Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN

SCIENZE CHIMICHE

CICLO XXVII

COORDINATORE Prof. Carlo Alberto Bignozzi

Bile acid and indole scaffolds for the synthesis of new biologically relevant compounds

Settore Scientifico Disciplinare CHIM/06

Dottorando

Dott. Massarenti Chiara

_____

(firma)

Tutore

Prof. Olga Bortolini

_____

(firma)

Anni 2012/2014

A mio padre

Sommario

In questo lavoro di Tesi, sono stati impiegati scaffolds di acidi biliari e di anelli indolici per la sintesi di diverse tipologie di molecole di interesse biologico in tre differenti progetti.

Il primo progetto riguarda la sintesi e la valutazione biologica di nuovi coniugati acidi biliari-deossiadenosine, ottenuti impiegando una reazione di 'click' chemistry per legare i due derivati biologicamente attivi. In particolare, la cicloaddizione azide-alchino catalizzata da Cu(I) è stata utilizzata per sintetizzare i nuovi coniugati, partendo da derivati azidici in posizione C-3 degli acidi biliari e da una serie di deossiadenosine funzionalizzate in posizione C-8 con residui alchinici terminali di diversa natura e lunghezza. L' attività anti-proliferativa delle nuove molecole sintetizzate è stata testata *in vitro* in quattro linee cellulari umane così come la citotossicità nei confronti di linee cellulari di fibroblasti umani. Vari derivati sintetizzati hanno dimostrato una forte attività anti-proliferativa nei confronti di alcune linee cellulari leucemiche umane e i risultati migliori sono stati ottenuti con un derivato basato sullo scaffold dell'acido chenodeossicolico in entrambe le linee leucemiche, con valori di IC_{50} fino a 8.51 μM. E' stata inoltre valutata l'attività apoptotica dei nuovi coniugati.

Il secondo progetto riguarda la sintesi di quattro scaffolds biliari ortogonalmente funzionalizzabili che presentano un gruppo azide in posizione C-3 ed una funzionalità tiolica in C-24 legata mediante un residuo di cisteammina. Lo scaffold steroideo dell'acido chenodesossicolico è stato impiegato per la sintesi di nuovi derivati monofunzionalizzati acido biliare-bis-fosfonato mediate reazioni ortogonali di 'click' chemistry. In particolare sono state utilizzate le reazioni di cicloaddizione azide-alchino catalizzata da Cu(I) e l'accoppiamento tiolo-alchene e tiolo-alchino. Inoltre, è stata sintetizzata una serie di nuovi derivati bifunzionalizzati acido biliare-bis-fosfonato impiegando un tag fluorescente, sfruttando la stessa strategia di 'click' chemistry utilizzata per la sintesi dei derivati monofunzionalizzati. Infine è stata studiata una metodologia adeguata, basata sulle condizioni di McKenna, per la deprotezione dell'estere del bis-fosfonato ad acido libero. Viste le problematiche riscontrate nella purificazione dei nuovi coniugati sintetizzati, è stato impiegato un tag fluorurato per semplificare e velocizzare questo processo.

Il terzo progetto, svolto presso il laboratorio del Professor Matteo Zanda all'Università di Aberdeen (UK), riguarda la sintesi di nuovi composti a base indolica modulatori allosterici positivi (PAMs) dei recettori del cannabinoidi CB_1 per il trattamento del dolore. Per evitare effetti collaterali di tipo psicoattivo, abbiamo deciso di concentrarci su il sito di legame

allosterico del recettore CB_1 usando un modulatore allosterico positivo che presenta il vantaggio di aumentare l'effetto degli endocannabinoidi solamente nel sito di rilascio, agendo quindi come pain relief, dove richiesto. ZCZ011 ha dimostrato un'eccellente attività *in vivo* nel trattamento del dolore non adeguatamente controllabile con i normali antidolorifici. Per poter studiare meglio la relazione struttura-attività di questo nuovo composto sono stati sintetizzati una serie di derivati che presentano modifiche a livello della posizione 5, 6 e 2 dello scaffold indolico. Inoltre è stata studiata anche l'importanza dei sostituenti sulla catena laterale. I nuovi derivati sintetizzati sono stati studiati *in vitro* per testarne la potenza e sono stati evidenziati risultati interessanti: cinque nuovi composti si sono dimostrati in grado di aumentare il livello massimo di stimolazione sul recettore CB_1 causato dal legando endogeno anandamide rispetto al lead compound ed un derivato in particolare, potrebbe aprire le porte allo sviluppo di un composto che agisca selettivamente a livello periferico.

Abstract

In this PhD Thesis work, bile acid and indole scaffolds were employed to synthetize new biologically relevant compounds in three different projects.

The first project is focused on the synthesis and biological evaluation of novel deoxyadenosine-bile acid conjugates linked through a 1,2,3-triazole ring. A 'click' chemistry reaction, namely the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC), was employed to achieve the new conjugates, starting from 3-azidobile acid derivatives and 8-alkynylated deoxyadenosines. All novel molecules were evaluated *in vitro* for their anti-proliferative activity against four human cell lines and for their cytotoxicity toward human fibroblast cells. Several conjugates exhibited strong anti-proliferative activity against human leukemia T cells. The best cytotoxicity was observed for a chenodeoxycholic acid-based derivative on both leukemia cell lines with IC_{50} up to 8.51 μM. Furthermore, the apoptotic activity of several conjugates was established. This work resulted in a scientific publication.

The second project is focused on the synthesis of four orthogonally functionalized bile acid scaffolds displaying an azido group at C-3 and a thiol moiety at C-24 through a cysteamine linker. The chenodeoxycholic-based modified scaffold was employed in 'click' chemistry orthogonal reactions to synthetize a collection of chenodeoxycholic acid-bisphosphonate tetraethyl esters. Notably, Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC), thiol-ene coupling (TEC) and thiol-yne (TYC) were used and their effectiveness and flexibility were demonstrated. Moreover, a series of fluorescently-labelled analogues of these new bile acid-bisphosphonate derivatives was accomplished with the same methodology. Deprotection of bisphosphonate esters was studied under McKenna conditions and a new bile acid-bisphosphonic acid analogue was achieved. On the last note, a fluorous tag strategy was successfully employed to overcome the purification and separation issues that limited the isolated yields of all the new bile acid-bisphosphonate derivatives synthetized.

The third project, developed in the laboratory of Professor Matteo Zanda at the University of Aberdeen (UK), is focused on the use of the indole scaffold for the synthesis of new positive allosteric modulators (PAMs) of cannabinoid receptors CB_1 for the treatment of pain. Targeting the allosteric binding site on the CB_1 receptors using positive allosteric modulators (PAMs) is a very promising approach because it would cause activation of the endocannabinoid system without causing the unwanted psychotropic effects, thus providing the potential of side-effect-free pain relief where required. In particular, the

structure-activity relationship for ZCZ011, an indole-based new PAM, was investigated replacing substituents at 2-position, 5-position and 6- position of the indole scaffold as well as the thienyl side chain. Hence, in order to easily access a good number of analogues for ZCZ011, a general synthetic strategy was developed. All the new analogues synthetized were evaluated *in vitro* for their ability to potentiate the maximum level of stimulation on CB_1 receptors caused by the endogenous agonist anandamide and some interesting results have been highlighted: five new compounds exhibited higher potency than the lead compound and one in particular could open the possibility to further development, in order to achieve a peripherally restricted positive allosteric modulator of CB_1 receptors.

Table of contents

List of acronyms:

AcOH Acetic acid

aq. Aqueous

DCM Dichloromethane

DIPEA *N,N*-Diisopropylethylamine

DMAP 4-Dimethylaminopyridine

DMF *N,N*-Dimethylformamide

DMSO Dimethyl sulfoxide

EtOAc Ethyl acetate

EtOH Ethanol

GPCRs G-Protein coupled receptors

MeOH Methanol

MTT 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide

MW Microwave

NH_4OH Ammonium hydroxide solution

o.n. Overnight

PEG Polyethylene glycol

r.t. Room temperature

TBAB Tetrabutylammonium bromide

TBTA *tris*-(Benzyltriazolylmethyl)amine

***t*BuOH** *tert*-Butanol

***t*-BuOK** Potassium *tert*-butoxide

TEA Triethylamine

TFA Trifluoroacetic acid

THF Tetrahydrofuran

TMEDA *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine

TMG *N*,*N*,*N*′,*N*′-Tetramethylguanidine

TMS-Br Bromotrimethylasilane

Trt-Cl Triphenylmethyl chloride

TsOH *p*-Toluenesulfonic acid

1 Introduction:

1.1 "Click chemistry":

In 2001, Sharpless *et al.*[1] published a landmark review describing the basic elements of a new style of organic synthesis, or as the authors also presented it, "the reinvigoration of an old style of organic synthesis". The name "click chemistry" was coined to describe this new approach, born to accelerate the discovery of new substances, especially in medicinal chemistry.

The approach derives from the authors' consideration that organic chemists have long made the error of thinking that they should mimic nature in finding ways of creating new C–C bonds. But most synthetic reactions that exist to do so, have only very modest thermodynamic driving forces, which means they are inefficient and give only low yields. That is a huge burden for pharmaceutical chemists because, every low-yield step in a multi-step synthesis of some biologically active molecules, reduces the final yield of product, making the synthesis wasteful and costly and slowing down the process of drug discovery. Hence, Sharpless and his co-workers proposed to focused their efforts on generating substances, in a very efficient way, by joining small units together with heteroatom links (C-X-C) while leaving the tough job of C-C bonds synthesis to nature.

A set of stringent criteria that a process must meet to be included in the context of "click chemistry" has been defined: the reaction must be modular, wide in scope, high yielding, create only inoffensive by-products and be stereospecific. Hence, a click process proceed rapidly to completion and it is highly selective for a single product. Moreover, the process must be orthogonal with other common organic synthesis reactions, simple to perform, in terms of reaction conditions, with readily available starting materials and reagents, employing neat conditions or using a solvent that is benign or easy to remove. Purification, if required, must be non-chromatographic, such as crystallization or distillation, and the product must be stable under physiological conditions. Last but not least, click reactions are strongly exothermic processes, either by virtue of highly energetic reactants or strongly stabilized products.[1]

To date, four major classifications of click reactions have been identified:[2]

- Cycloadditions reaction e.g., 1,3-dipolar cycloadditions and hetero-Diels-Alder cycloadditions;

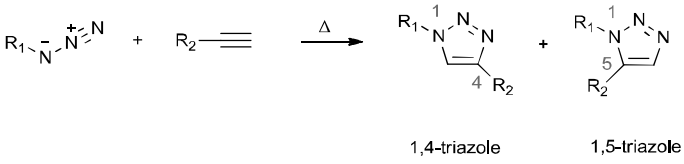
- Nucleophilic ring-openings reactions e.g., the openings of strained heterocyclic electrophiles, such as aziridines, epoxides, cyclic sulfates, aziridinium ions, episulfonium ions;
- Carbonyl chemistry of the non-aldol type e.g., the formations of oxime ethers, hydrazones and aromatic heterocycles;
- Additions to carbon-carbon multiple bonds e.g., Michael addition reactions, epoxidations, aziridinations, sulfenyl and nitrosyl halide additions.

1.1.1 The copper(I)-catalyzed azide-alkyne cycloaddition:

Among all the reactions that achieved the "click status", the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) has emerged as the quintessential example of a click reaction and it is often referred to simply as "the click reaction".

The Huisgen 1,3-dipolar cycloaddition reaction of organic azides and terminal alkynes[3] to afford 1,2,3-triazoles has gained considerable attention since the introduction of Cu(I) catalysis[4] that drastically changed the mechanism and the outcome of the reaction.

Azides and alkynes are easy to install and essentially inert to most biological and organic conditions, including molecular oxygen and water. In particular, kinetic factors allow organic azides to remain "invisible" until a good dipholarophile is present.[5] In fact, azides and alkynes, despite being among the most energetic species known, are also among the least reactive functional groups in organic chemistry, thus their uncatalyzed cycloaddition requires elevated temperature and long reaction times. Furthermore, the uncatalyzed Huisgen cycloaddition is not regioselective and usually give a mixture of the 1,4- and 1,5-regioisomers. (Scheme 1)



Scheme 1. Products of thermal 1,3-cycloaddition.

Cu(I)-catalysis dramatically improves regioselectivity to afford exclusively the 1,4-regioisomer and increases the rate of the reaction by a factor of 10^7 relative to the thermal process, eliminating the need for elevated temperatures. Moreover, CuAAC exhibits several other features that make it unique among other block-ligation reactions: it is not

significantly affected by the steric and electronic properties of the groups attached to the azide and alkyne centres, thus primary, secondary and even tertiary, electron-deficient and electron-rich, aliphatic, aromatic and heteroaromatic azides usually react well with variously substituted terminal alkynes;[6] it is unaffected by most organic functional groups therefore eliminating the need for protecting groups and proceeds in many protic and aprotic solvents. However, keeping the reactants soluble throughout the reaction is a key requirement for a successful outcome.[7] The 1,2,3-triazole unit that results from the reaction has a high chemical stability, in general, being inert to severe hydrolytic, oxidizing, and reducing conditions, even at high temperature, it has a strong dipole moment and a good hydrogen-bond-accepting ability. Thus, 1,2,3-triazole can be useful in medicinal chemistry serving as non-classical bioisostere of the amide bond.[8]

CuAAC is an extraordinarily robust reaction, which could be performed under a wide variety of conditions and with almost any source of solvated Cu(I). Indeed, different copper(I) sources can be utilised in this reaction: Cu(I) salts (iodide, bromide, chloride, acetate), *in situ* reduction of Cu(II) salts, copper coordination complexes such as $[Cu(CH_3CN)_4]PF_6$ or comproportionation of Cu(0) in the form of wires, turnings, and powder. Although the procedure based on the use of Cu(0) benefits from high selectivity that usually provides very pure triazoles with very low copper contamination, making it convenient for biological systems, it suffers from prolonged reaction times.[6]

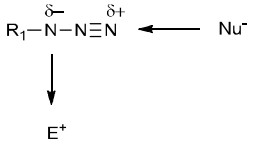
Among the three most common oxidation states of copper (0, +1, +2), the Cu(I) oxidation state is least thermodynamically stable and cuprous ion can be oxidized to catalytically inactive cupric species or can disproportionate to a mixture of Cu(II) and Cu(0). Hence, some precaution need to be taken to avoid this oxidation.

When the reaction is performed in organic solvents, the most common conditions employed are CuI in THF, CH_3CN, DMSO or DMF in the presence of a basic amine like DIPEA or TEA.[7] In this case, exclusion of oxygen may be required in order to avoid the oxidation of the reactive Cu(I) catalyst to unreactive Cu(II) species or a stabilizing ligand has to be added in the reaction mixture (e.g., TBTA).[9]

When the reaction is performed in aqueous conditions or in a water/alcohol mixtures (e.g., water/*tert*-butanol), most frequently, $CuSO_4$/ascorbate is employed as catalyst.[5] In this case, the catalytically active Cu(I) species is generated *in situ* by reduction with ascorbate, a mild reductant, providing a practical alternative to oxygen-free conditions. Of course, Cu(I) salts can also be used in combination with ascorbate, wherein it converts any oxidized Cu(II) species back to catalytically active ones.[6]

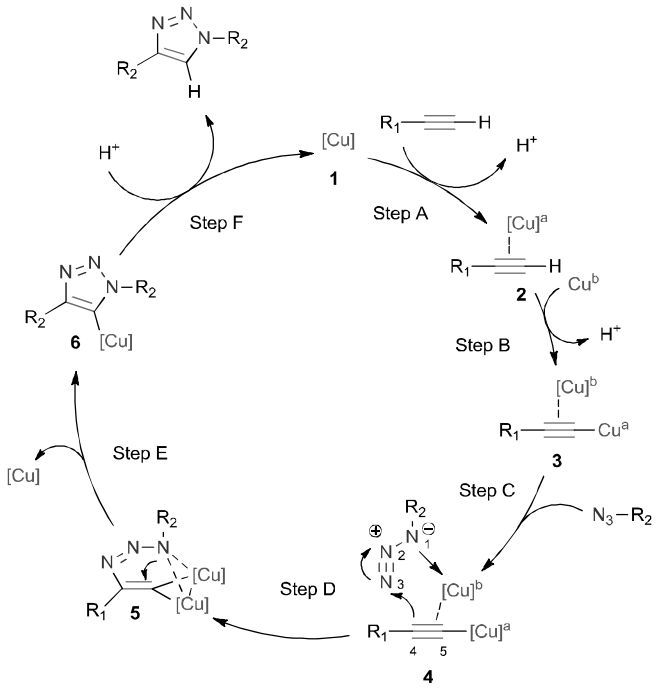
1.1.1.1 Mechanism of CuAAC:

Before considering the mechanistic pathways of the CuAAC process, it is important to consider the reactivity of the players: the organic azide and the copper(I) acetylide.
The reactivity of organic azides is dominated by reactions with nucleophiles at the terminal N-3 atom whereas electrophiles are attacked by N-1 centre.[10] (Scheme 2)



Scheme 2. Common reactivity patterns of organic azides.

Whereas ionic azides such as sodium azide are relatively stable compounds, covalently bound azides are thermally decomposable and in part explosive. For organic azides to be manipulable or non-explosive, the rule is that the number of nitrogen atoms must not exceed that of carbon but special care in handling them is always a good practice.[10] Low molecular weight azides should never be isolated from the solvent, therefore they should be generated *in situ* by SN_2 displacement from organic halides or arylsulfonates by sodium azide just before the click reaction, considering that CuAAC tolerates a lot of additives or spectator compounds, including inorganic NaN_3.[11]
Copper(I) acetylide species, deriving from Cu(I) insertion into terminal alkynes, are easily formed and are key components of the CuAAC reaction mechanism.
The proposed mechanism of CuAAC, outlined in Scheme 3 (next page), was recently elucidated by Worrel *et al.*[12] in a publication where it was highlighted the necessity of two chemically equivalent copper atoms working in concert for the regioselective formation of 1,4-substituted 1,2,3-triazoles. This was proven by the fact that monomeric copper acetylide complexes were not found able to react with organic azides, unless an exogenous copper catalyst was added. Hence, the first step (Step A) of the CuAAC reaction is the formation of the π-bound copper acetylide **2** from the alkyne and the catalytic copper species **1**, followed by the recruitment of a second σ-bound copper atom (step B), forming the catalytically active complex **3**.
Alkyne coordination with copper lowers its pka by 9.8 pH units, which explains the deprotonation of the π-alkyne-Cu^I intermediate in aqueous medium even without the presence of an additional base. Although, in organic solvents, the formation of **3** is unfavourable and a base is required for deprotonation.



Scheme 3. Proposed mechanism for CuAAC.

Step C of the catalytic cycle describes the coordination of the organic azide to the σ-bound copper acetylide bearing a second π-bound copper atom **3**, and subsequent formation of complex **4**. The following step (step D) is the nucleophilic attack by the acetylide carbon C-4 at the N-3 of the azide to form the first covalent C–N bond, generating the metallocycle **5**. This metallocycle positions the bound azide properly for subsequent ring contraction by a transannular association of the N-1 lone pairs with the C-5-Cu π* orbital to give the triazole-copper derivative **6** (step E). Protonation of the derivative **6** (step F) give the desired 1,2,3-triazole and it ends the reaction with regeneration of the catalysts, ready for another cycle.

1.1.1.2 When the click does not "click":

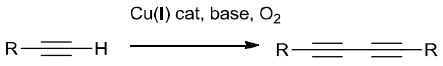
Although click reactions, by definition, should occur at room temperature, reach full conversion within a few minutes, and provide quantitative isolated product yields, the reality may be quite different and, in some cases, some form of process intensification will be advantageous.

Non-classical CuAAC conditions are commonly employed when the click process requires elevated temperature or prolonged reaction times to provide the desired 1,2,3-triazole. Controlled microwave heating under sealed vessel conditions, by virtue of the "microwave dielectric heating" phenomena, can dramatically reduce the reaction time and increase the

purity and the yield of the product compared to traditional CuAAC methods. The literature reports a vast amount of examples of microwave-assisted CuAAC in organic synthesis, also for the synthesis of peptides, oligonucleotides, carbohydrates and other macromolecules.[13]

Moreover, ultrasound irradiation or the use of ionic liquids as reaction medium, can be also grouped under non-classical CuAAC conditions.[13] The latter are a "green" alternative to water when the lack of solubility of the organic azide or alkyne in aqueous medium could prevent the success of the reaction.

In the case of a low-yielding CuAAC reaction, Cu-catalyzed acetylenic homocoupling should be taken into account. This Glaser-type homocoupling (Scheme 4) is very likely to happen in the presence of a significant amount of Cu(II) in the reaction mixture, deriving from the unwanted oxidation of Cu(I) catalytic species.



Scheme 4. Glaser coupling.

In order to avoid the formation these undesired by-products, which may impair the formation of the triazole product, the oxygen must be excluded from the reaction mixture. Furthermore, the presence of a small, unhindered amine such as TEA, pyridine or TMEDA can mediate the oxidative alkyne homocoupling, thus more sterically hindered bases should be employed.[14]

In the quite unique case of flexible polyalkynes, the proximity of the alkynes saturates Cu(I) atoms through chelation.[15] For a successful outcome of CuAAC reaction, Cu(I) requires labile ligands to enable competitive azide binding (Scheme 3); so in the presence of a chelating agent that saturates the Cu(I) catalyst, and thus precluding the binding of the azide, the triazole formation does not occur efficiently.

1.1.2 Thiol-ene coupling:

Thiol-ene coupling (TEC), known for over 100 years,[16] is simply the hydrothiolation of a carbon-carbon double bond and it has recently attracted researchers' attention due to the recognition of its "click" characteristics.

Two thiol-ene reactions particularly emerged: thiol-ene free radical addition to electron rich or electron poor C=C bonds (thermally or photochemically induced) and the catalysed

Michael addition to electron deficient carbon-carbon double bonds. There are several features associated with these thiol-click processes that make them particularly attractive, facile, and versatile. First, such hydrothiolation reactions can proceed under a variety of mild conditions, at room temperature, in highly polar solvents such as water or DMF, without expensive and potentially toxic metal-based catalysts. Second, they are tolerant to the presence of ambient oxygen and the radical-mediated process is insensitive to the presence of polar functional groups; they proceed more rapidly than many other click chemistry reactions, with near quantitative formation of the corresponding thioether in a regioselective fashion. Third, virtually any thiol can be employed, including highly functional species and a wide range of "enes" serve as suitable substrates, including activated and non-activated species as well as internal olefinic bonds. However, reactivity can vary considerably depending on reaction mechanism, the thiol employed, and the substitution pattern at the C=C bond.

What is necessary to bear in mind in dealing with thiol-based chemistry, is that not all thiols are equal. Essentially, there are four basic types of thiol structures typically encountered as shown in Figure 1: alkyl thiols, propionate thiols, acetate thiols and aromatic thiols. Each thiol will participate with different efficiency in radical and Michael addition reactions: the nucleophilicity of thiolate anions will increase going from alkyl to aromatic thiols while the electrophilicity of thiyl radical will increase going from aromatic to alkyl thiols.[17]

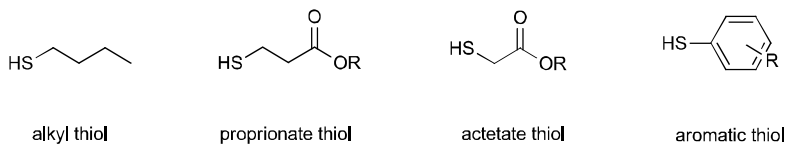


Figure 1. Structures of various thiols.

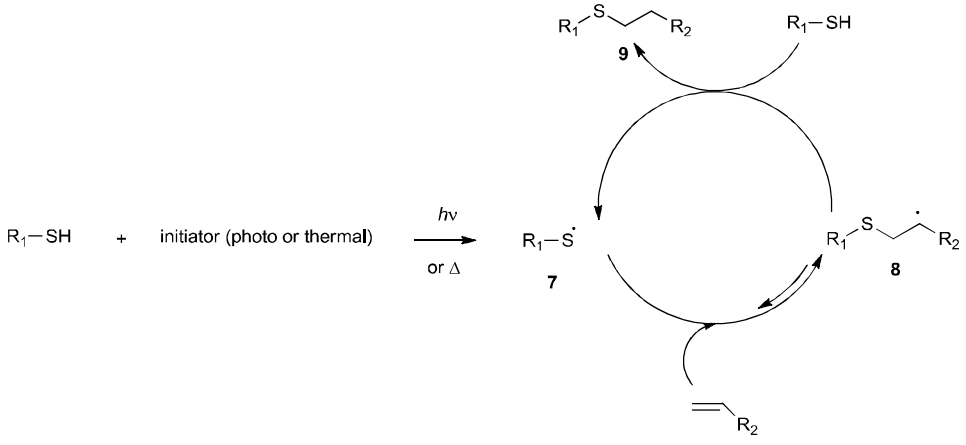
Some considerations concerning the structure of the alkene involved in the thiol-ene reaction need to be done: the ene reactivity decreases with decreasing electron density of the carbon-carbon double bond in the radical-mediated process and, generally, terminal enes are significantly more reactive towards hydrothiolation compared to internal ones. Norborene, methacrylate, styrene and conjugates dienes are special cases. Norbornene exhibits an exceptionally high reactivity toward thiols attributed to bond angle distortion in association with ring strain relief after TEC processes; methacrylate, styrene and

conjugates dienes produce a very stable radicals that show low hydrogen-abstraction rate constants.[18]

Although, in radical-mediated TEC processes, internal 1,2-disubstituted olefins generally show much lower reactivity towards the hydrothiolation than singly substituted ones, this is not uniquely due to steric hindrance effects for the approaching thiyl radical. An isomerization process could play an important role in this reduced reactivity of internal enes.[18] In fact, the addition of the thiyl radical to *cis*-double bond is reversible with efficient isomerization to the less reactive *trans* structure. This isomerization is best achieved under irradiation with UV-light and now chemists have taken advantage of this property to achieve the isomerization of *Z*-alkenes into the more stable *E*-isomers.[19,20]

1.1.2.1 Thiol-ene radical click reaction:

TEC reaction proceeds as a typical radical chain process with initiation, propagation and termination steps. The characteristic two-step mechanism for the hydrothiolation of a terminal alkene is represented in Scheme 5. The initiation step for the TEC process starts with the formation of the thiyl radical **7**, generated by different methods, that adds to the alkene with anti-Markovnikov orientation to give the carbon centred thioalkyl radical **8**. This radical abstracts a hydrogen from another thiol, thus forming the final thioether product **9** and a new thiyl radical, starting the propagation of the radical chain. Possible termination reactions involve typical radical-radical coupling processes.

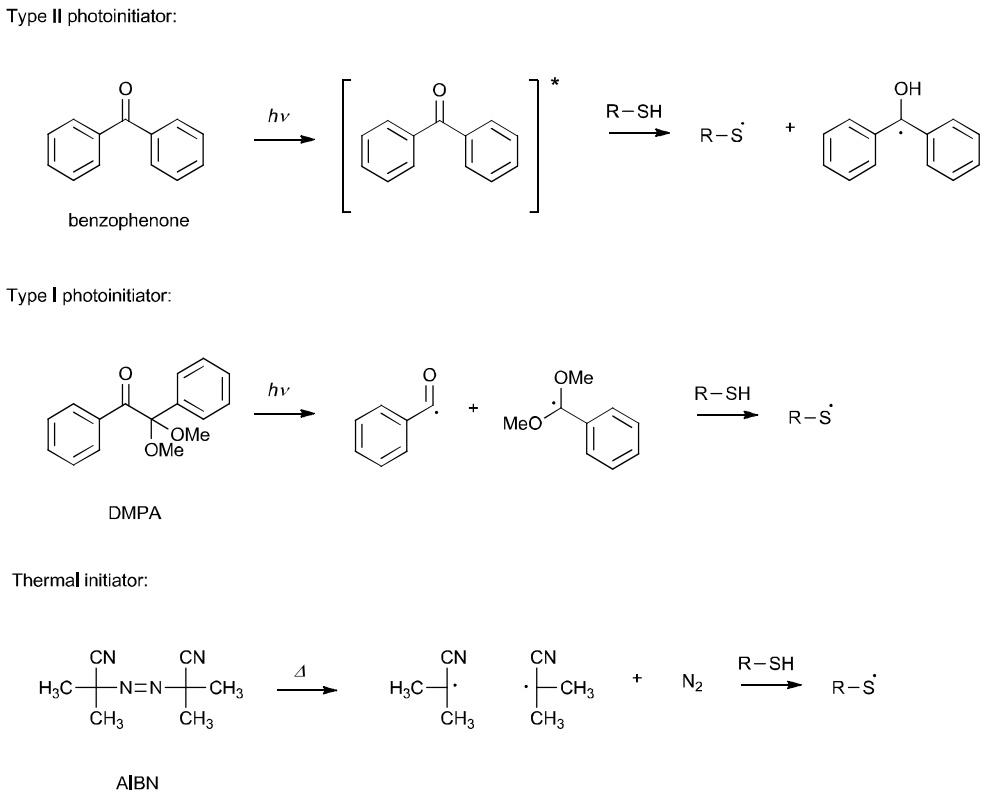


Scheme 5. The radical-mediated thiol-ene mechanism.

TEC reaction can be initiated by a variety of techniques including photochemical methods, with or without the addition of a photoinitiator, or under traditional thermal conditions with common azo species such as 2,2'-azobis(isobutyronitrile) (AIBN).

In its early implementation, TEC was initiated employing type II photoinitiator such as benzophenone (Scheme 6, type II photoinitiator). This diarylketone can be excited to its singlet state upon irradiation with UV light of sufficient energy. In the presence of thiols, there is a hydrogen transfer from the thiol to the excited state of benzophenone, resulting in the formation of a thiyl radical and a semipinacol radical.[18]

TEC reaction can also be initiated by cleavage photoinitiators (type I), such as 2,2-dimethoxyphenyl acetophenone (DMPA), which give a benzoyl radical and a tertiary carbon radical upon the absorption of a photon of light (Scheme 6, type I photoinitiator). These radicals may insert into a carbon-carbon bond directly or abstract a hydrogen from a thiol group, thus initiating the radical-chain process. Initiation with cleavage photoinitiator (type I) is more efficient than that with hydrogen-transfer photoinitiators (type II) because the quantum yield for the production of reactive radicals is higher.[18]



Scheme 6. Mechanism for the photoinitiation of thiol-ene coupling with type II and type I photoinitiators and thermal initiator.

One of the most unique aspects of the radical TEC click reaction is its ability to self-initiate the chain reaction in the presence of UV light, due to the homolysis of the labile sulphur-hydrogen bond; however, this alternative method of initiation often proceeds more slowly

than the initiation through the use of a photoinitiator, which results from lower rates of radical formation.

TEC reaction is a radical-mediated process and any technique that is appropriate for generating radicals could be suitable for initiating it. Indeed, TEC coupling induced by thermal initiators such as AIBN is not uncommon[21] (Scheme 6, thermal initiator). Nevertheless, the efficiency of thermally and photochemically initiated thiol-ene click coupling reactions was investigated and the photochemical coupling was found to proceed with higher efficiency and required shorter reaction time for complete conversion compared to the thermal counterpart.[22,23]

Despite the advantages of free radical TEC reactions that fit the "click" definition, there are potential limitations of this type of chemistry, which include the formation of unwanted disulphide by-products through thiyl-thiyl radical coupling or the head-to-head coupling of the carbon centred radicals. It should be noted, that in case of significant occurrence of side reactions, the whole process might not be considered a click process anymore, since the formation of by-products is a direct contradiction of the click concept.[24] Thus, TEC may only serve as an efficient conjugation tool if such reactions can be largely avoided.

Another drawback of TEC coupling is the reversibility of the thiyl radical addition to alkenes until proton radical abstraction.[25] This means that radical TEC often require an excess of either thiol or alkene to shift the equilibrium towards the sulfide product. Consequently, additional work up is necessary to get rid of that excess and this is not in line with the "click chemistry" principles.

1.1.2.2 Thiol-Michael addition click reaction:

Aside from the radical-mediated thiol-ene reaction, hydrothiolations can be readily accomplished under mild base or nucleophilic catalysis. Such reactions are commonly referred to simply as thiol-Michael addition reaction; they are slightly less versatile than the radical-mediated TEC reaction since to be effective the alkene must be activated, i.e. an electron-deficient alkene, but recently mild, solventless reaction conditions, using weak catalysts, have been employed and Michael-thiol reaction became a highly efficient, modular click method.[26]

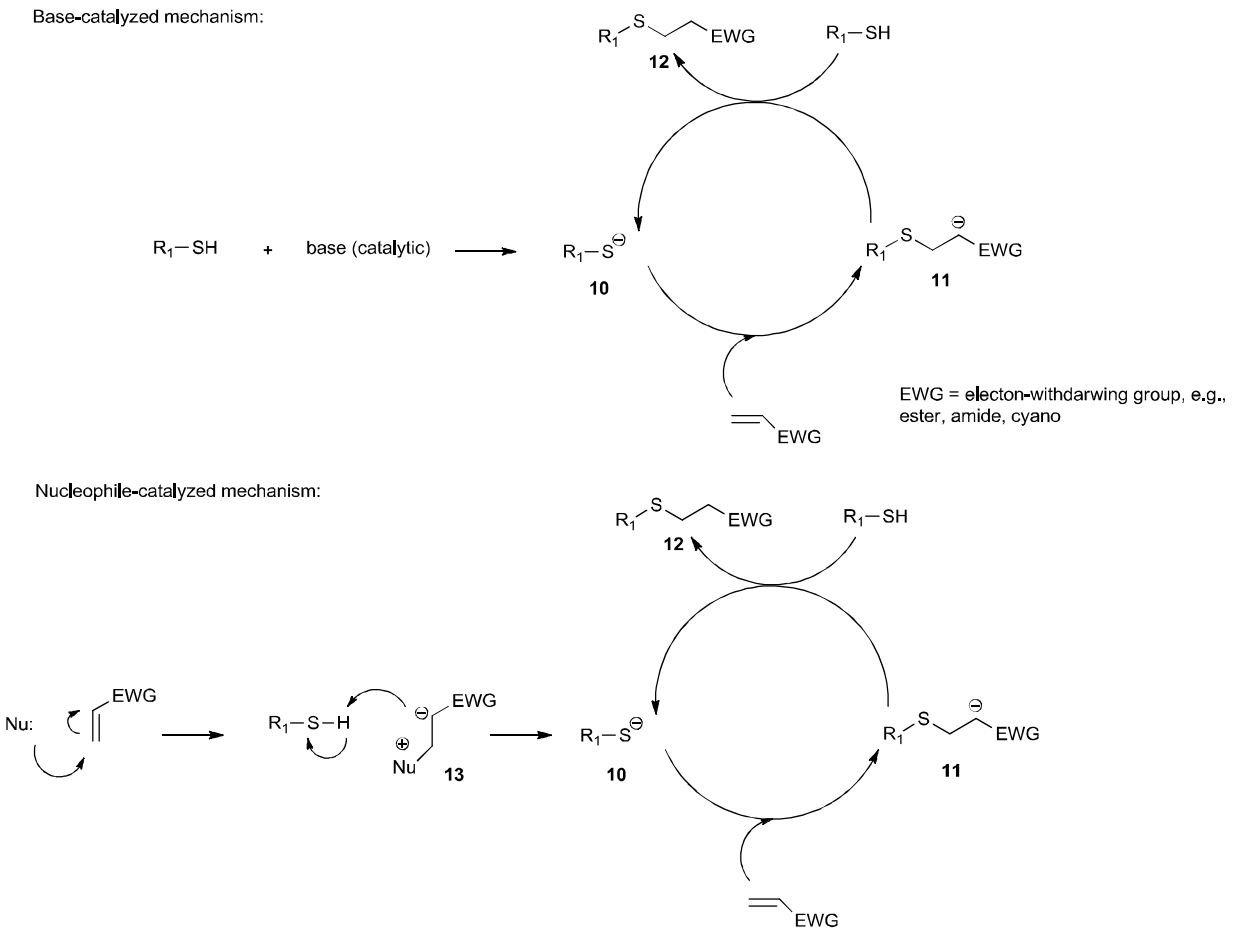
Thiol-Michael addition reactions proceed via ionic mechanism: the use of weak base catalyst, such as TEA, is usually enough to catalyse the process by virtue of the relatively low pK_a value of most thiols.

As shown in Scheme 7 (next page), the reaction of the thiol with the weak organobase (B) results in deprotonation of the thiol to the corresponding thiolate anion **10** that, being a

powerful nucleophile, attacks the electrophilic β-carbon of the C=C bond to form the intermediate enolate **11** which is a very strong base. This anion abstracts a proton from another thiol molecule or from the conjugate acid (BH^+), yielding the thiol-ene product **12**, again with regioselective formation of the anti-Markovnikov product, with concomitant regeneration of the base catalyst.[27]

So, in this process, a relatively weak base is used to generate a much stronger base (the carbanion **11**) in the catalytic cycle.

However, the overall rate and yield of such reactions can be influenced by the polarity of the solvent employed, the pK_a of the thiol or its steric bulkiness, the base catalyst strength and concentration, or the nature of the electron-withdrawing group (EWG) on the C=C bond.



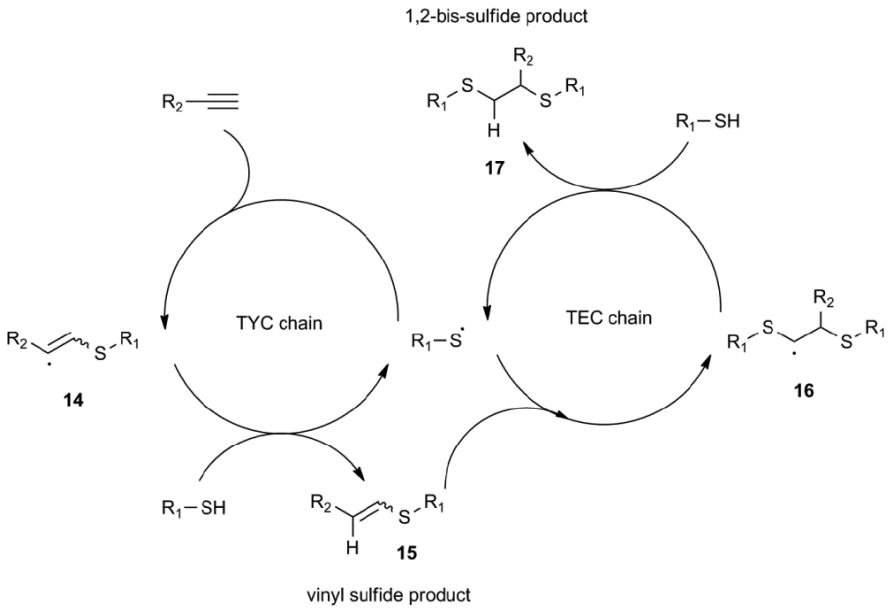
Scheme 7. The proposed base-catalyzed and nucleophile-catalyzed mechanism for the hydrothiolation of activated alkenes.

Recently, the nucleophilic thiol-Michael addition pathway has garnered attention as a means for improving various characteristics of the thiol-Michael reaction. Nucleophilic catalysts usually employed in nucleophilic thiol-Michael addition reaction are phosphine-centred nucleophiles such as tributylphosphine and dimethylphenylphosphine or secondary and tertiary amines such as *n*-dipropylamine.[28] Based on experimental observations,[26] the currently accepted mechanism for nucleophile-mediated thiol-Michael addition reactions is depicted in Scheme 7: the nucleophile initially undergoes conjugate addition to the activated C=C bond to generate the strong intermediate carbanion **13**, which, in turn, deprotonates the thiol to generate a thiolate anion **10**, which subsequently undergoes thiol-Michael addition. In this case, the nucleophile itself does not catalyse the reaction: it reacts with the electron-deficient carbon-carbon double bond to generate a strong base (**13**). Therefore, in this case, the reaction kinetics depend on the nucleophilicity of the catalyst, because the higher the nucleophilicity of the catalyst, the larger the number of active thiolate anion intermediates that can be generated. In comparison with the base-catalyzed mechanisms, the nucleophilic Michael addition reaction generally proceeds efficiently to high conversions with relatively low concentrations of the catalyst.

Noteworthy, both pathways, nucleophile- and base-catalyzed, could be affected by the presence of external acidic protons other than the thiol in the reaction, but the nucleophilic-mediated process seems to be more impaired by the presence of external proton species.[26] In fact, in extreme cases, the presence of protic species could completely inhibits the nucleophile-mediated thiol-Michael reaction.

1.1.3 Thiol-yne click reaction:

Thiol-yne coupling (TYC) is the radical addition of thiols to alkynes and it should be considered a sister reaction of the TEC reaction as well as a complimentary process to the CuAAC cycloaddition. This reaction, discovered in the 1930s,[29] allows for the introduction of two thiol fragments across a carbon-carbon triple bonds through the free-radical mechanism shown in Scheme 8.



Scheme 8. Accepted mechanism for the double hydrothiolation of a terminal alkyne bond under radical conditions.

The first step is the addition of a thiyl radical, generated by any of the conventional methods,[19] to the C≡C bond to afford an intermediate β-sulfanyl-substituted vinyl radical **14** that can abstract a hydrogen from the starting thiol to give the vinyl sulphide **15** (TYC product) and a new thiyl radical to continue the chain reaction. The vinyl thioether **15** is also highly reactive towards thiyl radicals and can undergo a subsequent addition of a second sulfanyl radical to give the bis-sulfide bis-adduct **17** as final product (TYC–TEC sequence, Scheme 8).[30] Indeed, kinetics indicate that the addition of a thiyl radical onto a vinylsulfide is very rapid, about 3 times faster than the addition onto the starting alkyne.[31] The structure of the alkyne employed plays a very important role on TYC outcome: the reaction is sensitive to steric hindrance with internal alkynes reacting more slowly than terminal ones, in addition, sulfanyl radicals are electrophilic in nature and react more

readily with electron-rich alkynes. However, such behaviour is not universal to all alkynes and some unexpected results have been found.[32]

Although the use of UV-light irradiation in the presence of a sensitizer is a very common way to initiate these coupling reactions, radical TYC can be initiated using a variety of radical initiation methods that have been recently reviewed.[19,30]

Some other considerations about the TYC reaction need to be done: the TYC process is usually poorly stereoselective, since it affords the vinyl sulfide product as mixtures of both *E*- and *Z*-stereoisomers losing one of the main requirements of click chemistry reactions, i.e. stereoselectivity. Furthermore, the double hydrothiolation (TYC-TEC sequence) generates a new chiral centre without any stereoselectivity, and this is an important thing to consider in the preparation of small molecules and especially those of biological relevance.

