39 (m, 16H, CH_2); 1.53-1.58 (m, 2H, CH_2); 2.14 (t, *J*=7.2 Hz, 2H, CH_2); 2.64 (m, 1H, OH); 3.44 (t, *J*=6.8 Hz, 2H, CH_2); 3.46-3.72 (m, 16H, CH_2); 7.18-7.29 (m, 9H, H_{arom}); 7.39-7.40 (m, 6H, H_{arom}).

Synthesis of 28-mercapto-4,4-dimethyl-6,9,12,16-tetraoxa-4-azoniaoctacosan-4-ium-1-sulfonate (103)

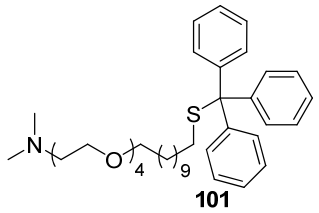
*26,26,26-triphenyl-2,5,8,12-tetraoxa-25-thiahexacosyl methanesulfonate (**100**)*



100

TEA (1.30 mL, 9.63 mmol) was added to a solution of **99** (3.00 g, 4.82 mmol) in 9 mL of CH_2Cl_2 at 0°C. At this temperature methanesulfonyl chloride (0.60 mL, 7.68 mmol) was added dropwise by a syringe. The reaction mixture was allowed to go to room temperature, stirred for 3h and then diluted with CH_2Cl_2. The solution was washed with a saturated solution of $NaHCO_3$ (4x100 mL) and water (2x100mL). The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated to give sulfonyl derivative **100** in 89% yield. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.10-1.39 (m, 16H, CH_2); 1.53-1.58 (m, 2H, CH_2); 2.05 (t, *J*=7.0 Hz, 2H, CH_2); 3.08 (s, 3H, CH_3); 3.44 (t, *J*=7.0 Hz, 2H, CH_2); 3.56-3.72 (m, 12H, CH_2); 3.76 (t, *J*=2.8 Hz, 2H, CH_2); 4.38 (t, *J*=2.8 Hz, 2H, CH_2); 7.16-7.20 (m, 3H, H_{arom}); 7.22-7.29 (m, 6H, H_{arom}); 7.39-7.42 (m, 6H, H_{arom}).

*N,N-dimethyl-26,26,26-triphenyl-2,5,8,12-tetraoxa-25-thiahexacosan-1-amine (**101**)*

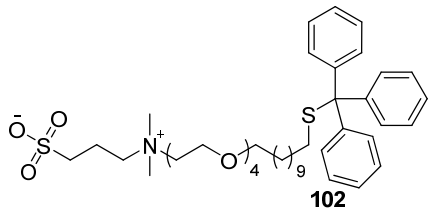


101

Dimethylamine hydrochloride (34.95 g, 428.00 mmol) and KOH were stirred as a mixture of solids in a flask connected by a canula to another flask with a solution of **100** (3.00 g, 4.28 mmol) in 100 mL of dry THF. The dimethylamine was bubbled as a gas into the solution for 5h, the reaction mixture was then stirred overnight. The solvent

was removed under reduced pressure and the crude product dissolved in CH_2Cl_2 and washed with water (6 times). The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated to give dimethylamine derivative **101** in 85% yield. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.40 (m, 18H, CH_2); 2.14 (t, *J*=7.3 Hz, 2H, CH_2); 2.53 (t, *J*=5.8 Hz, 2H, CH_2); 2.84 (s, 6H, $N(CH_3)_2$); 3.44 (t, *J*=6.8 Hz, 2H, OCH_2); 3.56-3.65 (m, 14H, CH_2), 7.16-7.20 (m, 3H, H_{arom}); 7.22-7.29 (m, 6H, H_{arom}); 7.39-7.42 (m, 6H, H_{arom}).^{13}C NMR, 400 MHz, $CDCl_3$, δ: 26.10, 28.60, 29.02, 29.19, 29.41, 29.48, 29.56, 29.65, 32.04, 45.81, 58.77, 66.36, 69.23, 70.07, 70.40, 70.61, 71.55, 126.49, 127.79, 129.62, 145.10.

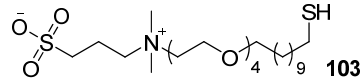
*27,27-dimethyl-1,1,1-triphenyl-15,19,22,25-tetraoxa-2-thia-27-azoniatriacontan-27-ium30sulfonate (**102**)*



102

An anhydrous acetone solution (100 mL) of compound **101** (2.20 g, 3.38 mmol) and 1,3-propanesultone (0.32 g, 6.44 mmol) was stirred at r.t. for 3h. The reaction mixture was filtered, and the resulting solid was washed with hexane/EtOAc=4/1 (8 times) and dried under vacuum to afford sulfonyl derivative **102** in 85% yield. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.40 (m, 18H, CH_2); 2.12 (t, *J*=7.3 Hz, 2H, SCH_2); 2.26 (m, 2H, CH_2); 2.88 (t, *J*=6.7 Hz, 2H, CH_2); 3.23 (s, 6H, $N(CH_3)_2$); 3.42 (t, J = 6.8 Hz, 2H, OCH_2); 3.56-3.71 (m, 14H, CH_2); 3.78 (m, 2H, NCH_2); 3.94 (m, 2H, OCH_2); 7.16-7.20 (m, 3H, H_{arom}); 7.22-7.29 (m, 6H, H_{arom}); 7.39-7.42 (m, 6H, H_{arom}). ESI(MS): 794.6 $[M+Na]^+$

*28-mercapto-4,4-dimethyl-6,9,12,16-tetraoxa-4-azoniaoctacosan-4-ium-1-sulfonate (**103**)*



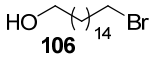
103

Compound **102** (0.60 g, 0.77 mmol) was dissolved in 8 mL of CH_2Cl_2 and TFA (1.80 mL, 15.54 mmol) was added, followed by TIPS (240 µL, 1.16 mmol). The mixture was stirred under argon overnight. The solvent was removed under vacuum and the residue was purified by washing with Hexane (7 times) and Et_2O (twice) to obtain thiol **103** with 74% yield. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.40 (m, 18H, CH_2); 2.26 (brs, 2H,

CH_2); 2.49 (q, *J*=7.2 Hz, 2H, $HSCH_2$); 2.90 (brs, 2H, CH_2); 3.04 (s, 6H, $N(CH_3)_2$); 3.41 (t, *J*=6.8 Hz, 2H, OCH_2); 3.52-3.79 (m, 16H, CH_2); 3.91 (m, 2H, OCH_2). ESI(MS): 552.4 $[M+Na]^+$.