On the other hand, although the radical addition to alkynes is generally slower than that to alkenes, it occurs in an irreversible manner and thus, it does not require an excess of thiol/alkyne to reach completion. In addition, the possibility of a TYC-TEC sequence is a very interesting feature of this process because it allows the preparation of highly crosslinked, polymeric networks in polymer science.[33] In fact, contrary to the related TEC reaction, which leads to a linear linkage between the thiol and the alkene counterpart, the bis-addition of thiols to alkynes gives a branched linkage. More interesting, under the appropriate reaction conditions, the reaction could be stopped after TYC sequence, at the vinyl sulfide stage, and a second, different thiol could be inserted through a subsequent TEC reaction to obtain a bis-functionalized molecule.[34,35] Indeed, it was demonstrated that the experimental conditions (e.g., temperature, solvent, alkyne/thiol ratio) can be properly adjusted to achieve selective production of the mono-coupling product followed by the insertion of a second thiol on the TYC product via TEC coupling.[35–37] This dual modification methods can be a very attractive tool for bioconjugation.

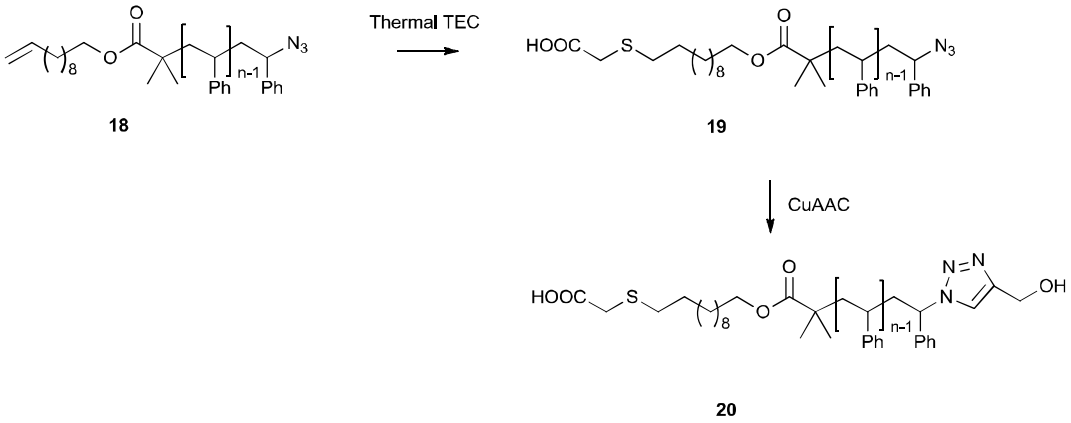
1.1.4 Orthogonal click reactions and biorthogonality:

The concept of orthogonality has been applied to many areas of chemistry. It was introduced by Barany and Merrifield[38] in 1977 as a protecting group removal strategy where each of the deprotection reactions occurs by a different mechanism, so a specific protecting group could be removed exclusively in the presence of all others. It was a simple but strong concept and since then, the use of the term orthogonality has broadened dramatically.

Avoiding the protection-deprotection steps can drastically improve the synthesis of complex molecules and the use of orthogonal coupling reactions is a powerful strategy to do that.

Click chemistry, in its definition, provides a set of powerful orthogonal coupling reactions with a high tolerance to broad range of reagents, solvents, and other functional groups. Several examples can be found in the literature describing the great potential of click chemistry, combining multiple click reactions, either performed simultaneously or in tandem, to synthesize complex structures and materials, hence demonstrating the orthogonality among them.[39]

In 2008, Campos and co-workers[22] investigated the efficiency and orthogonality of thiol-ene and CuAAC reaction. They synthetized the asymmetric polymer **18**, having a single alkene at one chain end and a single azide unit at the other chain end and they performed TEC under thermal conditions with thioglycholic acid followed by CuAAC with propargyl alcohol and vice versa, thus, demonstrating the othogonality of these two click processes (Scheme 9). The authors explained their choice of the thermal TEC reaction over the photochemical process due to the potential sensitivity of azides under UV illumination for an extended period of time.

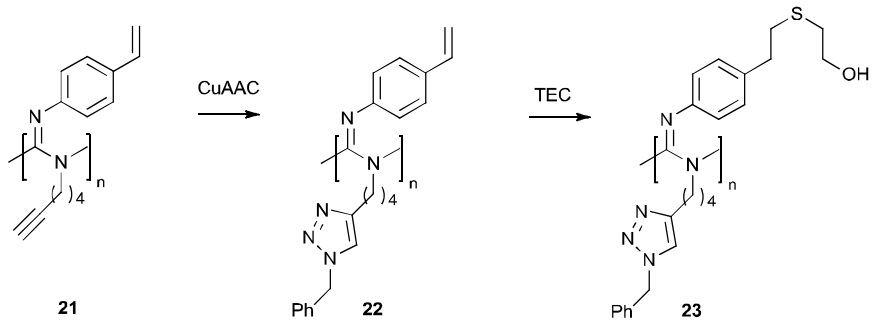


Scheme 9. Synthetic sequence TEC-CuAAC.

A couple of years later, Javakhishvili *et al.*[40] demonstrated the orthogonality of CuAAC and photochemically induced-TEC reaction in the preparation of a linear dendritic macromolecule. In this work, the starting substrate was bearing a C=C bond at one end and C≡C bond at the other end and CuAAC and TEC were performed sequentially in this order.

The same approach was used for the modification of alkyne and alkene side chain moieties of polycarbodiimide **21**: CuAAC with benzyl-azide and photochemical TEC with 2-

mercaptoethanol were employed in a consecutive manner yielding the new functional polycarbodiimide **23** in a short synthetic route.[41] (Scheme 10)



Scheme 10. Modification of the side chains of **21**.

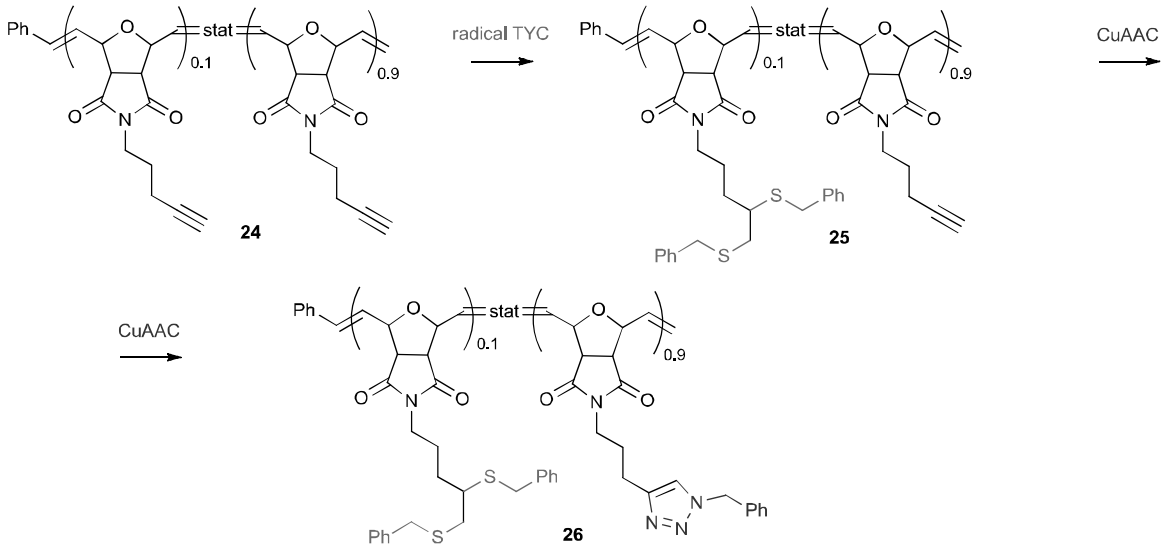
More recently,[42] bis-functionalized PEGs displaying alkenes and azides have been synthetized and used for the fabrication of bioactive hydrogels via CuAAC click chemistry and UV-initiated TEC chemistry, demonstrating once again the orthogonality and robustness of these methodology.

The ready available alkyne building blocks of CuAAC click chemistry can be employed to accomplish also sequential TYC and CuAAC reactions.

In a very elegant work, Hensbergen *et al.*[43] described the synthesis of a series of alkyne-containing copolymers. These copolymers were subjected to TYC obtaining quantitative modifications of the alkyne side groups in the presence of internal backbone alkenes. More interestingly, a TYC-CuAAC sequence to modify the alkynes in a different manner was accomplished. First, copolymer **24** was reacted with benzyl mercaptan under TYC radical conditions controlling stoichiometry and reaction time to limit conversion of the yne groups to *c.a.* 33%.

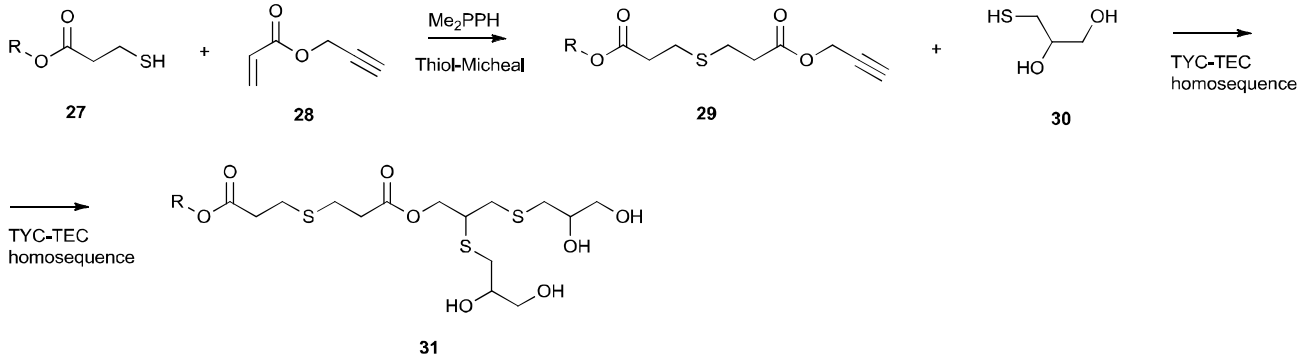
Second, a following CuAAC coupling of the residual yne groups of **25** with benzylazide was performed (Scheme 11, next page).

After the CuAAC reaction there was no evidence of remaining alkyne groups and polymer **26** was recovered. The authors were also very pleased to detect no significant change in the signals associated with the backbone-alkene functional groups, further highlighting the selectivity of this approach.



Scheme 11. Sequential radical thiol-yne and Cu-mediated alkyne-azide coupling reactions in copolymer post-polymerisation modification.

Due to different mechanisms for reactions within the thiol-click toolbox, it is expected that nucleophile-mediated and radical-mediated reactions of thiols can be conducted in an orthogonal fashion. Indeed, Lowe[44] and co-workers reported the first example of sequential thiol-Michael/TYC reactions to prepare highly functional materials under facile conditions (Scheme 12). The authors combined the extremely rapid and quantitative reaction of thiol **27** with the activated alkene **28** under nucleophile-initiated conditions, in the presence of dimethylphenylphosphine (Me_2PPh) as the initiator, to give product **29**, with the radical-mediated TYC reaction between **29** and **30**, which proceeds rapidly to yield the 1,2-addition product **31** exclusively and quantitatively.

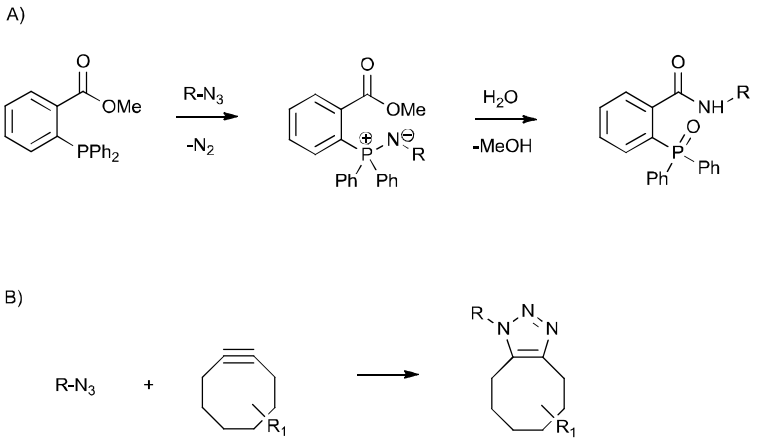


Scheme 12. Sequential thiol-Michael and thiol-yne reactions.

Bioorthogonal reactions are chemical reactions that neither interact with nor interfere with a biological system.

These reactions are now used for *in vivo* bioconjugation, therefore they must fulfil a number of requirements: they must form covalent linkages between two functional groups that are bioinert and ideally nontoxic, they must have fast kinetics even with very low reactant concentrations, as required in many biological labelling experiments, and they must be achieved in the physiological ranges of pH and temperature.[45]

A subclass of click reactions, whose components are inert to the surrounding biological environment, is termed bioorthogonal. Copper-free click chemistry methodologies that still take advantage of the azido group, a small, abiotic, and bioinert reaction partner, such as the Staudinger ligation and strain-promoted azide-alkyne cycloaddition (SPAAC) depicted in Scheme 13, are considered bioorthogonal and they avoid the presence of copper-catalysts that can have sever cytotoxic effects. These bioorthogonal reactions have been recently developed and reviewed.[45–48]



Scheme 13. A) Staudinger ligation, B) SPAAC

1.2 Bile acids:

1.2.1 Chemistry of primary and secondary bile acids:

Bile acids (BAs) are the end products of cholesterol metabolism with multiple physiological functions. All bile acids consist of a rigid steroid nucleus with polar hydroxyl functions and a short aliphatic side chain. The steroid nucleus is a saturated cyclopentanoperhydrophenanthrene containing three six-member rings (A, B, and C) and a five-member ring (D). In most natural bile acids, A and B rings are in a *cis*-fused configuration denoted by a 5β hydrogen atom (Figure 2). "Allo" bile acids, in which the A/B junction is *trans*, are much rarer and they are found mainly in lower vertebrates. The angular methyl groups at C-18 and C-19 positions determine the stereochemistry of substituent on the nucleus, whether it is α (in *trans* relationship, projecting in opposite direction as regard the general plane of the tetracyclic nucleus) or β (in *cis* relationship).

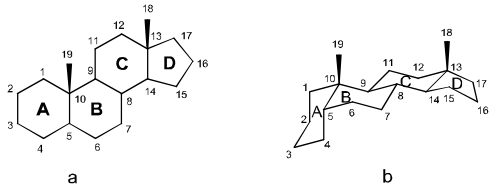


Figure 2. Numbering system of steroid nucleus. (a) chemical structure; (b) perspective structure (A/B *cis*).

Bile acids contain hydroxyl groups in the steroid nucleus mainly at positions C-3, C-7, C-6 or C-12 and they differ in the distribution, number, and stereochemistry among the common bile acids.

Reactions of the BAs might be expected at their various functional groups. The carboxylic acid group may be esterified, reduced, amidated, or subjected to salt formation with metal ions, alkaloids, or organic bases. The reactivity of the hydroxyl groups towards oxidation is C-7 > C-12 > C-3 and C-6 > C-3, while the order of acetylation, hydrolysis, vis-á-vis reduction, or hydrogenation is C-3 > C-7 > C-12.[49]

Natural bile acids are planar amphipathic molecules having a hydrophobic face (β side) containing no substituent and a hydrophilic face (α side) containing the hydroxyl groups combined with the negatively charged side chain.

The nomenclature of BAs is complex for historical reasons; trivial names were given in the 19th century long before their structures were determined. The bile acids and bile alcohols, also named cholanoid and cholestanoid, are divided in three great classes based on the side

chain structure: the C_{27} bile alcohols, the C_{27} bile acids, and the C_{24} bile acids (Figure 3). The C_{27} bile alcohols and the C_{27} bile acids contain the C_8 side chain of cholesterol while the C_{24} bile acids contains a saturated C_5 side chain. As suggested by their name, the side chain of each class may end in either a primary alcohol group or a carboxyl group. There is a clear evidence of evolution of bile acids through the stages: C_{27} alcohols → C_{27} acids → C_{24} acids.[50]

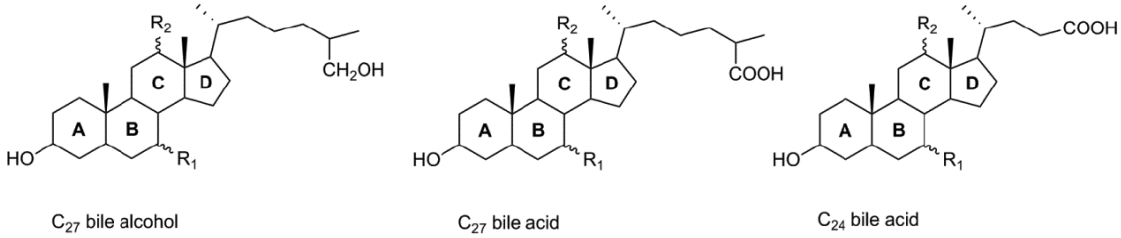


Figure 3. Chemical structure of C_{27} bile alcohols, C_{27} bile acids and C_{24} bile acids.

The BAs present in the human bile have two sources: those synthetized from cholesterol in the hepatocytes are called primary bile acids, while those produced from primary bile acids in the intestine by bacterial modification are called secondary bile acids.

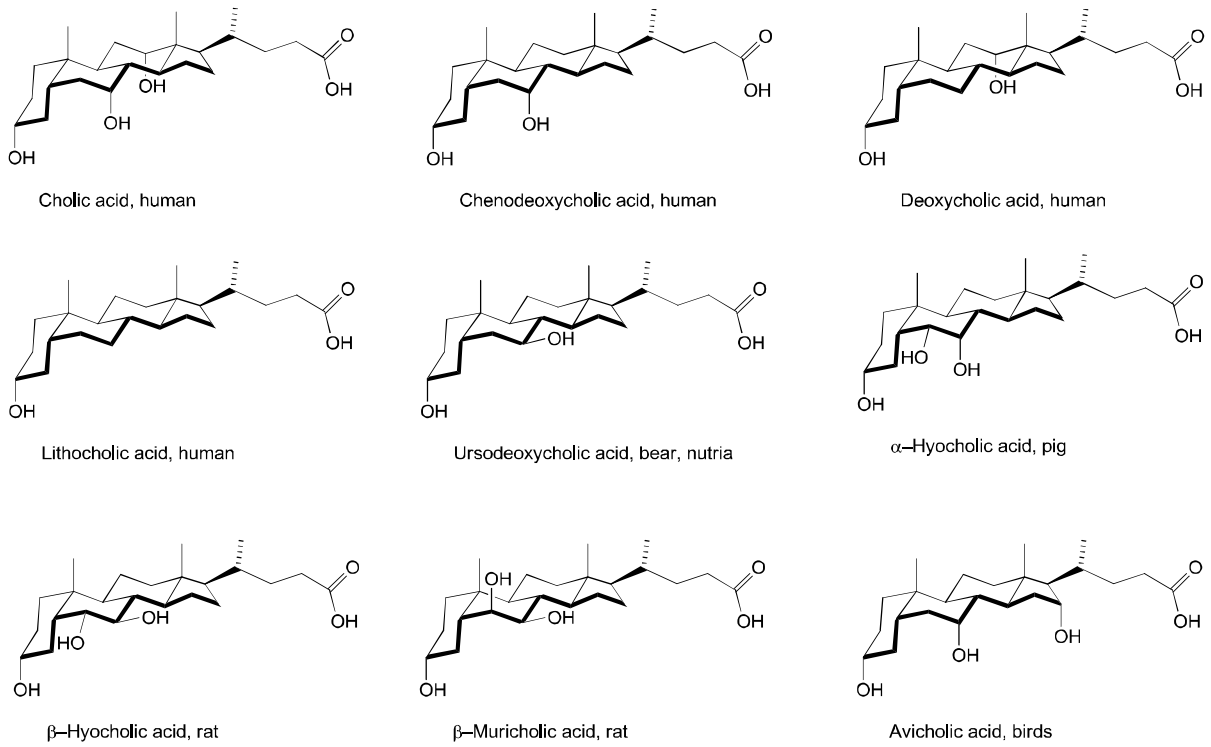


Figure 4. Chemical structure of some of the bile acids in vertebrate bile.

The most common primary BAs in man are cholic acid (CA, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) and chenodeoxycholic acid (CDCA, 3α,7α-dihydroxy-5β-cholan-24-oic acid). In the intestine, these BAs are transformed into secondary bile acids, such as deoxycholic acid (DCA, 3α,12α-dihydroxy-5β-cholan-24-oic acid) and lithocholic acid (LCA, 3α-hydroxy-5β-cholan-24-oic acid) by a bacterial 7α-dehydroxylase. Further removal, oxidation or epimerization of the nuclear hydroxyl groups or desaturation of the side chain, may occur in some species depending on the prevailing bacterial population, generating a novel "tertiary" bile acid.[51] (Figure 4)

Ursodeoxycholic acid (UDCA, 3α,7β-dihydroxy-5β-cholan-24-oic acid), as its name suggests, is prevalently found in the bile of bears and it plays a role in human cholesterol regulation and it has several medical applications.[52] Bile is thus a mixture of primary bile acids formed in the hepatocyte and secondary bile acids formed in the colon. All bile acids involved in the enterohepatic circulation, whether primary or secondary, do not occur as free carboxylic acids but they are conjugated with either the amino acid glycine or taurine. The taurine and glycine conjugates are often called bile salts by virtue of the fact that they are completely ionized at physiological pH. Conjugation of newly synthesized bile acids can occur in other four modes: sulfation, glucuronidation, conjugation with glutathione (GSH) and conjugation with *N*-acetylglucosamine.[53] In vertebrates, the only sulfate that occurs in appreciable proportions in biliary bile acids is the 3-sulfate of toxic lithocholic acid, combined with its amidation at C-24 position. Glutathione conjugates of bile acids (presumably as thioesters) have been identified in human infant bile, but the concentration of GSH conjugates of chenodeoxycholic and lithocholic acid was several orders of magnitude less than natural bile acid *N*-acylamidates.[54] Conjugation with *N*-acetylglucosamine was found at C-7 in UDCA, in addition to amidation with glycine or taurine at C-24.[55]

It is important to note that these modifications are essential in humans; in fact conjugations increase the polarity and the solubility of these molecules while in their unconjugated form, primary bile acids are only poorly soluble at physiological biliary pH. Furthermore, conjugations reduce the intrinsic toxicity of BAs, hence, conjugate bile acids are always negatively charged and therefore impermeable to cell membranes being too large to diffuse through the paracellular junctions of the biliary tract and small intestine. Moreover, conjugated bile acids are more efficient promoters of intestinal absorption of dietary lipid than unconjugated ones.

1.2.2 Micelle formation:

The essential physiological action of bile acids is to enhance the absorption of dietary lipids and fat-soluble vitamins by virtue of their ability to spontaneously form micelles in aqueous solutions. As aforementioned, bile acids are molecules with a rigid steroid backbone with methyl groups on the β-face and hydroxyl groups and the highly polar side chain on the α-face. Moreover, the 5β configuration of the junction between rings A and B bends the steroid nucleus into an L shape and enhances the separation of polar and nonpolar regions of the molecule. Bile acids therefore are amphiphilic and have detergent properties. Above a certain concentration, BAs aggregate to form micelles; the midpoint of this concentration range is called critical micellar concentration (CMC) and it is dependent on temperature, the chemical structure of the bile acid, and the electrolyte concentration.[56] In bile acids micelles, the hydrophobic portions of the bile acid molecule form the interior of the particle, while polar groups are at the surface and are free to interact with the aqueous environment. Micelles composed by only bile acid anions are called simple micelles but they do not occur in biological systems, where bile acids are mixed with other polar lipids to form mixed micelles. In the bile, the major component of mixed micelles is phosphatidylcholine. Through these mixed micelles, bile salts can solubilise important biological amphiphilic molecules that are otherwise water insoluble e.g., cholesterol, lipovitamins, lecithin, fatty acids, monoglycerides, etc. and thus greatly accelerate their absorption.[57]

1.2.3 Biosynthesis of bile acids:

Cholic acid and chenodeoxycholic acid are synthesized from cholesterol in the liver. Several structural modifications are necessary to convert cholesterol, with its 27 carbon atoms, C-5,6 double bond and 3β-hydroxyl group (Figure 5), into a C_{24}, saturated, 3α,7α,12α-trihydroxyl bile acid. The steps leading to the formation of these molecules can be divided into two broad categories: those that modify the steroid scaffold and those that oxidize and shorten the C_8 side chain of cholesterol.[58]

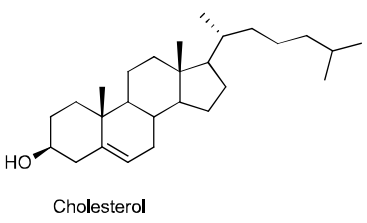


Figure 5. Chemical structure of cholesterol.

The rate-controlling step is the first introduction of a hydroxyl group in the axial configuration at C-7 position of cholesterol to give 7α-hydroxycholesterol. This reaction is catalysed by a cytochrome P-450 enzyme called cholesterol 7α-hydrolase. Further metabolism of 7α-hydroxy-cholesterol involves oxidation of the 3β- hydroxyl group and isomerization of the double bond from C-5,6 to C-4,5, yielding 7α-hydroxy-4-cholesten-3-one. (Figure 6)

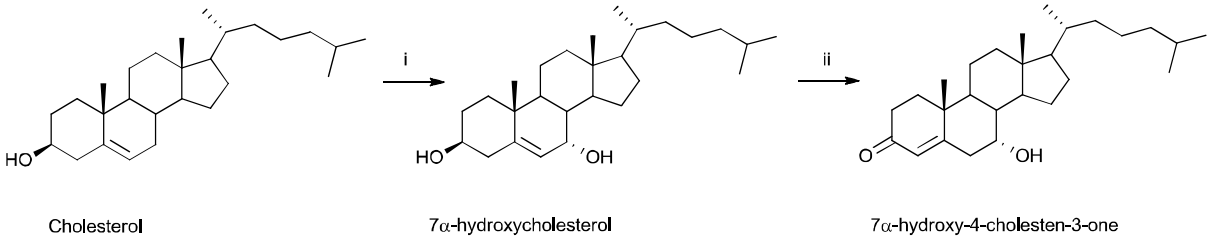


Figure 6. First steps of bile acid biosynthesis from cholesterol. (i) 7α-hydrolase; (ii) oxidation/isomerization.

This unsaturated oxo derivative is the branching point for cholic and chenodeoxycholic acid biosynthesis. If this intermediate undergoes a second hydroxylation at C-12, by a second microsomal enzyme, sterol 12α-hydroxylase, then the resulting product is ultimately converted into cholic acid. On the other hand, if 7α-hydroxy-4-cholesten-3-one serves as a substrate for a different enzyme, Δ^4-3-oxosteroid 5β-reductase, the sterol intermediate will be finally converted into chenodeoxycholic acid.[58] Further multiple sequential steps lead to the formation of the these primary bile acids.

Bile acid biosynthesis plays an important role in maintaining cholesterol homeostasis and it is regulated by the amount of bile acid returning to the liver via the enterohepatic circulation. Experimental evidences demonstrated that the biosynthetic pathway is controlled by a negative feedback mechanism involving the rate-limiting enzyme of bile acid synthesis, cholesterol 7α-hydroxylase.[59]

1.2.4 The enterohepatic circulation of bile acids:

After synthesis and conjugation, biliary BAs are stored into the gallbladder and discharged into the intestinal lumen upon ingestion of a meal. The bile acids are efficiently resorbed from the intestine, return to the liver and are resecreted into bile, resulting in the accumulation of a certain mass of BAs within the body, referred to as the BA pool. This cyclical movement is called the enterohepatic circulation (Figure 7).[60]

Intestinal conservation is extremely efficient and most of the bile acids present in bile have undergone multiple cycles of the enterohepatic circulation. Only a relatively small amount of these molecules is synthesized *de novo* each day to replace that which escape absorption and so are excreted into faeces. Indeed, almost 90% of bile acids entering the intestine are resorbed by active or passive mechanism.

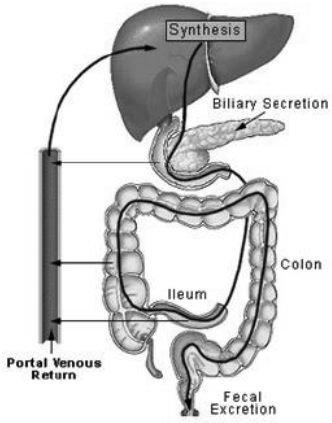


Figure 7. Schematic enterohepatic circulation of C_{24} bile acids in man.

The passive uptake of conjugates BAs in the ileum or jejunum depends on the dissociation constants of the individual bile acid and on the intraluminal pH while the active resorption requires, in the ileal enterocytes, an apical Na^+-dependent bile acid transport system (ASBT) and a basolateral anion exchanger, (Ost)α-Ostβ.[61] After resorption, bile acids are transported by portal venous blood, partially bound to albumin, back to the liver. Hepatocyte uptake is mediated by the Na^+-dependent bile acid transport system NTCP (sodium taurocholate cotransporting polypeptide) and by an Na^+-independent transport systems such as OATP (organic anion transport protein). These four transporters are involved and explain the enterohepatic cycling of conjugated bile acids.[62]

Nevertheless, a major fraction of bile acids returning from intestine escapes hepatic uptake and spills over into the systemic circulation.[63] However, these bile acids are again presented to the liver in the blood so the residence time of any of them in the systemic circulation is only few minutes.

During enterohepatic cycling, C_{24} primary bile acids are "damaged" by bacterial enzymes in the intestine to form secondary bile acids, and after their return to the liver, they are "repaired" partly or completely by hepatocyte enzymes. Intestinal microbial metabolism consists mainly of deconjugation and hydroxyl group oxidation.[64] Minor changes include epimerization of the hydroxyl groups, isomerization of the A/B ring juncture or desaturation of the side chain, which may happen in some species. The majority of

deconjugated bile acids are reabsorbed and return to the liver, where they are efficiently reconjugated with glycine or taurine. 7α-Dehydroxylated bile acids are not rehydroxylated but they are reabsorbed and transported to the liver to be conjugated with glycine or taurine. Furthermore, bile acids with 3β-hydroxyl group are re-epimerized to α-orientation and oxo groups are reduced to hydroxyl moieties. The end result of this "repairing" system is that, in the bile, BAs are mostly in amidated form and only trace amount of iso bile acids or hydroxy-oxo bile acids are present.

As previously mentioned, the enterohepatic circulation of bile acids and their biosynthesis are tightly connected. Indeed, bile acids must serve as the end products of cholesterol metabolism and their excretion is necessary for cholesterol balance. For this function, the enterohepatic cycling is irrelevant. On the other hand, the intestinal resorption of bile acids is needed to provide the BAs pool for efficient lipid digestion, and in this case their excretion is unimportant. Given these conflicting aims, the enterohepatic circulation of bile acids as well as their biosynthesis are strictly regulated processes. The present view is that bile acid synthesis is regulated at the level of the hepatocyte and their conservation is regulated at the level of the ileal enterocyte.[65]

1.2.5 Pharmacological application of bile acids, some examples:

The therapeutic use of bile acids has been recognised since ancient times. Bile acid therapy is based on their use as agonists or antagonists. Bile acid agonists (e.g., bile acids or their derivatives) are used in replacement therapy to correct a deficiency in bile acids because of defective biosynthesis or intestinal conservation and thereby to restore bile acid function.[66]

In contrast, bile acid antagonists are used in displacement therapy where the aim is to alter the composition of circulating bile acids and hence to modulate cholesterol metabolism and/or decrease the cytotoxicity of the circulating bile acid pool.[67] For example, UDCA was found to be effective in causing the dissolution of cholesterol gallstones in the gallbladder without hepatotoxicity.[68]

The literature describes a vast amount of pharmacological applications of bile acids and their derivatives. Some attractive recent developments in BAs medicinal chemistry have been reviewed.[69]

One of the most interesting therapeutic applications of bile acid derivatives and/or bile acid prodrugs are their employment as liver-specific drug carries taking advantage of the physiology of bile acid enterohepatic circulation, with the exclusive involvement of the liver and the small intestine. For this purpose, some crucial structural properties of BAs for

bile acid transporters recognition should be preserved: the negatively charged side-chain of the natural bile acids, the *cis*-configuration of rings A and B of the steroid skeleton, and at least one axial hydroxyl group on the steroid nucleus at position 3, 7 or 12.[70] Examples of bile acids as liver-specific carries of active principles could be found in the literature.[71–73]

BA derivatives were successfully employed to improve the intestinal absorption of poorly or non-absorbed drugs. For example, enzymatic hydrolysis and poor oral bioavailability are the major drawbacks, when considering the use of peptide drugs. Conjugation of peptides to bile acids was found to increase their metabolic stability as well as to improve their absorption from the intestine.[74] Other examples of drugs conjugated to bile acids in order to increase their bioavailability include anticancer treatments, antibiotics, GABA (γ-aminobutirric acid), L-DOPA (L-dihydroxyphenilalanine), as well as other active principles used in the treatment of Parkinson's disease.[75] Furthermore, bile acids conjugates were favourably employed for enhancing absorption of some antiviral treatment. [76]

Taken together, the efficiency and organ specificity of enterohepatic circulation of bile acids with its numerous transport proteins is an intriguing target in designing prodrugs with a view of improving intestinal absorption, increasing the metabolic stability of pharmaceuticals, specifically targeting drugs to organs involved in enterohepatic circulation, as well as reducing systemic side effects.

1.3 Bisphosphonates:

Bisphosphonates (BPs) are stable analogues of naturally occurring inorganic pyrophosphate (PPi). Stability is due to the replacement of the oxygen atom that connects the two phosphates of pyrophosphate (P-O-P) with a carbon atom (P-C-P). This render BPs resistant to chemical and enzymatic hydrolysis. Thus, all the BPs of clinical interest have the P-C-P backbone and two other substituents R_1 and R_2 sharing the central carbon atom. (Figure 8)

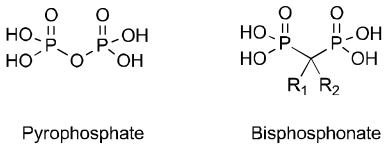


Figure 8. Chemical structure of pyrophosphate and bisphosphonate.

Bisphosphonates have high affinity for hydroxyapatite (HAP) and bone mineral. They inhibit the osteoclast-mediated resorption of living bone.[77] Over the past 40 years, BPs have revolutionised the treatment of patients suffering from bone resorption disorders, indeed they are now the gold standard for the treatment of osteoporosis, Paget's disease and other diseases in which there is an increase in the number or in the activity of osteoclasts, including tumour-associated osteolysis and hypercalcemia.[78,79]

Bisphosphonates have been known to chemists since the middle of the 19th century. They were employed in industrial processes mainly as corrosion inhibitors or as complexing agents in the textile, fertilizer and oil industries.[78] They were also used as "water softeners", due to their ability to inhibit calcium carbonate precipitation, in the prevention of scaling in domestic and industrial water installations. In the early 1960s, Fleisch *et al.*[80] studied the mechanism of calcification induced by collagen and proposed that naturally occurring polyphosphates might be the body's own natural "water softeners", preventing calcification of soft tissues and regulating bone mineralization. It subsequently become clear that calcification disorders might be linked to disturbances in inorganic pyrophosphate (PPi) metabolism.[81] However, when given by mouth, pyrophosphates and polyphosphates were inactive because of their fast hydrolysis in the gastrointestinal tract. During the search for more stable analogues of pyrophosphate, BPs became a very interesting class of compounds since they are characterized by the antimineralization properties typical of PPi and by a greater metabolic stability. In fact, early studies

demonstrated that these compounds bind to bone mineral and were found able to prevent calcification both *in vitro* and *in vivo* when orally administrated to rats.[82] This property of being active after oral administration, was the key to their future use in man. Nevertheless, the most important step toward the clinical use of BPs occurred when it was found that this class of new compounds, also showed a good ability to inhibit hydroxyapatite (HAP) crystals breakdown and bone resorption.[83] Consequently, BPs became the most widely used and effective antiresorptive agents for the treatment of osteoporosis and other bone disorders.

1.3.1 Classification of bisphosphonates:

The P-C-P moiety of bisphosphonates is responsible for their strong affinity and binding to bone mineral and allows for a number of variations in structure based on substitution in the R_1 and R_2 side chains which, in turn leads to the large range of activity observed among BPs. Structure-activity relationship (SAR) studies highlighted that: 1) the P-C-P backbone is necessary for *in vitro* and *in vivo* antiresorptive effect that indeed, cannot be achieved with monophosphates, P-C-C-P or P-N-P compounds;[84] 2) the presence of a hydroxyl group in the R_1 side chain enhances the ability of the BPs to bind to HAP crystals, preventing both crystal growth and dissolution, and increases the affinity for bone mineral through a tridentate binding with calcium;[85] 3) BPs antiresorptive potency is greatly enhanced by the presence of a primary, secondary or tertiary nitrogen function in the R_2 side chain.[84] Hence, now BPs are divided in two categories: nitrogen-containing bisphosphonates (N-BPs) and non-nitrogen-containing bisphosphonates (Figure 9).

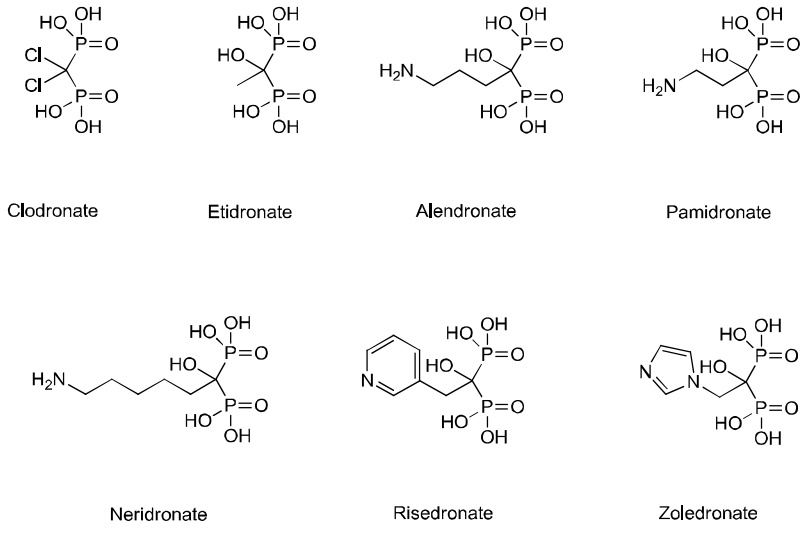


Figure 9. Clinically used bisphosphonates.

First-generation BPs, like clodronate and etidronate, contain simple substituents like short alkyl side chains or Cl atoms, and are now almost completely replaced by N-BPs for the treatment of osteoporosis. Second-generation compounds, such as alendronate and pamidronate, display alkyl chains with a basic NH_2 terminal group and are known as aminobisphosphonates (N-BPs). Risedronate and zoledronate, third-generation bisphosphonates, which are 10,000-fold more potent than first-generation BPs, are characterized by the presence of a nitrogen atom within a heterocyclic ring and so they are known as heterocyclic N-BPs.

The further analysis of SAR data, regarding the inhibitory potency of N-BPs, revealed that the basic nitrogen in the R_2 side chain must be in a specific spatial configuration and it must also have a critical distance from the P-C-P group. Furthermore, it was found that the geminal hydroxyl group has little or no influence on N-BPs inhibitory activity, in fact, analogues lacking this moiety retain biological activity but have reduced bone affinity. This characteristic may have therapeutic potential in situations when decreased drug retention is potentially advantageous, such as the treatment of bone disorders in childhood or metastatic breast cancer.[86]

1.3.2 Mechanism of action of bisphosphonates:

BPs are preferentially incorporated into active sites for bone remodelling in the skeleton. This selective uptake brings them in close contact with osteoclasts and some osteocytes but prevents prolonged contact with other type of cells. During bone resorption, the BPs are released from the bone surface and taken up by osteoclasts by endocytosis along with other products of resorption. Coxon *et al.*[87] confirmed that osteoclasts are able to take up large amounts of BPs by pinocytosis, due to their secretion of acid into the bone mineral as part of the active resorption process, causing BPs release from the dentine surface. By contrast, non-resorbing cells take up only small amounts of these drugs that become available due to natural desorption from the dentine surface.

Since the early 1990s there has been a systematic effort to elucidate the molecular mechanism of action of BPs and it has been found that they act on the osteoclast cells by two different mechanism, depending on whether they are nitrogen-containing or not.

Non-nitrogen containing BPs, such as clodronate and etidronate, are incorporated into a newly formed analogue of adenosine triphosphate (ATP) where the P-C-P moiety of the bisphosphonate is in place of the β,γ P-O-P moiety of ATP, resulting in the formation of a metabolite (AppCp-type) resistant to hydrolysis (Figure 10). The incorporations of simple

BPs to achieve AppCp-type nucleotides is carried out by members of the family of type II class aminoacyl-tRNA synthetases.[88]

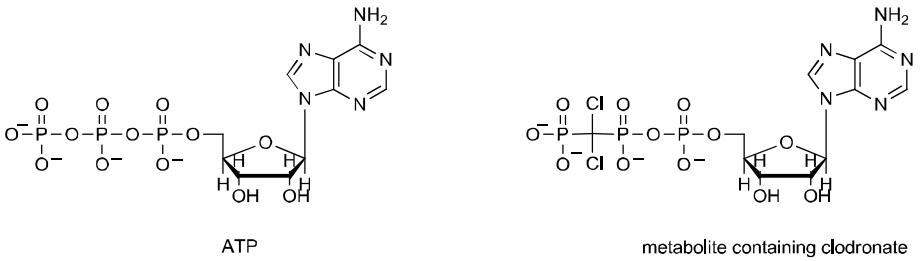


Figure 10. The structure of ATP and AppCp-type metabolite of clodronate.

Intracellular accumulation of these non-hydrolysable ATP analogues within osteoclasts is cytotoxic and cause the inhibition of ATP-dependent enzymes, such as adenine nucleotide translocase (ANT), a central component of the mitochondrial permeability transition pore.[89] This results in the collapse of the mitochondrial membrane potential, leading to the release of cytochrome-C, caspase activation and other steps of the apoptotic programme. Hence, first-generation BPs suppress bone resorption by causing osteoclast cells death without affecting other cell types due to their specific targeting to bone and selective uptake. The nitrogen-containing BPs (N-BPs), such as pamidronate, alendronate or risedronate, are not metabolised and do not inhibit the activity of aminoacyl-tRNA synthetase, still they are several orders of magnitude more potent than simple BPs at inhibiting bone resorption. This suggest that they work on a different molecular target with a different mechanism of action. N-BPs act, indeed, by inhibiting farnesyl diphopsphate synthase (FPPS), a key enzyme of the mevalonate pathway (Figure 11).

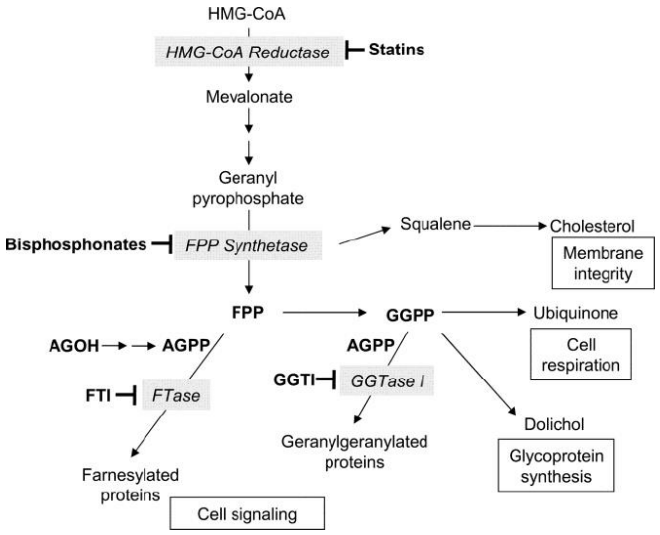


Figure 11. Schematic diagram of mevalonate pathway.

In 1992, Amin *et al.*[90] reported that ibandronate and incadronate inhibited squalene synthase and possibly other enzymes of the mevalonate pathway (cholesterol synthesis) in J774 macrophages. On the contrary, pamidronate and alendronate were not potent inhibitors of squalene synthase but they were able to inhibit cholesterol biosynthesis anyway, suggesting that an enzyme upstream in this pathway, controlling both cholesterol synthesis and isoprenylation, is the critical target of the N-BPs in the osteoclast. This study raised the possibility that cholesterol biosynthetic pathway could be involved in the effect of N-BPs on bone resorption, leading, only in the late 1990s-early 2000,[91–93] to the identification of farnesyl diphosphate synthase (FPPS) as the relevant molecular target for all N-BPs.

The primary function of mevalonate pathway is the production of cholesterol as well as the synthesis of isoprenoid lipids such as farnesyl diphosphate (FFP) and geranylgeranyl diphosphate (GGPP). These isoprenoids lipids are building blocks for the production of a variety of metabolites, but are also required for post-translational modification of proteins called prenylations.[94] The inhibition of FPPS prevents the prenylation of important signalling molecules (small GTPases such as Ras, Rac, Rho and Rab), thereby disrupting several pathways that are involved in cytoskeletal organization, cell survival and cell proliferation which lead to osteoclasts apoptosis. The importance of prenylated proteins for osteoclasts function has been confirmed using specific inhibitors to prevent either protein farnesylation or protein geranylgeranylation and it was found that loss of geranylgeranylated proteins causes disruption of actin rings leading to osteoclasts apoptosis, highlighting the fundamental importance of geranylgeranylated small GTPases rather than farnesylated proteins in these bone resorbing cells.[95]

More detailed studies,[93,96] displayed that N-BPs have an inhibition of FPPS in the nanomolar range of concentrations and that there is a highly significant correlation between the order of potency for inhibiting FPPS *in vitro* and anti-resorptive potency *in vivo*.

The incorporation in toxic, non-hydrolysable ATP analogues and the inhibition of FPP synthase are the two main mechanisms accounting for the anti-resorptive effects of BPs on osteoclasts and they also explain some of the adverse effects. Recent data[95] demonstrated that the inhibition of FPPS causes the accumulation of isopentenyl pyrophosphate (IPP), the metabolite immediately upstream in the mevalonate pathway. This accumulation causes the acute-phase reaction to N-BPs, a common side effect involving "flu-like" symptoms that are temporary and usually occurs soon after the first intravenous administration of these drugs.

1.3.3 The importance of fluorescent analogues:

In order to study uptake by osteoclasts, distribution in the skeleton and binding to hydroxyapatite, many fluorescently-labelled analogues of bisphosphonates have been developed.[97,98] One powerful method for producing fluorescent analogues was introduced by McKenna *at al.*[99] in 2008 and it employs an epoxide ring-opening reaction, under mild conditions, to introduce a primary amine functionality on the more recent heterocyclic N-BPs. This "magic linker" strategy is based on the concept of designing a universal linker which would permit a simple conjugation of N-BPs with any fluorophore activated ester while retaining significant affinity for bone mineral and ability to inhibit protein prenylation. (Figure 12)

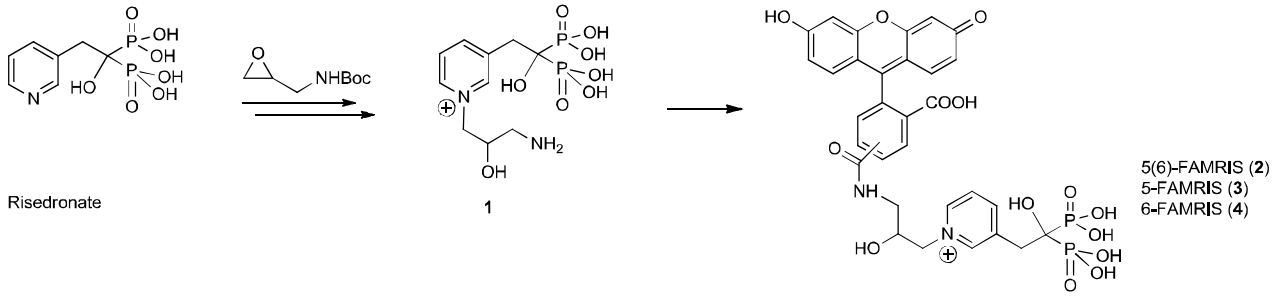


Figure 12. "Magic linker" synthesis of fluorescent risedronate conjugates. Λ few examples.

Another advantage of this linker is the permanent positive charge on the pyridinium nitrogen since recent investigations, using modern molecular modelling techniques, showed the importance of a positive charge feature at a relatively localized position in the bisphosphonate side chain and consequently, a number of pyridinium-bisphosphonates were synthetized.[100]

Fluorescent-analogues have been used to study the skeletal distribution and uptake of bisphosphonates by various cell types *in vivo*, giving insight into their mechanisms of action and potential effects.[101] Labelling of drugs using dyes with distinguishable fluorescent emission ranges allow simultaneous studies of multiple BPs with different bone affinities to elucidate their relative distributions in bone, bone tissues and cells. These kind of studies can shed light on reasons why different drugs have different efficacy or side-effect profiles. For example, fluorescently-labelled BPs were employed to study different responses of bone-site-specific osteoclast precursors (jaw and long bone) to bisphosphonates for better understanding a side-effect associated with bisphosphonates

which is the osteonecrosis of the jaw (ONJ).[102] This condition is defined as the persistence of exposed bone in the maxilla or mandible for at least 8 weeks and its prevalence is low in osteoporotic patients but a little higher in the oncological setting. The results obtained suggested that bisphosphonates have distinct effects on both populations of osteoclast precursors, indeed long-bone and jaw osteoclasts responded differently to BPs exposure.

With frequent use of BPs in multiple clinical settings, a growing body of evidence supports the notion that some N-BPs cause side-effects that are specific to soft tissues.[103] Recently, a fluorescent zoledronate analogue was employed to investigate whether N-BPs may have preferential cytotoxic effects to these tissues and indeed, some interesting evidences of N-BPs localization in soft tissue were provided.[104]

1.3.4 Bioavailability, drug delivery and complexation with bile acids:

The chemical structure of BPs, with two phosphate groups and two side chains (R_1 and R_2) bound to a central carbon atom, provides highly selective localization and retention in bone, and it also prevents enzymatic breakdown in the gastrointestinal tract by phosphatases. Despite these physiochemical advantages, the bioavailability remains a critical feature of BPs since they are poorly absorbed after oral administration, generally with absorption from 0.6% to 1.5% of the administered dose.[105] In general, a drug may be absorbed through the intestine by either transcellular or paracellular route depending on its physiochemical properties. In the transcellular transport, compounds cross the epithelial cells by traversing the intestinal cell membrane mainly by passive diffusion. Drugs with structures similar to those of nutrients may be taken up by carrier-mediated transport systems while in the paracellular route substances travel through epithelium tight junctions into the blood stream and can only move by passive diffusion. Bisphosphonates absorption occurs via the paracellular route since their hydrophilic, negative charged nature prevent transcellular transport, thus lowering absorbtion and oral bioavailability.[105] Moreover, oral administered BPs have gastrointestinal adverse effect like esophagitis, ulceration of esophagus, abdominal pain and nausea. The dose needs to be administered after overnight fasting with a glass of water at least 30 min before ingesting food, drinks, or other medications and additionally patients must remain upright for at least 30 minutes afterwards in order to prevent any gastroesophageal reflux. Bisphosphonates may be given also parenterally but, as previously mentioned, the main side effect of N-BPs given intravenously is an acute phase reaction (fever, myalgia, lymphopenia, etc.) due to the release of pro-inflammatory cytokines.[95]

A vast number of delivery systems have been investigated to overcome bioavailability limitations of BPs and to minimise their side effects making them more patient compliant. These novel drug delivery systems include nanoparticles and liposomes but also the co-administration of bisphosphonates with absorption enhancing agents such as bile salts and their analogues, chelating agents, salicilates and the design of lipophilic prodrugs have been investigated as well.[106]

Park *et al.*[107] developed a new ibandronate–DCK (N^{α}-deoxycholyl-L-lysyl- methylester) complex and evaluated the *in vitro* permeability and the *in vivo* oral bioavailability of this new formulation showing that these features were in fact enhanced by complex formation with DCK. In a different work[108], they employed DCK and another bile acid derivative, N^{α}-deoxycholyl-L-lysyl- hydroxide (HDCK) as oral absorption enhancers for risedronate. (Figure 13)

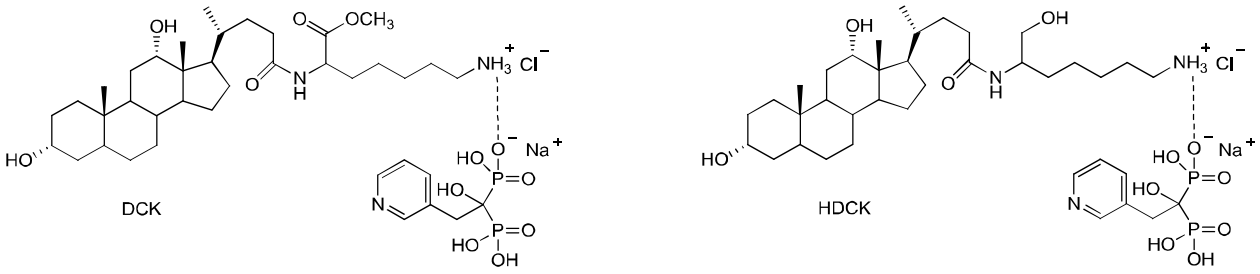


Figure 13. Structural diagram of ionic complexation of risedronate with DCK and HDCK.

The best results were obtained with risedronate/DCK complexed. In *vitro* permeability of this complex was significantly enhanced due to its higher lipophilicity. Furthermore, the complexed molecule interacted well with bile acid transporters, present mainly in the ileum, increasing the concentration gradient of the drug across the intestinal membrane. As a result, the oral bioavailability increased by 267% compared with that of the free risedronate.

In this work, it was demonstrated that drug modification by attaching a recognition carrier molecule, such as a bile acid, can specifically deliver target molecules. Moreover, this ionic complex approach has some notable advantages such as, the possibility to be prepared as enteric-coated oral formulation, which will not disintegrate in the stomach and will not cause irritation of mucous membranes. Besides, this delivery strategy does not require the employment of an absorption enhancer that modifies junctions or epithelial

layers causing irritation because of their surfactant properties or absorption of undesired substances.

In conclusion, this strategy may improve patient compliance and therapeutic efficacy by reducing dose and/or frequency and adverse reactions.

1.4 The indole scaffold and its reactivity:

Indole is an aromatic organic compound consisting of a six-membered benzene ring fused to the α,β-positions of a five-membered pyrrole ring (Figure 14). It is a heterocyclic system with ten π-electrons free to circulate throughout the molecule which originate from four double bonds and the lone pair of the nitrogen atom. The participation of the nitrogen lone pair in the aromatic ring means that indole is not a base, and it does not behave like a simple amine.

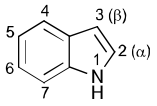


Figure 14. Indole chemical structure.

The chemistry of indole is extremely vast and, from a certain point of view, well established and consolidated.[109,110] Indoles typically function as excellent nucleophiles that readily participate in electrophilic aromatic substitutions that constitute by far the largest and most important group of indole reactions. These reactions commonly occur in a high regioselective fashion leading to C-3 substituted indoles. However, if the indole already bears a substituent in that position, then products of electrophilic substitution at C-2 are generally found. The N-1 position is next most active, but many of the reactions on the nitrogen are reversible. Thus, the chemistry of indole functionalization is based on the enhanced reactivity toward electrophiles[111] and commonly employed reaction in indole functionalization are Friedel-Crafts acylation using electron-poor alkenes, which may require a tunable activation by Lewis or Brønsted acids depending on the electrophilic properties of the olefin used, the Vilsmeier-Haack reaction, and Mannich and related reactions. The Friedel-Crafts-type 1,4-addition of indoles to unsaturated carbonyl compounds and their derivatives can be considered as Michael reaction[112] and recently enantioselective alkylations of indoles have gained attention.[113,114]

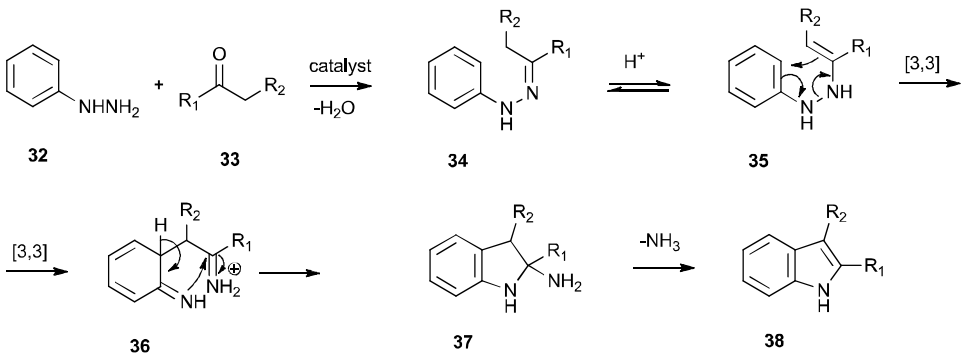
1.4.1 Methods for indole synthesis, two examples:

1.4.1.1 The classical Fischer indole synthesis:

For over a hundred years, the synthesis and functionalization of indoles has been a major area of focus for synthetic organic chemists, and numerous methods for the preparation of indoles have been developed and reviewed.[115–120] Key factors, including starting material

availability and functional group tolerance, often dictate which particular indole synthesis will be suitable.

The Fischer indole synthesis, first reported in 1883,[121,122] remains an essential methods for the synthesis of indoles and it is routinely employed in the large-scale production of indole pharmaceutical intermediates. This reaction consists of the condensation of an aromatic hydrazine with a ketone to form an arylhydrazone, followed by a [3,3]-sigmatropic rearrangement, subsequent ammonia elimination and aromatization. In many cases, the reaction is carried out by simply heating the ketone and the arylhydrazine in acidic solution without isolation of the intermediate hydrazone. In details, the mechanism of the Fischer indole cyclization depicted in Scheme 14 begins with formation of the phenylhydrazone **34** through the acid catalysed reaction of the phenyhydrazine **32** with the ketone **33**. Then, the phenylhydrazone get protonated and rearranges to the ene-hydrazine **35**. A [3,3]-sigmatropic rearrangement leads to the formation of the imine **36** that undergoes re-aromatization to give the aminal **37**. Finally, acid-catalysed decomposition of the aminal and subsequent loss of ammonia provide the indole product **38**.[123]



Scheme 14. Mechanism of Fisher indole synthesis.

This reaction is often catalysed by acids, including: mineral acids such as polyphosphoric acid (PPA), weak acids i.e. acetic acid, solid acids such as montmorillonite, KSF clay or acidic Zeolite catalysts, and Lewis acids. The most frequently employed Lewis acid is zinc chloride, either with no solvent or in ethanol or acetic acid. The use of a Lewis acids allows to run reactions at room temperature and therefore these conditions are much milder than other Fischer protocols. Moreover, Fischer methods for indole synthesis has also been applied to solid-phase synthesis.[124]

Advantages of the Fischer reaction include the easy functionalization of an inactivated aromatic C-H position by virtue of the [3,3]-sigmatropic shift and the acceptance of a wide range of compatible functional groups around the aromatic ring. Even though the Fischer indole synthesis is one of the most common methods for indole preparation, it also has

some potential problems and it is not suitable for all indoles. The major drawbacks of the Fischer indole reaction are the relatively small range of arylhydrazines that are commercially available and the low yields with numerous by-products often formed if the reaction involves unsymmetrical hydrazines or ketones. In this case, usually mixtures of regioisomers are obtained. For examples, when the phenylhydrazine is meta-substituted both the 6- and 4-substituted indoles will be achieved in an average ratio of 7:3, respectively.[124] Because indole is ubiquitous among a wide range of natural products, this reaction has been extensively investigated, ameliorated, and widely applied to pharmaceutical and medicinal chemistry as well as organic synthesis.[125–127] Asymmetric versions of the Fischer indole synthesis have been developed and optimised. Key features of this process was the introduction of the new and powerful chiral phosphoric acid catalyst as well as the identification of a cation exchange resin for the efficient removal of ammonia from the reaction mixture.[128] Indeed, Brønsted acidic catalysts must be employed in stoichiometric amount because of the stoichiometric formation of ammonia during the reaction, causing the poisoning of catalyst by salt formation. Furthermore, new mild, efficient and environmentally-friendly catalysts for Fischer indole synthesis have been studied. For example, Li *et al.*[129] employed an acidic ionic liquid as catalysts while Gore and co-workers[130] used low melting L-(+) tartaric acid (TA)-dimethyl urea (DMU) mixture in which the melt plays a dual role as solvent and as catalyst.

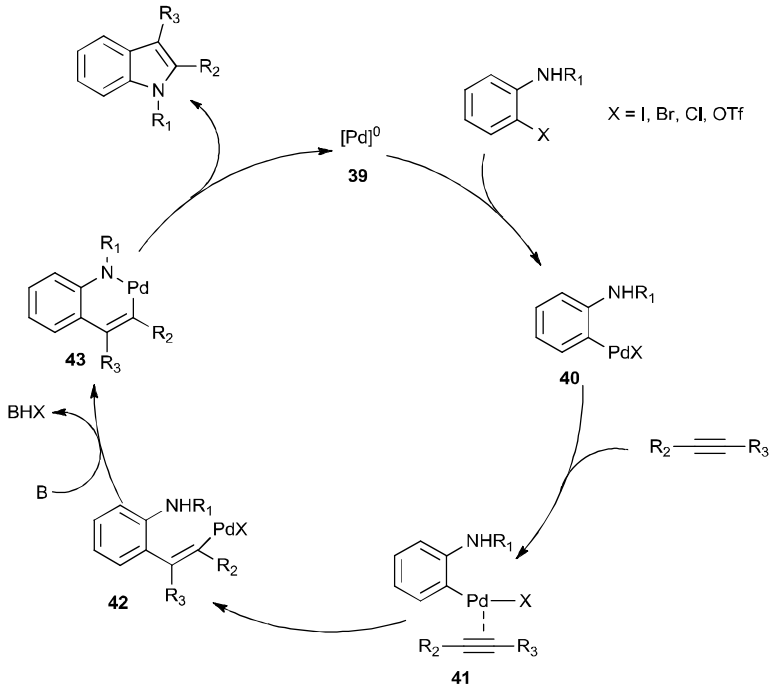
1.4.1.2 The Larock indole synthesis:

Since its first report in 1991,[131] the Larock indole synthesis has become one of the most attractive and practical method for the preparation of 2,3-disubstituted indoles and it has been extensively studied.[127] This approach to indoles involves a palladium-catalysed heteroannulation of internal alkynes using *o*-iodoanilines in the presence of a base and a lithium halide. As shown in Scheme 15 (next page), the catalytic cycle begins with the reduction of the palladium-catalyst to Pd(0), then the oxidative addition of the aryl halide to Pd(0) catalyst gives the intermediate **40**.

The next step is the π-coordination of the palladium to the alkyne (**41**) and subsequent regioselective *syn*-insertion into the arylpalladium bond to afford the vinylic palladium intermediate **42**. The following nitrogen displacement of the halide, bound to the Pd, results in the formation of the palladacycle **43** and the final reductive elimination affords the desired 2,3-substituted indole product regenerating the Pd(0) catalyst.

This reaction is affected by various factors, including an added base, lithium halide and its stoichiometry, the presence or absence of ligand PPh_3, and the reaction temperature.[132]

Alternative methods which enable the use of *o*-bromo- or *o*-chloroanilines as well as triflate as precursors have been developed to overcome the problem of the high cost and the low stability of *o*-iodoanilines.[133]



Scheme 15. Mechanism of Larock indole synthesis.

Other alternatives to the original Larock synthesis are based on the utilization of *o*-iodobenzoic acid[134] or *N*-aroylbenzotriazole[135] as synthetic equivalents of *o*-iodoanilines. A major drawback of Larock protocol arises in the control of the regioselectivity when unsymmetrical alkynes are used. The carbopalladation step is crucial to the regiochemical outcome of the reaction that depends on the nature of the substituents bound to the carbon-carbon triple bond. Normally, the more sterically bulky group ends up attached at position C-2 in the indole product.[136] Nevertheless, with alkynes bearing substituents with similar steric demands, mixtures of regioisomers are obtained. Despite the possible disadvantages, the Larock annulation reaction was widely employed for the preparation of indole derivatives and it is especially useful for preparing acid-sensitive substances where Fischer indole synthesis, for instance, would fail.[137]

Larock synthesis and other palladium-catalysed methods are particularly useful approaches to the synthesis of substituted indoles, and a large number of applications were developed based on this synthetic protocol. Furthermore, palladium is not the only metal found to be effective for indole synthesis but various transition-metal-catalysed synthesis of indoles have recently gained considerable attention.[138]

1.4.2 Indole as a privileged scaffold and its biomedical importance:

The term privileged structure was introduced in medicinal chemistry by Evans *et al.*[139] in 1988 and it was defined as "a single molecular framework capable of providing useful ligands for more than one receptor and that judicious modification of such structures could be a viable alternative in the search for new receptor agonists and antagonists". Hence, privileged structures are able to bind with a given protein family, i.e. GPCRs, and the generation of vast libraries based on these structures could allow the discovery of new biologically relevant compounds, interacting with different members of the same protein family, in a shorter time. Since 1988, the concept has been widely exploited in the literature,[140] some organic scaffold were classified as privileged structures and indoles fall into this category.[141]

The indole scaffold is probably the most widely distributed among heterocyclic compounds in nature having medicinal importance. Notably, the indole structure is found in naturally occurring molecules such as the essential amino acid tryptophan that is also the precursor of the neurotransmitter serotonin. Moreover, the indole scaffold is found in the hormone melatonin that plays an important role in the circadian rhythm regulation (Figure 15).

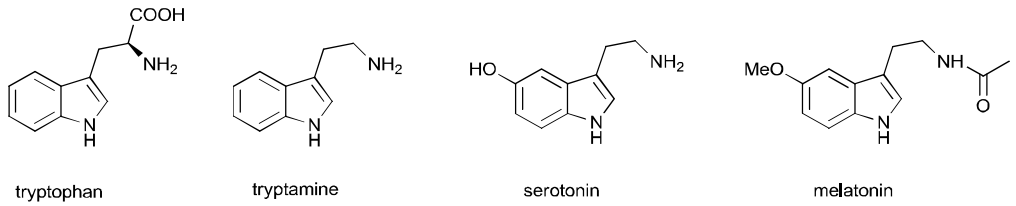


Figure 15. Structure of some naturally occurring indoles.

Metabolic decarboxylation of tryptophan leads to tryptamine which is very important in the plant kingdom, since it represents the biosynthetic precursor of many indole alkaloids.[142] Most of these alkaloids are biologically active with complicated frameworks and, together with their synthetic derivatives, show remarkable pharmacological activities. An interesting review[143] highlights all the different medical purposes for indole alkaloids that go from drugs for the treatment of Parkinson's disease such as bromocriptine to ergotamine for treatment of acute migraine attacks or vincristine and vinblastine that are antineoplastic agents. Thus, the indole scaffold has attracted a great deal of attention among the scientific community and the indole-based pharmaceuticals constitute a very important class of therapeutic molecules.

Table 1 lists some important indole ring-containing marketed drugs and their associated biological activities.[144]

Drug	Application	Drug	Application	Drug	Application
Vincristine	Anticancer	Vincamine	Antihypertensive	Roxindole	Antipsychotic
Vinblastine	Anticancer	Reserpine	Antihypertensive	Delavirdine	Anti-HIV
Vinorelbine	Anticancer	Peridopril	Antihypertensive	Atevirdine	Anti-HIV
Vindesine	Anticancer	Pindolol	Antihypertensive	Arbidol	Antiviral
Mitraphylline	Anticancer	Binedaline	Antidepressant	Zafirlukast	Anti-asthmatic
Etodolac	Antiarthritis	Amedalin	Antidepressant	Bucindol	β-Blocker
Panobinostat	Anti-leukamic	Oxypertine	Antipsychotic	Pericine	Opioid agonist
Tropisetron	Antiemetic	Siramesine	Anxiolytic	Mitragyne	Opioid agonist
Ondansetron	Antiemetic	Indolmycin	Antibiotic	Pravadoline	Analgesic
Sumatriptan	Antimigraine	Yohimbine	Sexual disorder	Bufotenine	Toxin
Oglufanide	Immunomodulatory	Indomethacin	NSAID	Proamanullin	Toxin

Table 1. Indole-containing drug molecules.

1.5 The endocannabinoid system:

The endogenous cannabinoid system, named after the plant that led to its discovery, is one of the most important physiologic systems involved in establishing and maintaining human health. Cannabinoids are a class of chemical compounds that include the phytocannabinoids, synthetic cannabinoids, and endogenous cannabinoids also called endocannabinoids.

Phytocannabinoids, found in *Cannabis sativa*, encompass Δ^9-THC, that is the primary psychoactive componentof the cannabis plant, [145] cannabidiol (CBD), and cannabinol (CBN). They are the most prevalent natural cannabinoids and have been largely studied.[146] Synthetic cannabinoids include a variety of distinct chemical classes: the classical cannabinoids structurally related to Δ^9-tetrahydrocannabinol (Δ^9-THC), the non-classical cannabinoids including the aminoalkylindoles, 1,5-diarylpyrazoles, quinolines, and arylsulfonamides, as well as eicosanoids related to the endocannabinoids.[147] The two best-studied endocannabinoids isolated to date are arachidonoylethanolamine (anandamide or AEA) and 2-arachidonoylglycerol (2-AG) (Figure 16).

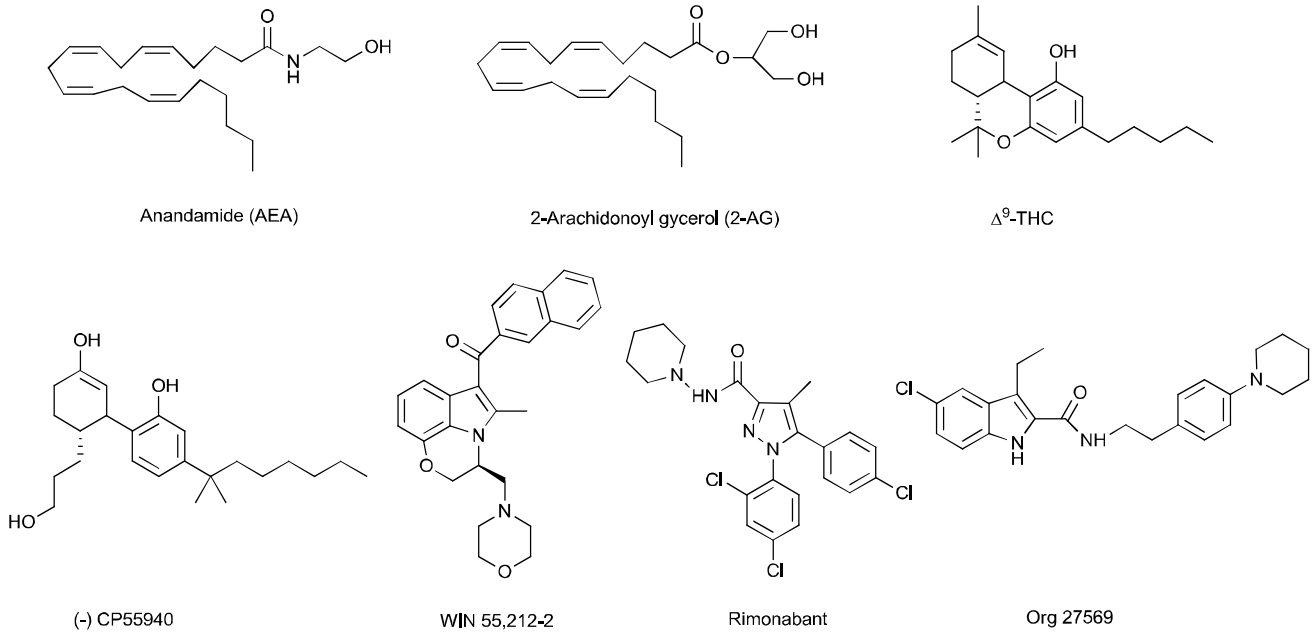


Figure 16. Chemical structures of the major plant cannabinoid (Δ^9-THC), the two best-studied endocannabinoids (AEA and 2-AG) and some synthetic cannabinoids.

The endocannabinoid system is implicated in different physiological and pathological functions such as nociception (pain sensation), appetite, lipid metabolism, gastrointestinal motility, cardiovascular modulation, motor activity, mood, and memory.[148] Pain

management is the most important among the medicinal purposes, and has been drawing intense attention following the discovery of cannabinoid receptors and their endogenous ligands.

1.5.1 Cannabinoid receptors:

Cannabinoids produce their effects through the activation of various receptors. Classical cannabinoid receptors are the CB_1 and CB_2 receptors that have been identified as members of the superfamily of seven-transmembrane-spanning G protein-coupled receptors.[149]

CB_1 receptors are found predominantly in the central nervous systems (CNS) and, to a lesser extent, in certain peripheral tissues. They are highly expressed in regions of the brain, such as the cortex, limbic system, hippocampus, cerebellum, brainstem, and several nuclei in the basal ganglia that are associated with emotion, cognition, memory, motor and executive function. More specifically, they are expressed in brain areas involved in pain transmission and modulation, in addition to the dorsal horn of the spinal cord and dorsal root ganglion (DRG).

CB_2 receptors are found mainly, but not exclusively, outside the CNS, in fact, they are primarily localized on cells of the immune system. CB_2 receptors in the brain can be found in the perivascular microglial cells and astrocytes, where they modulate the immune response.

Activation of both cannabinoid receptor subtypes triggers a number of signal transduction pathways via the $G_{i/o}$ family of G proteins. CB_1 receptors generally mediate the inhibition of neurotransmitter release by the inhibition of adenylate cyclase, and consequent inhibition of cyclic adenosine monophosphate (cAMP) production, through the α subunit of the G-protein-signalling complex while they mediate the activation of extracellular-signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) through the βγ subunit of this complex.[150] Furthermore, CB_1 receptors modulate synaptic transmission by inhibiting calcium channels and activating potassium channels on presynaptic terminals. Similar to CB_1 receptors, CB_2 receptors are also able to inhibit adenylate cyclase, but they are not involved in the regulation of calcium and potassium conductance.

Pharmacological evidence also supports the existence of one or more additional receptors for cannabinoids distinct from CB_1 and CB_2 receptors, such as the orphan-related receptors GPR55 and GPR18 that are also seven-transmembrane GPCRs and have been reviewed.[150–152]

1.5.2 Endocannabinoids:

The endocannabinoids are a family of compounds which may be agonists, antagonists or even inverse agonists depending on the dose, cannabinoid receptor, conformational state of the receptor, disease state as well as interactions with various ion channel. They include ethanolamine derivatives, such as anandamide (AEA) that was the first endocannabinoid isolated from the brain, and glycerol derivatives, i.e. 2-arachidonoyl glycerol (2-AG) shown in Fig. 16. 2-AG is a full agonist at CB_1 and CB_2 receptors while AEA is a partial agonist when intracellular calcium is the measured response.[153] Other endocannabinoids have been isolated, such as N-arachidonoyldopamine (NADA), 2-arachidonoylglyceryl ether (2-AGE, noladin ether), and O-arachidonoylethanolamine (OAE, virodhamine).[147]

Strong pharmacological and biochemical evidences have demonstrated that endocannabinoids are also able to interact with non-cannabinoids receptors, increasing the complexity of endocannabinoid system and thus its molecular pathways. In particular, their best known non-cannabinoid target is the transient receptor potential vanilloid type 1 (TRPV1) channel, which is activated by both AEA and 2-AG. Other receptors activated by endocannabinoids are peroxisome proliferator-activated receptor (PPAR), the aforementioned orphan-related receptors and others.[154]

The endocannabinoids are endogenous metabolites generated from hydrolysis of membrane polyunsaturated fatty acids and are not stored in vesicles but synthetized on demand in postsynaptic neurons and released immediately upon neuron activation. In the CNS, they act as neurotransmitters, thus they are released from depolarized postsynaptic neurons and travel to presynaptic terminals where they activate CB_1 receptors through a retrograde signalling mechanism.[155] Their overall effect may be excitatory or inhibitory, mainly resulting in suppression of neurotransmitters release, especially GABA (γ-aminobutirric acid) and glutamate.

A two-step process has been suggested for the biosynthesis of AEA. First, phosphatidylethanolamine, a membrane phospholipid, is converted to N-acyl-phosphatidylethanolamine (NAPE) by a calcium-dependent N-acyltransferase (NAT). Then, NAPE is hydrolysed to AEA by a specific phospholipase D (NAPE-PLD).[156]

For 2-AG, the major pathway also consists of a two-step process: diacylglicerol (DAG) is initially produced from inositol phospholipids by the phospholipase C enzyme, and subsequently hydrolysed to 2-AG by diacylglycerol lipase (DAGL).[157] (Figure 17)

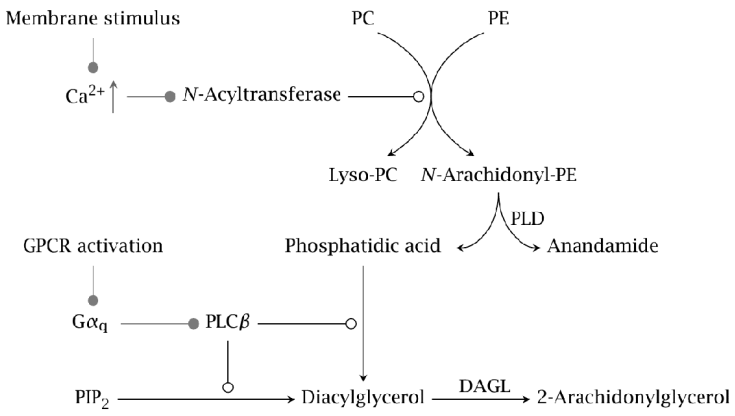


Figure 17. Endocannabinoids synthesis.

After activating cannabinoid receptors, endocannabinoid signalling functions are efficiently terminated by cellular uptake and rapid, enzyme-hydrolytic inactivation. Neuronal uptake for AEA occurs by facilitated diffusion rather than employing a specific transport mechanism.[158] This process is saturable, temperature-dependent, does not require ATP and is driven by a transmembrane concentration gradient. However, the existence of an endocannabinoid-specific transporters remains controversial and the mechanism of re-uptake is not fully understood. After re-uptake, anandamide is rapidly catabolyzed by fatty acid amide hydrolase-1 (FAAH-1) to arachidonic acid and ethanolamine. Not much is known about the uptake of 2-AG but, after exerting its activity, it is rapidly metabolized to yield arachidonic acid and glycerol by intracellular monoacylglycerol lipase (MGL).[159]

1.5.3 Modulation of endocannabinoid system:

Analgesia is one of the principal therapeutic targets of cannabinoids. Animal studies have firmly established cannabinoid-induced analgesia in a wide array of pain models.[147] In models of acute or physiological pain, cannabinoids are highly effective against thermal, mechanical, and chemical pain, and typically, cannabinoids are comparable with opiates, both in potency and efficacy, in producing antinociception. On the other hand, in models of chronic pain, both inflammatory and neuropathic, cannabinoids have shown even greater potency and efficacy. Neuropathic pain is defined as pain arising as a direct consequence of a lesion or disease affecting the somatosensory system. It can be caused by several disorders, like nerve injury, diabetes, viral infection, and chemotherapic agents. Currently available compounds (cannabinoid agonists) include Sativex®, a cannabis extract approved in UK and Canada for the treatment of spasticity and pain in multiple sclerosis, and medical cannabis, approved for prescription by Health Canada. Nevertheless, central

psychoactive side effects of these exogenous cannabinoids, most of which are related to CB_1 receptors activation in the CNS, directed researchers to look for alternative strategies. One approach may be to develop cannabinoid receptor agonist that selectively target non-CNS cannabinoid receptors. For example peripherally restricted CB_1 agonists[160,161] were designed to control neuropathic pain. Targeting of non-CNS cannabinoid receptors could be also desirable when antagonism is the required effect. Rimonabant, an anti-obesity CB_1 inverse agonist depicted in Figure 16, was launched in Europe in 2006 but soon withdrawn in 2008 due to severe CNS adverse effects, including depression, anxiety, and stress disorders. Peripherally selective CB_1 antagonists for the treatment of obesity could cause weight loss and improve related metabolic disorders without additional CNS-mediated behavioural effects.[162,163]

Selective CB_2 receptor agonists also deserve attention, since they are expected to minimise unwanted CNS effects mediated via CB_1 receptors activation, and this make them very promising candidates for the treatment of pain. Thus, selective CB_2 agonists with analgesic effects in human chronic inflammatory and joint related pain have been developed.[164,165]

Another approach for increasing the level of endocannabinoids may be to inhibit their uptake. Thus, inhibitors of endocannabinoid cellular uptake have been identified.[166] However, the major issues concerning a better development of these inhibitors is the fact that the mechanism underlying endocannabinoid cellular uptake has not been fully elucidated yet.

Furthermore, compounds which increase levels of endocannabinoids by preventing their breakdown (FAAH and MGL inhibitors)[167] have aroused a lot of interest, hence more and more of such inhibitors have been developed, including both reversible and irreversible inhibitors. Unfortunately, there are several drawbacks to cannabinoid-hydrolyzing enzyme inhibitors. One of these worth mentioning is the analgesic tolerance as a consequence of chronic administration of these inhibitors, through plastic changes in the endocannabinoid system.[153]

1.5.3.1 Allosteric modulation of cannabinoid receptors:

It is now acknowledged that numerous GPCRs contain allosteric binding sites for endogenous and/or synthetic ligands, which are topographically distinct from the agonist-binding site, known as the orthosteric site. The binding of allosteric modulators delivers a conformational change which affects the affinity and/or efficacy of the orthosteric ligand, thereby fine-tuning the pre-existing actions of the endogenous ligands, in fact allosteric

modulators have no effect in the absence of agonist. The first evidence of an allosteric binding site at the cannabinoid CB_1 receptor was reported by Price *et al*.[168] in 2005.

The identification of the CB_1 allosteric binding site prompted the search for allosteric ligands as new therapeutic tools since allosteric enhancement has the advantage of increasing endocannabinoid effects only at the site of release (e.g., in the spinal cord or at the site of inflammation), thus providing side-effect-free pain relief where required. Positive allosteric modulators (PAMs; allosteric enhancers) of the CB_1 receptor are predicted to have therapeutic potential similar to that of cannabinoid agonists, but again lacking the side effects of indiscriminate systemic activation of the cannabinoid system.

To date, a number of PAMs of the CB_1 receptor have been identified.

For example, Org 27569[169] shown in Figure 16, was proved to be a PAM modulator, its binding site of CB_1 receptor was recently elucidated and its activity evaluated in *in vivo* models.[170,171] Furthermore, indole-based positive allosteric modulators of CB_1 receptors have been identified and patented.[172,173] (Figure 18)

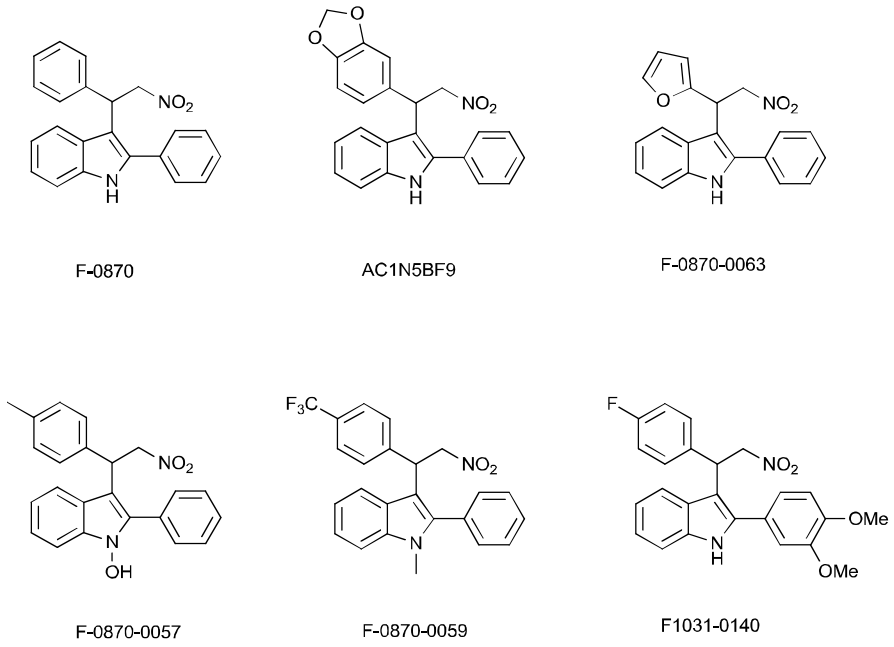


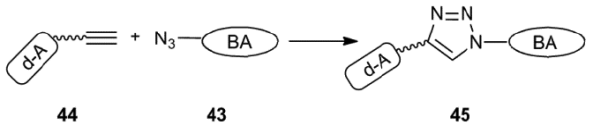
Figure 18. Chemical structure of some of the indole-based patented PAMs of cannabinoid CB_1 receptors.