Synthesis of 16-(tritylthio)hexadecan methanesulfonate (108)

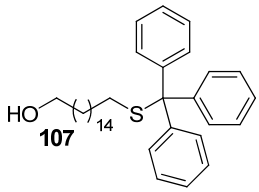
*16-bromohexadecan-1-ol (**106**)*



106

To a solution of hexadecane-1,16-diol (0.75 g, 2.90 mmol) in 7.5 mL of cyclohexane, HBr (7.5 mL) was added. The reaction mixture was refluxed for 6h, then diluted with hexane and washed with a saturated solution of $NaHCO_3$ (3x50 mL) and water. The organic layer was dried over Na_2SO_4, filtered and evaporated to give bromide **106** in 99% yield. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.25-1.42 (m, 22H, CH_2); 1.49-1.55 (m, 4H, CH_2); 1.80 (m, 2H, CH_2); 3.39 (t, *J*=6.8 Hz, 2H, OCH_2); 3.62 (t, *J*=6.8 Hz, 2H, $BrCH_2$).

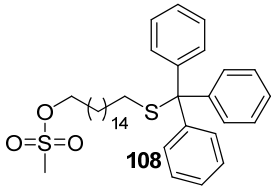
*12-Tritylthio hexadecan-1-ol (**107**)*



107

Triphenylmethanethiol (1.58 g, 5.73 mmol) was dissolved in EtOH/toluene (10 mL/10 mL). To this solution 0.6 mL of NaOH 12M (0.29 g, 7.16 mmol) and a solution of **106** (0.93 g, 2.58 mmol) in EtOH/toluene (4 mL/4 mL) were added. The red mixture was stirred at 60°C for 4h, then the solvent was removed. The oil obtained was diluted with CH_2Cl_2 (150 mL) and a saturated solution of $NaHCO_3$ (150 mL). The two phases were separated and the aqueous layer was extracted with CH_2Cl_2 (3x50mL). The combined organic layers were washed with water (3x100mL), dried over Na_2SO_4, filtered and evaporated. The crude product was purified by flash chromatography with 3/1=hexane/EtOAc as eluent to give thioether **107** in 30% yield. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.15-1.42 (m, 22H, CH_2); 1.49-1.55 (m, 4H, CH_2); 1.80 (m, 2H, CH_2); 2.05 (t, *J*=6.8 Hz, 2H, CH_2); 3.03 (t, *J*=6.8 Hz, 2H, OCH_2); 7.18-7.25 (m, 3H, H_{arom}); 7.26-7.46 (m, 12H, H_{arom}).

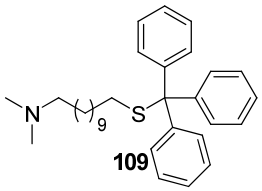
*16-(tritylthio)hexadecan methanesulfonate (**108**)*



TEA (0.22 mL, 1.16 mmol) was added to a solution of **107** (0.40 g, 0.78 mmol) in 10 mL of CH_2Cl_2 at 0°C. At this temperature methanesulfonyl chloride (0.09 mL, 1.16 mmol) was added dropwise by a syringe. The reaction mixture was allowed to go to room temperature, stirred for 5h and then diluted with CH_2Cl_2. The solution was washed with a saturated solution of $NaHCO_3$ (4x20 mL) and water (2x20mL). The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated to give sulfonyl derivative **108** in 64% yield. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.15-1.42 (m, 26H, CH_2); 1.80 (m, 2H, CH_2); 2.08 (t, *J*=6.8 Hz, 2H, SCH_2); 3.00 (s, 3H, CH_3); 4.22 (t, *J*=8.8 Hz, 2H, OCH_2); 7.16-7.20 (m, 3H, H_{arom}); 7.22-7.29 (m, 6H, H_{arom}); 7.39-7.42 (m, 6H, H_{arom}).

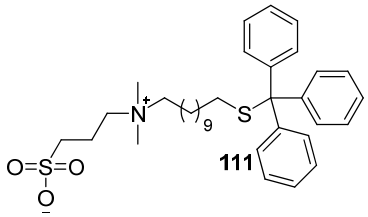
Synthesis of 3-((13-mercaptotridecyl)dimethylammonio)propane-1-sulfonate (104)

*Tritylthio-13-(dimethylamino)tridecane-1-thiol (**109**)*



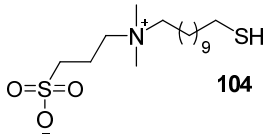
Dimethylamine hydrochloride (28.00 g, 343.35 mmol) and KOH were stirred as a mixture of solids in a flask connected by a canula to a solution of **98** (1.80 g, 3.43 mmol) in 50 mL of dry THF. The dimethylamine was bubbled as a gas into the solution for 8h under vigorous stirring, the reaction mixture was then stirred overnight. The solvent was removed under reduced pressure and the crude product dissolved in CH_2Cl_2 and washed with water (6 times). The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated to give dimethylamine derivative **109** in 98% yield. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.15-1.28 (m, 16H, CH_2); 1.36-1.38 (m, 2H, CH_2); 2.12 (t, *J*=7.6 Hz, 2H, SCH_2); 2.33 (s, 6H, CH_3); 2.34-2.38 (m, 2H, CH_2); 7.18-7.35 (m, 10H, H_{arom}); 7.35-7.50 (m, 5H, H_{arom}).

*Tritylthio-3-((13-mercaptotridecyl)dimethylammonio)propane-1-sulfonate (**111**)*



An anhydrous acetone solution (80 mL) of compound **109** (1.60 g, 3.38 mmol) and 1,3-propanesultone (0.78 g, 6.42 mmol) was stirred overnight at r.t.. The reaction mixture was filtered, and the resulting solid was washed with hexane/EtOAc=4/1 (8 times) and dried under vacuum to afford sulfonyl derivative **111** in 75% yield. ^{1}H NMR, 400 MHz, CD_3OD, δ: 1.15-1.42 (m, 16H, CH_2); 1.72-1.80 (m, 2H, CH_2); 2.10-2.30 (m, 4H, CH_2); 2.51-2.55 (m, 2H, CH_2); 2.88 (t, *J*=6.7 Hz, 2H, CH_2); 3.07 (s, 6H, $N(CH_3)_2$); 3.20-3.30 (m, 2H, CH_2); 3.48-3.54 (m, 2H, CH_2); 7.16-7.20 (m, 3H, H_{arom}); 7.22-7.29 (m, 6H, H_{arom}); 7.39-7.42 (m, 6H, H_{arom}). ^{13}C NMR, 400 MHz, $CDCl_3$, δ: 19.1, 22.5, 23.5, 26.1, 28.4, 28.8, 29.0, 29.2, 29.3, 31.8, 42.9, 43.9, 47.6, 50.5, 53.4, 62.9, 64.3, 66.1, 68.8, 126.3, 127.6, 129.4, 144.8. ESI(MS) : 618.4 $[M+Na]^+$.