2 Project 1: "Synthesis and in vitro cytotoxicity of deoxyadenosine-bile acid conjugates linked with 1,2,3-triazole"

2.1 Aim of the project:

Among the variety of molecular scaffold employed as building block for the design and synthesis of novel conjugates, bile acids and their derivatives have received a great deal of attention in many fields.[49,71,174–177] The interest in this class of natural compounds is explained by the peculiar combination of features as rigidity, chirality, amphiphilicity and modularity, together lipophilicity another well-known feature of bile acid structure, that is believed to be correlated with induction of cytotoxicity and apoptosis in several human cancer cells. Indeed, synthetic derivatives of chenodeoxycholic acid and ursodeoxycholic acid conjugates, significantly inhibit cell growth and induce apoptosis in various human cancer cells.[178–181] The literature also describes a vast amount of bile acid conjugates with improved pharmacological profiles in terms of bioavailability and biostability which demonstrate the further potential of modified bile acids to act as hybrid molecules and prodrug moieties in drug discovery.[71,75] The expanding number of applications employing bile acid templates has stimulated the development of new conjugates with enhanced properties, obtained using specific and in some cases orthogonal reactions. The copper(I)-catalyzed Huisgen-1,3-dipolar cycloaddition[182] between alkynes and azides (CuAAC) to form 1,4-disubstituted 1,2,3-triazoles linker, is a well- established reaction for the preparation of bioconjugates and 1,2,3-Triazole moieties are attractive linkers, because they are stable under typical physiological conditions and form hydrogen bonds which can be suitable for solubility enhancement and for binding of biomolecular targets.[2] Few examples of bile acid-nucleoside conjugates have been reported in literature,[76,183,184] and to the best of our knowledge there are no synthetic approaches with 1,2,3-triazoles as connecting units, nor about the cytotoxic evaluation of bile acid-nucleoside conjugates as new potential anticancer drugs.Purine nucleoside analogues are a pharmacologically interesting class of compounds with a long history for treatment of various viral and tumor diseases.[185–187] Particularly, several deoxyadenosine nucleoside analogues represent a group of cytotoxic agents with high activity in lymphoid and myeloid malignancies.[188,189]

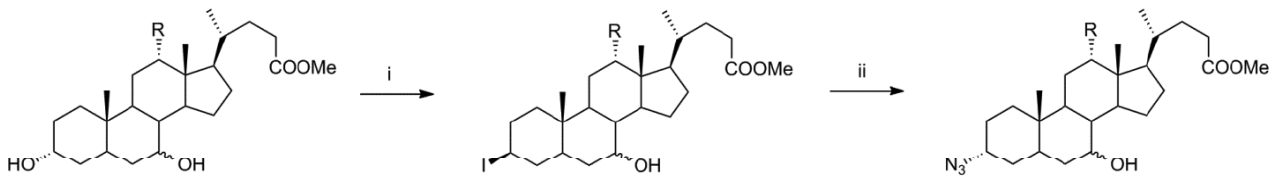
With all this in mind, we considered of interest the synthesis and biological evaluation of bile acid-deoxyadenosine conjugates **3** (Scheme 16).



Scheme 16. d-A = deoxyadenosine derivative, BA = Bile Acid part.

2.2 Synthesis of C-3α-azide derivatives:

In our approach to synthesize these new conjugates using "click" chemistry, three bile acids with different hydrophobic nature were converted into their 3α-azido derivatives. As outlined in Scheme 17, azido derivatives **51**, **52**, and **53** were prepared in two steps starting from the methyl esters[190] of commercially available cholic acid (CA), chenodeoxycholic acid (CDCA), and ursodeoxycholic acid (UDCA), respectively. Introduction of 3α-azides was accomplished through a double nucleophilic displacement, i.e., with retention of configuration, according to a literature procedure,[191] with small modifications. Thus, 3-iodides **48**, **49**, and **50** were generated upon treatment of the corresponding unprotected bile acid methyl esters with I_2 and Ph_3P in the presence of imidazole and 1,3-dioxolane.



45 R = OH, C_7 α-OH **CA-OMe**
46 R = H, C_7 α-OH **CDC-OMe**
47 R = H, C_7 β-OH **UDC-OMe**

48 R = OH, C_7 α-OH **I-CA-OMe**
49 R = H, C_7 α-OH **I-CDC-OMe**
50 R = H, C_7 β-OH **I-UDC-OMe**

51 R = OH, C_7 α-OH **N_3-CA-OMe**
52 R = H, C_7 α-OH **N_3-CDC-OMe**
53 R = H, C_7 β-OH **N_3-UDC-OMe**

Scheme 17. Synthetic sequence for preparation of 3-α azide derivatives.(i) I_2, PPh_3, imidazole, 1,3-dioxolane, 25 °C, 30 min, 55-67% ; (ii) NaN_3, DMF, 25 °C, 6 h, 80-94%.

Although the conversion of bile acid methyl ester substrates was almost quantitative in all the cases, the lengthy separation of 3β-iodides from small quantities of not well-recognized by-products lowered the isolated yields ranging from 55 to 67%. However, the nucleophilic displacement with iodide was selective for the less hindered 3α-OH of CA and CDCA esters, as previously reported[174,192,193] for the introduction of methane-sulfonic acid as a nucleophile, under Mitsunobu conditions. In contrast, in the case of UDCA methyl ester **47**, the formation of small amounts (less than 10%) of a diiodide derivative

was pointed out according to the increased reactivity of the equatorial 7β-OH, and the further lowering of isolated yield (55%). The 3β-iodides **48-50** were used for back displacement with NaN_3 in DMF to give the desired 3α-azides **51-53** in 80–94% isolated yields (Scheme 2).

2.3 Synthesis of 8-aklynyl deoxyadenosine:

For the "click" conjugation, a series of three deoxyadenosines bearing a different alkynylic substituent on the C-8 position was selected (Fig. 19). Lastly, in order to further explore the requirements for bile acid-based conjugate cytotoxicity, propargyl alcohol **57** was added to the series of terminal alkynes.

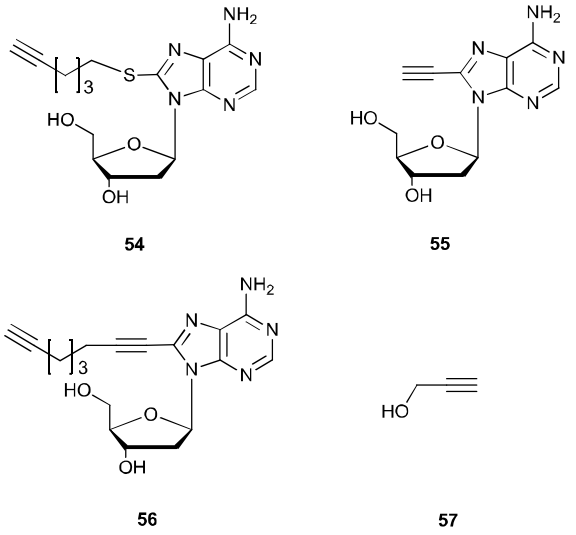
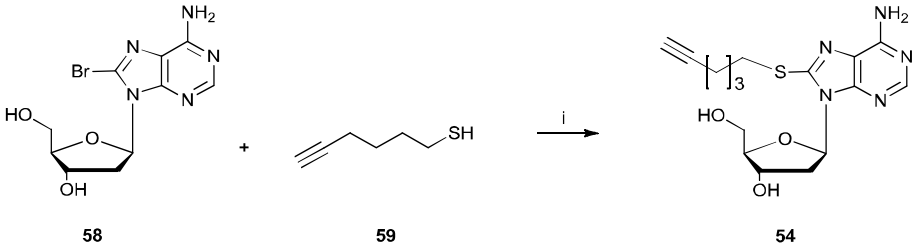


Figure 19. Alkynes used for click reactions.

The thiopurine **54** was prepared by coupling of 8-bromodeoxyadenosine **58** with hexyne thiol as shown in Scheme 18.

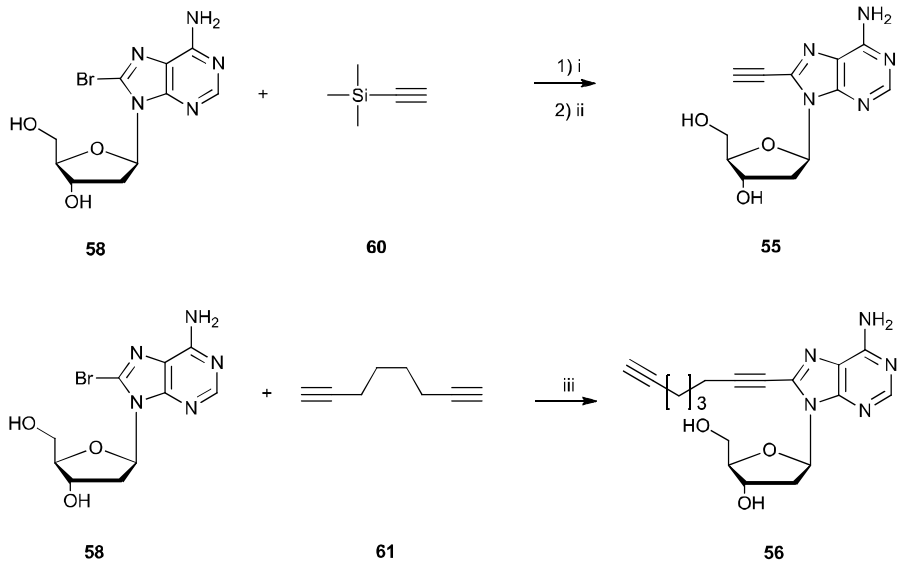


Scheme 18. Synthesis of alkynyl-deoxyadenosine **54**. (i) H_2O, TEA, 100 °C, 2h, 30%.

This reaction can be smoothly performed in water and leads to a simple purification of the thioalkylated nucleoside **54** that only requires the extraction of the compound with warm EtOAc from the aqueous crude mixture and the removal of the solvent by evaporation

under reduced pressure. However, the desired product was recovered in low yield, probably due to the poor hydrofilicity of the hex-5-yne-1-thiol **59**.

The synthesis of the 8-substituted adenosine **55** and **56** were carried out by a palladium catalysed cross-coupling reaction starting from commercially available 8-bromoadenosine **58** as reported in a literature procedure.[194] Thus, a Sonogashira coupling[195] of **58** with protected alkynes **60** in DMF in the presence of 0.06 equivalents of $Pd(PPh_3)Cl_2$, 0.16 equivalents of CuI, and 10 equivalents of TEA afforded the corresponding protected alkynyl-adenosine that was desilylated with NH_4OH in MeOH to obtain the desired product **55** in 63% isolated yield. As mentioned above, the same reaction conditions were applied for the synthesis of modified adenosine **56** that was obtained in 73% yield. (Scheme 19)



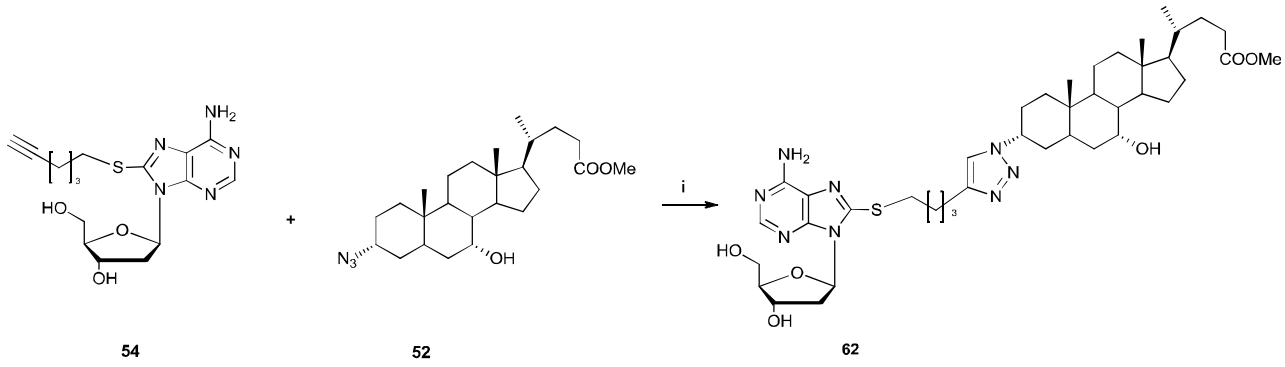
Scheme 19. Synthesis of alkynyl-adenosine **55** and **56**. (i) dry DMF, TEA, CuI, $Pd(PPh_3)Cl_2$, 50 °C, 15 min, 60%; (ii) NH_4OH, MeOH, 10 min, 50 °C, 63 %, (iii) dry DMF, TEA, CuI, $Pd(PPh_3)Cl_2$, 50 °C, 15 min, 73%.

2.4 Synthesis of bile acid-deoxyadenosine conjugates:

Having the corresponding sets of 3-azidobile acids **51-53** and 8-alkynylated deoxyadenosines **54-56** in hand, our next aim was to synthesize target bile acid-deoxyadenosine conjugates linked with a 1,2,3-triazole moiety.

Azide **52** and terminal alkyne **54** were selected to explore the feasibility of the "click" chemistry for the construction of novel conjugates. The cycloaddition reaction was attempted under commonly used conditions: the *t*BuOH-H_2O mixture in the presence of the $CuSO_4$ catalyst and sodium ascorbate.[5] At first, using 0.04 molar equivalents of the

catalyst and 2 molar excess of sodium ascorbate, only a small amounts of conversion compound was observed by TLC and ^{1}H-NMR analysis, even after 72 h at room temperature. An analogous experiment with CuI and DIPEA[4] did not improve the conversion. The best result were obtained using 0.4 molar equivalents of $CuSO_4 \cdot 5H_2O$ relative to the alkyne, in the THF-*t*BuOH-H_2O mixture (Scheme 20).



Scheme 20. (i) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, THF/*t*BuOH/H_2O (1.5:1:1), 25 °C, 18 h, 70 %; or microwave 80 °C, 30 min, 73%.

THF was the most effective co-solvent tested to dissolve lipophilic azide **52** in combination with water and *t*BuOH. The conversion was complete after 18 h and allowed the synthesis of the desired conjugate **62** in 70% yield after chromatographic purification. The same protocol to perform "click" conjugation was carried out for all terminal alkynes shown in Fig. 19 with azidobile acid derivatives shown in Scheme 17. All reactions proceeded to completion with isolated yields of conjugates ranging from 68 to 85% as summarized in Table 2.

Azide **Alkyne**	**51** **(N_3-CA-OMe)**	**52** **(N_3-CDC-OMe)**	**53** **(N_3-UDC-OMe)**
54	**63** (68%)	**62** (70%)	**64** (73%)
55	**65** (75%)	**66** (80%)	**67** (75%)
56	**68** (70%)	**69** (75%)	**70** (68%)
57	**71** (85%)	**72** (80%)	**73** (83%)

Table 2. Isolated yields (%) of conjugate compounds.

Despite the satisfying results, the “click” reaction investigated was under microwave conditions with the aim to improve the yields of conjugate compounds.

The CuAAC reaction of **54** with **52** under microwave irradiation at 80 °C for 30 min with a 1:1.5:0.4:2 molar ratio of alkyne, azide, $CuSO_4 \cdot 5H_2O$, and sodium ascorbate in the THF-*t*BuOH-H_2O mixture (1.5:1:1, *v/v*) proceeded with complete conversion, nevertheless affording conjugate **62** in 73% isolated yield. Other optimization attempts failed, so only a rate enhancement of microwave assisted conjugation reaction was observed.

2.5 Biological evaluation:

The conjugated compounds reported in Table 1, as well as the free alkynyl deoxyadenosines **54**, **55**, and **56** shown in Fig. 19, were evaluated for their cytotoxic activity against human cells of different histopathological origin: two leukemia cell lines (Jurkat and K562), a colon cancer cell line (HCT116), an ovarian cancer cell line (A2780) and, as a control, normal human skin fibroblast cells. Cisplatin served as a reference compound. The cytotoxicity was evaluated using MTT assay[196] and cell growth inhibition was determined at concentrations of 200, 100, 50, and 10 μM for all compounds after 72 h. The estimated IC_{50} values in comparison with that for cisplatin are shown in Table 3.

Compound	Log *P*[b]	K562 IC_{50} (μM)	Jurkat IC_{50} (μM)	HCT116 IC_{50} (μM)	A2780 IC_{50} (μM)	Human Fibroblast IC_{50} (μM)
CA series						
68	2.97	141.79 ± 2.49	168.97 ± 1.99	>200	>200	>200
65	4.77	131.90 ± 9.29	86.18 ± 7.32	>200	>200	>200
63	4.62	172.36 ± 9.60	35.86 ± 11.60	>200	>200	>200
71	2.35	51.06 ± 4.24	77.80 ± 0.54	96.93 ± 9.00	84.47 ± 3.90	128.79 ± 19.09
CDC series						
69	4.28	23.25 ± 4.32	21.88 ± 1.16	146.32 ± 7.34	>200	155.2 ± 3.15
66	6.08	8.51 ± 4.05	10.47 ± 2.64	>200	>200	>200
62	5.94	22.05 ±0.61	16.76 ± 3.06	>200	>200	>200
72	3.66	38.84 ± 2.50	51.43 ± 7.57	75.49 ± 11.70	69.88 ±13.85	79.01 ± 3.90
UDC series						
70	4.28	35.65 ± 2.23	36.17 ± 1.51	>200	>200	36.36 ± 1.21
67	6.08	102.35 ± 2.05	24.57 ± 2.31	>200	>200	>200
64	5.94	125.33 ± 26.87	23.93 ± 0.98	>200	>200	>200
73	3.66	47.23 ±0.52	36.44 ± 18.07	65.37 ± 26.02	84.61 ± 1.39	80.18 ± 1.47
Alkynyl-deoxyadenosine						
55	0.19	>200	>200	>200	>200	>200
56	2.24	156.63±14.76	123.48±19.09	112.58±5.75	107.01±1.27	>200
54	2.09	174.87±15.06	183.35±17.06	110.69±3.44	131.67±1.94	>200
Cisplatin[c]		5.35±1.01	2.21±1.51	8.47±1.20	0.95±0.35	25.38±3.51

[a] IC_{50} values were determined from the dose-response curves using MTT assay. Results are expressed as the mean of three independent experiments ± SD. [b] Log P was determined by MarvinSketch program. [c] Used as a reference compound.

Table 3. Cytotoxic activity of bile acid-based conjugates and alkynyl deoxyadenosines **54-56** on human cancer cell lines and human fibroblast cells.

Data for all the conjugated compounds against the K562 line as the mean of three independent experiments are graphically represented in Fig. 20.

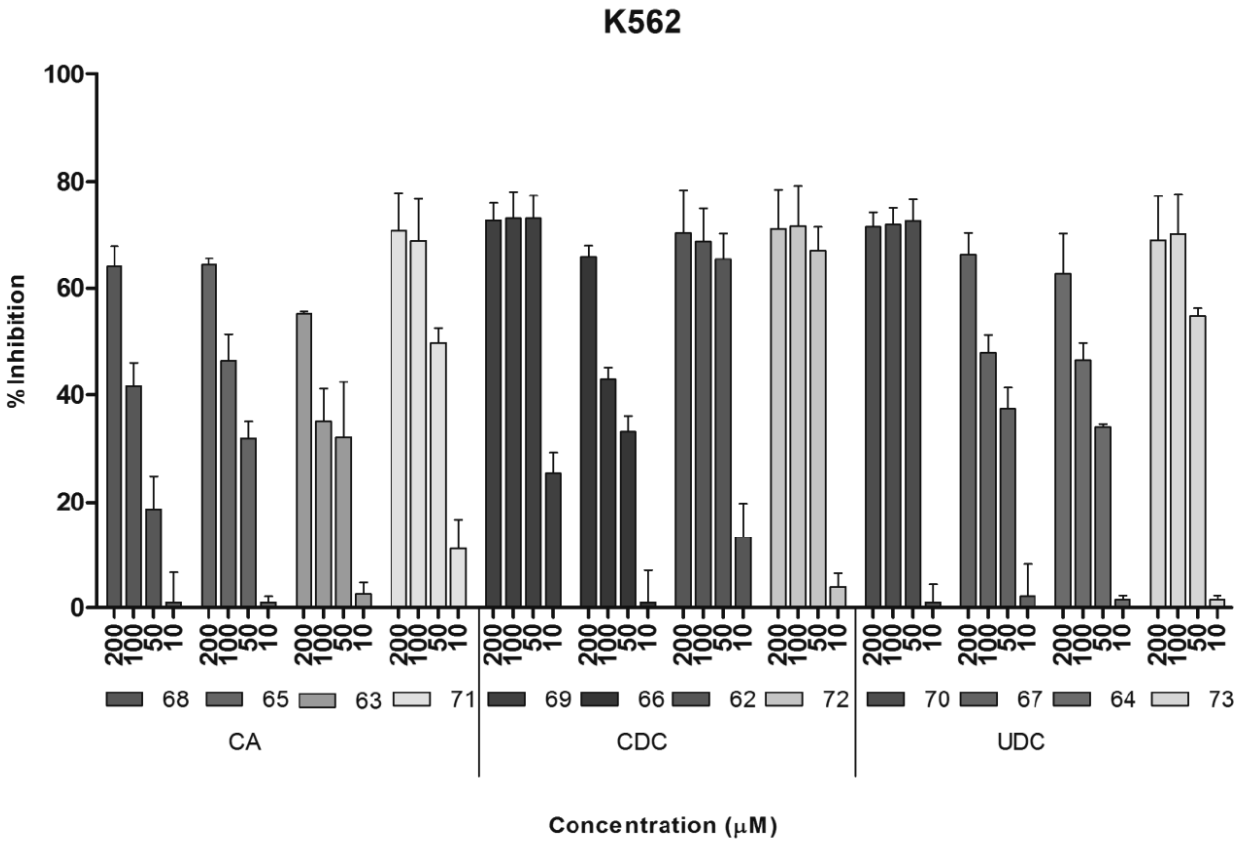


Figure 20. Anti-proliferative activity of conjugated compounds against the K562 cell line. Mean of three independent experiments ± SD.

Similar results were obtained for the Jurkat cell line as shown in Figure 21 (next page).
No cytotoxicity was observed with modified deoxyadenosine-bile acid conjugates **62-70** on HCT116 and A2780 cell lines up to 200 mM concentration. In contrast, some of these conjugates showed a significant concentration-dependent anti-proliferative effect on both leukemia cell lines. The highest activity was obtained with CDC-derivatives. Particularly, the best cytotoxicity was observed for **69** on Jurkat and K562 cell lines with IC_{50} values of 10.47 and 8.51 μM respectively. These values were quite close to the cisplatin ones, whereas **69** was not toxic against healthy fibroblast cells. Conjugate **62** also displayed a substantial activity with IC_{50} 16.76-22.05 μM. Otherwise, UDC-based conjugates **64**, **67** and **70** showed good cytotoxicity only on Jurkat line.

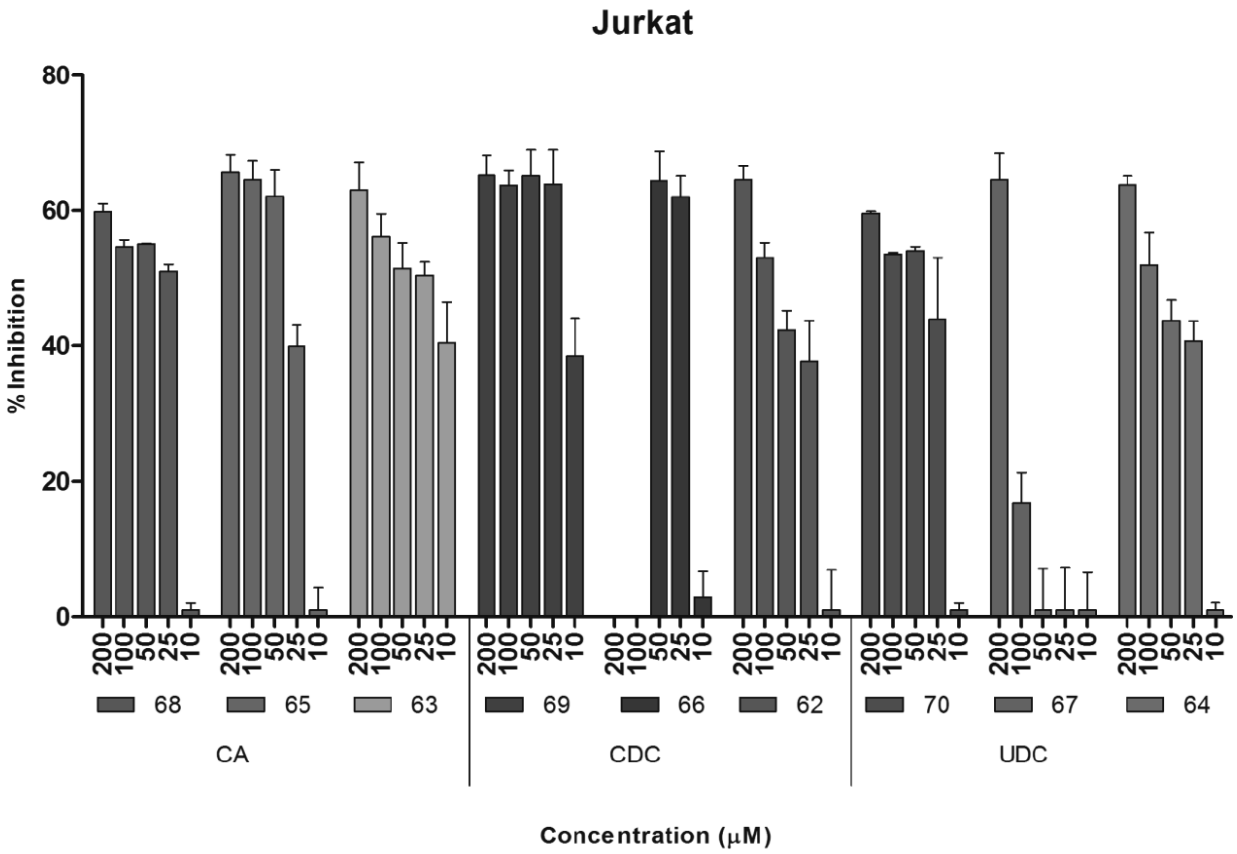


Figure 21. Anti-proliferative activity of conjugated compounds against the Jurkat cell line. Mean of three independent experiments ± SD.

A poor anti-proliferative effect was observed on the leukemia cell lines with compounds **63**, **65**, and **68** including a cholic acid scaffold. On the other hand, the lipophilicity (log *P*) of all bile acid conjugates was predicted from structures using the MarvinSketch program (Table 2) and the data found showed that compounds **62** and **69** containing CDC scaffold, and **64** and **70** containing UDC scaffold, have the highest lipophilicity, suggesting that a relationship between bile acid lipophilicity and cytotoxic activity of these conjugates can be assumed. This is consistent with several studies on bile acid derivatives, recently reported in the literature.[178,181,197] Furthermore, it is worth noting that, in the test on human fibroblast cells, no significant cytotoxic effect was observed for modified deoxyadenosine-bile acid conjugates **62-70**, with compound **67** as the only exception (IC_{50} 36.36 μM). As far as **54**, **55**, and **56** are concerned, no considerable cytotoxic activity was detected in all lines tested (IC_{50} ranging from 107.01 to >200 μM).

Finally, it can be seen that propargyl alcohol-bile acid conjugates **71**, **72** and **73** caused cell death at a quite similar concentration in all cell lines tested including healthy fibroblast cells, suggesting that these conjugates showed a non-structure-specific cytotoxic

mechanism. Taken together these results, the following general structure-cytotoxicity observations can be made: (i) the activity of conjugated compounds was suppressed when the steroid moiety possessed the hydroxyl group in the C-12 position (cholic acid structure); (ii) comparing CDC-based with UDC-based conjugates the activity was enhanced with the 7-OH group in the alpha stereochemistry; (iii) the substitution of a sulphur with an alkynyl moiety increased the cytotoxic activity against the K562 cell line; (iv) comparing propargyl acohol-bile acid with deoxyadenosine-bile acid compounds, it can be concluded that the conjugation to a C-8 alkynylated deoxyadenosine derivative played a crucial role in the selectivity of the cytotoxic process. Following the results obtained, the mechanism of K562 cell death was analysed. For this purpose, it was investigated whether the anti-proliferative effects of **62** and **69** and propargyl acohol-based compound **72** were due to apoptosis.

As shown by the annexin V test (Fig. 3), the percentage of dead cells significantly increased after 24 h treatment with 50 μM of **62** and **69**, but this effect was not present with **72**. The percentage of specific apoptosis was found to be 96% for **69** and 46% for **62**. These data are in accordance with IC_{50} values. The low percentage of specific apoptosis of **72** (9%) suggested that this conjugate induced K562 cell death through a necrosis process.

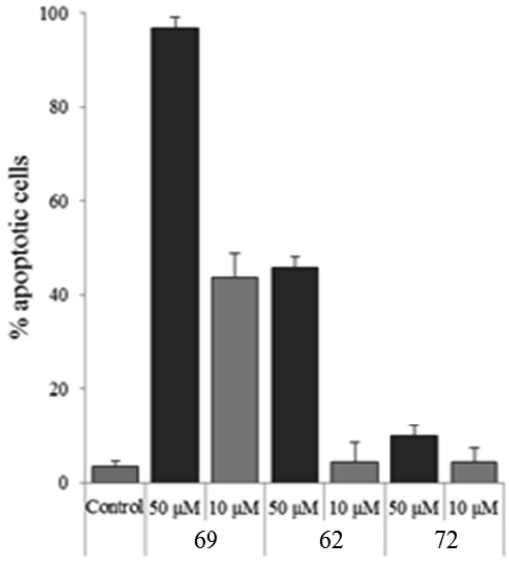


Figure 22. Percentage of apoptotic K562 cells determined after 24h treatment with **62**, **69** and **72** (50-10 μM) by annexin V staining.

2.6 Conclusions:

In conclusion, twelve novel bile acid-based conjugates were synthesized by using a "click" chemistry approach. Their *in vitro* anti-proliferative activity on four human cell lines and cytotoxicity toward human fibroblast cells were evaluated. Some of them strongly and selectively inhibited cell proliferation and induced apoptosis in cultured human leukemic K562 cells. The best activity shown by CDC-based derivatives **62** and **69** depicted in Figure 23, seemed to be correlated to the lipophilicity and to the 7α-OH group orientation. Furthermore, except conjugate **67** (UDC-series) and propargyl alochol-bile acid series, all new conjugates did not show any significant cytotoxicity to the human fibroblast cells.

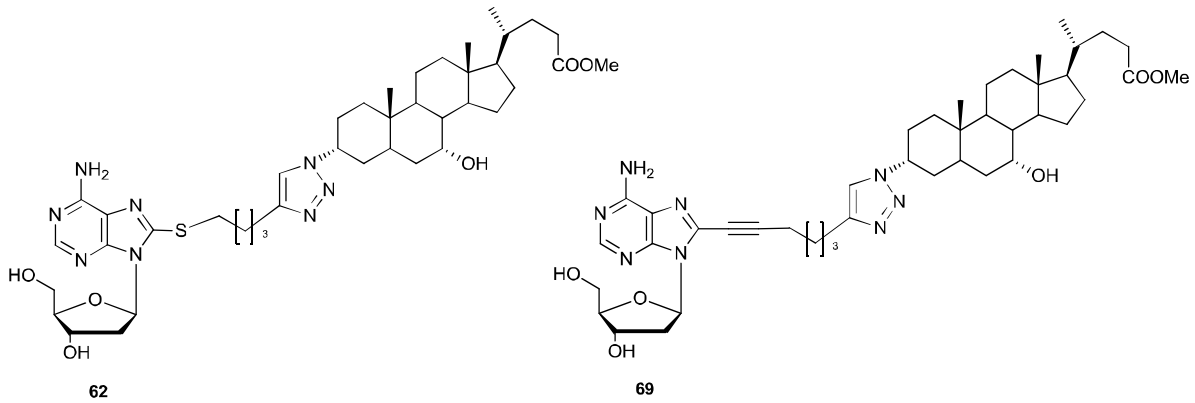


Figure 23. Bile acid-deoxyadenosine conjugate **62** and **69**.

Therefore, these derivatives constitute a starting lot of candidate drugs toward the development of promising chemical entities that, including a nucleoside-bile acid moiety in their structure, specifically target human cancer cell lines by triggering an apoptotic process.

This work resulted in a scientific publication. For all the details concerning the experimental part, see the paper in attachment.

3 Project 2: "Use of steroid scaffold in "click chemistry" orthogonal reactions for the synthesis of new fluorescent bisphosphonate conjugates"

3.1 Aim of the project:

Bisphosphonates are metabolically stable analogues of pyrophosphates. These compounds are formed by formal replacement of the P-O-P linkage with the P-C-P bond which allows additional structural modifications that would be impossible in the pyrophosphate structure. These agents, by virtue of their backbone P-C-P structure and ability to chelate calcium ions, target rapidly to bone mineral hydroxyapatite (HAP), moreover they are powerful inhibitors of bone resorption[77] affecting osteoclast recruitment, differentiation, resorptive activity and apoptosis. For this reason, bisphosphonates are used in both prevention and treatment of osteoporosis and other bone diseases such as Paget's disease, myeloma and hypercalcemia.[78,79]

Bortolini *et al.*[198] recently reported the synthesis of a series of bile acid-derived bisphosphonates and demonstrated that these new conjugates exhibit an affinity to HAP much higher than neridronate or clodronate (Fig.24), drugs currently approved for the treatment of bone diseases.

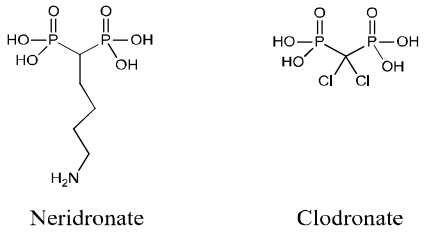


Figure 24. Chemical structure of bisphosphonates currently approved for the treatment of bone diseases.

Furthermore, cytotoxicity screening performed on these new analogues showed that bile acid-containing bisphosphonate salts are significantly less cytotoxic than neridronate in L929 murine fibroblast culture cells. These new compounds also decreased the formation of human osteoclasts, increasing the percentage of apoptotic cells compared to neridronate.

The chenodeoxycholic-derived bisphosphonate salt reported in Figure 25 showed the highest affinity to hydroxyapatite and the best biological activities.

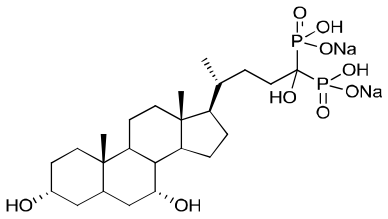


Figure 25.

Following up these new results, we envisaged the synthesis of four orthogonally functionalized bile acid scaffolds (*cholic*, *chenodeoxycholic*, *lithocholic*, and *ursodeoxycholic* series) to achieve a small collection of new bile acid-based bisphosphonates by using a "click chemistry" approach.

3.2 Synthesis of bile acid scaffolds:

3.2.1 Synthesis of C-3α-azide derivatives:

In our approach to synthetize new bile acid-bisphosphonate conjugates, two human primary bile acids, cholic acid and chenodeoxycholic acid, one secondary human bile acid, lithocholic acid, and the ursodeoxycholic acid (Fig. 26) were selected for the scope. Bile acids were functionalized at C-3 with an azide moiety and C-24 with a thiol group through a cysteamine linker.

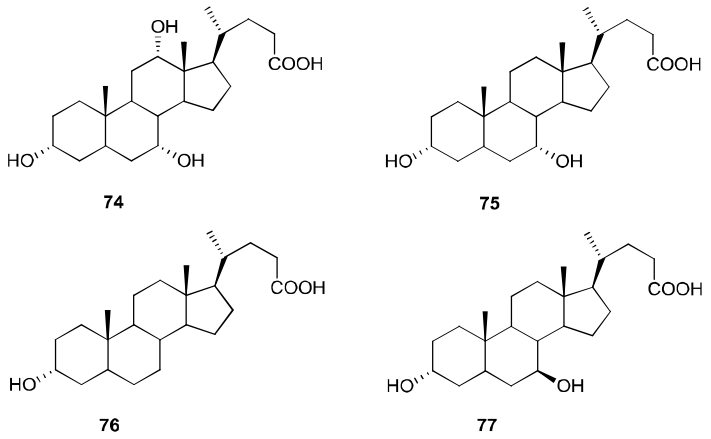
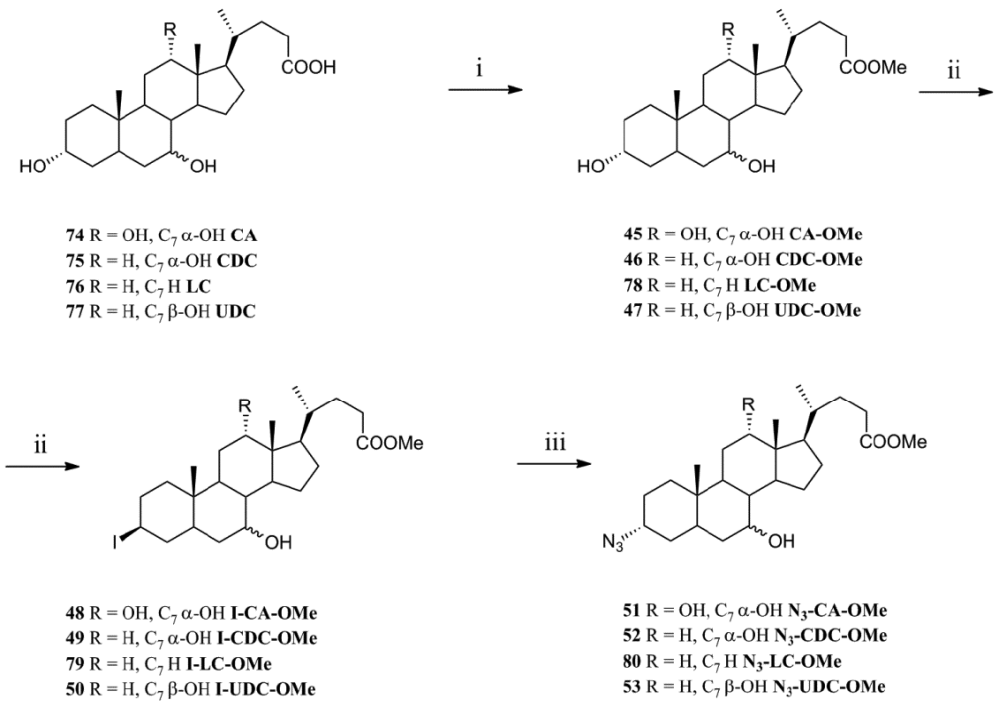


Figure 26: cholic acid (**74**), chenodeoxycholic acid (**75**), lithocolic acid (**76**), urodeoxycholic acid (**77**)

Introduction of 3-α-azide functionality was accomplished through a double nucleofilic displacement, with retention of configuration, according to our previous procedure,[199] with small modifications.

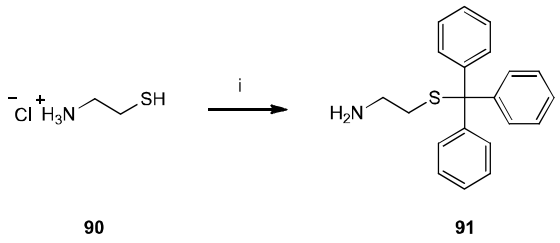
First, the bile acids **74-77** (Figure 26) were converted into the corresponding methyl esters **45**, **46**, **47** and **78** using the classical Fisher esterification method; then, the 3-β-iodides **48-50** and **79** were generated upon treatment of these esters with the triphenylphosphine/iodine/imidazole reagent systemin 1,3-dioxolane.[200] Although the conversion into 3-β-iodides was almost quantitative, the difficulty in chromatographic purification of **48-50** and **79** from not well-recognized byproducts lowered the isolated yields. To overcome this limitation, the expensive and time-consuming chromatographic step was replaced by a crystallization procedure in MeOH/EtOAc that led to a significant improvement of the isolated yields. The resulting 3-β iodides **48-50** and **79** were then subjected to the second nucleophilic substitution with sodium azide in DMF at room temperature to give the 3-α-azide compounds **51-53** and **80** in very good yields (Scheme 21).



Scheme 21. Synthetic sequence for preparation of 3-α azide derivatives: (i) c.H_2SO_4, MeOH, 100 °C, 1 h, quantitative yields; (ii) I_2, PPh3, imidazole, 1,3-dioxolane, 25 °C, 30 min, 55–67%; (iii) NaN_3, DMF, 25 °C, 6 h, 80–94%.

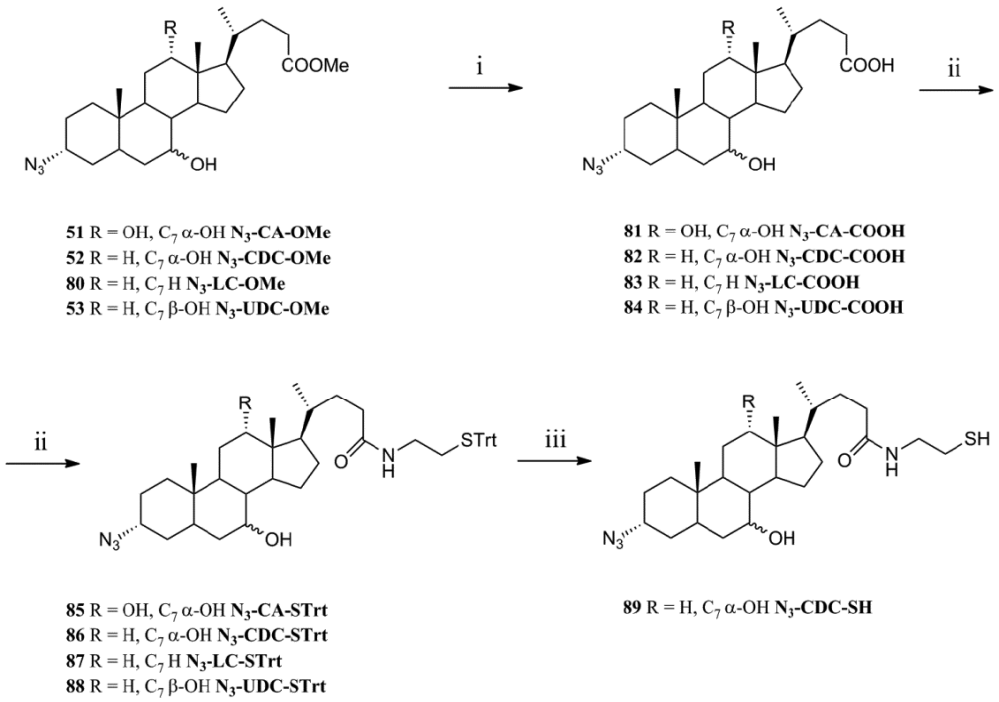
3.2.2 Introduction of the C-24-*S*-Trityl-cysteamine moiety:

The synthetic route to the C-24-*S*-trityl-cysteamine functionalized bile acids **85-88** is described in Scheme 23 (next page). First, C-24 methyl esters **51-53** and **80** were hydrolysed using a 0.5 M LiOH solution in MeOH to give the corresponding free acids **81-84** in almost quantitative yields. In parallel, cysteamine hydrochloride **90** was *S*-tritylated as shown in Scheme 22; resulting amine **91** was next coupled with bile acids **81-84** via mixed anhydride method, which involved carboxylic group activation by treatment with ethyl chloroformate in the presence of triethylamine as the base.



Scheme 22. Protection of cysteamine hydrochloride: Trt-Cl, dry DCM/DMF (5:1), r.t., 14 h, 86%.

The formation of each mixed anhydride was conducted at 0 °C for 45 minutes and it was followed by the coupling with the previously protected cysteamine **91** to give the target bile acids **85-88**. These derivatives constitute a set of activated bile acid compounds ready for further elaborations via CuAAC and either thiol-ene or thiol-yne orthogonal click reactions. Deprotection of the *S*-trityl group to liberate the reactive thiol functionality was planned to be performed just before the TEC/TYC step to avoid undesired sulphur oxidation side-reactions.



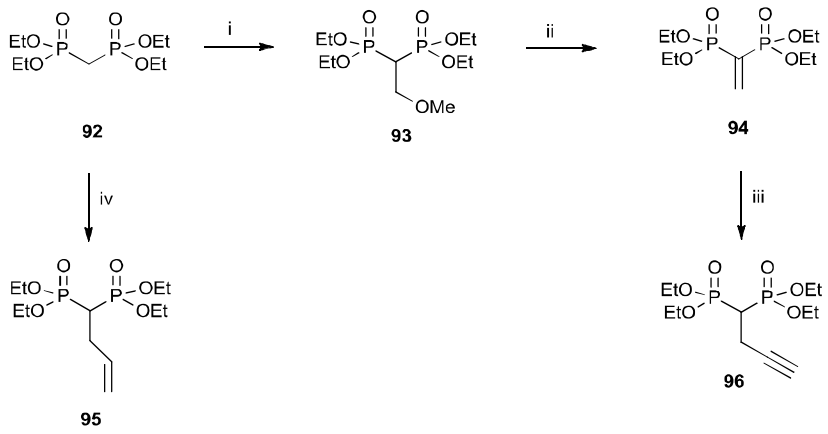
Scheme 23. Synthetic sequence for preparation of C-26-thiol derivatives: (i) LiOH, MeOH, 50 °C, o.n., 77%-quantitative yields; (ii) ClCOOEt, TEA, dry THF, 0 °C, 45 min then protected cysteamine, r.t., o.n., 45-83%; (iii) TFA/ dry DCM (1:20), r.t., 30 min, 93%.

The feasibility of the *S*-deprotection step was tested using the chenodeoxycholic derivative **86** because of the already demonstrated biological relevance of bisphosphonate salts conjugated to the chenodeoxycholic scaffold.[198] Hence, removal of the trityl group from compound **86** by TFA/triethylsilane afforded the target compound C-26-SH **89** suitable for orthogonal functionalization by the planned click reactions.

3.3 Synthesis of "clickable" bisphosphonate synthons:

In order to achieve a small library of new bile acid-bisphosphonate derivatives, three 'clickable' bisphosphonates were synthetized as described in Scheme 24.

Tetraethyl ethane-1,1-bisphosphonate **94** was synthetized according to a literature-known procedure.[201] Thus, the tetraethyl methylenediphosphonate **92** was treated with paraformaldehyde and diethylamine in methanol to produce tetraethyl methoxyethane-1,1-bisphosphonate **93** which in turn was dehydrated under acidic conditions to give **94** in 80% overall yield. A chromatography purification of the crude alkene **94** was attempted using EtOAc/MeOH (95:5) as the elution system as described in the literature.[202]



Scheme 24. Synthetic route to functionalized bisphosphonates: (i) Et_2NH, $(CH_2O)_n$, dry MeOH, 80 °C, o.n., 97%; (ii) cat. TsOH, dry toluene, 4Å molecular sieves, 120 °C, o.n., 80% (iii) sodium acetylide, dry THF, -15 °C to r.t., o.n., 40%; (iv) *t*-BuOK, allyl iodide, dry toluene, 0 °C, 3h, 40%.

Nevertheless, tetraethyl methoxyethane-1,1-bisphosphonate **93** resulted as the only detectable product during chromatography: indeed, the very fast addition of methanol to the Michael acceptor **94** made purification of this compound impracticable under these conditions. Hence, a purification step was avoided at this point of the synthetic sequence and tetraethyl ethane-1,1- diylbisphosphonate **94** was used in its crude form after the removal of any residual methanol by a co-evaporation process in toluene.[203]

The tetraethyl but-1-yne-4,4-diylbisphosphonate **96** was prepared by selective addition of sodium acetylide to bisphosphonate **94** at -15 °C in THF.[204] After ordinary work-up and purification, the target compound **96** was obtain in 40% yield.

Although the synthesis of the tetraethyl but-1-ene-4,4-diylbisphosphonate **95** via direct alkylation of the tetraethyl-methylenedisphosphonate **92** by allyl iodide is reported in the literature,[205] in our experience this reaction produced the undesired double alkylation product almost exclusively. In order to contain this side-reaction, few modifications to the published procedure were made: thus, the *C*-allyl bisphosphonate **95** was prepared by treatment of starting methylene compound **92** with potassium *tert*-butoxide and allyl iodide in dry toluene at 0 °C for 3 hours. The desired mono-alkylation reaction was still

accompanied by double-alkylated by-product, whose separation from **95** by column chromatography was troublesome, due to the similar *Rf* values of these compounds. However, reduction of the temperature and the reaction time allowed us recover the target compound **95** in a satisfactory 40% yield.

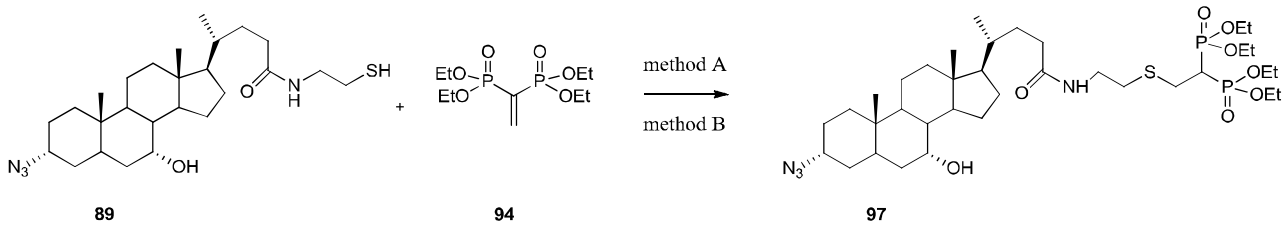
3.4 Synthesis of new chenodeoxycholic acid-bisphosphonate derivatives:

3.4.1 Synthesis of the tail-conjugated bile acid-bisphosphonate 97:

Having the orthogonally functionalized chenodeoxycholic acid derivative **89** and a set of modified bisphosphonates in hands, our next aim was to synthetize a series of new bile acid-bisphosphonates analogues the "click chemistry" approach; accordingly, thiol-ene coupling (TEC),[18] thiol-yne coupling (TYC)[30] and copper(I)-catalyzed Huisgen–1,3-dipolar cycloaddition (CuAAC)[182] were considered for this purpose.

In 2001, Sharpless *et al.*[1] described the concept of "click chemistry". Several efficient reactions, which are capable of producing a wide catalogue of synthetic molecules and organic materials, have been grouped accordingly under this term. Within this field of research, one reaction is emerging as an attractive click process: the century-old addition of thiols to alkenes,[16] which is currently called thiol-ene coupling (TEC). This reaction, whether proceeding by a radical or anionic chain (thiol Michael addition) carries many of the attributes of click chemistry and it is now routinely referred to in the literature as thiol click reaction.[18]

Based on the premise established for click reactions, the bile acid thiol **89** was coupled with bisphosphonate alkene **94** to obtain the first new bile-acid bisphosphonate derivative **97** as shown in Scheme 25.



89 **94** **97**

Scheme 25. Synthesis of compound **97**. Method A: dry DMF, DMAP, hν (365nm), r.t., 1 h, 68%; Method B: TEA, CH_2Cl_2, r.t., o.n., 62%.

The reaction was carried out in DMF at room temperature for 1h by irradiation with a UV-lamp (λ_{max} 365 nm) using 2,2-dimethoxy-2-phenylacetophenone (DMPA,10 mol%) as the photosensitizer (method A, Scheme 25); these are the conditions normally employed in this kind of radical couplings.[35,44] The ^{1}H NMR analysis of the crude reaction mixture revealed the consumption of all the starting alkene, indicated by the absence of the characteristic alkene proton signals at 6.99 ppm. Despite the fact that the reaction reached completion with an estimated NMR yield >95%, the isolated yield of compound **97** was only 68%. This derivative showed a high polar nature and even if the bisphosphonate moiety was employed in its ester-protected form, it was strongly retained on silica gel stationary phase determining the lower value of the isolated yield.

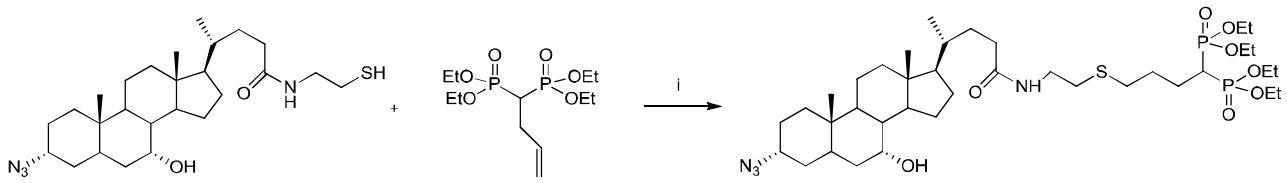
The bisphosphonate alkene **94** can easily undergo thiol Michael addition, therefore the coupling between bile acid **89** and compound **94** to achieve the derivative **97** was also performed under basic conditions (method B, Scheme 25). More precisely, 1.5 equivalents of triethylamine in CH_2Cl_2 were employed to facilitate the deprotonation of the sulphur nucleophile **89**. Again, although the product **97** was formed in almost quantitative yield, as determined by ^{1}H NMR analysis, the isolated yield was still lower than expected due to the difficulty in purification mentioned above.

Both types of thiol click reactions were efficient but they suffered from significant problems of purification; this limitation was observed for all the bile acid-bisphosphonate conjugates synthetized (*vide infra*).

3.4.2 Synthesis of the tail-conjugated bile acid-bisphosphonate 98:

The photoinduced thiol-ene coupling (TEC) was the reaction of choice for the synthesis of compound **98** as described in Scheme 26.

Chenodeoxycholic acid derivate **89** was coupled with allyl-bisphosphonate **95** using the aforementioned thiol-ene radical conditions that involve 2,2-dimethoxy-2-phenylacetophenone (DMPA, 10 mol%) as the photosensitizer. The thiol-ene coupling, whether proceeding through a radical or ionic mechanism, is certainly influenced by the structure of the alkene partner: the terminal alkene **95** easily undergoes the radical-mediated thiol-ene process while it is not a suitable substrate for the thiol Michael addition contrary to the α,β-unsaturated bisphosphonate **94**, which can be engaged in both types of TECs.



89 95 98

Scheme 26. Synthesis of compound **98**. (i) dry DMF, DMAP, hν (365nm), r.t., 1 h, 64%.

In order to establish the best conditions, two different thiol/alkene ratio were employed. First, the TEC reaction was carried out using an excess of alkene **95**. ^{31}P NMR analysis of the crude material revealed the only presence of the starting bisphosphonate together with the desired product **98**. Unfortunately, the chromatographic separation of the desired product **98** from the starting *C*-allyl bisphosphonate was troublesome due to the lengthy elution from the column that is typical of bisphosphonate-containing products, resulting in the collection of a mixture of **95** and **98**.

The thiol/alkene ratio was then reversed: an excess of SH was employed in order to overcome the purification problems. Also in this case, ^{1}H NMR analysis of the crude material revealed that reaction reached completion and TLC analysis disclosed that the main side product was the disulphide compound arising from thiol homocoupling. Chromatographic separation of the bile acid-bisphosphonate new derivative **98** from the bile acid disulphide was easy to achieved due to the very different *Rf* values of these compounds. However, as mentioned before, compound **98** suffered from a long retention time on silica gel column, even using a 2:1 mixture of acetone/CH_2Cl_2 as eluent, resulting in only 64% of isolated yield of pure product.

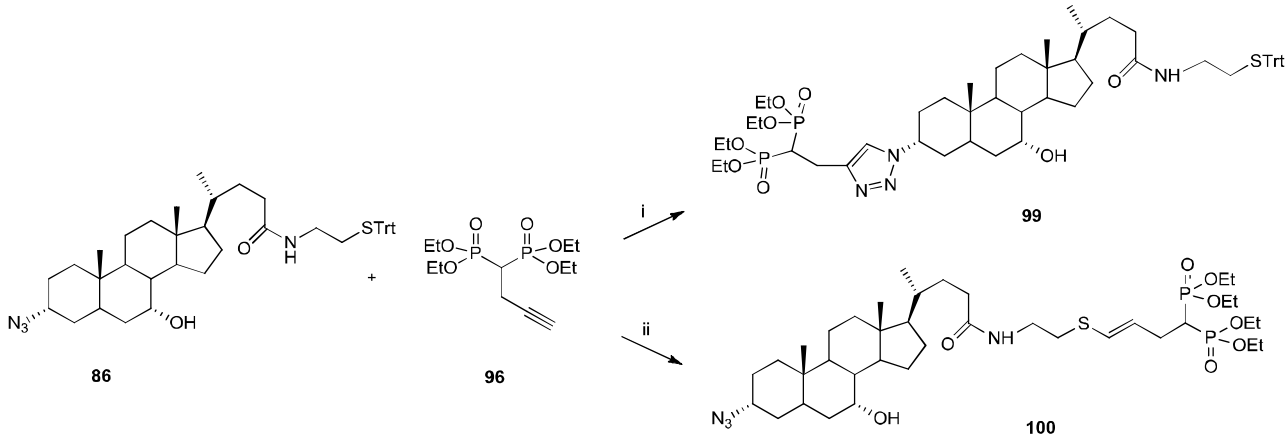
3.4.3 Synthesis of the head-conjugated bile acid-bisphosphonate 99 and the tail-conjugated bile acid-bisphosphonate 100:

The new bile acid-bisphosphonate conjugates **99** and **100** were synthetized exploiting copper(I)-catalyzed Huisgen-1,3-dipolar cycloaddition (CuAAC) and photoinduced thiol-yne coupling (TYC), respectively. (Scheme 27, next page)

Copper(I)-catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) takes advantage of the formation of a copper acetylide to activate terminal alkynes toward reaction with azides and it holds all the properties of a click reaction: efficiency, simplicity, and selectivity. In fact, it has become the quintessential click reaction and is often referred to simply as "click chemistry".[182] Noteworthy, CuAAC conditions are fully compatible with the majority of

biological functions and their common protective groups,[4] thus making the click chemistry approach a valuable tool in bioconjugation studies.

Although the unprotected thiol moiety on the modified chenodeoxycholic acid would be unable to react with the bisphosphonate-alkyne using CuAAC conditions, we chose to perform the coupling between the alkyne **96** and the *S*-tritylated chenodeoxycholic acid **86** hoping to reduce the polar nature of the product **99** and so, to facilitate the tedious purification process. Thus, these compounds were allowed to react overnight at room temperature in the presence of CuI and DIPEA in a mixture toluene and pyridine to obtain the new bile acid-bisphosphonate derivative **99** in 64% isolated yield. In this case, the bisphosphonate was linked to the "head" of the bile acid, while the previously reported examples[198] displayed the bisphosphonate linked to the "tail".



Scheme 27. Synthesis of compounds **99** and compound **100**. (i) dry toluene/pyridine (10:1), DIPEA, CuI, r.t., o.n., 64%, (ii) 1) TFA/ dry DCM (1:20), r.t., 30 min, 93%, 2) dry DMF, DMAP, hν (365nm), r.t., 1 h, 53%.

The tetraethyl but-1-yne-4,4-diylbisphosphonate **96** and the modified bile acid **86** can also be conjugated using the photoinduced thiol-yne (TYC) coupling after removal of trityl protective group. Hence, compound **86** was treated with TFA in the presence of triethylsilane and, after filtration on a silica plug, it was subjected to thiol-yne coupling with alkyne **96** using UV irradiation and the DMPA photosensitizer employed for TECs. After chromatographic purification, the new bile acid-bisphosphonate **100** was recovered in 53% isolated yield. Interesting feature of the thiol-yne coupling procedure is the possibility to modulate the reactivity of the alkyne substrate towards either one or two thiol molecules to produce multifunctional derivatives with increasing complexity.[30] The reaction conditions could be changed in order to favour the formation of bis-adduct (TYC-TEC sequence) or to stop the reaction at the vinyl sulphide stage (TYC).[35] For the

synthesis of compound **100**, an excess (2 equivalents) of bisphosphonate alkyne **96** was employed in order to introduce only one thiol per alkyne (TYC). A second and different thiol could be introduced on compound **100** by a subsequent thiol-ene coupling leading to a non-symmetric bis-functionalization. Unfortunately, the introduction of a second thiol on vinyl sulphide **100** is yet to be performed.

3.5 Synthesis of fluorescent analogues:

Over the past 40 years, bisphosphonates have revolutionised the treatment of patients suffering from disorders of bone resorption but their precise biochemical target were not known until the late 1990s when it was identified in FPP synthases, a key enzyme of the mevalonate pathway. Despite extensive studies,[86] how N-BPs reach their intracellular target, their skeletal distribution as well as their cellular localization are not completely defined. In the past few years, a series of fluorescent bisphosphonates[97,98] and related analogues have been synthetized and have become a powerful tool for these research studies.[101] These fluorescent analogues were used to highlight the different affinity that bisphosphonates have for bone mineral and to investigate skeletal and cellular distribution. Moreover, fluorescently-labelled bisphosphonates have been very useful to investigate the localization of BPs in both hard and soft tissues for a better understanding of their major side effects such as gastrointestinal inflammation and ulceration, and osteonecrosis of the jaw (ONJ).

On this basis, we decided to take advantage of our orthogonally modified chenodeoxycholic acid scaffold to introduce a fluorescent tag and synthetize a series of new fluorescent bile acid-bisphosphonate conjugates via a "click chemistry" approach (Figure 27).

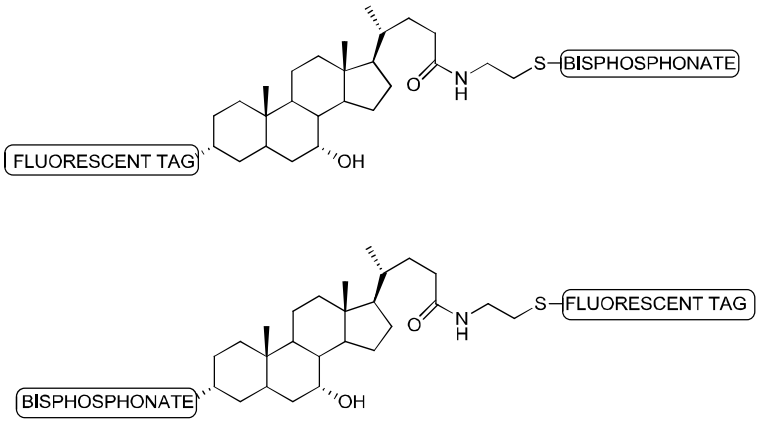
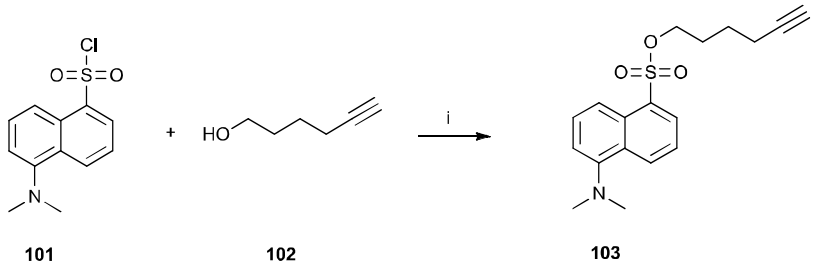


Figure 27. Fluorescent chenodeoxycholic acid-bisphosphonate conjugates.

3.5.1 Synthesis of the fluorescent building block 103:

The 5-dimethylamino-1-naphthalenesulfonyl chloride (dansyl chloride) is a fluorescent dye commonly used for labelling primary and secondary amines. Dansyl chloride was first applied for fluorescence labelling of albumin[206] and later routinely used in protein analysis for the determination of terminal amino acids.

For our purposes, a dansyl-alkyne derivative was prepared (Scheme 28) according to a published protocol.[207]



101 102 103

Scheme 28. Route to fluorescent dansyl-alkyne **103**. (i) CH_2Cl_2/pyridine (24:1), r.t., 24 h, 62%.

Dansyl chloride **101** was reacted with hex-5-yn-1-ol **102** in a mixture of CH_2Cl_2/pyridine for 24 hours in the dark to give the fluorescent alkyne **103** in good yield (62%), suitable CuAAC and TYC couplings.

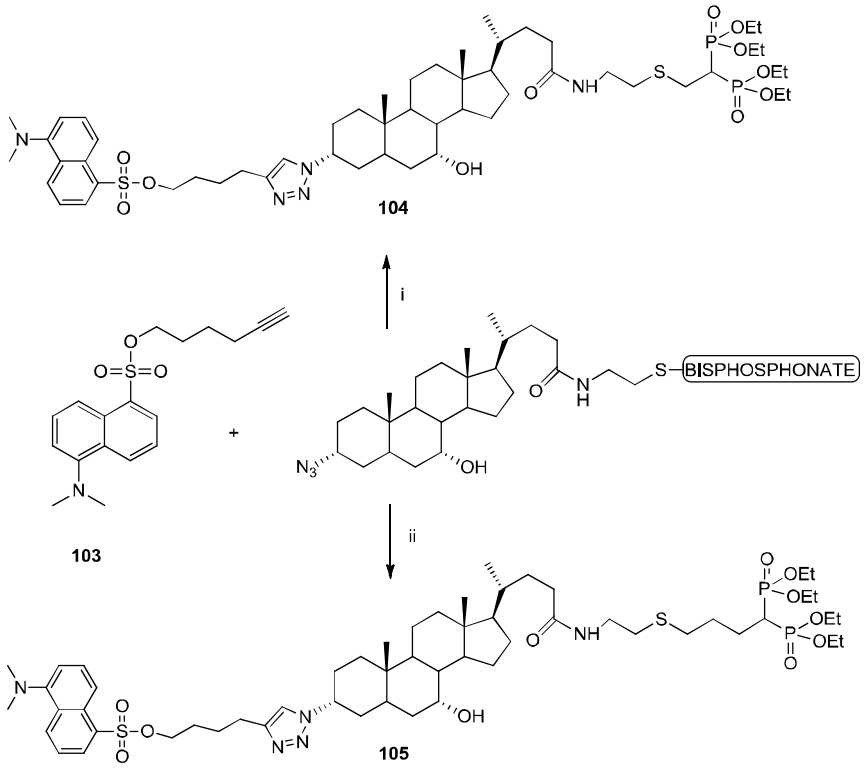
3.5.2 Synthesis of fluorescent conjugates 104 and 105:

The first new fluorescent chenodeoxycholic acid-bisphosphonate derivatives were synthetized exploiting CuAAC click reaction to link the dansyl-alkyne **103** through a 1,2,3-triazole ring to the bile acid-bisphosphonate compounds **97** and **98** prepared beforehand as shown in Scheme 29 (next page).

Azide **97** and a slightly excess (1.2 equivalents) of alkyne **103** were coupled in the presence of a catalytic amount of CuI and 4 equivalents of DIPEA in a mixture of toluene and DMF. Reaction proceeded smoothly at room temperature for 18 h and the crude material was then subjected to chromatographic purification to recover the target compound **104** in a 61% yield.

The same reaction conditions were also applied to conjugate the dansyl-alkyne **103** to azide **98** affording the fluorescent conjugate **105** (Scheme 29, next page) in 75% isolated yield.

The previously discussed issue concerning the lengthy and difficult purification of bisphosphonate-containing bile acids affected the fluorescent conjugates **104** and **105**, too.



Scheme 29. Synthesis of compound **104** and compound **105**. (i) **97**, dry toluene/pyridine (10:1), DIPEA, CuI, r.t., o.n., 61%; (ii) **98**, dry toluene/pyridine (10:1), DIPEA, CuI, r.t., o.n., 75%.

Medium-pressure liquid chromatography (MPLC) was used in order to solve the major drawbacks of flash chromatography when applied to our compounds.

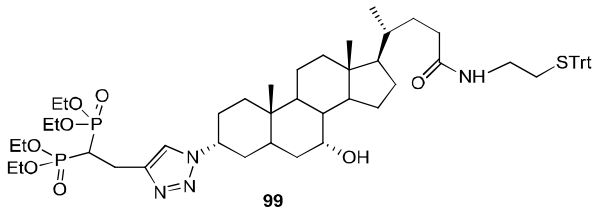
Medium-pressure liquid chromatography (MPLC) is one of the various preparative column chromatography techniques and it was introduced in the 1970s as an efficient technique for preparative separations of organic compounds. MPLC allows to obtain faster and improved separations compared to flash chromatography. Like in other chromatographic techniques, the column is the central point when optimizing a preparative chromatographic separation and criteria such as amount of sample to be purified, amount of packing material and column diameter, have to be carefully considered. A stationary phase with lower particle size, under pressure, enhances separation quality and moreover the solid phase can be reused. With regard to cost-effectiveness, the most frequently utilised stationary phase is silica gel. Despite its economic advantages, the risk of irreversible absorption is a possible major disadvantage of the utilization of this support.

Therefore, despite ^{1}H NMR analysis of crude reaction mixtures revealed complete consumption of starting azides, as can be seen by the disappearance of the C3-βH multiplet around 3.00 ppm and by the appearance of a new C3-βH multiplet around 4.30 ppm, the

isolated yield of fluorescently-labeled conjugates **104** and **105**, was lower than expected. Unluckily, we have reasons to believe that part of our new compounds remained irreversibly attached to the silica gel stationary phase even using the MPLC technique.

3.5.3 Synthesis of the fluorescent conjugate 107:

The alkyne fluorescent building block **103** may be used to fluorescently label also the bile acid-bisphosphonate derivative **99** (Fig.28) which in turn is obtained by chemoselective reaction of the azide group of **86** with the bisphosphonate alkyne **96**.

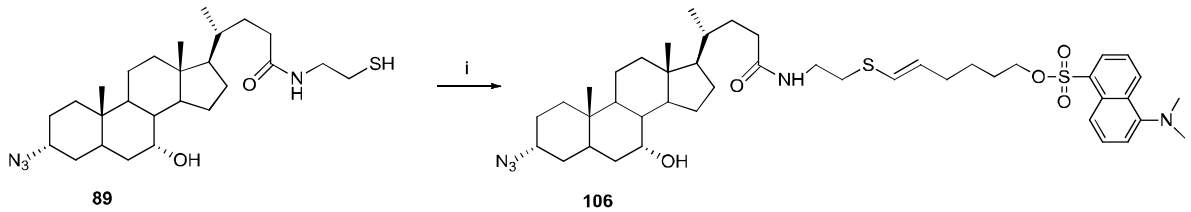


99

Figure 28. Compound **99**.

Even though the literature[208] reports examples of dendrimers synthetized by CuAAC click reactions followed by photochemical TEC couplings, in our personal experience the best results could be achieved when the photochemical click reaction was performed before the azide-alkyne coupling. In our support, some examples[209–211] were found in the literature where the synthetic strategy followed this order.

Thus, the TYC coupling between the dansyl-alkyne **103** and N_3-CDC-SH **89** was carried out irradiating at 365 nm UV light in the presence of DMPA as the photosensitizer to obtain the intermediate **106** in a reasonable yield (43%, Scheme 30).

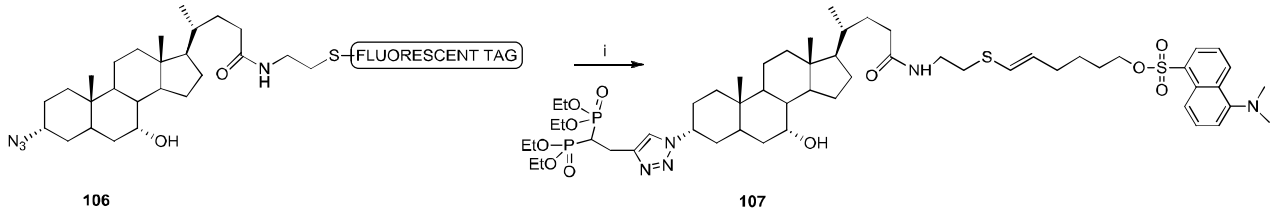


89 **106**

Scheme 30. Synthesis of compound **106**. (i) **103**, dry DMF, DMPA, hν (365 nm), r.t., 1 h, 43%.

Then, the fluorescent azide **106** was subjected to CuAAC with bisphosphonate-alkyne **96** in a 10:1 mixture of toluene and pyridine, using a catalytic amount of CuI (2 mol%) and an

excess of DIPEA as described in Scheme 31. The fluorescent derivative **107** was recovered in 55% isolated yield after MPLC purification that unfortunately encountered the same aforementioned limitations.



Scheme 31. Synthesis of compound **107**. (i) **96**, dry toluene/pyridine (10:1), DIPEA, CuI, r.t., o.n., 55%.

3.6 Deprotection of bisphosphonate tetraethyl esters:

Tetraesters can function as protecting groups when modifying the bisphosphonate moiety. They are synthetically useful precursors of bisphosphonic acids that make possible to perform synthesis and conjugations in organic solvents, which is mandatory in some cases. If needed, they can be removed afterward to produce the corresponding bisphosphonic acids by either acid hydrolysis,[212] basic hydrolysis[198] or through a silylation/desilylation procedure (McKenna reaction)[213] under mild conditions using bromotrimethylsilane.

3.6.1 Synthesis of the bisphosphonic acid derivative 108:

Compound **98** (Fig. 29) was chosen to investigate the feasibility of deprotection on our new bile acid-bisphosphonate analogues using the mild silylation/desilylation procedure mentioned above.

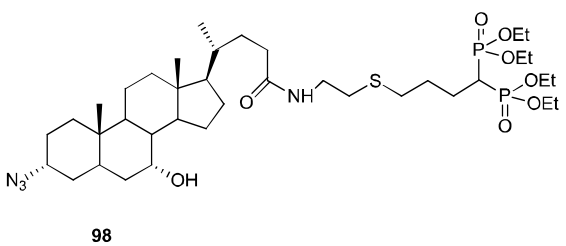
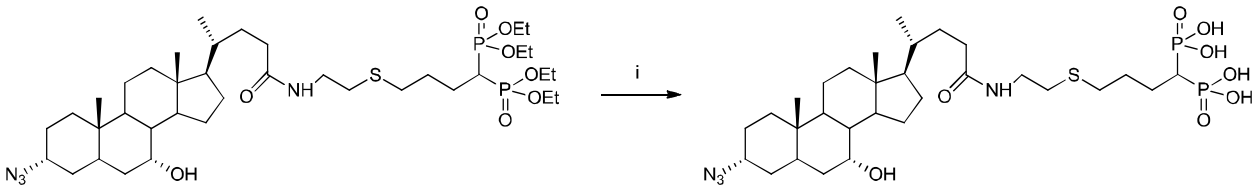


Figure 29. Compound **98**.

At first, the reaction was attempted treating compound **98** with a 10-fold excess of bromotrimethylsilane in dichloromethane at 0 °C under argon atmosphere, as often reported in the literature.[214] The reaction mixture was allowed to react overnight, then methanol was added in order to protonate the intermediate silyl ester and free the target bisphosphonic acid. Unfortunately, this attempt failed due to an unexpected and not-well understood break of the molecule. MS-analysis led us to believe that the amide bond was somehow affected by the reaction conditions used. So, the second attempt was carried out reducing the excess of trimethylsilyl bromide to 5-fold, but also this time the reaction was unsuccessful and the material recovered was a complex mixture of products, without predominance of the desired free acid. The sterically hindered weak bases 2,6-lutidine and 2,4,6-collidine are reported in the literature[215,216] in combination with TMS-Br in this type of deprotection reaction in order to reduce the acidity of the medium. Accordingly, the reaction was next performed using 5 equivalents of TMS-Br and adding 13 equivalents of 2,6-lutidine to the reaction mixture at 0 °C. These conditions led only to a mixture of products originated by the partial deprotection of esters, as detected by MS-analysis. It should be noted that difficulty was encountered using the published conditions, however, when the equivalents of TMS-Br were increased, the hydrolysis finally proceeded cleanly and gave the target compound **108**.

The optimized conditions for the synthesis of compound **108** are shown in Scheme 32.



98 **108**

Scheme 32. Deprotection of bisphosphonate esters. (i) TMS-Br, 2,6-ludine, dry CH_2Cl_2, from 0°C to r.t., 24 h then MeOH, r.t., 1h, 53%.

Thus, the starting ester **98** was successfully deprotected in the presence of a 10-fold excess of TMS-Br and a 13-fold excess of 2,6-lutidine in CH_2Cl_2 for 24 hours. Methanol was then added and the reaction mixture was stirred at ambient temperature for 1 hour. Concentration and purification by size exclusion chromatography (Sephadex LH-20) gave the chenodeoxycholic-bisphosphonic acid derivative **108** in 53% yield.

Established the best conditions for the tetraethyl bisphosphonate ester cleavage on the model compound **98**, the next step was the investigation of the feasibility of the silylation/desilylation procedure on our fluorescently-labelled derivatives.

The McKenna reaction[213] is one of the most widely used methods in the synthesis of bisphosphonic acids: the procedure is convenient, mild and efficient and can tolerate a variety of additional functional groups in the molecule. Compound **105** (Fig. 30) was chosen for proving the selectivity of the McKenna reaction towards bisphosphonate esters in the presence of the dansyl-sulfonyl ester moiety.

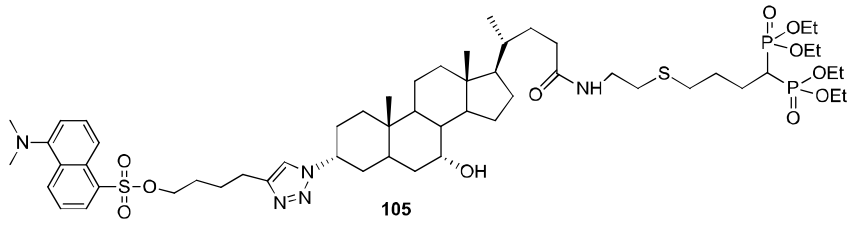


Figure 30. Compound **105**

Therefore, the fluorescent derivative **105** was subjected to bisphosphonate ester removal with TMS-Br in the presence of 2,6-lutidine. Unluckily, even after 72 hours, reaction did not reach completion and MS-analysis of the crude material revealed a mixture of partially deprotected products.

Even though the hydrolysis was incomplete, no by-products deriving from cleavage of sulfonyl ester were detected and the selectivity of this procedure was confirmed.

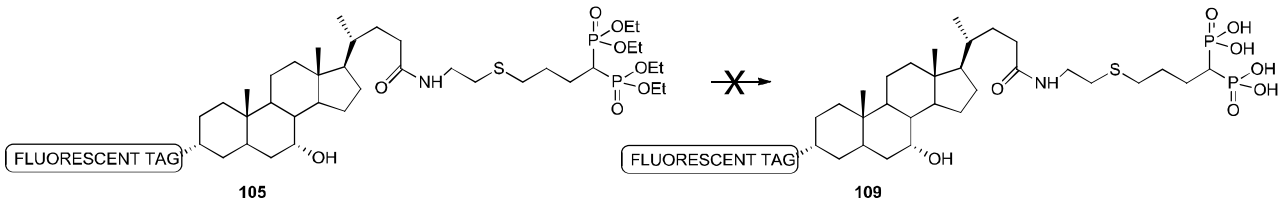


Figure 31. Cleavage of bisphosphonate esters.

The click chemistry reactions, namely the TEC, TYC, and CuAAC, employed to synthetize our collection of new bile acid-bisphosphonate compounds and their fluorescent analogues,

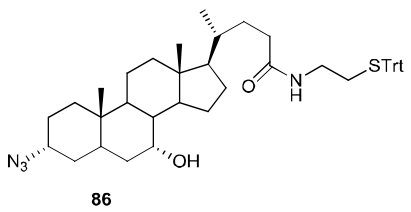
were proven to be a very efficient set of powerful, highly reliable, and selective reactions. In our experience though, ^{1}H NMR analysis revealed that the best performances were reached with a little deviation from equimolar amounts of building blocks in play. The most challenging problem of this double click reaction strategy were the complicated purification steps of our new compounds and the resulting lowered isolated yields. This is in contrast with the definition of a click process that should give very high yields, with a variety of starting materials, under simple reaction conditions and with simple product isolation processes, ideally by non-chromatographic methods.[1]

3.7 Fluorous-tagging strategy:

The term fluorous chemistry was not in the chemists vocabulary before 1994 when Horvàth and Rabai[217] introduced the concept of "fluorous biphase catalysis" and the concept of "ponytail".

A "ponytail" or "fluorous tag" is a portion or domain of a molecule that is rich in carbon-fluorine bonds and exerts primary control over the separability characteristics of the molecule in fluorous separation techniques.[218,219] These are most commonly fluoroalkyl moieties of the formula $(CH_2)_m(CF_2)_{n-1}CF_3$, with at least six fully fluorinated carbons. The fluorous chain can be attached to reagents, scavengers, and catalysts and it has some unique features. For instance, the fluorous tags are inert to chemical reactions and have minimal effect on the reactivity of the attached molecules, the reaction process can be monitored by conventional analytical methods, both intermediates and final products can be purified by fluorous separations as well as by normal and reverse phase chromatography; moreover literature reaction conditions can be easily applied with little or no modifications.

With all this in mind, we decided to take advantage of the C-7-OH group on the bile acid scaffold **86** (Figure 32) to introduce a perfluoroalkyl group with the aim to facilitate the separation process.



86

Figure 32. Orthogonal scaffold **86**.

3.7.1 Synthesis of the fluorous-tagged scaffold 114:

The functionalization of the chenodeoxycholic acid scaffold **86** was first attempted using the Yamaguchi esterification,[220] commonly used in the synthesis of macrolactones. The Yamaguchi esterification is a mild method that involves the reaction of an aliphatic acid with 2,4,6-trichlorobenzoyl chloride to form the mixed aliphatic 2,4,6-trichlorobenzoyl anhydride. This mixed anhydride, upon reaction with an alcohol, in the presence of DMAP, produces the aliphatic ester regioselectively. So, the bile acid **86** and heptadecafluorononanoic acid were mixed together in anhydrous THF in the presence of 2,4,6-trichlorobenzoyl chloride (Yamaguchi reagent) and triethylamine at room temperature. After 5 minutes, a catalytic amount of DMAP was added to the reaction mixture (Fig. 33). The reaction was monitored by TLC analysis but no desired product was detectable.

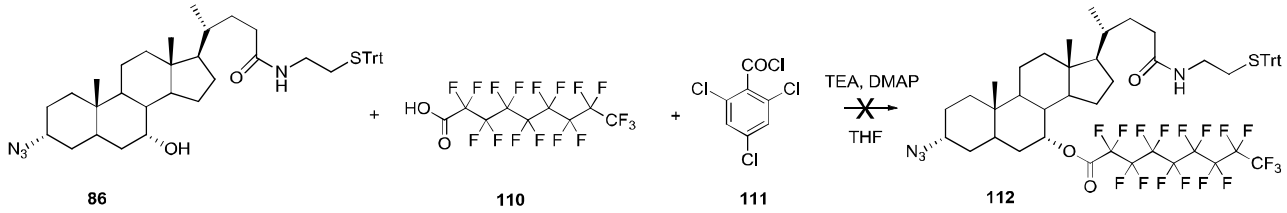
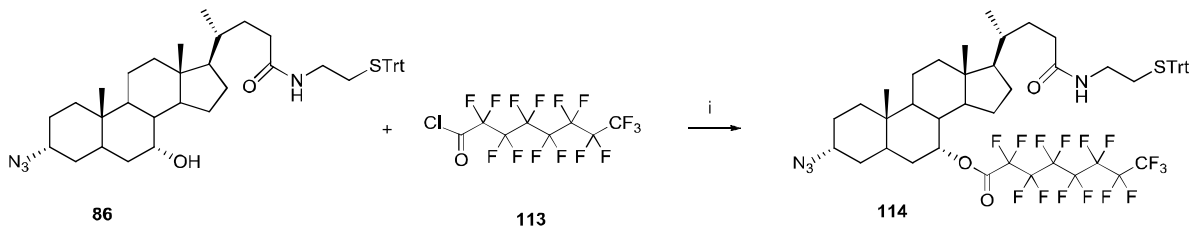


Figure 33.