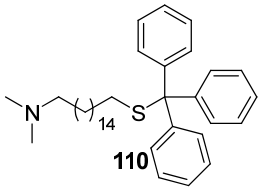
*3-((13-mercaptotridecyl)dimethylammonio)propane-1-sulfonate (**104**)*



Compound **111** (0.45 g, 0.75 mmol) was dissolved in 7 mL of CH_2Cl_2 and TFA (1.80 mL, 15.00 mmol) was added, followed by TIPS (240 μL, 1.13 mmol). The mixture was stirred under argon for 32h. The solvent was removed under vacuum and the residue was purified by washing with hexane (3 times) and Et_2O (4 times) to obtain thiol **104** with 98% yield. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.19-1.39 (m, 14H, CH_2); 1.54-1.78 (m, 4H, CH_2); 2.18-2.30 (m, 2H, CH_2); 2.49 (q, *J*=7.2 Hz, 2H, $HSCH_2$); 2.90 (brs, 2H, CH_2); 3.10-3.23 (s, 6H, $N(CH_3)_2$); 3.24-3.30 (m, 2H, CH_2); 3.58-3.74 (m, 2H, CH_2). ESI(MS): 354.2 $[M+Na]^+$.

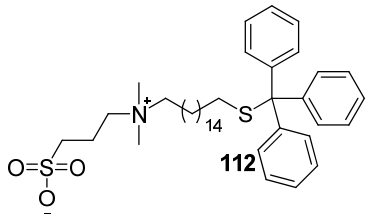
Synthesis of 3-((16-mercaptohexadecyl)dimethylammonio)propane-1-sulfonate (105)

*Tritylthio-16-(dimethylamino)hexadecane-1-thiol (**110**)*



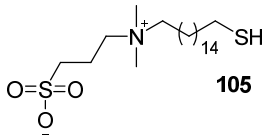
Dimethylamine hydrochloride (4.08 g, 50.00 mmol) and KOH were stirred as a mixture of solids in a flask connected by a canula to a solution of **108** (0.30 g, 0.50 mmol) in 10 mL of dry THF. The dimethylamine was bubbled as a gas into the solution for 8h under vigorous stirring, the reaction mixture was then stirred overnight. The solvent was removed under reduced pressure and the crude product dissolved in CH_2Cl_2 and washed with water (6 times). The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated to give dimethylamine derivative **110** in 96% yield. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.15-1.42 (m, 26H, CH_2); 1.80 (m, 2H, CH_2); 2.12 (t, *J*=7.2 Hz, 2H, SCH_2); 2.40 (s, 6H, CH_3); 2.49 (t, *J*=7.6 Hz, 2H, OCH_2); 7.18-7.35 (m, 10H, H_{arom}); 7.35-7.50 (m, 5H, H_{arom}).

*Tritylthio-3-((16-mercaptohexadecyl)dimethylammonio)propane-1-sulfonate (**112**)*



An anhydrous acetone solution (10 mL) of compound **110** (0.26 g, 0.48 mmol) and 1,3-propanesultone (0.11 g, 0.90 mmol) was stirred overnight at r.t.. The reaction mixture was filtered, and the resulting solid was washed with hexane/EtOAc=4/1 (8 times) and dried under vacuum to afford sulfoxide derivative **112** in 63% yield. ^{1}H NMR, 400 MHz, CD_3OD, δ: 1.15-1.42 (m, 30H, CH_2); 2.10-2.30 (m, 4H, CH_2); 2.55-2.65 (m, 2H, CH_2); 2.88 (t, *J*=6.7 Hz, 2H, CH_2); 3.07 (s, 6H, $N(CH_3)_2$); 3.48-3.54 (m, 4H, CH_2); 7.16-7.20 (m, 3H, H_{arom}); 7.22-7.29 (m, 6H, H_{arom}); 7.39-7.42 (m, 6H, H_{arom}). ESI(MS): 688.4 $[M+Na]^+$.

*3-((16-mercaptohexadecyl)dimethylammonio)propane-1-sulfonate (**105**)*

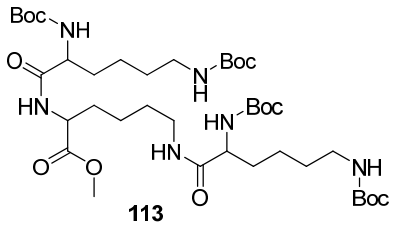


105

Compound **112** (0.20 g, 0.30 mmol) was dissolved in 2 mL of CH_2Cl_2 and TFA (0.70 mL, 6.00 mmol) was added, followed by TIPS (100 µL, 0.45 mmol). The mixture was stirred under argon for 32h. The solvent was removed under vacuum and the residue was purified by washing with hexane (7 times) and Et_2O (twice) to obtain thiol **105** with 79% yield. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.19-1.39 (m, 26H, CH_2); 1.54-1.78 (m, 4H, CH_2); 2.18-2.30 (m, 2H, CH_2); 2.49 (q, *J*=7.2 Hz, 2H, $HSCH_2$); 2.90 (brs, 2H, CH_2); 3.10-3.35 (s, 10H, $N(CH_3)_2$ + CH_2); 3.58-3.74 (m, 2H, CH_2). ESI(MS): 446.2 $[M+Na]^+$.

Synthesis of 23-mercapto-3,6,9,12-tetraoxatricosyl-2,6-bis(2,6-diaminohexanamido)hexanoate (116)

methyl 16-(tert-butoxycarbonylamino)-6-(4-(tert-butoxycarbonylamino)butyl)-2,2,24,24-tetramethyl-4,7,15,22-tetraoxo-3,23-dioxa-5,8,14,21-tetraazapentacosane-9-carboxylate (**113**)

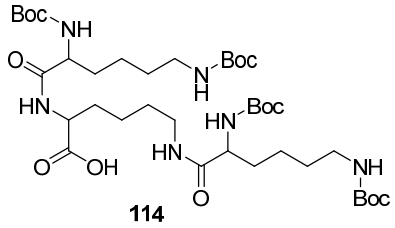


113

L-Lysine methyl ester dihydrochloride (2.33 g, 10.00 mmol) was suspended in 83 mL of EtOAc. TEA (3.00 mL, 22.00 mmol) was added, followed by Boc-Lys(Boc)-OH.DCHA (12.60 g, 22.00 mmol). The mixture was stirred under argon for 2 minutes and then cooled to 0°C. Hydroxybenzotriazole (HOBt, 2.97 g, 22.00 mmol) and dicyclohexyl carbodiimmide (DCC, 4.54 g, 22.00 mmol) were added simultaneously as a mixture of solids. The reaction mixture was allowed to warm at rt and stirred for 24h. The precipitate was removed by filtration and discarded. The filtrate was washed with a saturated solution of $NaHCO_3$ (3x100 mL) and water (1x100mL). The organic phase was dried over Na_2SO_4, filtered and concentrated to give a white solid. This residue was purified by flash chromatography with 30/1=CH_2Cl_2/MeOH to obtain G1(COOMe) **113** with 97% yield. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.35-1.85 (m, 54H, CH_2 and CH_3);

2.94-2.99 (m, 4H, CH_2NH); 2.94-3.20 (m, 4H, CH_2NH); 3.47-3.52 (m, 2H, CH_2NH); 3.71 (s, 3H, CH_3); 4.08-4.13 (m, 1H, CH); 4.25-4.39 (m, 2H, CH); 4.75 (br s, 1H, NH-Boc); 4.90 (br s, 1H, NH-Boc); 5.55 (br s, 1H, NH-Boc); 5.90 (br s, 1H, NH-Boc); 6.92 (br s, 1H, CONH); 7.35 (br s, 1H, CONH).

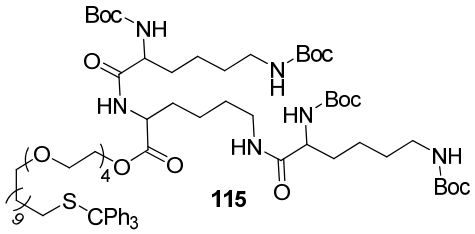
16-(tert-butoxycarbonylamino)-6-(4-(tert-butoxycarbonylamino)butyl)-2,2,24,24-tetramethyl-4,7,15,22-tetraoxo-3,23-dioxa-5,8,14,21-tetraazapentacosane-9-carboxylic acid (**114**)



114

1M NaOH (26.60 mL, 26.60 mmol) was added to a solution of **113** (7.50 g, 9.20 mmol) in 375 mL of CH_2Cl_2 at 0°C, under argon atmosphere. The reaction mixture was then stirred at r.t. for 24h, concentrated and, after the addition of water (200 mL), acidified to pH 3 with aqueous $NaHSO_4$. The product was extracted with EtOAc (5x150 mL) and washed with water and brine. The resulting solution was dried over Na_2SO_4, filtered and concentrated to give the desired dendrimer **114** in 95% yield. ^{1}H NMR, 400 MHz, $(CD_3)_2CO$), δ: 1.35-1.85 (m, 54H, CH_2 and CH_3); 2.94-2.99 (m, 4H, CH_2NH); 2.94-3.20 (m, 4H, CH_2NH); 3.47-3.52 (m, 2H, CH_2NH); 3.71 (s, 3H, CH_3); 4.08-4.13 (m, 1H, CH); 4.25-4.39 (m, 2H, CH); 4.75 (br s, 1H, NH-Boc); 4.90 (br s, 1H, NH-Boc); 5.55 (br s, 1H, NH-Boc); 5.90 (br s, 1H, NH-Boc); 6.92 (br s, 1H, CONH); 7.35 (br s, 1H, CONH).

*Tritylthio-23-mercapto-3,6,9,12-tetraoxatricosyl-2,6-bis(2,6-diaminohexanamido)hexanoate (**115**)*

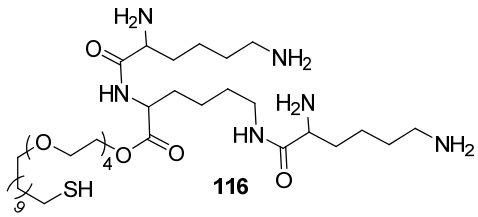


115

G1-COOH **114** (1.00 g, 1.25 mmol) was dissolved in 15 mL of CH_2Cl_2 and cooled to 0°C under argon. DMAP (0.05 g, 0.40 mmol) was added, followed by DCC (0.403 g,

1.94 mmol). The mixture was stirred under argon for 10 minutes. A solution of **99** (0.554 g, 0.89 mmol) in 8 mL of CH_2Cl_2 was added. The reaction mixture was allowed to warm at rt and stirred for 24h. The solvent was removed under vacuum and the residue was purified by flash chromatography with 80/1=CH_2Cl_2/MeOH as eluent to obtain G1-conjugate **115** with 99% yield. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.10-1.95 (m, 73H, CH_2 and CH_3); 2.12 (t, *J*=7.2 Hz, 2H, CH_2); 2.84-3.15 (m, 6H, CH_2NH); 3.43 (t, *J*=6.8 Hz, 2H, CH_2); 3.55-3.73 (m, 16H, CH_2); 4.06-4.12 (m, 1H, CH); 4.25-4.30 (m, 2H, CH); 4.75 (br s, 1H, NH-Boc); 4.90 (br s, 1H, NH-Boc); 5.55 (br s, 1H, NH-Boc); 5.90 (br s, 1H, NH-Boc); 6.60 (br s, 1H, CONH); 6.92 (br s, 1H, CONH); 7.15-7.29 (m, 9H, H_{arom}); 7.39-7.40 (m, 6H, H_{arom}).