A few trials to obtain the perfluoroalkylated compound **112** using Yamaguchi esterification were made increasing the amount of DMAP from catalytic to stoichiometric and/or using a slightly higher excess of perfluorinated acid **110**.Unfortunately, according to chromatographic analysis, they did not result in the formation of the desired product.

Another strategy was then employed: the activated pentadecafluorooctanoyl chloride **113** was used in place of the heptadecafluorononanoic acid **110**: the fluorous tag was then successfully inserted in the bile acid scaffold (Scheme 33) following a published procedure[221] with little modifications.



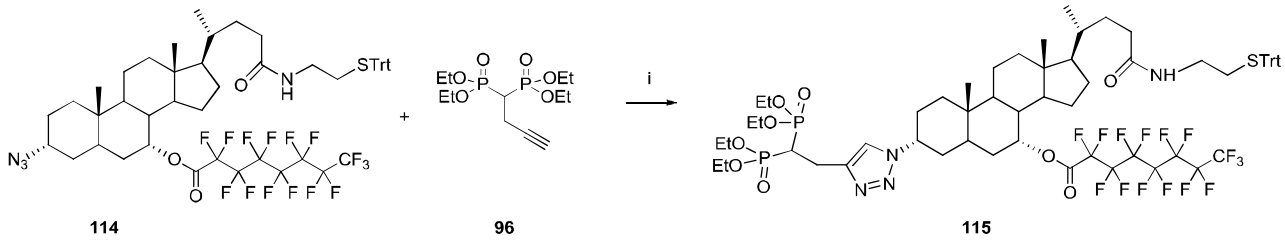
Scheme 33. Insertion of fluorous tag. (i) TEA, DMAP, dry CH_2Cl_2, 0 °C, 1 h, 71%.

The reaction of bile acid **86** with a slight excess of perfluorinated chloride **113** in an ice-cooled solution of CH_2Cl_2 in the presence of TEA and 10 mol% of DMAP gave the fluorous-tagged scaffold **114** in 71% isolated yield. Esterification proceeded smoothly and, after 1 hour at 0 °C, it reached completion. Indeed, the 7β-H broad singlet shifted from 3.85 ppm to 5.19 ppm as revealed by ^{1}H NMR analysis of crude material. At this stage, a flash chromatographic purification on silica gel was successfully performed to isolate the desired product.

3.7.2 Synthesis of the fluorous-tagged bile acid-bisphosphonate derivative 115:

The CuAAC between the perfluoroalkylated bile acid scaffold **114** and the tetraethyl but-1-yne-4,4-diylbisphosphonate **96** was chosen as model reaction to test if, in the presence of the fluorous tag, the separation process could become easier and reliable according to the "fluorous chemistry" principles.

As expected, the same reaction conditions used for the synthesis of nonfluorous-tagged bile acid-bisphosphonate compound **99** were successfully employed also for the synthesis of the perfluorinated analogue **115** as shown in Scheme 34.



Scheme 34. Synthesis of perfluoroalkylated compound **115**. (i) dry toluene/pyridine (10:1), DIPEA, CuI, r.t., o.n., 78%.

The success of fluorous synthesis largely depends on the efficiency of fluorous separations. Fluorous separations are based on fluorine-fluorine interactions between the fluorous molecules and the fluorous separation media that could be fluorous solvents or fluorous silica gel.

Fluorous solid-phase extraction (F-SPE) was used to separate the desired product **115** from the unreacted excess of bisphosphonate alkyne **96**. As in a typical F-SPE separation, the crude reaction mixture was loaded with a minimum amount of organic solvent (CH_2Cl_2) onto the SPE cartridge, packed with FluoroFlash^{TM} silica gel. The organic components quickly eluted with 80:20 MeOH/H_2O, whereas the fluorous compound was retained on

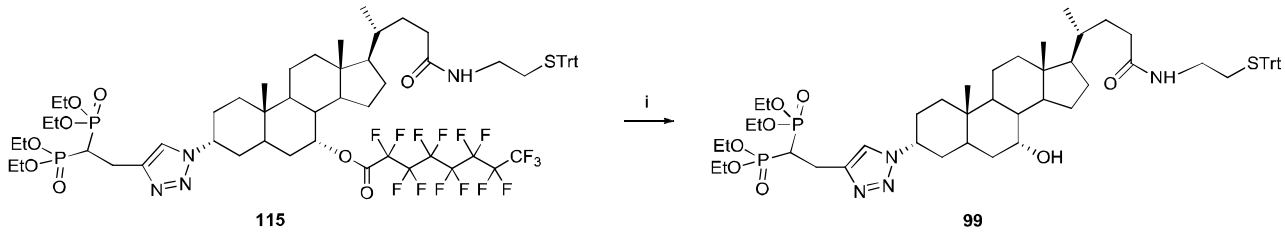
the cartridge until elution with 100% MeOH. In a simple two-step elution, fluorous compound **115** and non-fluorous compound **96** were separated into two fractions. Moreover, the isolated yield improved from 64% to 78% avoiding a long, time-consuming, expensive chromatographic process.

Having demonstrated that the fluorous tag is a very useful tool for improving the purification of our new bile acid-bisphosphonate derivatives, our next aim was to investigate the possibility of a selective detagging procedure.

3.7.3 Selective detagging:

Fluorous tags should be installed and removed from a substrate using mild reaction conditions.

A selective hydrolysis of the perfluorinated ester **115** allowed the removal of the tag to give the corresponding non-fluorinated compound **99**.



Scheme 35. Detagging. (i) 0.5 M NaOH, EtOH/H_2O (8:1), r.t., 1 h, 92%

Fluorous compound **115** was treated with 0.5 M NaOH in a mixture of EtOH/H_2O at room temperature for 1 hour. ^{1}H NMR analysis of the crude reaction mixture revealed that a selective removal of the tag occurred: the 7β-H broad singlet shifted back from 5.19 ppm to 3.85 ppm and the integration of ethyl ester signals of the bisphosphonate was consistent with the presence of 4 esters meaning that a selective cleavage of the fluorous tag was accomplished.

Elution with the "fluorophobic solvent" 80:20 MeOH/H_2O of the FluoroFlash^{TM} cartridge allowed to recover desired product **99** in 92% yield while the fluorinated tag remained on the column. This was then recovered by fluorophilic elution with MeOH.

3.8 Conclusions and perspectives:

In conclusion, four orthogonally functionalized bile acid scaffolds, based on cholic acid, chenodeoxycholic acid, lithocholic acid and ursodeoxycholic acid, were designed and synthetized.

The orthogonal chenodeoxycholic acid scaffold was employed to synthetize a collection of chenodeoxycholic acid-bisphosphonate tetraethyl esters conjugates at C-3 and C-24 positions of bile acid by using a "click chemistry" approach. Moreover, a series of fluorescently-labelled analogues of these new bile acid-bisphosphonate derivatives was achieved with the same methodology (Figure 34). McKenna reaction for bisphosphonate ester deprotection was studied on our new substrates and its selectivity toward bisphosphonate ester was established.

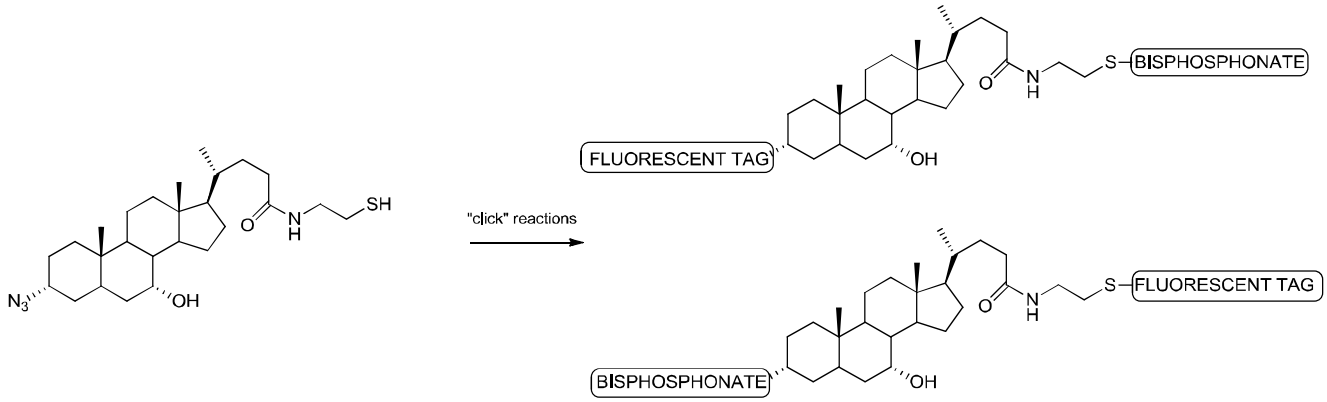


Figure 34.

The effectiveness and flexibility of the orthogonal azide-alkyne and thiol-ene or thiol-yne "click" reactions was demonstrated. Unfortunately, recovery and purification difficulties limited the yield and the utility of this otherwise successful "click" strategy.

Tagging bile acid-bisphosphonate derivatives with a fluorous tail such as the perfluorooctyl (C_8F_{15}) group followed by product purification using fluorous solid-phase extraction (F-SPE) provided a powerful way of addressing the separation issue. A fluorous-tagged analogue of orthogonal chenodeoxycholic acid scaffold was synthetized as a model and the CuAAC with alkyne bisphosphonate **96** was chosen to test the efficiency of the fluorous separation. The fluorous-tagged bile acid-bisphosphonate derivative **115** (Scheme 34) was purified using F-SPE cartridges in a simple, quick, and efficient fashion. Furthermore, after purification, the fluorous tail was easily and selectively removed from the target compound. (Scheme 35)

This “tagging-detagging” protocol could be further developed and employed for expanding and optimizing our small collection of new compounds without the use of a troublesome and time-consuming silica gel chromatography.

Moreover, the vinyl sulphide bile acid-bisphosphonate conjugate **100** as well as its fluorescent analogue **107** (Scheme 31) are promising substrates for non-symmetric bis-functionalizations by thiol-ene couplings with a second thiol molecule such as the SH-cysteamine-based bisphosphonate **116**.

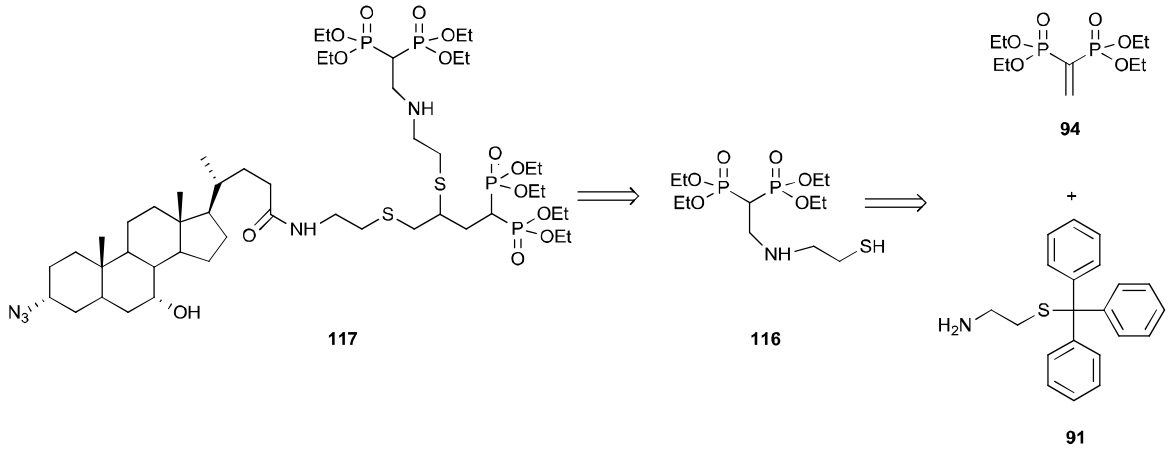


Figure 35. Possible retrosynthetic strategy to non-symmetric bis-functionalized compound **117**.

A series of preliminary *in vitro* test will be performed to evaluate the relative affinity of the newly synthetized derivatives toward hydroxyapatite and establish their cytotoxicity and ability to inhibit osteoclastogenesis.

Chenodeoxycholic acid-bisphosphonate compounds synthetized by “click chemistry” approach are very useful models for further studies: their orthogonally functionalized scaffold can be exploited for a further modification, with a second active ingredient, for a specific delivery of active principles to the bone with the aim to maximize the effect of the agent on the bone and limit any adverse effect.

The presence of the bisphosphonate portion could impart bone affinity to a specific drug such as proteins, anti-neoplastic, anti-infammatory and statins,[222] while the bile acid scaffold could improve the bioavailability of the conjugates by increasing its lipophilicity and affinity to bile transporters.[107,108]

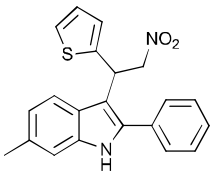
4 Project 3: "Development and testing of small molecules CB_1 positive allosteric modulators for the treatment of pain"

4.1 Aim of the project:

The endocannabinoid system is one of the body's most important natural painkilling systems; the endocannabinoids are released on demand as and when they are required. These compounds act on cannabinoid CB_1 and CB_2 receptors which are located on pain processing pathways both central, in the brain, and in peripheral nerve endings in tissues and organs. Cannabinoids receptor agonists have shown therapeutic value for a number of important medical conditions besides pain, including anxiety, depression, glaucoma, nausea, emesis, muscle spasms, and wasting diseases.

Global activation of the cannabinoid CB_1 receptor by direct agonists such as Sativex®, a cannabis extract which has been approved in the UK and Canada for the treatment of spasticity and pain in multiple sclerosis, leads to psychoactive side effects and abuse liability. On the contrary, targeting the allosteric binding site on the CB_1 receptor using positive allosteric modulators (PAMs) is a very promising approach because it would cause activation of the endocannabinoid system without causing the unwanted psychotropic effects, thus providing the potential of side-effect-free pain relief where required.

ZCZ011 has been developed in the laboratories of Professor Matteo Zanda at the University of Aberdeen, UK and it was identified as a potent CB_1 PAM (Figure 36). This compound showed great activity in *in vitro* tests increasing the maximum level of stimulation (Emax) caused by the endogenous cannabinoid agonist anandamide (AEA) of 126% at concentration of 1 μM. Moreover, it was demonstrated to be effective *in vivo*, enhancing the pain-relieving effects of endocannabinoids in an animal model of acute pain. Notably, it was shown that ZCZ011 does not induce the catalepsy in the mouse model, an effect that is characteristic of psychoactive CB_1 receptor orthosteric agonists.



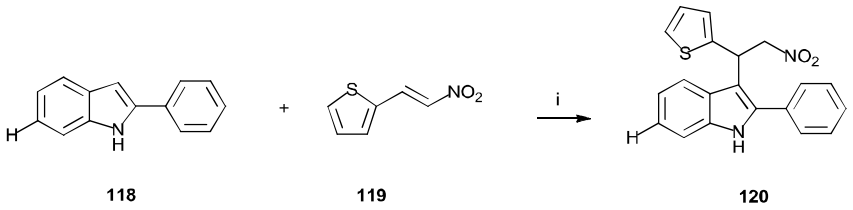
ZCZ011

Figure 36. Chemical structure of the new PAM ZCZ011.

The aim of the project was to investigate the structure-activity relationship (SAR) for ZCZ011 in order to identify regions of its structure that may be modified to improve its activity, and produce a more stable and efficacious PAM, and eventually identify a better candidate for *in vivo* testing. In fact, the tool compound, ZCZ011 lacks *in vivo* potency, being effective only at 40 mg/kg. Thus, in order to conduct our SAR investigation, we synthetized a series of analogues replacing substituents
at 2-position, 5-position and 6- position of the indole scaffold as well as the thienyl side chain. Furthermore, the new analogues developed were *in vitro* screened for potency. This project has been developed in the laboratories of Professor Matteo Zanda at the University of Aberdeen, UK. The potency experiments were conducted by Dr Gemma Baillie.

4.2 Modification of 6-position of indole scaffold:

We began our SAR investigation replacing the 6-methyl moiety of ZCZ011 with a hydrogen atom and a series of electron-withdrawing groups.
Compound **120**, in which the CH_3 group is replaced by a hydrogen atom, was synthetized following a literature procedure[223] with small modifications; as described in Scheme 36. Compound **120** was prepared starting from commercially available 2-phenylindole **118** through conjugate addition with 2-(2-nitrovinyl) thiophene **119**. This coupling step was carried out under microwave irradiation for 15 minutes at 150 °C in a mixture of H_2O/*tert*-butanol.



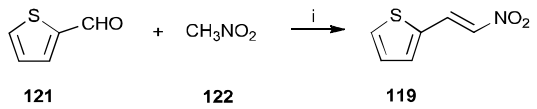
118 119 120

Scheme 36. Synthesis of compound **3**. (i) H_2O/*t*BuOH 5:1, MW 150°C, 15 min, 77%.

This procedure represent a significant improvement of the original conditions used for the synthesis of ZCZ011. In fact, the lead compound was synthetized following a published procedure[224] optimised for the synthesis of indolyl-nitroalkane derivatives catalysed by *N*-bromosuccinimide (NBS) using conventional heating at 40 °C in DCM as the solvent. The main problems of this protocol were long reaction time (24 hours), relative low yield and the presence of brominated by-products deriving from the use of NBS as reaction catalyst. On the contrary, under microwave irradiation, the reaction was clean, proceeded to completion and no main by-products were detected.

Furthermore, for the synthesis of ZCZ011, the commercially available and expensive 2-(2-nitrovinyl) thiophene was employed.

Hence, in order to develop a more cost-effective synthesis of new analogues, the required nitroalkene **119** was synthetized following a literature procedure[225] as described in Scheme 37.



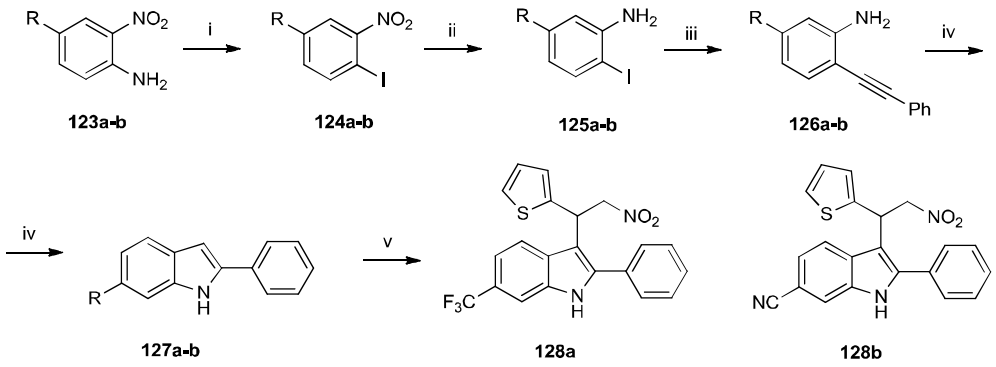
Scheme 37. Synthesis of 2-(2-nitrovinyl) thiophene **119**. (i) piperidine, $FeCl_3$, toluene, 120°C, 3 h, 95%.

Next, the 6-position of the indole scaffold was modified with a CF_3, a CN and a Cl moiety. As there are few known literature[226,227] reactions to functionalize the 6-position of an indole and the starting materials for the introduction of the required 6-substituents are not always commercially available, the indole core had to be built up from a 5-substituted-2-iodoaniline.

Hence, in order to easily access a good number of analogues for ZCZ011, a general synthetic method was developed and this is outlined in Scheme 38. Thus, the sequence of reactions employed started with a Sandmeyer reaction[228] with commercially available 2-nitroanilines to obtain the required aromatic iodo compounds, then reduction of the nitro group and a subsequent Sonogashira coupling[195] with the appropriate alkyne, led to the required alkyne-anilines. A Larock-type heteroannulation[131] reaction gave the desired indole derivatives that underwent conjugative addition with 2-(2-nitrovinyl)thiophene **119** to give the final ZCZ011 analogues.

Few modifications to this general procedure were made depending on the substrate employed, the availability of starting materials and their reactivity. Every detail will be discussed case by case.

Scheme 38 describes the synthesis of compound **128a**, bearing a trifluoromethyl group at 6-position and compound **128b**, bearing a cyano moiety in place of the methyl one of ZCZ011.

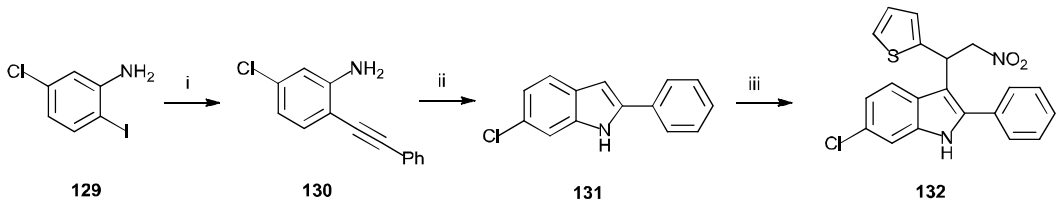


Scheme 38. Synthesis of analogues **128a** and **128b**. (i) 1) aq $NaNO_2$, HCl, 100 °C – 0 °C, 1h (a), aq $NaNO_2$, DMSO/HCl, (b) 2) aq KI, 0°C – r.t., 2 h, 74% (a), 73% (b); (ii) Fe, EtOH/AcOH 1:1, r.t., 18 h, quantitative yield; (iii) phenylacetylene, CuI, $PdCl_2(PPh_3)_2$, TEA, dry THF, 70°C, 1 h, 87% (a), 95% (b); (iv) $InBr_3$, toluene, 120°C, 18 h, 80% (a), 48% (b); (v) **119**, $InBr_3$, H_2O/*t*BuOH 5:1, MW 150°C, 15 min + 15 min, 74% (a), 13% (b).

Starting from the appropriate 4-sustituted-2-nitroaniline, a Sandmayer reaction was performed to achieve the desired 1-iodo-2-nitro-4-(trifluoromethyl)benzene (**124a**) and 4-iodo-3-nitrobenzonitrile (**124b**): in general, the iodine atom was introduced into the aromatic compound through treatment of the arenediazonium salt, generated from 2-nitroanilines with $NaNO_2$ in concentrated HCl, with potassium iodide. In order to avoid the acidic hydrolysis of the nitrile group in starting material **123b**, the arenediazonium salt was generated upon treatment of a DMSO solution of **123b**, with the minimum amount of conc.HCl and under controlled heating. Then, the reduction of the nitro group in the presence of iron powder in a 1:1 AcOH/EtOH solution followed by a Sonogashira coupling with phenylacetylene, in the presence of a palladium source such as $PdCl_2(PPh_3)_2$ as catalyst combined with a co-catalytic amount of CuI and 5 equivalents of TEA in THF as solvent, gave the required 2-(phenylethynyl)anilines **126a** and **126b**. It is well known that electron-withdrawing groups present on the aromatic halide facilitate the palladium-catalysed Sonogashira coupling, while electron-donating groups impart adverse effects.[229] In these particular cases, the CF_3 and CN on the anilines are electron-withdrawing groups, activating the halides toward the Sonogashira coupling, thus the reduction step of the nitro group to amine can be performed before the palladium-catalysed reaction without affecting the outcome of the latter. Afterwards, intermediates **126a** and **126b** were used for a 5-*endo-dig* cyclisation, employing $InBr_3$ as catalyst, to produce the desired indoles **127a** and

127b. Lastly, analogues **128a** and **128b** were obtained through conjugate addition of the previously synthetized indoles with nitroalkene **119**, that was carried out employing the conditions described for the synthesis of analogue **120**, with the addition of a catalytic amount of $InBr_3$, a Lewis acid that allows the process to be carried out in aqueous media.[230]

The synthesis of the analogue **132**, bearing a Cl atom at 6-position, is described in Scheme 39.

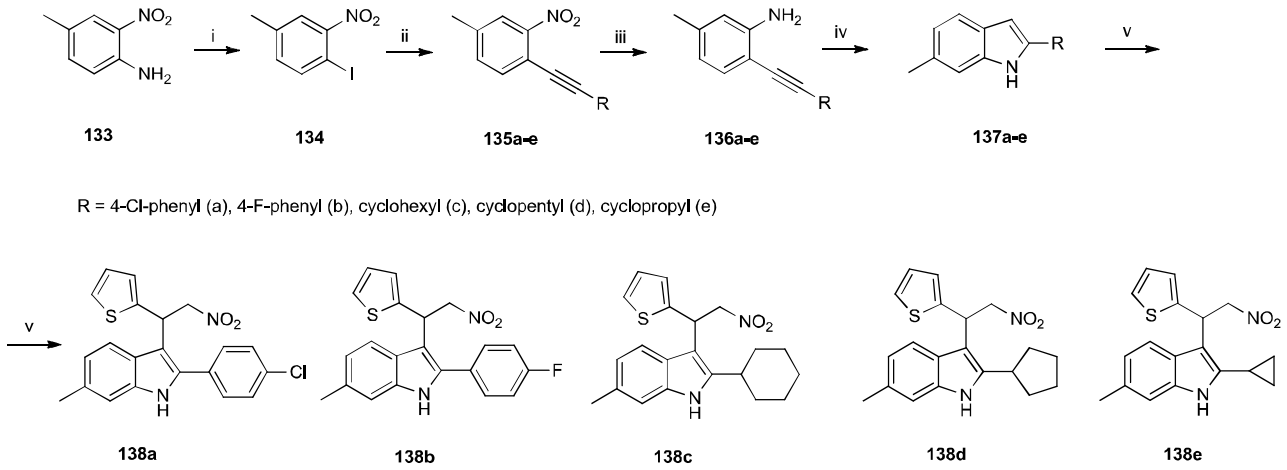


Scheme 39. Synthesis of analogue **132**. (i) Phenylacetylene, CuI, $PdCl_2(PPh_3)_2$, TEA, dry THF, 70°C, 1 h, 90%; (ii) $InBr_3$, toluene, 120°C, 18 h, 81%; (iii) **119**, $InBr_3$, H_2O/*t*BuOH 5:1, MW 150°C, 15 min + 15 min, 77%.

For the synthesis of this analogue, the Sandmeyer reaction and the reduction step were not necessary since the 5-chloro-2-iodoaniline **129** was commercially available. Hence, the Sonogashira coupling, performed under the previously described reaction conditions, gave the phenylethynylaniline **130** in 90% isolated yield. Ring closure to form the 6-chloro-2-phenylindole **131** was accomplished by addition of a catalytic amount of $InBr_3$ in toluene at 120 °C and subsequent conjugate addition, again catalysed by the presence of $InBr_3$ in the reaction mixture, afforded the final 6-Cl analogue **132** in 77% isolated yield.

4.3 Modification of 2-position of indole scaffold:

We next sought to modify the 2-phenyl portion of the indole ring. To prepare these analogues, the five-step synthetic route, previously used for the synthesis of 6-modified analogues, was slightly adapted. As depicted in Scheme 40, the 1-iodo-4-methyl-2-nitrobenzene **134**, obtained through a Sandmeyer reaction, was the starting point for the synthesis of all 2-modified analogues.

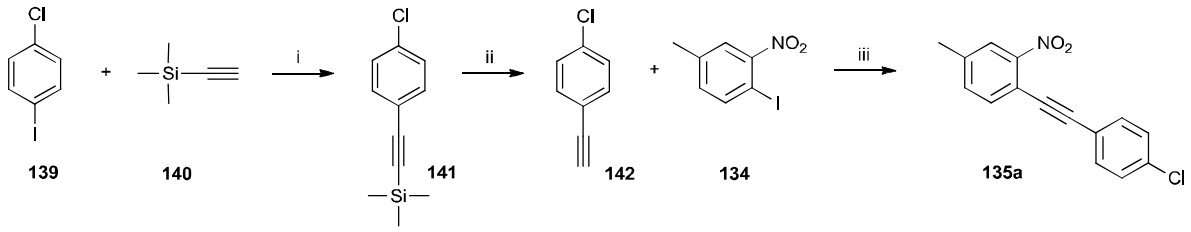


Scheme 40. Synthesis of 2-modified analogues. (i)1) aq $NaNO_2$, HCl 2) aq KI, 0°C – r.t., 2 h, 84%; (ii) alkyne, CuI, $PdCl_2(PPh_3)_2$, TEA, dry THF or dry DMF, 70°C or 50°C, 1 h or o.n, 73-96%; (iii) Zn or $SnCl_2$, EtOH/AcOH 1:1 or Fe, EtOH/c.HCl, r.t. or 50°C, 18 h or 3h, 31-79%; (iv) $InBr_3$, toluene, 120°C, 18 h, 26-70%; (v) **119**, $InBr_3$, H_2O/*t*BuOH 5:1, MW 150°C, 15 min + 15 min, 32-88%.

Notably, for the synthesis of analogue **138a**, intermediate **135a** was prepared as described in Scheme 41 (next page).

Commercially available 1-chloro-4-iodobenzene **139** was reacted under Sonogashira conditions with (trimethylsilyl)acetylene to afford the protected compound **141**. The crude material was filtrated on silica plug to remove the catalysts and it was submitted to deprotection under basic conditions.

Thus, after stirring for 1 hour in a KOH solution, the reaction mixture containing the *in situ* generated free alkyne **142** was poured into a second reaction mixture containing 1-iodo-4-methyl-2-nitrobenzene **134** and all the required catalysts for a second Sonogashira coupling that allowed to obtain the needed intermediate **135a** in 76% yield.



Scheme 41. Synthesis of intermediate **135a**. (i) CuI, $PdCl_2(PPh_3)_2$, TEA, dry THF, 70 °C, 1h; (ii) KOH, MeOH, r.t., 1 h; (iii) CuI, $PdCl_2(PPh_3)_2$, TEA, dry THF, 70 °C, 2 h, 76%.

This result clearly indicates that the desilylation conditions which rely on the inexpensive base KOH are compatible with the catalyst system used for the subsequent Sonogashira coupling.[231]

The other required intermediates **135b-e** were readily prepared in 73-96% range yield by treating compound **134** with the corresponding alkyne under the palladium-catalysed reaction conditions described before (Scheme 40). However, when the alkyne coupling partner was either cyclohexylacetylene, cyclopentylacetylene or cyclopropylacetylene, better outcomes of the Sonogashira coupling were found using a more polar solvent such as DMF at 50 °C instead of THF. Moreover, these alkynes required longer reaction times than their aromatic counterparts; in fact reactions generally proceeded overnight.

It is necessary to point out that, as aforementioned, the Sonogashira coupling is facilitated by the presence of electron-withdrawing groups on the aromatic halide ring, and for this reason, this coupling was performed before the reduction of the nitro group to amine because, otherwise 5-CF_3 and 5-CN anilines, the 5-methylaniline does not provide the electronic deficiency required. Hence, reduction of the nitro group was performed after the Sonogashira coupling, and different protocols were employed depending on the substrate involved. Specifically, the reduction of aromatic alkynes **135a** and **135b**, bearing a Cl and a F group on *para*-position of 2-phenyl ring, respectively, and also cyclohexyl compound **135c** was accomplished using zinc in a 1:1 mixture of EtOH/AcOH at room temperature for 2 hours, while the reduction of compound **135d**, containing a cyclopentyl alkyne, gave better results in the presence of 5 equivalents $SnCl_2$ and 4 equivalents of conc.HCl in ethanolic solution at 50 °C.[232]

Unfortunately, the reduction of 1-(cyclopropylethynyl)-4-methyl-2-nitrobenzene **135e** was the most problematic. First, the reduction was attempted using the same conditions involving $SnCl_2$ as described for compound **135d** but, ^{1}H-NMR and ^{13}C-NMR analysis, revealed that the isolated compound after chromatographic purification was not the expected alkyne-aniline but the ketone **143** (Figure 37, next page). Then, the reaction was

attempted employing $NiCl_2 \cdot 6H_2O$ and $NaBH_4$ as reducing agents in a cold methanolic solution.[233] Nevertheless, it was not possible to recover the desired product and the fully reduced compound **144** was obtained, instead (Figure 37). Finally, aniline **136e** was synthetized upon reduction of the starting nitro compound, in the presence of iron powder in a 1:1 mixture of EtOH/AcOH at 40 °C, but only in 31% isolated yield.

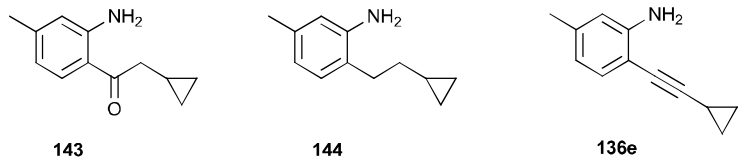
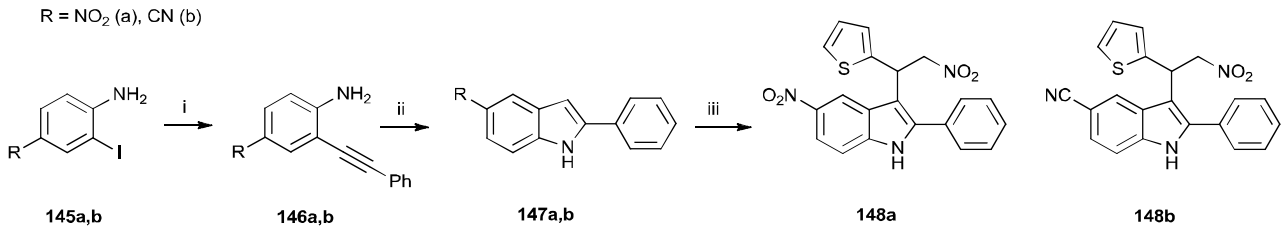


Figure 37. Chemical structure of by-products **143** and **144** and the desired intermediate **136 e**.

In conclusion, indoles **137a-e** were obtain through a Larock-type heteroannulation as previously described, and a following Michael addition with 2-(2-nitrovinyl)thiophene **119** under microwave irradiation, afforded the final analogues **138a-e** (Scheme 40), bearing aromatic and non-aromatic moieties in place of the 2-phenyl group of ZCZ011.

4.4 Modification of 5-position of indole scaffold:

With ZCZ011 exhibiting interesting biological activity, an effort was taken to improve its potency exploring the effect of various substituents at different positions of the indole core. Therefore, three different moieties were introduced at 5-position, that displays no functional group in the lead compound, to evaluate the SAR effect of electron-withdrawing groups such as NO_2 and CN, and of a weak electron-donating group such as a methyl group, in this position.

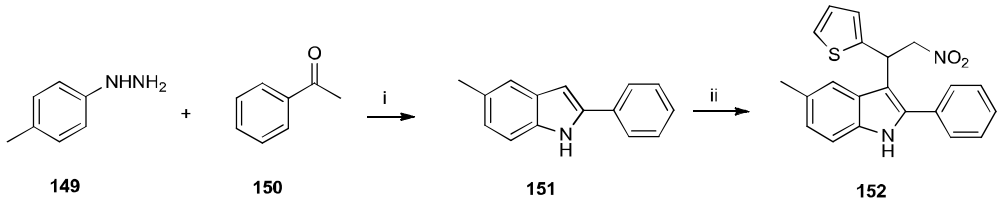


Scheme 42. Synthesis of analogues **148a** and **148b**. (i) Phenylacetylene, CuI, $PdCl_2(PPh_3)_2$, TEA, dry THF, 70°C, 1 h, 95% (a), 88% (b); (ii) $InBr_3$, toluene, 120°C, 18 h, 74% (a), 93% (b); (iii) **119**, $InBr_3$, H_2O/*t*BuOH 5:1, MW 150°C, 15 min + 15 min, 41% (a), 36% (b).

The synthesis of the analogues **148a** and **148b**, bearing electron-withdrawing groups at 5-position on the indole scaffold, is outlined in Scheme 42.

Thus, the palladium-catalysed coupling step between 2-iodoanilines **145a** and **145b** and phenylacetylene was completed within an hour, as expected for this kind of activated substrates, giving intermediates **146a** and **146b** in excellent isolated yields. Besides, the ring closure reaction, catalysed by $InBr_3$ in refluxing toluene, was also very satisfactory, yielding the desired indoles **147a** and **147b** in 74% and 93%, respectively. On the contrary, the final Michael addition, leading to 5-modified analogues **148a** and **148b,** gave only poor results; the reaction did not reach completion even after more than two microwave runs (15 minutes, 150 °C each) with freshly added catalyst, leading only to the formation of more by-products. In fact, the desired analogues were recovered only in 41% yield for the nitro-containing analogue, and in 36% yield, for its 5-CN counterpart. The yield decrement may be due to steric and electronic factors, as reported in the literature.[234] In fact, the presence of these electron-withdrawing groups, which decrease the electron density of the indole scaffold, combined with the steric hindrance of the 2-phenyl substituent, resulted in lower yield of the corresponding products.

The synthetic sequence for the 5-modified analogue with a weak electron-donating group is depicted in Scheme 43. A Fischer indole synthesis was employed to synthetize 5-methyl-2-phenylindole **151**: acetophenone **150** was reacted with 4-methylphenylhydrazine **149** to produce the intermediate hydrazone, which was used without purification for the following indole formation process, that was accomplished by heating the reaction mixture at 120 °C in toluene, in the presence of Lewis acid $ZnBr_2$ as catalyst. The application of the Fischer indole reaction to the synthesis of indole **151** was a very convenient method as the starting materials were readily available and no other possible regioisomer was formed as by-product.



149 150 151 152

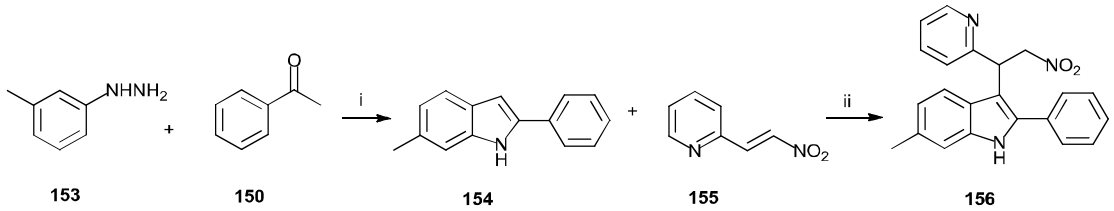
Scheme 43. Synthesis of analogue **152**. (i) 1) EtOH, r.t., 18 h; 2) $ZnBr_2$, toluene, 120°C, 18 h, 67%; (ii) **119**, $InBr_3$, H_2O/*t*BuOH 5:1, MW 150°C, 15 min + 15 min, 79%.

Finally, the 5-methyl-bearing analogue **152** was synthetized using the Michael reaction conditions described before. In this case, the desired product was obtain in a very good

79% yield, supporting the theory that the lower yields gained with the electron-withdrawing 5-NO_2 and 5-CN analogues, were indeed due to electronic factors.

4.5 Modification of the thienyl moiety in the side chain:

To further understand the SAR of the lead compound ZCZ011, a new analogue was synthetized by altering the thienyl-containing side chain. The synthetic sequence leading to the synthesis of analogue **156**, containing a pyridyl moiety in the side chain, is described in Scheme 44.

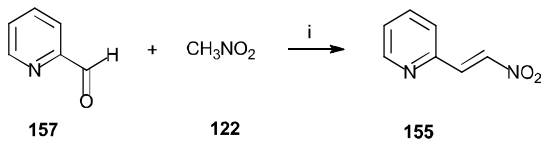


Scheme 44. Synthesis of analogue **156**. (i) 1) EtOH, r.t., 18 h; 2) PPA, toluene, 120°C, 18 h, 45%; (ii) TBAB, H_2O/dioxane 1:3, 100°C, 18 h, 32%.

A Fischer indole synthesis was chosen to synthetize the 6-methyl-2-phenylindole **154**, following the same reaction conditions employed for the synthesis of the lead compound. The *meta*-substituted hydrazine **153** produced both 6- and 4-subtituted indoles, and this is explained simply by Fischer reaction mechanism in which two different ene-hydrazine could be formed and undergo [3,3]-sigmatropic rearrangement.[124] ^{1}H-NMR analysis of the crude reaction mixture revealed that the ratio of 6-methyl to 4-methyl indoles was 10:1, respectively. Hence, the desired 6-methyl substituted indole **154** was isolated by crystallization and subsequently coupled with 2-[(*E*)-2-nitroethenyl]pyridine **155**.

Nitroethylene derivatives can be obtained from aryl aldehydes and nitroalkanes under Henry condensation conditions followed by an elimination reaction with removal of water when an acidic α-proton is available.

Thus, 2-pyridinecarboxyaldehyde **157** and nitromethane **122** were combined together via TMG-catalysed Henry reaction, as shown in Scheme 45, to obtain the intermediate β-nitroalcohol, that underwent elimination of water promoted by addition of methanesulfonyl chloride (MsCl). Therefore, the required 2-[(*E*)-2-nitroethenyl]pyridine **155** was synthetized in a one-pot two-step process but, in a modest 36% isolated yield.[235]



157 122 155

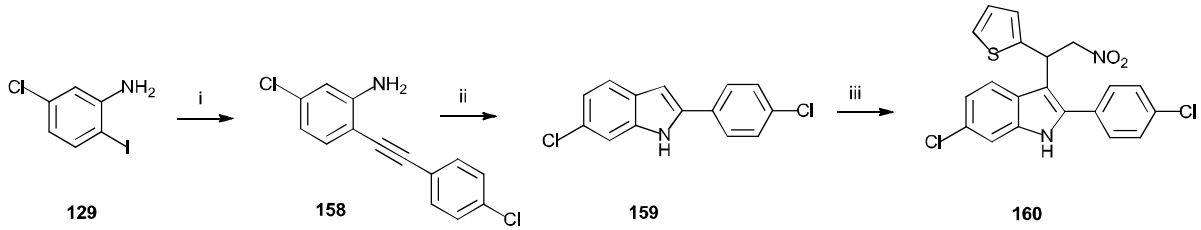
Scheme 45. Synthesis of compound **155**. (i) 1) TMG, toluene, r.t., 5 min 2) MsCl, TEA, r.t., 30 min, 36%.

A final conjugative addition between 6-methyl-2-phenylindole **154** and nitroalkene **155** led to analogue **156** containing a pyridinyl side chain in place of the thienyl one of ZCZ011 (Scheme 44, previous page). The coupling reaction was, at first, unsuccessfully attempted using the same microwave conditions described in the analogue general synthesis and only a complex mixture of products was recovered, probably caused by the degradation of the starting materials under water "superheated conditions". Thus, the reaction was carried out as described in a literature procedure[172] employing conventional heating (100 °C) in a mixture of water/dioxane and TBAB as catalyst. However, **156** was recovered only in a modest 32% isolated yield.