23-mercapto-3,6,9,12-tetraoxatricosyl-2,6-bis(2,6-diaminohexanamido)hexanoate ***(116)***



116

Compound **115** (0.25 g, 0.18 mmol) was dissolved in 10 mL of CH_2Cl_2 and TFA (1 mL) was added, followed by triisopropylsilane (TIPS) (60 µL). The mixture was stirred under argon for 24h. The solvent was removed under vacuum and the residue was washed with Hexane (6 times) and Et_2O (twice) to obtain G1-conjugate **116** with 99% yield. ^{1}H NMR, 400 MHz, CD_3OD, δ: 1.00-1.95 (m, 36H, CH_2 and CH_3); 2.50 (t, *J*=7.2 Hz, 2H, CH_2); 2.90-2.93 (m, 4H, CH_2NH_2); 3.15-3.26 (m, 2H, CH_2); 3.41-3.47 (m, 2H, CH_2); 3.56-3.81 (m, 16H, CH_2); 3.96 (br s, 1H, CH); 4.43 (br s, 1H, CH); 4.79 (br s, 1H, CH).

Synthesis of Gold (~ 2 nm) nanoparticles

C5 AuNP were prepared by following the Brust-Schiffrin two-phase method using 1-pentanethiol as capping ligands.

Operatively a solution of tetrabutylammonium bromide (TOAB, 4.20 g, 7.62 mmol) in 150 mL of toluene was mixed with a solution of $HAuCl_4$ (2.00 g, 5.08 mmol) in 150 mL of water. The mixture was stirred for 10 min while the toluene layer became red. Pentanethiol (0.74 g, 7.11 mmol) was then added and the reaction stirred for 10 min, cooled and a solution of $NaBH_4$ (3.84 g, 101.60 mmol) in 12 mL of water was added dropwise. The two-phase mixture was stirred overnight at r.t., then separated and the organic layer was evaporated at 30 °C under vacuum, diluted with CH_2Cl_2 and the AuNPs precipitated by the addition of diethyl ether. After washing several times with ethanol the C5 AuNPs were dried and stored at r.t.

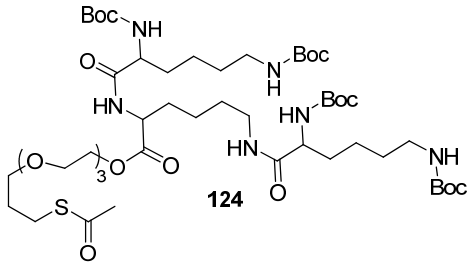
To fabricate **117**, **118**, **119** and **120** via ligand place exchange reaction, 30 mg of C5 NP were mixed with 100 mg of **103**, **104**, **105** and **116** respectively in dichloromethane (10 mL). The solution was stirred at room temperature for 24 h. Solvent was removed by rotary evaporator and the nanoparticles were washed with diethyl ether (20 mL x 5). The nanoparticles were further purified by dialysis in MiliQ water using SnakeSkin pleated dialysis tubing (10,000 MWCO) for 1 day.

Preparation and characterization of AuNPZwit complexes (AuNPZwit-dye/drug conjugates)

In order to prepare AuNPZwit complexes, solvent displacement method was carried out using water and acetone. An acetone solution of each guest compound (10 mg) and aqueous solution of **117** (80 μM) were well mixed. Acetone was then removed by rotary evaporator or slow evaporation at room temperature. During the evaporation of acetone, some guest compounds were entrapped in interior of AuNPZwit due to hydrophobic interaction and the rest of guest compounds were precipitated out. The mixture was then filtered using a vacuo filter (0.2 μm pore) to remove the precipitate. For further purification, the AuNPZwit **117** complexes solution was washed with distilled water and centrifuged with Amicon Ultra-4 tube (10,000 MWCO) several times (2 mL x~5 times) until no absorbance of guest molecules in filtrate was detected by spectrophotometer.

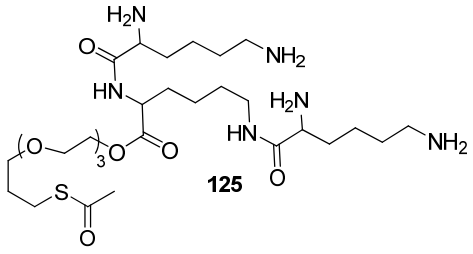
Synthesis of 2-(2-(2-(3-mercaptopropoxy)ethoxy)ethoxy)ethyl 2,6-bis(2,6-diaminohexanamido)hexanoate (122)

*13-oxo-3,6,9-trioxa-12-thiatetradecyl 16-(tert-butoxycarbonylamino)-6-(4-(tert-butoxycarbonylamino)butyl)-2,2,24,24-tetramethyl-4,7,15,22-tetraoxo-3,23-dioxa-5,8,14,21-tetraazapentacosane-9-carboxylate (**124**)*



G1-COOH (**114**) (1.00 g, 1.25 mmol) was dissolved in 15 mL of CH_2Cl_2 and cooled to 0°C under argon. DMAP (0.05 g, 0.40 mmol) was added, followed by DCC (0.40 g, 1.94 mmol). The mixture was stirred under argon for 10 minutes. A solution of **123** (0.554 g, 0.89 mmol) in 8 mL of CH_2Cl_2 was added. The reaction mixture was allowed to warm at rt and stirred for 24 h. The solvent was removed under vacuum and the residue was purified by flash chromatography with 80/1=CH_2Cl_2/MeOH followed by 20/1 to obtain G1-conjugate **124** with 99% yield. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.30-1.95 (m, 56H, CH_2 and CH_3); 2.30 (s, 3H, CH_3); 2.86-3.00 (m, 6H, CH_2NH); 3.24-3.30 (m, 2H, CH_2S); 3.48-3.73 (m, 14H, CH_2); 4.06-4.12 (m, 1H, CH); 4.25-4.30 (m, 2H, CH); 4.75 (br s, 1H, NH-Boc); 4.90 (br s, 1H, NH-Boc); 5.55 (br s, 1H, NH-Boc); 5.90 (br s, 1H, NH-Boc); 6.60 (br s, 1H, CONH); 6.92 (br s, 1H, CONH).

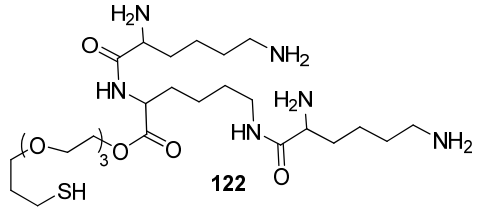
*2-(2-(2-(2-mercaptoethoxy)ethoxy)ethoxy)ethyl 16-(tert-butoxycarbonylamino)-6-(4-(tert-butoxycarbonylamino)butyl)-2,2,24,24-tetramethyl-4,7,15,22-tetraoxo-3,23-dioxa-5,8,14,21-tetraazapentacosane-9-carboxylate (**125**)*



Compound **124** (0.20 g, 0.19 mmol) was dissolved in 5 mL of CH_2Cl_2 and TFA (1 mL) was added, followed by triisopropylsilane (TIPS) (40 μL). The mixture was stirred

under argon overnight. The solvent was removed under vacuum and the residue was washed with Hexane (7 times) and Et_2O (twice) to obtain G1-conjugate **125** with 99% yield. ^{1}H NMR, 400 MHz, CD_3OD, δ: 1.30-1.95 (m, 20H, CH_2 and CH_3); 2.30 (s, 3H, CH_3); 2.86-3.00 (m, 6H, CH_2NH); 3.24-3.30 (m, 2H, CH_2S); 3.48-3.73 (m, 12H, CH_2); 3.85 (t, 2H, CH_2); 3.90-4.00 (m, 1H, CH); 4.20-4.35 (m, 1H, CH); 4.35-4.50 (m, 1H, CH).

*2-(2-(2-(3-mercaptopropoxy)ethoxy)ethoxy)ethyl 2,6-bis(2,6-diaminohexanamido)hexanoate (**122**)*

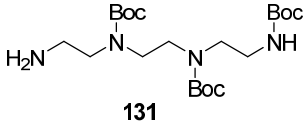


122

Compound **125** (0.12 g, 0.19 mmol) was dissolved in 10 mL of MeOH, the solution was cooled at 0°C and acetyl chloride (0.22 g, 2.85 mmol) was added slowly under argon atmosphere. The reaction mixture was allowed to rise automatically to r.t. and stirred for 3h. The solvent was removed under reduced pressure to obtain thiol **122** with 86% yield. ^{1}H NMR, 400 MHz, CD_3OD, δ: 1.40-2.00 (m, 20H, CH_2 and CH_3); 2.58 (t, 2H, CH_2); 2.88-3.02 (m, 6H, CH_2NH); 3.22-3.28 (m, 2H, CH_2); 3.55-3.73 (m, 12H, CH_2); 3.89-3.93 (m, 2H, CH_2); 3.95-4.09 (m, 2H, CH); 4.30-4.50 (m, 1H, CH). ESI(MS): 609.5 $[M+Na]^+$.

Synthesis of N-(2-(2-(2-aminoethylamino)ethylamino)ethyl)-4-((4-(3,4-dihydroxyphenethylamino)-4-oxobutyl)disulfanyl)butanamide (126) and N1-(2-(2-(2-aminoethylamino)ethylamino)ethyl)-N10-(3,4-dihydroxyphenethyl)decanediamide (127)

*N1, N2, N3-tris(tert-butoxycarbonyl)-triethylenetetramine (**131**)*

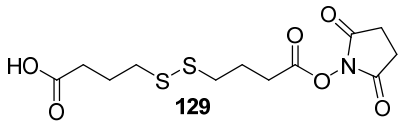


131

To a solution of triethylenetetramine (1.00 g, 6.84 mmol) in 80 mL of dry MeOH at -78 °C under N_2, ethyltrifluoroacetate (0.97 g, 6.84 mmol) was added dropwise over 45 mins. Stirring was continued for another 45 min at -78 °C, the temperature was increased to 0 °C and the mixture was further stirred for 1h. Without isolation of the

mono-trifluoroacetamide derivative obtained, the remaining amino groups were protected by dropwise addition of di-*tert*-butyl dicarbonate (5.56 g, 25.64 mmol) in 10 mL of dry MeOH over 30 min at 0 °C. The reaction was then allowed to go to r.t. and stirred for 20 h before removing the solvent. The residue was dissolved in CH_2Cl_2 and washed with a saturated solution of $NaHCO_3$ and water. The organic layer was dried, filtered and evaporated. The crude residue was purified by column chromatography with 50/1= CH_2Cl_2/MeOH as eluent to give the protected oligoethyleneamine in 90% yield. The next reaction was carried out directly on a solution of thw fully protected compound (3.00 g, 6.00 mmol) in 79 mL of MeOH where potassium carbonate (0.88 g in 7.6 mL of H_2O) was added and refluxed for 20 h. The solvent was removed under reduced pressure and the residue was diluted with CH_2Cl_2 and washed with H_2O. The organic layer was dried over Na_2SO_4 and concentrated to yield the crude product, that was then purified by column chromatography with 80/20/1= CH_2Cl_2/MeOH/NH_4OH as eluent to obtain amine **131** with 30% yield. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.39 (s, 2H, NH_2), 1.43 (s, 27H, $C(CH_3)_3$), 2.79 (bt, 2H, CH_2NH_2), 3.23–3.30 (m, 10H, CH_2N and CH_2NH).

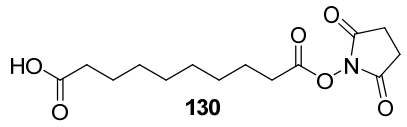
*4-((4-(2,5-dioxopyrrolidin-1-yloxy)-4-oxobutyl)disulfanyl)butanoic acid (**129**)*



129

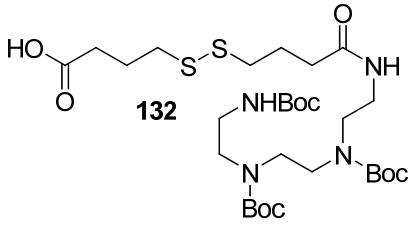
To a solution of dithiodibutyric acid **34** (2.00 g, 8.40 mmol) in dry THF (15 mL), N-hydroxysuccinimide (1.16 g, 10.08 mmol) was added followed by a solution of DCC (1.77 g, 8.57 mmol) in 5 mL of dry THF at 0°C. The mixture was stirred for 15 min, then DMAP (0.01, 0.08 mmol) was added. The reaction mixture was allowed to go to r.t. and stirred overnight. The white precipitate was filtered and the solvent was removed from the filtrate to give a residue that was then dissolved in CH_2Cl_2 and washed with water. The organic layer was dried over Na_2SO_4, filtered and evaporated. The crude product obtained was purified by flash chromatography with 1/1=EtOAc/CH_2Cl_2 as eluent to give activated acid **129** in 72% yield. ^{1}H NMR, 400 MHz, CD_3OD, δ: 1.98 (quintet, *J*=7.2 Hz, 2H, CH_2); 2.14 (quintet, *J*=7.2 Hz, 2H, CH_2); 2.42 (t, *J*=7.2 Hz, 2H, CH_2); 2.72-2.89 (m, 10H, CH_2).