4.6 Combined modifications of indole scaffold:

In addition to the above analogues, four new compounds presenting combined modifications were successfully synthetized. The first two analogue display combined modifications of 6-position and 2-position of the indole scaffold.

The synthesis of analogue **160** involves 3 steps (Scheme 46). In the first step, commercially available aniline **129** is coupled with 2-chlorophenylacetylene **142**, prepared following the protocol developed for the synthesis of intermediate **135a** (Scheme 41), in the presence of $PdCl_2(PPh_3)_2$ as the palladium-source in THF at 70 °C for 1 hour.

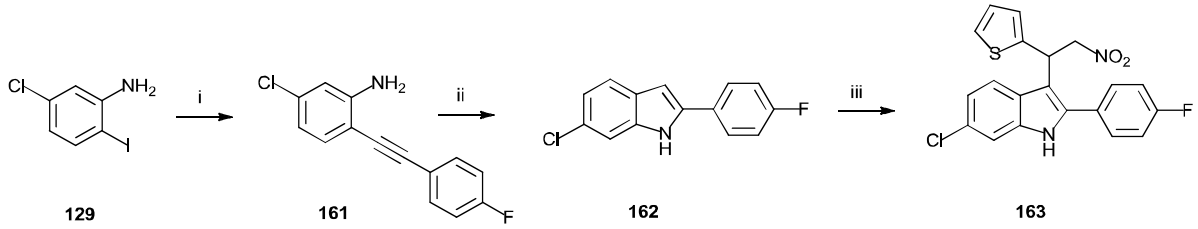


129 158 159 160

Scheme 46. Synthesis of analogue **160**. (i) **142**, CuI, $PdCl_2(PPh_3)_2$, TEA, dry THF, 70°C, 1 h, 80%; (ii) $InBr_3$, toluene, 120°C, 18 h, 68%; (iii) **119**, $InBr_3$, H_2O/*t*BuOH 5:1, MW 150°C, 15 min + 15 min, 55%.

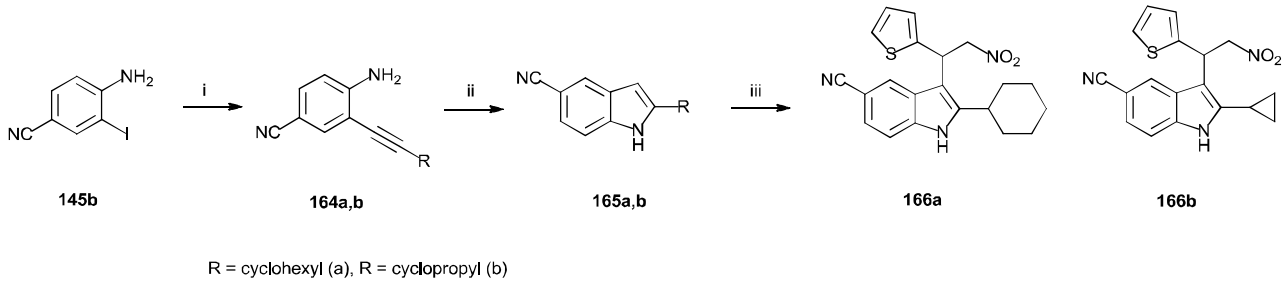
The required intermediate **158** was achieved in a very good 80% isolated yield and the subsequent 5-*endo-dig* cyclisation led to indole **159** in a still satisfactory 68% isolated yield. The final conjugate addition under microwave condition allowed to obtain the final analogue **160** presenting a chlorine atom at 6-position of the indole scaffold and at *para*-position of the 2-phenyl moiety.

As described in Scheme 47, commercially available aniline **129** was the starting material also for the synthesis of analogue **163**, which bears a chlorine atom at 6-position of indole scaffold and a fluorine atom at *para*-position of the 2-phenyl group. The synthesis followed the general procedure without variations.



Scheme 47. Synthesis of analogue **163**. (i) 4-Fluoro-phenylacetylene, CuI, $PdCl_2(PPh_3)_2$, TEA, dry THF, 70°C, 1 h, 86%; (ii) $InBr_3$, toluene, 120°C, 18 h, 83%; (iii) **119**, $InBr_3$, H_2O/*t*BuOH 5:1, MW 150°C, 15 min + 15 min, 63%

To complete this series of ZCZ011 analogues, two additional compounds, showing combined modification at 5-position and 2-position of the indole core, were designed and synthetized.The synthesis of the last two analogues is described below in Scheme 48 and it followed the general procedure. In particular, both compounds are characterised by the presence of a CN moiety at 5-position while the 2-phenyl group of the lead compound, is replaced by a non-aromatic cyclohexyl moiety in **166a**, and by a non-aromatic smaller cyclopropyl group in compound **166b**.



Scheme 48. Synthesis of analogues **166a** and **166b**. (i) alkyne, CuI, $PdCl_2(PPh_3)_2$, TEA, dry THF, 70°C, 18 h, 93% (a), 61%(b); (ii) $InBr_3$, toluene, 120°C, 18 h, 71% (a), 60% (b); (iii) **119**, $InBr_3$, H_2O/*t*BuOH 5:1, MW 150°C, 15 min + 15 min, 55% (a), 80% (b).

4.7 Biological evaluation:

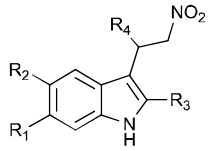
Cannabinoid receptor allosteric ligands may be classified on their overall functional effects: ligands which amplify the effect of an agonist are known as allosteric enhancers or positive allosteric modulators while ligands which suppress the effect of an agonist are known as allosteric inhibitors or negative allosteric modulators.

The initial screening of all new ZCZ011 analogues synthetized was performed using an *in vitro* assay to determine their functional characteristics. PathHunter™ β-Arrestin assay developed by DiscoveRX is a revolutionary high-throughput screening assay for monitoring GPCR activation following ligand stimulation, without an imaging instrument, fluorescent protein tag, or radioactivity. This assay platform uses an enzyme complementation system to detect the interaction between the GPCR C-terminus of interest and β-arrestin.[236] Indeed, it represents an excellent starting point for G-protein coupled receptor based drug discovery where the main purpose of drug discovery is to find compounds that exert functional effects. Moreover, PathHunter™ β-arrestin assay system is found in the literature as a useful system to screen library of compounds and it was used to identify interesting cannabinoid CB_2 receptor ligands.[237]

Using the PathHunter™ CB_1 kit from DiscoveRX, a cannabinoid CB_1 receptor agonist (anandamide, AEA) was added and it activated the receptor which result in a fluorescence signal that was recorded by a luminescence plate reader. The fluorescence signal is directly related to the activation of the receptor, therefore a higher concentration of agonist will yield a larger fluorescence signal. The cannabinoid agonist was added in increasing concentrations to obtain an agonist dose-response curve. With pre-incubation of a potential allosteric modulator, either an enhancement of agonist signalling with an allosteric enhancer or a decrease in the maximal response of the agonist with an allosteric inhibitor, is expected.

All raw data, from the screening of ZCZ011 analogues synthetized, were obtained as luminescence (relative light units). They were normalised and presented as a percentage of the maximal response for the endogenous cannabinoid CB_1 receptor agonist anandamide (AEA).

Results are shown in Table 4 along with data from the lead compound ZCZ011 and the patented F-0870 as a comparison.



Entry	*Compound*	*R_1*	*R_2*	*R_3*	*R_4*	*Potency % Enhancement*	
						100 nM	**1000 nM**
1	F-0870	H	H	Ph	Ph	-	76.8
2	ZCZ011	Me	H	Ph	thienyl	56.8	126.0
3	**120**	H	H	Ph	thienyl	55	inactive
4	**128a**	CF_3	H	Ph	thienyl	69	215
5	**128b**	CN	H	Ph	thienyl	51.7	122.8
6	**132**	Cl	H	Ph	thienyl	117.1	102.1
7	**138a**	Me	H	4-Cl-Ph	thienyl	-	inactive
8	**138b**	Me	H	4-F-Ph	thienyl	-	inactive
9	**138c**	Me	H	cyclohexyl	thienyl	-	inactive
10	**138d**	Me	H	cyclopentyl	thienyl	-	inactive
11	**138e**	Me	H	cyclopropyl	thienyl	140.1	142.3
12	**148a**	H	NO_2	Ph	thienyl	-	inactive
13	**148b**	H	CN	Ph	thienyl	-	inactive
14	**152**	H	Me	Ph	thienyl	62.8	167.4
15	**156**	Me	H	Ph	pyridyl	-	inactive
16	**160**	Cl	H	4-Cl-Ph	thienyl	-	inactive
17	**163**	Cl	H	4-F-Ph	thienyl	-	59.7
18	**166a**	H	CN	cyclohexyl	thienyl	-	inactive
19	**166b**	H	CN	cyclopropyl	thienyl	-	inactive

Table 4. Potency enhancement (%) of AEA signal values for the substitution of indole ring and side chain.

As it can be seen from the table, the replacement of the methyl group at 6-position of the indole scaffold with an electron-withdrawing group such as CF_3, CN or Cl (entry 4,5,6

compounds **128a**, **128b** and **132**) increased the potency compared to parent compound ZCZ011, while removing the functional group in this position led to an inactive compound (entry 3, compound **120**). Moreover, analogue **128b**, containing a polar 6-CN moiety, is a very interesting example and it could be further developed and investigated in order to obtain peripherally restricted drugs.

Moving the methyl group to another position on the indole ring (entry 14, compound **152**, 5-methyl substituted) also resulted in an AEA-induced signalling enhancement. In fact, 5-methyl analogue **152** is able to enhance anandamide signal better than ZCZ011. On the contrary, inserting an electron-withdrawing moiety at 5-position (entry 12,13 compounds **148a** and **148b**) led to inactive analogues. The importance of the phenyl-moiety at 2-position was also evaluated. Results clearly indicate that, in order to retain some biological activity, this phenyl ring cannot display a halogen at *para*-position, in fact analogues **138a** and **138b** (entry 7 and 8) are inactive. Similar results were obtained with analogues **138c** and **138d** (entry 9 and 10) in which the 2-phenyl group of the lead compound was replaced by a cyclohexyl or a cyclopentyl moiety. Surprisingly, analogue **138e** (entry 11), containing a 2-cyclopropyl group is active and also quite potent.

On the basis of this series of modifications on the aromatic indole ring, it could be concluded that replacing the 6-methyl group with an electron-withdrawing one increases the potency, while at 5-position, only the presence of a methyl group is beneficial. Furthermore, the 2-phenyl ring should not be replaced by a more sterically hindered cycloalkane or modified at *para*-position, as these modifications, probably, render the substituent at 2-position too bulk to fit the binding pocket that the 2-phenyl normally occupies. This theory could explain why compound **132** (entry 6) was able to act as a positive allosteric modulators of CB_1 receptors while its 2-(4-Cl)phenyl analogue **160** (entry 16) and its 2-(4-F)phenyl analogue **163** (entry 17) were found inactive. However, the unexpected result obtained with analogue **138 e** (entry 11), could be explained supposing that the 2-cyclopropyl moiety also fits into this binding pocket.

Finally, it was discovered the necessity of a thienyl moiety in the side chain by replacing it with a pyridine ring (entry 15, compound **156**), which resulted in a loss of activity.

4.8 Conclusions and perspectives:

In conclusion, 17 new structural analogues of ZCZ011 were designed, synthetized and fully characterized by taking advantage of a highly efficient general synthetic approach. All the reactions were optimised case by case, to achieve the best yields depending on the substrate involved.

The new analogues have been evaluated for their ability to potentiate the maximum level of stimulation on CB_1 receptors caused by the endogenous agonist anandamide and some interesting results have been highlighted. It was found that the insertion of a trifluoromethyl group at 6-position of the indole scaffold, results in a more potent analogues of ZCZ011 (compound **128a**) while the insertion of a nitrile at that position, results in the analogue **128b**, with promising properties for the development of a peripherally restricted compound (Figure 38). Notably, it was found that the 2-phenyl group cannot tolerate further modification and the thienyl moiety on the side chain is crucial for the activity.

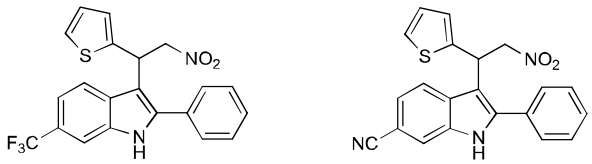


Figure 38. Chemical structure of analogues **128a** and **128b**.

The SAR investigations demonstrated that simple modification of the parent structure of ZCZ011 can produce some potentially important derivatives, which may improve PAM potency and affinity towards CB_1 receptors. However, results from a more extensive investigation using a greater number of derivatives are needed for SAR study, for the design and synthesis of a more effective positive allosteric modulator for *in vivo* assessments.

5 Experimental part:

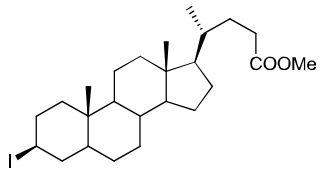
5.1 Experimental part project 2:

All reactions were performed under a nitrogen atmosphere using oven-dried glassware. Anhydrous solvents were dried over standard drying agents30 and freshly distilled prior to use. Reactions were monitored by TLC on silica gel 60 F254 with detection by charring with phosphomolybdic acid. Flash column chromatography was performed on silica gel 60 (230–400 mesh). ^{1}H (400 MHz), ^{13}C (101 MHz) and ^{31}P (122 MHz) NMR spectra were recorded on Varian VNMRS-400 spectrometer in $CDCl_3$ or CD_3OD solutions at room temperature. Medium-pressure (8 bar) chromatography was performed on silica gel 60 (230 mesh) using a Chromatospac Prep 100.

General procedure for the synthesis of 3β-iodides:

To a solution of bile acid ester (12.30 mmol) in 1,3-dioxolane (100 mL), PPh_3 (18.45 mmol) and imidazole (36.90 mmol) were added. After 5 min, I_2 (18.45 mmol) was added portionwise. The resulting solution was stirred at room temperature for 30 min. The reaction mixture was poured into water containing few drops of 30% H_2O_2 and extracted with EtOAc. The organic layer was washed with aq. $Na_2S_2O_5$ and dried over anhydrous Na_2SO_4, filtered and concentrated under reduce pressure. The crude product was dissolved in MeOH/EtOAc and allowed to stand overnight. The
crystallized desired product was recovered as a white solid.

Methyl 3β-iodo-5β−cholan-24-oate, 79:

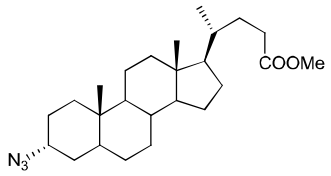


78 (1.50 g, 3.84 mmol), PPh_3 (3.02 g, 11.52 mmol), imidazole (1.05 g, 15.36 mmol) and I_2 (2.92 g, 11.52 mmol) were employed. Compound **79** was crystallized from MeOH/EtOAc as a white amorphous solid (1.75 g, yield 92%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.99 (bs, 1 H, H-3α), 3.65 (s, 3 H, OCH_3), 1.01 (s, 3 H, H-19), 0.90 (d, *J* = 6.4 Hz, 3 H, H-21), 0.64 (s, 3 H, H-18); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 174.8, 56.6, 55.9, 51.5, 42.7, 41.7, 40.1, 39.5, 39.0, 36.9, 35.7, 35.7, 35.4, 32.70, 31.2, 31.0, 30.9, 28.6, 27.0, 26.4, 24.1, 23.7, 20.8, 18.7, 12.0.

General procedure for the synthesis of 3α-azides:

Iodo-derivate bile ester (1.94 mmol) was dissolved in DMF (10 ml) and NaN_3 (378.4 mg, 5.8 mmol) was added. The reaction mixture was stirred at room temperature for 6 h and then poured into water (8 ml) and extracted twice with a mixture of Et_2O (12 ml) and EtOAc (3 ml). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* to give azido-compounds that were used without further purifications.

Methyl 3α-azido-5β-cholan-24-oate, 80:



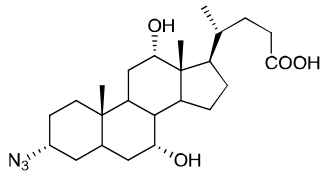
79 (504.3 mg, 1.01 mmol) and NaN_3 (194 mg, 3.03 mmol) were employed. Compound **80** was obtained as a white amorphous solid (367.8 mg, yield 87%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.66 (s, 3 H, OCH_3), 3.36-3.25 (m, 1 H, H-3β), 0.93 (s, 3 H, H-19), 0.91 (d, *J* = 6.4 Hz, 3 H, H-21), 0.64 (s, 3 H, H-18); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 174.8, 61.6, 56.4, 55.9, 51.5, 42.7, 42.3, 40.4, 40.0, 35.8, 35.5, 35.4, 34.6, 32.4, 31.0, 30.9, 28.2, 27.1, 26.7, 26.3, 24.2, 23.4, 20.8, 18.3, 12.0.

General procedure for ester hydrolysis:

To a solution of N_3-bile acid ester (1.74 mmol) in MeOH (10 mL), 1.5 M LiOH (28.88 mmol) was added and the reaction mixture was heated at 50 °C for 24 h and then cooled to room temperature.

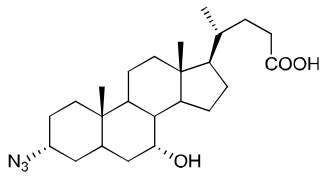
1 N HCl was added until pH= 4, the mixture was extracted with EtOAc (2 × 20 mL) and washed with water (2 × 15 mL). After drying over Na_2SO_4, the solvent was evaporated under reduced pressure and the crude product was used without further purifications.

3α-Azido-7α,12α-dihydroxy-5β-cholan-24-oic acid, 81:



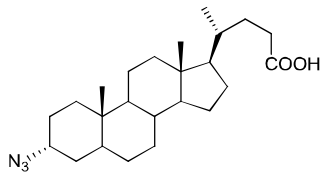
51 (193.6 mg, 0.43 mmol) and 1.5 M LiOH (3.44 mL, 5.16 mmol) were employed. Compound **81** was obtained as a white amorphous solid (143.5 mg, yield 77%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.00 (bs, 1 H, H-12β), 3.87-3.86 (m, 1 H, H-7β), 3.18-3.11 (m, 1 H, H-3β), 0.98 (d, *J* = 6.0 Hz, 3 H, H-21), 0.90 (s, 3 H, H-19), 0.69 (s, 3 H, H-18); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 179.3, 73.1, 68.4, 61.3, 47.1, 46.5, 41.9, 41.8, 39.3, 35.4, 34.8, 34.5, 31.0, 30.7, 28.1, 27.6, 26.8, 26.5, 23.2, 22.5, 17.3, 12.5.

3α-Azido-7α-hydroxy-5β-cholan-24-oic acid, 82:



52 (751 mg, 1.74 mmol) and 1.5 M LiOH (13.92 mL, 20.88 mmol) were employed. Compound **82** was obtained as a white amorphous solid (724.3 mg, quantitative yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.87-3.85 (m, 1 H, H-7β), 3.19-3.11 (m, 1 H, H-3β), 0.93 (d, *J* = 6.4 Hz, 3 H, H-21), 0.91 (s, 3 H, H-19), 0.66 (s, 3 H, H-18); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 179.8, 68.4, 61.23, 55.72 50.3, 42.7, 41.8, 39.5, 39.4, 35.5, 35.4, 35.3, 35.1, 34.4, 32.7, 30.9, 30.7, 28.1, 26.8, 23.7, 22.8, 20.6, 18.2, 11.8.

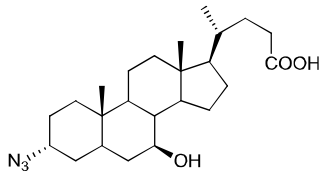
3α-Azido-5β-cholan-24-oic acid, 83:



80 (363.8 mg, 0.88 mmol) and 1.5 M LiOH (7 mL, 10.56 mmol) were employed. Compound **83** was obtained as a white amorphous solid (273.9 mg, yield 77%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.33-3.26 (m, 1 H, H-3α), 0.92 (overlap. 6 H, H-21, H-19), 0.64 (s, 3 H, H-18); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 180.2, 61.2, 56.3, 55.9, 42.7, 42.3, 40.4,

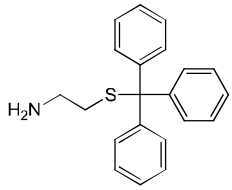
40.0, 35.8, 35.5, 35.3, 34.6, 32.4, 30.9, 30.7, 28.1, 27.1, 26.7, 26.3, 24.2, 23.4, 20.8, 18.2, 12.04.

3α-Azido-7β-hydroxy-5β-cholan-24-oic acid, 84:



53 (81.1 mg, 0.18 mmol) and 1.5 M LiOH (1.5 mL, 2.26 mmol) were employed. Compound **84** was obtained as a white amorphous solid (61.2 mg, yield 80%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.61-3.55 (m, 1 H, H-7α), 3.31-3.23 (m, 1 H, H-3β), 0.96 (s, 3 H, H-19), 0.93 (d, *J* = 6.4 Hz, 3H, 21-CH3), 0.67 (s, 3 H, H-18); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 179.6, 71.2, 60.8, 55.6, 54.8, 43.7, 43.6, 42.7, 39.9, 39.1, 36.6, 35.2, 35.1, 34.1, 33.4, 30.9, 30.8, 28.6, 26.8, 23.4, 22.3, 21.2, 18.9, 18.3, 12.1.

Synthesis of 2-(Tritylsulfanyl)ethanamine, 91:

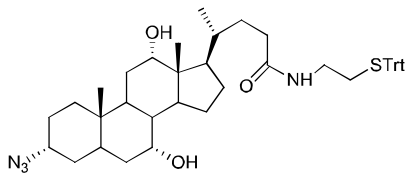


To a solution of cysteamine hydrochloride **90** (1 g, 8.8 mmol) in CH_2Cl_2/DMF (5:1, *v/v*, 72 mL), trityl chloride (2.45 g, 8.8 mmol) was addeda and the resulting mixture was stirred at room temperature for 14 h. The solution was concentrated under reduced pressure and the crude residue was triturated with Et_2O. The crude material was suspended with 2M KOH/Et_2O (1:1, *v/v*, 30 mL) and extracted with Et_2O (2 × 15 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under reduce pressure to give compound **91** as a white solid (2.47 g, yield 86%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.46-7.41 (m, 6 H, H-Ar), 7.31-7.25 (m, 6 H,H-Ar), 7.24-7.17 (m, 3 H, H-Ar), 2.59 (t, *J* = 6.5 Hz, 2 H, CH_2-N), 2.32 (t, *J* = 6.5 Hz, 2 H, CH_2-S); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 144.9, 129.6, 127.9,126.7, 66.5, 41.0, 36.3.

General procedure for the synthesis of bile acid–2-(tritylsulfanyl)ethanamine conjugates:

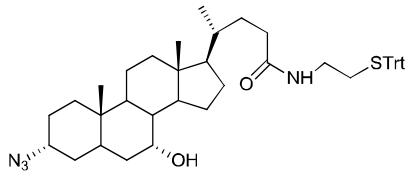
A solution of bile acid (0.96 mmol) in THF (12 mL) was cooled to 0 °C then triethylamine (1.44 mmol) and ethyl chloroformate (1.44 mmol) were added dropwise. The resulting mixture was stirred at 0 °C for 45 min. 2-(Tritylsulfanyl)ethanamine (1.44 mmol) was the added to the freshly prepared bile acid anhydride and the stirring continued for 20 h at r.t. The crude product obtained after the evaporation of the volatiles was dissolved in CH_2Cl_2 (30 mL) and washed with water (2 × 15 mL), 0.1 N HCl solution (2 × 15 mL), water (2 × 15 mL), and finally with brine (2 × 15 mL). After drying over Na_2SO_4, the solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography (silica gel).

***N*-(2-Tritylsulfanylethyl)-3*α*-azido-7*α*,12*α*-hydroxy-5β-cholan-24-amide, 85:**



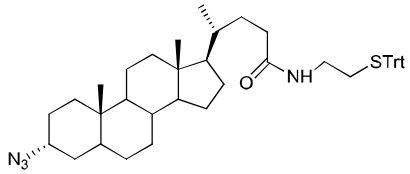
81 (126 mg, 0.29 mmol), triethylamine (60 μL, 0.44 mmol), ethyl chloroformate (41 μL, 0.44 mmol) and 2-(tritylsulfanyl)ethanamine **91** (139 mg, 0.44 mmol) were employed. Purification by flash chromatography (CH_2Cl_2/EtOAc 2:1, MeOH 1 %) gave compound **85** as a white amorphous solid (156.9 mg, yield 76%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.43-7.38 (m, 6 H, H-Ar), 7.32-7.19 (m, 9 H, H-Ar), 5.71 (bs, 1 H, N*H*), 3.98-3.96 (bs, 1 H, H-12β), 3.84 (m, 1 H, H-7β), 3.20-3.03 (m, 3 H, H-3β,H-25), 2.41 (t, *J* = 6.3 Hz, 2 H, H-26), 0.95 (d, *J* = 6.4 Hz, 3 H, H-21), 0.90 (s, 3 H, H-19), 0.67 (s, 3 H, H-18); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 173.6, 144.6, 132.5, 129.5, 127.8, 126.6, 72.9, 68.1, 61.3, 46.8, 46.5, 41.9, 41.8, 39.4, 38.1, 35.5, 35.3, 34.7, 34.5, 32.9, 31.9, 31.4, 28.2, 27.5, 26.8, 26.6, 23.2, 22.6, 17.4, 12.5.

***N*-(2-Tritylsulfanylethyl)-3α-azido-7α-hydroxy-5β-cholan-24-amide, 86:**



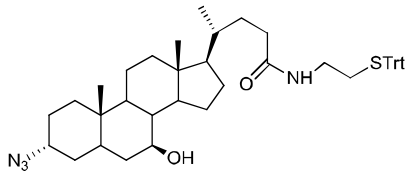
82 (400 mg, 0.96 mmol)), triethylamine (199 μL, 1.44 mmol), ethyl chloroformate (137 μL, 1.44 mmol) and 2-(tritylsulfanyl)ethanamine **91** (460 mg, 1.44 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc 3:1, CH_2Cl_2 5%) gave compound **86** as a white amorphous solid (574.2 mg, yield 83%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.43-7.39 (m, 6 H, H-Ar), 7.31-7.26 (m, 6 H, H-Ar), 7.24-7.19 (m, 3 H, H-Ar), 5.47-5.45 (m, 1 H, N*H*), 3.85 (brs, 1 H, H-7β), 3.20-3.02 (m, 3 H, H-3β, H-25), 2.41 (t, *J* = 6.3 Hz, 2 H, H-26), 0.92 (d, *J* = 2.6 Hz, 3 H, H-21), 0.91 (s, 3 H, H-19), 0.64 (s, 3 H, H-18); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 173.2, 144.6, 129.5, 127.9, 126.8, 68.3, 61.4, 55.8, 50.3, 42.7, 41.8, 39.5, 39.7, 38.0, 35.6, 35.4, 35.1, 34.4, 33.5, 32.8, 32.1, 31.6, 28.2, 26.8, 23.7, 22.9, 20.6, 18.4, 11.8.

***N*-(2-Tritylsulfanylethyl)-3α-azido-5β-cholan-24-amide, 87:**



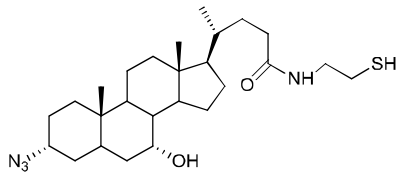
83 (157.2 mg, 0.39 mmol)), triethylamine (81 μL, 0.58 mmol), ethyl chloroformate (56 μL, 0.58 mmol) and 2-(tritylsulfanyl)ethanamine **91** (186.8 mg, 0.58 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc 5:1) gave compound **87** as a white amorphous solid (122.4 mg, yield 43.5%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.43-7.39 (m, 6 H, H-Ar), 7.31-7.26 (m, 6 H, H-Ar), 7.24-7.19 (m, 3 H, H-Ar), 5.46 (bs, 1 H, N*H*), 3.35-3.37 (m, 1 H, H-3β), 3.10 - 3.06 (m, 2 H, H-25), 2.41 (t, *J* = 6.3 Hz, 2 H, H-26), 0.93 (s, 3 H, H-19), 0.90 (d, *J* = 6.5 Hz, 3 H, H-21), 0.63 (s, 3H, H-18); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 173.3, 144.6, 129.5, 127.9, 126.8, 61.3, 56.4, 55.9, 42.7, 42.4, 40.4, 40.1, 38.0, 35.8, 35.5, 35.4, 34.6, 33.5, 32.4, 32.1, 31.6, 28.2, 27.1, 26.7, 26.3, 24.2, 23.4, 20.8, 18.4, 12.1.

***N*-(2-Tritylsulfanylethyl)-3α-azido-7β-hydroxy-5β-cholan-24-amide, 88:**



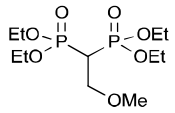
84 (297.5 mg, 0.71 mmol)), triethylamine (148 μL, 1.07 mmol), ethyl chloroformate (102 μL, 1.07 mmol) and 2-(tritylsulfanyl)ethanamine (341 mg, 1.07 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc 1.5:1) gave compound **88** as a white amorphous solid (305.7 mg, yield 60%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.43-7.39 (m, 6 H, H-Ar), 7.31-7.26 (m, 6 H, H-Ar), 7.22 (m, 3 H, H-Ar), 5.51 (bs, 1 H, N*H*), 3.60-3.54 (m, 1 H, H-7α), 3.31-3.23 (m, 1 H, H-3β), 3.07 (m, 2 H,H-25), 2.41 (t, *J* = 6.3 Hz, 2 H, H-26), 0.96 (s, 3 H, H-19), 0.91 (d, *J* = 6.5 Hz, 3 H, H-21), 0.66 (s, 3 H,H-18); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 173.3, 144.6, 129.5, 127.9, 126.8, 71.1, 60.9, 55.6, 54.9, 43.7, 43.7, 42.7, 40.0, 39.1, 38.1, 36.6, 35.3, 35.1, 34.1, 33.5, 33.4, 32.1, 31.7, 28.7, 26.8, 26.6, 23.5, 21.2, 18.5, 12.2.

Synthesis of *N*-(2-Thioethyl)-3α-azido-7α-hydroxy-5β-cholan-24-amide, 89:



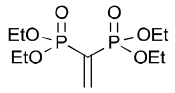
To a solution of compound **86** (100 mg, 0.14 mmol) in CH_2Cl_2 (2 ml), TES (0.42 mmol) and TFA (200 μL) were added and the reaction mixture was stirred at r.t.. After 15 min, the solvent was evaporated under reduced pressure, and the crude residue was washed three times with toluene and after each washing it was evaporated to dryness. The crude produce was subjected to a short plug of silica gel and eluted with CH_2Cl_2 and then with CH_2Cl_2/MeOH (95:5) to obtain compound **89** as a white amorphous solid (60 mg, yield 93%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.85 (bs, 1 H, N*H*), 3.85 (bs, 1 H, H-7β), 3.46 – 3.39 (m, 2 H, H-25), 3.19 – 3.10 (m, 1 H, H-3β), 2.71 – 2.64 (m, 2 H, 2 H-26), 0.94 (d, *J* = 6.5 Hz, 3 H, H-21), 0.91 (s, 3 H, H-19), 0.66 (s, 3 H, H-18); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 173.7, 68.3, 61.3, 55.8, 50.3, 42.7, 42.3, 41.8, 39.5, 39.4, 35.5, 35.4, 35.1, 34.4, 33.5, 32.8, 31.7, 28.2, 26.8, 24.7, 23.7, 22.8, 20.6, 18.4, 11.8.

Synthesis of Tetraethyl (2-methoxyethane-1,1-diyl)bisphosphonate 93:



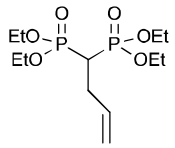
Tetraethyl methylene bisphosphonate **92** (0.86 mL, 3.47 mmol) was added to a stirred solution of paraformaldehyde (521 mg, 17.35 mmol) and diethylamine (0.36 mL, 3.47 mmol) in methanol (6 mL) at room temperature. The mixture was heated to 80 °C and refluxed for two hours. The clear solution was then stirred overnight at 65 °C. The solvent was removed under reduced pressure, toluene (10 mL) was added, and removed again under reduced pressure in order to coevaporate residual paraformaldehyde and methanol. This dissolution and evaporation process was repeated twice. The crude intermediate was purified by flash chromatography (CH_2Cl_2/MeOH 95:5) to obtain compound **93** as a clear viscous oil (1.08 g, yield 97%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.03 (m, 8 H,OC*H2*CH_3); 3.74 (dt, *J* = 5.5 Hz, *J* = 16.2 Hz, 2 H, CH_2); 3.22 (s, 3 H, OCH_3); 2.54 (tt, *J* =5.5 Hz, *J* = 23.8 Hz, 1 H, CH); 1.19 (t, J = 7.1 Hz, 12 H, OCH_2C*H3*).[202]

Synthesis of Tetraethyl ethene-1,1-diylbisphosphonate 94:



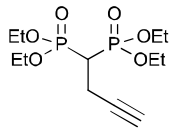
To a stirred solution of tetraethyl (2-methoxyethane-1,1-diyl)bisphosphonate **93** (505 mg, 1.52 mmol) in toluene (10 mL), *p*-toluenesulfonic acid (8.94 mg, 0.05 mmol) was added in the presence of 4Å molecular sieves. The mixture was refluxed over night at 110 °C and concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 (5 ml) and extracted with H_2O (5 ml). The organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* to afford bisphosphonate **94** as a colorless oil (366.8 mg, yield 80%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.99 (dd, *J* = 4.0 Hz, *J* = 71.6 Hz, 2 H, C*H2*), 4.22 – 4.06 (m, 8 H, OC*H2*CH_3), 1.35 (t, *J* = 7.1 Hz, 12 H, OCH_2C*H3*); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 149.3, 131.9 (t, J_{CP} = 166.7 Hz) 62.6, 16.3; ^{31}P NMR (122 MHz, $CDCl_3$): δ = 13.1.

Synthesis of Tetraethyl but-1-ene-4,4-diylbisphosphonate 95:



A solution of tetraethyl methylene bisphosphonate **92** (0.62 mL, 3.47 mmol) in dry toluene (7 mL) was slowly added under N_2 to a stirred suspension of potassium *tert*-butoxide (428 mg, 3.82 mmol) in toluene (7 mL) at 0° C. After stirring for 1 h, allyl iodide (0.32 mL, 3.47 mmol) was added to the reaction mixture and stirred for 3 h at 0 °C. The reaction mixture was quenched with a pH 7 phosphate buffer (30 mL) and extracted with EtOAc (15 mL). The organic layer was then washed with brine and dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography using cyclohexane/acetone (2:1 then 1:1 then 1:2) as eluent to give compound **95** as a colourless oil (455.7 mg, yield 40%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.02-5.90 (m, 1 H, *H*C=CH_2), 5.16-5.01 (m, 2 H, HC=C*H*$_2$), 4.17 (q, *J* = 6.8 Hz, 8H, OC*H*$_2$$CH_3$), 2.68 (tt, *J* = 17.1, 6.6 Hz, 2 H, CH_2), 2.38 (tt, *J* = 23.9, 6.2 Hz, 1 H, CH), 1.30 (t, *J* = 6.8 Hz, 12 H, OCH2C*H3*); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 135.9 (t, J_{CP} = 7.1 Hz), 116.6, 62.5 (dd, J_{CP} = 9.7, 6.9 Hz), 37.05 (t, *J* = 133.3 Hz), 29.8, 16.4, ^{31}P NMR (122 MHz, $CDCl_3$): δ = 23.1.

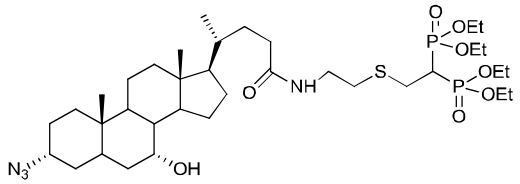
Synthesis of Tetraethyl but-1-yne-4,4-diylbisphosphonate 96:



A stirred solution of tetraethyl ethene-1,1-diylbisphosphonate **94** (347.3 mg, 1.16 mmol) in THF (3.5 mL) was cooled to -15 °C and sodium acetylide (18% in xylene, 0.45 mL, 1.51 mmol) was added dropwise over a period of one hour. The mixture was stirred at room temperature for 18 h and was subsequently quenched with a pH 7 phosphate buffer (15 mL) then the volatiles were evaporated and the residue diluted with EtOAc (15 mL) and extracted with H_2O (10 mL). The aqueous phase was extracted again with EtOAc (15 mL) and the combined organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography using CH_2Cl_2/ EtOAc/MeOH (4.5:4.5:0.5) as eluent to afford compound **96** as a pale yellow oil (151.3 mg, yield 40%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.19 (p, *J* = 7.2 Hz, 8 H, OC*H*$_2$$CH_3$), 2.81 (tdd, *J* = 16.3, 6.1, 2.7 Hz, 2 H, C*H*$_2$-C≡C-H), 2.54 (tt, *J* = 23.4, 6.2 Hz, 1

H, CH), 2.05 (t, J = 2.7 Hz, 1 H, CH_2-C≡C-*H*), 1.33 (t, J = 7.1 Hz, 12 H, OCH_2CH_3); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 81.2 (t, J_{CP} = 11.1 Hz), 62.9, 62.8 (d, J_{CP} = 7.0 Hz), 36.5 (t, J_{CP} = 134.3 Hz), 16.3 (d, J_{CP} = 5.1 Hz), 15.5 (t, J_{CP} = 4.9 Hz); ^{31}P NMR (122 MHz, $CDCl_3$): δ = 21.4.

Synthesis of 97:



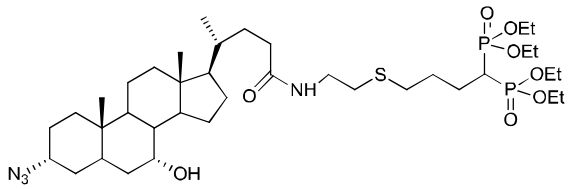
Method A:

To a solution of **89** (110 mg, 0.23 mmol) in DMF (1.5 mL), bisphosphonate **94** (34.6 mg, 0.12 mmol) and 2,2-dimethoxy-2-phenyl-acetophenone (2.5 mg, 0.01 mmol) were added. The resulting mixture was irradiated at r.t. for 1 h under vigorous magnetic stirring, then concentrated and purified by flash chromatography (CH_2Cl_2/acetone 1:2) to obtain compound **97** as a light yellow oil (63.4 mg, yield 68%).

Method B:

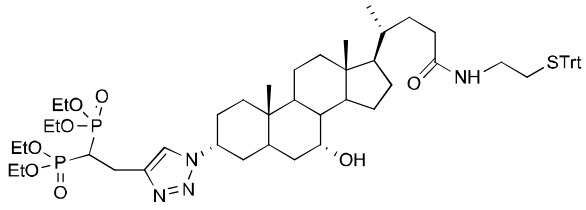
To a solution of **89** (112.3 mg, 0.19 mmol) in CH_2Cl_2 (3 mL), bisphosphonate **94** (37.8 mg, 0.13 mmol) and TEA (26μL, 0.19 mmol) were added. The resulting mixture was stirred at r.t. for 18 h and then concentrated. The crude residue was purified by flash chromatography (CH_2Cl_2/acetone 1:2) to obtain **97** as a light yellow oil (60 mg, yield 62%). ^1H NMR (300 MHz, $CDCl_3$): δ = 6.75 (bs, 1 H, N*H*), 4.26-4.12 (m, 8 H, OCH_2CH_3), 3.83 (bs, 1 H, H-7β), 3.51-3.41 (m, 2 H, H-25), 3.19-2.97 (m, 3 H, H-3β, H-27), 2.74-2.48 (m, 3 H, H-26, H-28), 0.92 (d, J = 6.4 Hz, 3 H, H-21), 0.90 (s, 3 H, H-19), 0.64 (s, 3 H, H-18); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 173.8, 68.2, 62.9 (dd, J_{CP} = 22.1, 6.3 Hz), 61.3, 55.8, 50.3, 42.6, 41.8, 39.5, 39.4, 38.6 (t, J_{CP} = 132.5 Hz), 38.0, 35.5, 35.5, 35.4, 35.1, 34.4, 33.3, 32.8, 32.7, 31.7, 28.2, 27.2, 26.8, 23.7, 22.8, 20.6, 18.3, 16.4, 16.4, 11.8; ^{31}P NMR (122 MHz, $CDCl_3$): δ = 21.5.

Synthesis of 98:



To a solution of **89** (67.5 mg, 0.14 mmol) in DMF (0.7 mL), bisphosphonate **95** (23 mg, 0.07 mmol) and 2,2-dimethoxy-2-phenyl-acetophenone (1.8 mg, 0.007 mmol) were added. The resulting mixture was irradiated at r.t. for 1 h under vigorous magnetic stirring, then concentrated and purified by flash chromatography (CH_2Cl_2/acetone 1:2) to obtain compound **98** as a light yellow oil (32.6 mg, yield 64%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.30 (bs, 1 H, N*H*), 4.24-4.12 (m, 8 H, OC*H*$_2$CH$_3$), 3.85 (bs, 1 H, H-7β), 3.45-3.40 (m, 2 H, H-25), 3.18-3.10 (m, 1 H, H-3β), 2.70-2.65 (m, 2 H, H-26), 2.57-2.50 (m, 2 H, H-27), 0.92 (d, *J* = 6.4 Hz, 3 H, H-21), 0.91 (s, 3 H, H-19), 0.66 (s, 3 H, H-18); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 174.3, 68.5,62.9, 62.8, 62.6 (dd, J_{CP}= 20.8, 5.4 Hz), 61.6, 56.0, 50.6, 42.9, 42.0, 39.8, 39.6, 38.6, 38.5 (t, J_{CP} = 132.7 Hz), 36.6, 35.7, 35.3, 34.6, 33.4, 32.9, 32.2, 31.9, 31.6, 29.9, 28.4, 27.0, 24.8, 23.9, 23.1, 20.8, 18.6, 16.6, 14.3, 12.0; ^{31}P NMR (122 MHz, $CDCl_3$): δ = 23.5; MS (ESI-LCQ): $[MH]^+$ = 805.5, $[M + Na^+]$ = 827.6; $[M + K^+]$ = 843.5.