*10-(2,5-dioxopyrrolidin-1-yloxy)-10-oxodecanoic acid (**130**)*



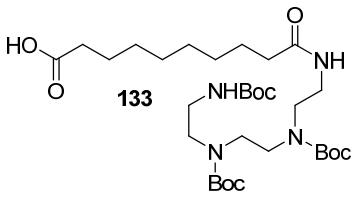
Sebacic acid **128** (2.00 g, 9.89 mmol) was dissolved in dry THF (25 mL). To this solution N-hydroxysuccinimide (1.36 g, 11.87 mmol) was added followed by a solution of DCC (2.02 g, 10.09 mmol) in 5 mL of dry THF at 0°C. The mixture was stirred for 15 min, then DMAP (0.01 g, 0.08 mmol) was added. The reaction mixture was allowed to go to r.t. and stirred for 7 h. The white precipitate was filtered and the solvent was removed from the filtrate to give a residue that was then re-dissolved in CH_2Cl_2 and washed with water. The organic layer was dried over Na_2SO_4, filtered and evaporated and the crude product obtained was purified by flash chromatography with 1/2=EtOAc/CH_2Cl_2 as eluent to give activated acid **130** in 72% yield. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.20-1.42 (m, 12H, CH_2); 1.55 (quintet, *J*=7.2 Hz, 2H, CH_2); 1.70 (quintet, *J*=7.2 Hz, 2H, CH_2); 2.30 (t, *J*=7.2Hz, 2H, CH_2); 2.30 (t, *J*=7.2Hz, 2H, CH_2); 2.56 (t, *J*=7.2Hz, 2H, CH_2); 2.80 (s, 4H, CH_2).

*8,11-bis(tert-butoxycarbonyl)-2,2-dimethyl-4,15-dioxo-3-oxa-19,20-dithia-5,8,11,14-tetraazatetracosan-24-oic acid (**132**)*



TEA was added to a solution of **131** (0.20 g, 0.45 mmol) in DMF/H_2O (5 mL/5 mL), until pH 9 is reached. This basic solution was then added to a solution of **129** (0.15 g, 0.45 mmol) in 15 mL of DMF. The reaction mixture was stirred for 18 h under argon, then poured onto water and extracted with EtOAc (6x100 mL). The recollected organic layers were washed with water, dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash chromatography with 25/1=CH_2Cl_2/MeOH → 15/1 as eluent to yield amide **132** in 98% yield. ^{1}H NMR, 400 MHz, CD_3OD, δ: 1.43 (s, 9H, CH_3); 1.47 (s, 18H, CH_3); 1.96-2.01 (m, 5H, CH_2 + NH); 2.29-2.31 (m, 2H, CH_2); 2.42 (t, *J*=7.2 Hz, 2H, CH_2); 2.69-2.76 (m, 4H, CH_2); 3.18 (t, *J*=6.0 Hz, 2H, CH_2); 3.26-3.37 (m, 10H, CH_2).

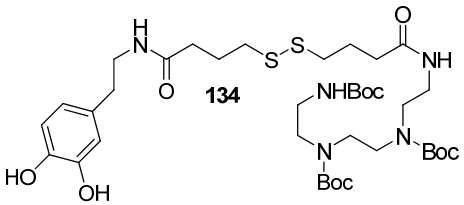
*8,11-bis(tert-butoxycarbonyl)-2,2-dimethyl-4,15-dioxo-3-oxa-5,8,11,14-tetraazatetracosan-24-oic acid (**133**)*



133

TEA was added to a solution of **131** (0.20 g, 0.45 mmol) in DMF/H_2O (5 mL/5 mL), until pH 9 is reached. This basic solution was then added to another solution of **130** (0.15 g, 0.45 mmol) in 11 mL of DMF. The reaction mixture was stirred for 18 h under argon, then poured onto water and extracted with EtOAc (6x100 mL). The recollected organic layers were washed with water, dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash chromatography with 50/1=CH_2Cl_2/MeOH as eluent to yield amide **133** in 99% yield. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.20-1.30 (m, 12H, CH_2); 1.43 (s, 9H, CH_3); 1.47 (s, 18H, CH_3); 1.52-1.61 (m, 1H, NH); 2.13 (at, *J*=7.2 Hz, 2H, CH_2); 2.30 (t, *J*=7.2 Hz, 2H, CH_2); 3.18-3.45 (m, 12H, CH_2).

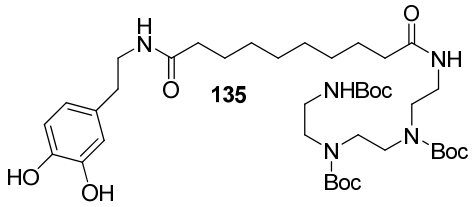
*Dopamine-8,11-bis(tert-butoxycarbonyl)-2,2-dimethyl-4,15-dioxo-3-oxa-19,20-dithia-5,8,11,14-tetraazatetracosan-24-oic amide (**134**)*



134

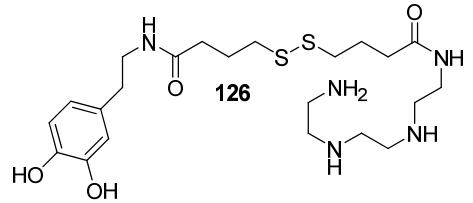
Compound **132** (0.37 g, 0.56 mmol) and dopamine hydrochloride (0.10 g, 0.61 mmol) were dissolved in 10 mL of DMSO. The solution was degassed under argon and cooled to 0 °C before adding HBTU (0.26 g, 0.70 mmol) and DIPEA (380 µL, 2.22 mmol). The reaction mixture was then stirred at r.t. for 64 h, concentrated and, after the addition of EtOAc washed with HCl 1M and water. The resulting solution was dried over Na_2SO_4, filtered and concentrated to give the desired dopamine derivative **134** in 79% yield without further purification. ^{1}H NMR, 400 MHz, $(CD_3)_2CO$, δ: 1.43 (s, 9H, CH_3); 1.47 (s, 18H, CH_3); 1.95-2.01 (m, 4H, CH_2); 2.24-2.28 (m, 24H, CH_2); 2.61-2.72 (m, 4H, CH_2); 3.19 (t, *J*=6.0 Hz, 2H, CH_2); 3.26-3.37 (m, 10H, CH_2), 6.51 (dd, J_1 =8.0 Hz, J_2 =1.8 Hz, 1H, H_{arom}); 6.32-6.68 (m, 2H, H_{arom}).

*Dopamine-,11-bis(tert-butoxycarbonyl)-2,2-dimethyl-4,15-dioxo-3-oxa-5,8,11,14-tetraazatetracosan-24-oic amide (**135**)*



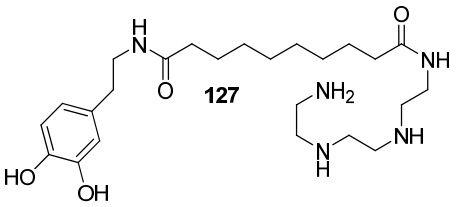
Compound **133** (0.28 g, 0.44 mmol) and dopamine hydrochloride (0.08 g, 0.49 mmol) were dissolved in 8 mL of DMSO. The solution was degassed with argon and cooled to 0 °C before adding HBTU (0.21 g, 0.56 mmol) and DIPEA (0.24 g, 1.79 mmol). The reaction mixture was then stirred at r.t. for 64 h, concentrated and, after the addition of EtOAc, washed with HCl 1M and water. The resulting solution was dried over Na_2SO_4, filtered and concentrated to give the desired dopamine derivative **135** in 99% yield without further purification. ^{1}H NMR, 400 MHz, $CDCl_3$, δ: 1.21-1.29 (m, 12H, CH_2); 1.35-1.59 (m, 31H, CH_3 + CH_2); 2.11-2.17 (m, 4H, CH_2); 2.62 (at, *J*=7.2 Hz, 2H, CH_2); 3.19 (bs, 2H, CH_2); 3.27-3.41 (m, 10H, CH_2); 6.51 (d, *J*=8.0 Hz, 1H, H_{arom}); 6.66-6.75 (m, 2H, H_{arom}).

*N-(2-(2-(2-aminoethylamino)ethylamino)ethyl)-4-((4-(3,4-dihydroxyphenethylamino)-4-oxobutyl)disulfanyl)butanamide (**126**)*



Compound **134** (0.34 g, 0.43 mmol) was dissolved in 15 mL of CH_2Cl_2 and TFA (5.00 mL, 43.17 mmol) was added. The mixture was stirred under argon for 4 h, then the solvent was removed under vacuum and the residue was purified by washing with Hexane (7 times) and Et_2O (twice) to obtain amine **126** with 90% yield. ^{1}H NMR, 400 MHz, CD_3OD, δ: 1.87-2.03 (m, 4H, CH_2); 2.25 (t, *J*=7.2 Hz, 2H, CH_2); 2.34 (t, *J*=7.2 Hz, 2H, CH_2); 2.64 (at, *J*=7.2 Hz, 2H, CH_2); 2.71 (at, *J*=7.2 Hz, 2H, CH_2); 3.25 (bs, 2H, CH_2); 3.27-3.41 (m, 14H, CH_2); 6.51 (d, *J*=8.0 Hz, 1H, H_{arom}); 6.66-6.75 (m, 2H, H_{arom}).

*N1-(2-(2-(2-aminoethylamino)ethylamino)ethyl)-N10-(3,4-dihydroxyphenethyl)decanediamide (**127**)*



127

Compound **135** (0.34 g, 0.45 mmol) was dissolved in 15 mL of CH_2Cl_2 and TFA (5.00 mL, 43.17 mmol) was added. The mixture was stirred under argon for 4 h. The solvent was removed under vacuum and the residue was purified by washing with Hexane (3 times) and Et_2O (twice) to obtain amine **127** in 99% yield. PM=466

^{1}H NMR, 400 MHz, CD_3OD, δ: 1.21-1.29 (m, 8H, CH_2); 1.51-1.62 (m, 4H, CH_2); 2.13 (t, *J*=7.2 Hz, 2H, CH_2); 2.22 (t, *J*=7.6 Hz, 2H, CH_2); 2.61-2.69 (m, 2H, CH_2); 3.23 (bs, 2H, CH_2); 3.27-3.41 (m, 12H, CH_2); 6.51 (d, *J*=8.0 Hz, 1H, H_{arom}); 6.66-6.75 (m, 2H, H_{arom}).

Synthesis of FePt (~ 4 nm) nanoparticles[99]

One typical synthetic procedure is as follows: under airless condition, platinum acetylacetonate (197 mg, 0.5 mmol), 1,2-hexadecanediol (390 mg, 1.5 mmol), and dioctylether (20 ml) were mixed and heated to 100°C. Oleic acid (0.16 ml, 0.5 mmol), oleyl amine (0.17 ml, 0.5 mmol), and $Fe(CO)_5$ (0.13 ml, 1 mmol) were added, and the mixture was heated to reflux (297°C). The refluxing was continued for 30 min. The heat source was then removed, and the reaction mixture was allowed to cool to room temperature. The inert gas protected system could be opened to ambient environment at this point. The black product was precipitated by adding ethanol (40 ml) and separated by centrifugation. Yellow-brown supernatant
was discarded. The black precipitate was dispersed in hexane (25 ml) in the presence of oleic acid (0.05 ml) and oleyl amine (0.05 ml) and precipitated out by adding ethanol (20 ml) and centrifuging. The product was dispersed in hexane (20 ml), centrifuged to remove any unsolved precipitation (almost no precipitation was found at this stage), and precipitated out by adding ethanol (15 ml) and centrifuging. The materials were redispersed and stored in hexane solution.

Synthesis of water soluble FePtNPs **136** and **137**, 5 mg of oleic acid and oleylamine stabilized FePt NPs were mixed with 10 mg of deprotected derivative of **99** (SH-C11-TEG-OH) in CH_2Cl_2. The mixture was stirred overnight then to the resulting SH-C_{11}-TEG-OH functionalized FePtNPs, 7.5 mg of DOP-PEI (**126** or **127**) were added and the reaction mixture stirred for 2 days at 35 °C. The functionalized NPs were then purified by multiple filtrations through a molecular weight cut-off filter and dissolved in water.

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