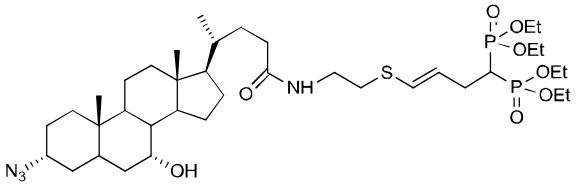
Synthesis of 99:



To a solution of **86** (40 mg, 0.05 mmol) in toluene/DMF (10:1, *v/v*, 0.55 mL), bisphosphonate **96** (27 mg, 0.08 mmol), DIPEA (38 μL, 0.22 mmol) and CuI (5 mg, 0.03 mmol) were sequentially added. The resulting mixture was stirred in the dark for 18 h, then concentrated and purified by flash chromatography (CH_2Cl_2/MeOH 9:1) to obtain compound **99** as a white amorphous solid (33.4 mg, yield 64%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.53 (s, 1 H, H-triazole), 7.44-7.38 (m, 6 H, H-Ar), 7.33-7.27 (m, 6 H, H-Ar), 7.25-7.18 (m, 3 H, H-Ar), 5.47 (bs, 1 H, N*H*), 4.33 (bs, 1 H, H-3β), 4.23-4.03 (m, 8 H, OC*H*$_2$CH$_3$), 3.86 (bs, 1 H, H-7β), 3.32 (td, *J* = 16.1, 6.3 Hz, 2 H, H-27), 3.13-2.81 (m, 3 H, H-25, H-28), 2.41 (t, *J* = 6.2 Hz, 2 H, H-26), 0.98 (s, 3 H, H-19), 0.93 (d, *J* = 6.4 Hz, 3 H, H-21), 0.66 (s, 3 H, H-18); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 173.2, 146.8, 144.6,

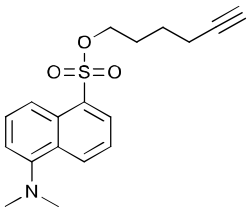
129.5, 127.9, 127.9, 127.2, 126.8, 120.7, 68.2, 66.8, 63.1, 62.8 (dd, J_{CP} = 35.0, 6.4 Hz), 61.5, 55.9, 55.5, 50.3, 42.7, 42.1, 39.4, 38.1, 37.8, 36.9, 36.6 (t, J_{CP} = 130.2 Hz), 35.7, 35.4, 35.2, 35.1, 34.2, 33.5, 33.1, 32.8, 32.1, 31.6, 28.2, 28.1, 23.7, 22.8, 21.9, 20.6, 18.4, 16.3, 11.8; ^{31}P NMR (122 MHz, $CDCl_3$): δ = 22.2.

Synthesis of 100:



To a solution of **89** (50 mg, 0.10 mmol) in DMF (0.5 mL), bisphosphonate **96** (68.4 mg, 0.20 mmol) and 2,2-dimethoxy-2-phenyl-acetophenone (2.6 mg, 0.01 mmol) were added. The resulting mixture was irradiated at r.t. for 1 h under vigorous magnetic stirring, then concentrated and purified by MPLC (cyclohexane/acetone 1:1) to obtain compound **100** as a light yellow oil (43.6 mg, yield 53%) as a mixture of *E* and *Z* isomers. ^{1}H NMR (300 MHz, $CDCl_3$, mixture of *E* and *Z* isomers): δ = 6.52 (bs, 1 H, N*H*), 5.96 (d, *J* = 9.3 Hz, 1 H, H-27), 5.80 (dd, *J* = 16.1, 7.0 Hz, 1 H, H-28), 4.26-4.07 (m, 8 H, OC*H*$_2$CH$_3$), 3.84 (bs, 1 H, H-7β), 3.43-3.36 (m, 2 H, H-25), 3.19-3.05 (m, 1 H, H-3β), 2.9- 2.72 (m, 4 H, H-26, H-29), 0.92 (d, *J* = 6.3 Hz, 3 H, H-21), 0.90 (s, 3 H, H-19), 0.64 (s, 3 H, H-18); ^{13}C NMR (76 MHz, $CDCl_3$, mixture of E and Z isomers): δ = 174.7, 129.0, 126.3, 68.4, 63.0 (dd, J_{CP} = 25.5, 5.7 Hz), 62.9, 61.6, 60.6, 56.0, 50.6, 42.9, 42.0, 39.8, 39.6, 39.5, 38.7 (t, J_{CP} = 133.2 Hz), 35.8, 35.7, 35.3, 34.6, 34.1, 33.0, 32.3, 32.0, 29.9, 29.6, 28.4, 27.0, 25.3, 23.9, 23.1, 21.3, 20.8, 18.6, 16.7, 16.6, 14.4, 12.0; ^{31}P NMR (122 MHz, $CDCl_3$): δ = 22.7.

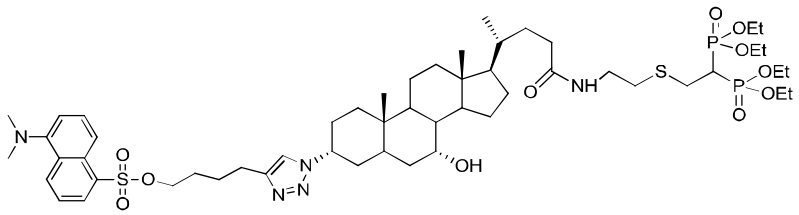
Synthesis of Hex-5-yn-1-yl 5-(dimethylamino)naphthalene-1-sulfonate 103:



To a solution of dansyl chloride (200 mg, 0.74 mmol) in CH_2Cl_2/pyiridine (24:1, *v/v*, 2.5 mL), 5-hexyn-1-ol (98 μL, 0.89 mmol) was addede and the resulting mixture was allowed to stir for 24 h at room temperature. The precipitate was filtered and the resulting clear solution was concentrated and purified by flash chromatography (cyclohexane/EtOAc 8:1) to obtain compound **103** as a fluorescent oil (153.9 mg, yield 62%). ^{1}H NMR (400 MHz,

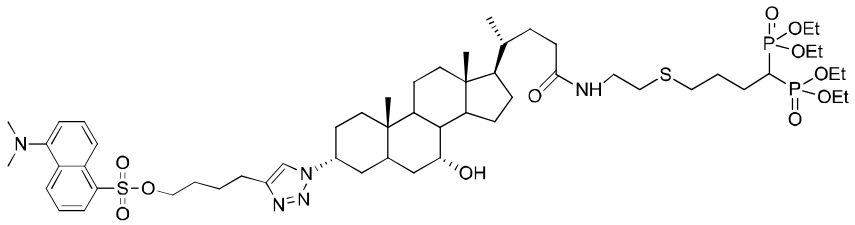
$CDCl_3$): δ = 8.69 (d, *J* = 8.1 Hz, 1 H, H-Ar), 8.34-8.29 (m, 2 H, H-Ar), 7.61-7.53 (m, 2 H, H-Ar), 7.21-7.19 (m, 1 H, H-Ar), 4.02 (t, *J* = 6.2 Hz, 2 H, OCH_2), 2.89 (s, 6 H, CH_3), 2.10-2.04 (m, 2 H, CH_2C≡CH), 1.86 (t, *J* = 2.6 Hz, 1 H, C≡C*H*), 1.75-1.68 (m, 2 H, CH_2), 1.55-1.47 (m, 2 H, CH_2); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 167.8, 150.8, 131.5, 131.3, 130.9, 130.5, 129.6, 123.4, 120.1, 115.9, 83.3, 70.3, 68.9, 45.6, 27.7, 24.2, 17.6.

Synthesis of 104:



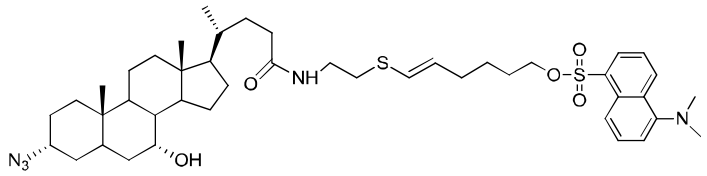
To a solution of **97** (57 mg, 0.07 mmol) in toluene/DMF (10:1, *v/v*, 1.1 mL), dansyl-alkyne **103** (28 mg, 0.08 mmol), DIPEA (49 μL, 0.28 mmol) and CuI (2.6 mg, 0.02 mmol) were sequentially added. The resulting mixture was stirred in the dark for 18 h, then concentrated and purified by MPLC (CH_2Cl_2/acetone 1:1) to obtain compound **104** (47.3 mg, yield 61%). ^{1}H NMR (300 MHz, $CDCl_3$, slightly impure): δ = 8.75-8.65 (m, 1 H, H-Ar), 8.35-8.24 (m, 2 H, H-Ar), 7.6-7.54 (m, 2 H, H-Ar), 7.35-7.18 (m, 2 H, H-Ar, H-triazole), 6.64 (bs, 1 H, N*H*), 4.38-4.25 (m, 1 H, H-3β), 4.26-4.10 (m, 8 H, OCH_2CH_3), 4.02-3.97 (m, 2 H, SO_2OCH_2), 3.88 (bs, 1 H, H-7β), 3.51-3.42 (m, 2 H, H-25), 3.06 (td, *J* = 17.0, 6.4 Hz, 2 H, H-27), 2.95 (s, 6 H, NCH_3), 2.75-2.66 (m, 2 H, H-26), 0.98 (s, 3 H, H-19), 0.92 (d, *J* = 6.2 Hz, 3 H, H-21), 0.66 (s, 3 H, H-18), ^{13}C NMR (101 MHz, $CDCl_3$): δ = 173.9, 149.4, 131.5, 130.6, 128.7, 128.4, 123.2, 118.3, 115.6, 70.7, 68.4, 68.1, 65.3, 63.0 (dd, *J* = 21.2, 6.3 Hz), 62.7, 61.0, 58.2, 56.0, 55.8, 50.4, 50.3, 49.0, 45.5, 42.8, 42.7, 42.2, 41.7, 39.4, 38.7 (t, *J* = 132.6 Hz), 38.1, 37.0, 35.6, 34.5, 34.2, 33.5, 32.9, 31.8, 30.4, 29.8, 28.3, 27.5, 27.2, 25.3, 25.0, 23.7, 23.7, 22.9, 22.6, 21.8, 20.8, 18.4, 16.5, 16.4 11.9; ^{31}P NMR (122 MHz, $CDCl_3$): δ = 21.5.

Synthesis of 105:



To a solution of **98** (30.4 mg, 0.04 mmol) in toluene/DMF (10:1, *v/v*, 0.55 mL), dansyl-alkyne **103** (43.2 mg, 0.05 mmol), DIPEA (28 μL, 0.16 mmol) and CuI (1.5 mg, 0.01 mmol) were sequentially added. The resulting mixture was stirred in the dark for 18 h, then concentrated and purified by MPLC (CH_2Cl_2/MeOH 9:1) to obtain compound **105** as a yellow oil (29.7 mg, yield 75%). ^{1}H NMR (300 MHz, CDCl3): δ = 8.60-8.58 (m, 1 H, H-Ar), 8.28-8.21 (m, 2 H, H-Ar), 7.62-7.49 (m, 2 H, H-Ar), 7.27 (s, 1 H, H-triazole), 7.27-7.18 (m, 1 H, H-Ar), 6.16-6.10 (m, 1 H, N*H*), 4.36-4.26 (m, 1 H, H-3β), 4.23-4.10 (m, 8H, OC*H*$_2$CH$_3$), 4.00-3.97 (m, 2H, SO_2O C*H*$_2$), 3.87 (bs, 1 H, H-7β), 3.44 – 3.39 (m, 2 H,H-25), 2.88 (s, 6 H, NC*H*$_3$), 2.70-2.65 (m, 2 H, H-26), 2.57-2.50 (m, 2 H, H-27), 0.98 (s, 3 H, H-19), 0.93 (d, *J* = 6.4 Hz, 3 H, H-21), 0.66 (s, 3 H, H-18); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 173.8, 146.9, 131.7, 130.7, 128.9, 123.3, 119.6, 118.5, 115.8, 70.8, 68.5, 62.81 (dd, J_{CP}= 14.9, 6.5 Hz), 61.1, 56.1, 50.6, 45.7, 42.9, 42.4, 39.7, 39.6, 38.6 (t, J_{CP} = 131.4 Hz), 38.4, 37.2, 36.6, 36.1, 35.7, 35.5, 34.8, 34.6, 33.7, 33.1, 32.2, 31.9, 31.5, 28.9, 28.5, 25.4, 25.1, 24.8, 23.9, 23.1, 22.8, 20.9, 18.6, 16.7, 16.6, 12.0; ^{31}P NMR (122 MHz, $CDCl_3$): δ = 23.5; MS (ESI-LCQ): [M + Na^+] = 1158.40.

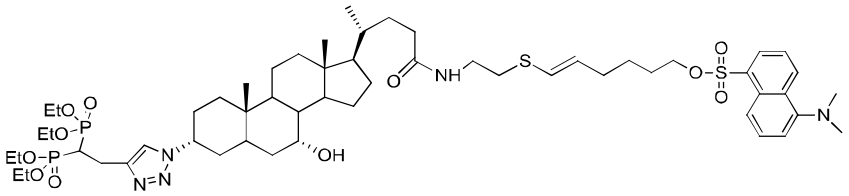
Synthesis of 106:



To a solution of **89** (94 mg, 0.20 mmol) in DMF (0.5 mL), dansyl-alkyne **103** (132.6 mg, 0.40 mmol) and 2,2-dimethoxy-2-phenyl-acetophenone (5.1 mg, 0.02 mmol) were added. The resulting mixture was irradiated at r.t. for 1 h under vigorous magnetic stirring, then concentrated and purified by flash chromatography (CH_2Cl_2 then CH_2Cl_2/MeOH 98:2) to obtain compound **106** as a yellow oil (70.9 mg, yield 43%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.60 (d, *J* = 8.6 Hz, 1 H, H-Ar), 8.30-8.22 (m, 2 H, H-Ar), 7.63-7.51 (m, 2 H, H-Ar), 7.20 (d, *J* = 7.5 Hz, 1 H, H-Ar), 5.93-5.73 (m, 2 H, H-27, N*H*), 5.61-5.36 (m, 1 H, H-28), 4.04-3.95 (m, 2 H, SO_2OC*H*$_2$), 3.84 (bs, 1 H, H-7β), 3.46-3.37 (m, 2 H, H-25), 3.20-3.08

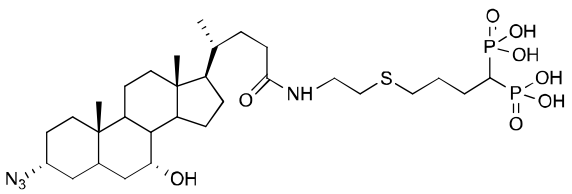
(m, 1 H, H-3β), 2.89 (s, 6 H, NC*H*$_3$), 2.80-2.62 (m, 2 H, H-26), 0.91 (d, *J* = 3.1 Hz, 3 H, H-21), 0.90 (s, 3 H, H-19), 0.63 (s, 3 H, H-18); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 173.6, 131.4, 131.3, 130.4, 129.9, 129.5, 128.6, 124.9, 123.1, 122.3, 119.4, 115.5, 70.6, 68.3, 61.4, 55.7, 50.3, 45.4, 42.3, 42.2, 41.8, 39.5, 39.4, 39.3, 38.4, 35.5, 35.4, 35.1, 34.4, 33.7, 33.5, 33.4, 32.8, 32.2, 31.6, 28.2, 26.8, 24.9, 24.6, 23.7, 22.8, 20.6, 18.4, 11.8.

Synthesis of 107:



To a solution of **106** (13.6 mg, 0.02 mmol) in toluene/DMF (10:1, *v/v*, 275 μL), bisphosphonate **96** (6.7 mg, 0.03 mmol), DIPEA (19 μL, 0.07 mmol) and CuI (0.7 mg, 0.003 mmol) were sequentially added. The resulting mixture was stirred in the dark for 18 h, then concentrated and purified by MPLC (CH_2Cl_2/MeOH 95:5) to obtain a yellow oil (10.6 mg, yield 55%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.62 (d, *J* = 7.9 Hz, 1 H, H-Ar), 8.32-8.21 (m, 2 H, H-Ar), 7.65-7.50 (m, 2 H, H-Ar), 7.25 (s, 1 H, H-triazole), 7.22 (d, *J* = 6.7 Hz, 1 H, H-Ar), 5.94-5.70 (m, 2 H, H-27, N*H*), 5.61-5.37 (m, 1 H, H-28), 4.39-4.26 (m, 1 H, H-3β), 4.24-4.04 (m, 8 H, OC*H*$_2$CH$_3$), 4.04-3.95 (m, 2 H, SO_2OC*H*$_2$), 3.85 (bs, 1 H, H-7β), 3.49-3.23 (m, 4 H, H-25, H-27), 2.90 (s,6 H, NC*H*$_3$), 2.81-2.70 (m, 2 H, H-26), 0.97 (s, 3 H, H-19), 0.92 (d, *J* = 6.2 Hz, 3 H, H-21), 0.66 (s, 3 H, H-18), ^{13}C NMR (101 MHz, $CDCl_3$): δ = 173.7, 144.4, 131.7, 131.3, 130.7, 130.0, 129.5, 128.6, 125.0, 123.8, 122.4, 116.1, 70.8, 68.3, 63.0, 62.6, 61.2, 55.9, 50.4, 45.8, 42.8, 42.2, 39.5, 39.4, 38.0 (t, J_{CP} = 133.4 Hz), 37.1, 35.9, 35.5, 35.3, 34.3, 33.7, 33.5, 32.9, 32.7, 32.3, 31.7, 29.8, 28.3, 28.2, 25.0, 24.6, 23.8, 23.0, 22.2, 20.7, 18.5, 16.4, 11.9; ^{31}P NMR (122 MHz, $CDCl_3$): δ = 22.8; MS (ESI-LCQ): $[M]^+$ = 1134.96, $[M + Na^+]$ = 1157.24.

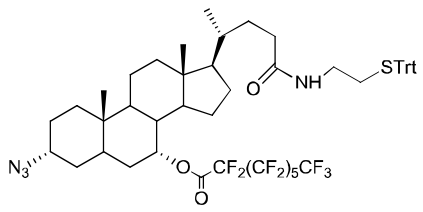
Synthesis of 108:



A solution of **98** (54 mg, 0.07 mmol) in CH_2Cl_2 (0.5 mL) was cooled at 0 °C then 2,6-lutidine (106 μL, 0.91 mmol) and TMSBr (0.7 mmol, 92 μL) were added dropwise. The

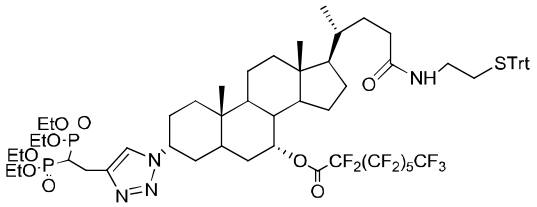
reaction mixture was stirred at room temperature for 24 h. After cooling at 0 °C, MeOH(1 mL) was added and the resulting mixture was allowed to reach room temperature. The solution was then concentrated under reduced pressure. The residue was dissolved in MeOH (1 mL) and subsequently concentrated twice under reduced pressure. The solvent was evaporated and the residue was purified by size exclusion chromatography (Sephadex G20, eluent: MeOH) to obtain compound **108** as a white amorphous solid (25.7 mg, yield 53%). ^{1}H NMR (400 MHz, cd_3od): δ = 3.80 (bs, 1 H, H-7β), 3.37-3.31 (m, 2 H, H-25), 3.22-3.11 (m, 1 H, H-3β), 2.67-2.60 (m, 2 H, H-26), 2.60-2.54 (m, 2 H, H-27), 0.97 (d, *J* = 6.5 Hz, 3 H, H-21), 0.94 (s, 3 H, H-19), 0.69 (s, 3 H, H-18); ^{13}C NMR (101 MHz, cd_3od): δ = 176.8, 68.9, 62.7, 61.5, 57.3, 51.4, 43.6, 43.3, 40.9, 40.7, 40.1 (t, J_{CP} = 132.2 Hz), 36.8, 36.6, 36.6, 36.2, 35.6, 34.1, 34.0, 33.3, 32.3, 31.9, 30.4, 29.2, 27.9, 25.7, 23.3, 21.7, 20.8,18.9, 12.2; ^{31}P NMR (122 MHz, cd_3od): δ = 21.6.

Synthesis of 114:



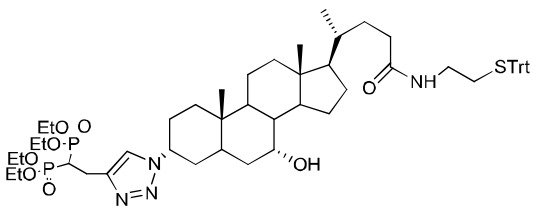
A solution of **86** (50 mg, 0.07 mmol) in CH_2Cl_2 (1 mL) was cooled at 0 °C then pentadecafluorooctanoyl chloride (21 µL, 0.08 mmol), triethylamine (30 µL, 0.21 mmol) and 4-(dimethylamino)pyridine (0.85 mg, 0.007 mmol) were added. The resulting mixture was stirred for 1 h at 0 °C then diluted with CH_2Cl_2 (10 mL) and washed with sat. $NaHCO_3$ (2 × 5 mL), H_2O (2 × 5 mL) and brine. After drying over Na_2SO_4, the solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography (cyclohexane/EtOAc 8:1, acetone 2%) to obtain compound **114** as a white amorphous solid (51 mg, yield 71%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.43-7.38 (m, 6 H, H-Ar), 7.31-7.25 (m, 6 H, H-Ar), 7.24-7.19 (m, 3 H, H-Ar), 5.45 (bs, 1 H, N*H*), 5.19 (s, 1 H, H-7β), 3.18-3.04 (m, 3 H, H-3β, H-25), 2.41 (t, *J* = 6.3 Hz, 2 H, H-26), 0.96 (s, 3 H, H-19), 0.91 (d, *J* = 6.5 Hz, 3 H, H-21), 0.63 (s, 3 H, H-18); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 173.1, 157.8 (t, J_{CF} = 29.4 Hz), 144.6, 129.5, 127.9, 126.8, 77.6, 66.8, 60.5, 55.8, 49.8, 42.7, 41.0, 39.2, 38.1, 38.0, 35.4, 35.3, 34.7, 34.4, 33.8, 33.6, 32.0, 31.5, 31.4, 27.8, 26.9, 26.7, 23.4, 22.7, 20.5, 18.3, 11.7, ^{19}F NMR (376 MHz, $CDCl_3$): δ = -80.7 (t, J_{CF} = 9.9 Hz), -117.8 (t, J_{CF} = 11.9 Hz), -118.1 (t, J_{CF} = 13.6 Hz), -121.5, -122.0, -122.7, -126.1 (d, J_{CF} = 5.9 Hz).

Synthesis of 115:



To a solution of **114** (45 mg, 0.04 mmol) in toluene/DMF (10:1, *v/v*, 0.44 mL), bisphosphonate **96** (20 mg, 0.06 mmol), DIPEA (28 μL, 0.16 mmol) and CuI (4 mg, 0.02 mmol) were sequentially added. The resulting mixture was stirred in the dark for 18 h, then concentrated. The residue was taken up in CH_2Cl_2 (0.2 mL) and applied to a small column containing fluoroFlash Fluorous silica (4g) which was pre eluted with MeOH/H_2O 8:2. The column was eluted with MeOH/H_2O 8:2 until all the non fluorous byproducts were removed. Subsequently the fluorous product was eluted from the column with MeOH 100% to obtain compound **115** as a white amorphous solid (44.9 mg, yield 78%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.49 (s, 1 H, H-triazole), 7.44-7.37 (m, 6 H, H-Ar), 7.31-7.26 (m, 6 H, H-Ar), 7.23-7.19 (m, 3 H, H-Ar), 5.44 (bs, 1 H, N*H*), 5.20 (bs, 1 H, H-7β), 4.48-4.32 (m, 1 H, H-3β), 4.21-4.00 (m, 8 H, OCH_2CH$_3$), 3.37-3.23 (m, 2 H, H-27), 3.11-2.94 (m, 3 H, H-25, H-28), 2.41 (t, *J* = 6.3 Hz, 2 H, H-26), 1.03 (s, 3 H, H-19), 0.91 (d, *J* = 6.5 Hz, 3 H, H-21), 0.65 (s, 3 H, H-18); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 173.0, 157.5 (t, *J* = 23.6 Hz), 144.6, 129.5, 127.9, 126.8, 119.9, 62.8, 62.5, 60.8, 55.9, 49.7, 42.7, 41.4, 39.1, 38.1, 38.0, 36.6 (t, J_{CP} = 130.2 Hz), 35.8, 35.6, 35.4, 34.9, 34.0, 33.7, 32.0, 31.4, 31.3, 29.7, 28.4, 27.8, 23.4, 22.7, 21.9, 20.5, 18.3, 16.2, 11.7; ^{31}P NMR (122 MHz, $CDCl_3$): δ = 22.3; ^{19}F NMR (376 MHz, $CDCl_3$): δ = -80.66 (t, *J* = 9.9 Hz), -118.13 (t, *J* = 12.6 Hz), -121.39, -121.94, -122.11 – -122.37 (m), -122.67, -126.01.

Detagging procedure:



To a solution of **115** (19 mg, 0.013 mmol) in EtOH/H_2O (8:1, *v/v*, 0.45 mL), 0.5 M NaOH (52 μL, 0.026 mmol) was added and the resulting mixture was allowed to stir at room temperature for 1 hour and then concentrated under reduced pressure. The crude material was loaded into a fluoroFlash Fluorous silica cartridge (4g) using CH_2Cl_2 (0.2 mL). The

column was pre eluted with MeOH/H_2O 8:2 and then it was eluted with MeOH/H_2O 8:2 to provide the non-fluorous desired product **99** as a white amorphous solid (13 mg, yield 92%). Subsequently the column was eluted with MeOH 100% to remove the fluorous residues.

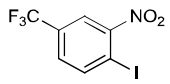
5.2 Experimental part project 3:

Commercially available reagents were purchased from Sigma-Aldrich, Fisher, Acros Organic, Merck and VWR Prolabo. All of these chemicals were the highest grade and used without further purification. TLCs were performed on Merck silica gel glass plates (60 F254). Visualisation was accomplished by irradiation with a UV lamp and/or staining with a ceric ammonium molybdate or $KMnO_4$ solution. Flash column chromatography was performed on silica gel 60 (230–400 mesh). NMR data were recorded on Bruker ADVANCE III for ^{1}H at 400 MHz, for ^{13}C at 101 MHz and for ^{19}F at 376 MHz.

General procedure for Sandmeyer reaction:

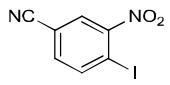
A suspension of 2-nitroaniline (3.29 mmol) in c.HCl (7 mL) was heated at 100°C for 5 min. Then the solution was cooled to 0°C and a solution of $NaNO_2$ (3.95 mmol) in 1 mL H_2O was added dropwise. The reaction mixture was stirred at 0°C. After stirring for 1 h, a solution of KI (4.94 mmol) in 1 mL H_2O was added dropwise at 0°C and the resulting mixture was stirred at 23°C for an additional hour. H_2O (20 mL) was added and the crude product was extracted with EtOAc (2 × 15 mL). The combined organic layers were washed with 2N HCl, sat. $NaHCO_3$, sat. Na_2SO_3 and brine. After drying over Na_2SO_4, the solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography (silica gel).

1-Iodo-2-nitro-4-(trifluoromethyl)benzene 124 a:



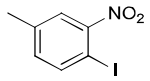
2-Nitro-4-(trifluoromethyl)aniline **123 a** (700 mg, 3.40 mmol), $NaNO_2$ (281.5 mg, 4.08 mmol) and KI (846.6 mg, 5.10 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 9:1) gave compound **124 a** as an orange solid (803.6 mg, yield 74%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.24 (d, J = 8.3 Hz, 1 H), 8.13 (d, J = 1.7 Hz, 1 H), 7.54 (dd, J = 8.3, 2.0 Hz, 1 H);[238] ^{19}F NMR (376 MHz, $CDCl_3$): δ = -63.22.

4-Iodo-3-nitrobenzonitrile 124 b:



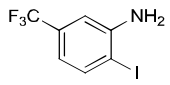
To solution of 4-Amino-3-nitrobenzonitrile **123 b** (250 mg, 1.53 mmol) in DMSO (1.5 mL), c.HCl (1 mL) was added and the resulting mixture was heated at 100°C for 5 min. Then the solution was cooled to 0°C and a solution of $NaNO_2$ (126.7 mg, 1.84 mmol) in 0.5 mL H_2O was added dropwise. The reaction mixture was stirred at 0°C. After stirring for 1 h, a solution of KI (381 mg, 2.30 mmol) in 0.5 mL H_2O was added dropwise at 0°C and the resulting mixture was stirred at 23°C for an additional hour. H_2O (20 mL) was added and the crude product was extracted with EtOAc (2 × 15 mL). The combined organic layers were washed with 2N HCl, sat. $NaHCO_3$, sat. Na_2SO_3 and brine. The crude 4-iodo-3-nitrobenzonitrile **124 b** was used in the next step without further purifications (297 mg, yield 73%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.25 (d, *J* = 8.2 Hz, 1 H), 8.14 (d, *J* = 1.8 Hz, 1 H), 7.54 (dd, *J* = 8.2, 1.9 Hz, 1 H).[239]

1-Iodo-4-methyl-2-nitrobenzene 134:



4-Methyl-2-nitroaniline **133** (500 mg, 3.29 mmol), $NaNO_2$ (273 mg, 3.95 mmol) and KI (820 mg, 4.94 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 15:1) gave compound **134** as an orange solid (726.9 mg, yield 84%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.91 (d, *J* = 8.1 Hz, 1 H), 7.70 (d, *J* = 1.4 Hz, 1 H), 7.11 (dd, *J* = 8.1, 1.5 Hz, 1 H), 2.42 (s, 3 H).[238]

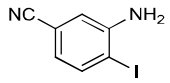
Synthesis of 2-iodo-5-(trifluoromethyl)aniline 125 a:



To a solution of **124 a** (803.6 mg, 2.54 mmol) in EtOH-AcOH (1:1, v/v, 16 mL), Fe (709 mg, 12.7 mmol) was added and the resulting mixture was stirred at 23°C for 18h. The crude reaction mixture was neutralised with sat. $NaHCO_3$ (50 mL) and extracted with EtOAc (3 × 30 mL). The combined organic extracts were dried over Na_2SO_4 and evaporated *in vacuo*. The crude material **125 a** was used in the next step without purification. (734.4 mg, quantitative yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.76 (dd, *J* =

8.2, 0.7 Hz, 1 H), 6.96 (d, *J* = 1.7 Hz, 1 H), 6.72 (ddd, *J* = 8.2, 2.0, 0.6 Hz, 1 H), 4.32 (bs, 2 H).[238]

Synthesis of 3-amino-4-iodobenzonitrile 125 b:

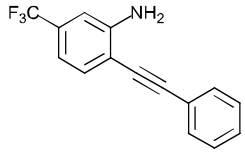


To a solution of **124 b** (290.8 mg, 1.06 mmol) in EtOH-AcOH (1:1, v/v, 10 mL), Fe (296.3 mg, 5.3 mmol) was added and the resulting mixture was stirred at 23°C for 18h. The crude reaction mixture was neutralised with sat. $NaHCO_3$ (50 mL) and extracted with EtOAc (3 × 30 mL). The combined organic extracts were dried over Na_2SO_4 and evaporated *in vacuo.* The crude material **125 b** was used in the next step without purification. (260 mg, quantitative yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.76 (d, *J* = 8.1 Hz, 1 H), 6.96 (d, *J* = 1.6 Hz, 1 H), 6.73 (dd, *J* = 8.1, 1.6 Hz, 1 H), 4.36 b(s, 2 H).

General procedure for Sonogashira reaction:

To a solution of 2-iodoaniline (0.7 mmol) in dry THF (10 mL) under N_2 atmosphere, CuI (0.04 mmol), $PdCl_2(PPh_3)_2$ (0.04 mmol), alkyne (0.84 mmol) and triethylamine (2.5 mmol) were added and the resulting mixture was heated at 70°C and monitored by TLC-analysis. After all starting material has been consumed the reaction mixture was cooled to 23°C and diluted with water (15 mL) and extracted with EtOAc (2 × 15 mL). The combined organic phases were washed with brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure to obtain a dark solid which was purified by flash chromatography (silica gel) to give the desired product.

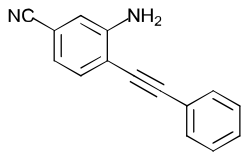
2-(Phenylethynyl)-5-(trifluoromethyl)aniline 126 a:



Compound **125 a** (250 mg, 0.87 mmol), CuI (8.28 mg, 0.04 mmol), $PdCl_2(PPh_3)_2$ (30.5 mg, 0.04 mmol), phenylacetylene (115 µL, 1.04 mmol) and triethylamine (0.6 mL, 4.35 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 10:1) gave compound **126 a** as a pale yellow solid (198 mg, yield 87%). ^{1}H NMR (400 MHz, $CDCl_3$):

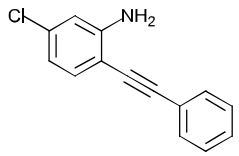
δ = 7.56 (m, 2H), 7.47 (dd, *J* = 8.5, 0.8 Hz, 1H), 7.42 – 7.38 (m, 3H), 6.98-6.96 (m, 2H), 4.48 (bs, 2H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 147.8, 132.5, 131.6 (2C), 131.5 (d, J_{CF} = 32.2 Hz), 128.7, 128.5 (2C), 124.0 (q, J_{CF} = 272.3 Hz), 122.6, 114.3 (q, J_{CF} = 3.8 Hz), 111.2, 110.7 (q, J_{CF} = 4.0 Hz), 96.5, 84.6; ^{19}F NMR (376 MHz, $CDCl_3$): δ = -63.13.

3-Amino-4-(phenylethynyl)benzonitrile 126 b:



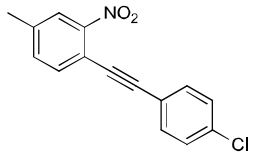
Compound **125 b** (260 mg, 1.06 mmol) CuI (10 mg, 0.05 mmol), $PdCl_2(PPh_3)_2$ (35 mg, 0.05 mmol), phenylacetylene (140 µL, 1.28 mmol) and triethylamine (0.7 mL, 5.3 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 5:1) gave compound **126 b** as a yellow solid (222.3 mg, yield 95%). ^{1}H NMR (400 MHz, $CDCl_3$): = δ 7.58 – 7.54 (m, 2 H), 7.46 – 7.39 (m, 4 H), 7.01 – 6.98 (m, 2 H), 4.51 (bs, 2 H);[240] ^{13}C NMR (101 MHz, $CDCl_3$): δ = 147.8, 132.7, 131.6 (2C), 129.0, 128.6 (2C), 122.3, 121.0, 118.9, 116.9, 112.5, 112.4, 98.1, 84.3.

5-Chloro-2-(phenylethynyl)aniline 130:



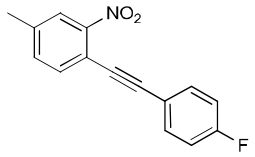
5-Chloro-2-iodoaniline **129** (50 mg, 0.2 mmol), CuI (1.9 mg, 0.01 mmol), $PdCl_2(PPh_3)_2$ (7.01 mg, 0.01 mmol), phenylacetylene (26 µL, 0.24 mmol) and triethylamine (138 µL , 1 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 10:1) gave compound **130** as a light brown solid (41.7 mg, yield 90%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.54 (dd, *J* = 6.5, 3.2 Hz, 2 H), 7.40 – 7.35 (m, 3 H), 7.30 (d, *J* = 8.2 Hz, 1 H), 6.75 (d, *J* = 2.0 Hz, 1 H), 6.71 (dd, *J* = 8.2, 2.0 Hz, 1 H), 4.36 (bs, 2 H).[238]

1-[(4-Chlorophenyl)ethynyl]-3-methyl-2-nitrobenzene 135 a:



To a solution of 1-chloro-4-iodobenzene (136 mg, 0.57 mmol) in dry THF (5 mL) under N_2 atmosphere, CuI (1.9 mg, 0.03 mmol), $PdCl_2(PPh_3)_2$ (20 mg, 0.03 mmol), (trimethylsilyl)acetylene (242 µL, 1.71 mmol) and triethylamine (0.4 mL, 2.85 mmol) were added and the resulting mixture was heated at 70°C for 1h. After 1 h, solvent was removed under reduced pressure. Crude material was filtrated through a short silica plug and dissolved in a solution of KOH (63.8 mg, 1.14 mmol) in MeOH (5 mL). The resulting mixture was stirred at 23°C for 1h. After 1h, this mixture was added to a solution of 1-iodo-4-methyl-2-nitrobenzene **134** (100 mg, 0.38 mmol), CuI (3.6 mg, 0.02 mmol), $PdCl_2(PPh_3)_2$ (13.3 mg, 0.02 mmol) in dry THF (5 mL). Then triethylamine (263 µL, 0.57 mmol) was added and stirring was continued at 70°C for 2h. After that, the reaction mixture was cooled to 23°C and diluted with water (15 mL) and extracted with EtOAc (2 × 15 mL). The combined organic phases were washed with brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure to obtain a dark solid which was purified by flash chromatography (Hexane/CH_2Cl_2, 3:1) to give compound **135 a** (80.4 mg, yield 76%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.93 (s, 1 H), 7.61 (d, *J* = 7.9 Hz, 1 H), 7.54 (d, *J* = 8.6 Hz, 2 H), 7.44 (d, *J* = 7.9 Hz, 1 H), 7.37 (d, *J* = 8.6 Hz, 2 H), 2.49 (s, 3 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 149.4, 139.8, 135.1, 134.3, 133.8, 133.1 (2C), 128.8 (2C), 125.1, 121.1, 115.5, 94.9, 85.9, 21.3.

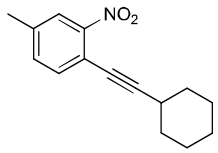
1-[(4-Fluorophenyl)ethynyl]-4-methyl-2-nitrobenzene 135 b:



1-Iodo-4-methyl-2-nitrobenzene **134** (200 mg, 0.76 mmol), CuI (7.2 mg, 0.04 mmol), $PdCl_2(PPh_3)_2$ (26.6 mg, 0.04 mmol), 4-fluorophenylacetylene (174 µL, 1.52 mmol) and triethylamine (0.52 mL , 3.8 mmol) were employed. Purification by flash chromatography (Hexane/CH_2Cl_2, 3:1) gave compound **135 b** as a pale yellow solid (165.2 mg, yield 96%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.92 (s, 1 H), 7.63 – 7.56 (m, 3 H), 7.43 (d, *J* = 7.9 Hz, 1 H), 7.13 – 7.06 (m, 2 H), 2.49 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 163.0 (d, J_{CF} =

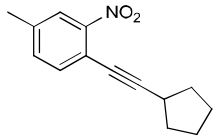
250.7 Hz), 149.5, 139.6, 134.2, 133.9 (d, J_{CF} = 8.6 Hz) (2C), 133.7, 125.1, 118.7 (d, J_{CF} = 3.7 Hz), 115.8 (d, J_{CF} = 22.2 Hz) (2C), 95.1, 84.7, 84.6, 21.2; ^{19}F NMR (376 MHz, $CDCl_3$): δ = -109.55 (tt, *J* = 8.7, 5.4 Hz).

1-(Cyclohexylmethyl)-4-methyl-2-nitrobenzene 135 c:



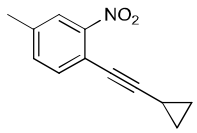
1-Iodo-4-methyl-2-nitrobenzene **134** (250 mg, 0.95 mmol, 1 eq), CuI (18 mg, 0.01 mmol, 0.1 eq), $PdCl_2(PPh_3)_2$ (33 mg, 0.05 mmol, 0.05 eq), cyclohexylacetylene (250 µL, 1.9 mmol, 2 eq) and triethylamine (1.3 mL , 9.5 mmol, 10 eq) in dry DMF (7 mL) at 50°C were employed. Purification by flash chromatography (Hexane/EtOAc, 15:1) gave compound **135 c** as a light brown oil (219 mg, yield 94%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.79 (d, *J* = 0.7 Hz, 1 H), 7.46 (d, *J* = 7.9 Hz, 1 H), 7.33 (ddd, *J* = 7.9, 1.7, 0.7 Hz, 1 H), 2.73 – 2.64 (m, 1 H), 2.43 (s, 3 H), 1.94 – 1.84 (m, 2 H), 1.84 – 1.74 (m, 2 H), 1.66 – 1.52 (m, 3 H), 1.47 – 1.34 (m, 3 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 150.0, 138.6, 134.4, 133.3, 124.7, 116.5, 102.1, 76.0, 32.2 (2C), 29.9, 25.9 (2C), 24.7, 21.1.

1-(Cyclopentylmethyl)-4-methyl-2-nitrobenzene 135 d:



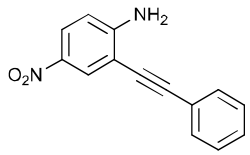
1-Iodo-4-methyl-2-nitrobenzene **134** (200 mg, 0.76 mmol), CuI (7.2 mg, 0.04 mmol), $PdCl_2(PPh_3)_2$ (26.6 mg, 0.04 mmol), cyclopentylacetylene (264 µL, 2.3 mmol, 3 eq) and triethylamine (0.52 mL , 3.8 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 20:1) gave compound **135 d** as a light brown oil (144 mg, yield 83%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.79 (s, 1 H), 7.46 (d, *J* = 7.9 Hz, 1 H), 7.33 (dd, *J* = 7.9, 1.0 Hz, 1 H), 2.95 – 2.86 (m, 1 H), 2.44 (s, 3 H), 2.06 – 1.96 (m, 2 H), 1.87 – 1.72 (m, 4 H), 1.71 – 1.59 (m, 2 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 149.9, 138.5, 134.3, 133.4, 124.6, 116.5, 102.3, 75.5, 33.6 (2C), 31.0, 25.1 (2C), 21.1.

1-(Cyclopropylethynyl)-4-methyl-2-nitrobenzene 135 e:



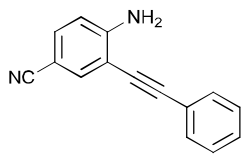
1-Iodo-4-methyl-2-nitrobenzene **134** (300 mg, 1.14 mmol, 1 eq), CuI (22 mg, 0.12 mmol, 0.1 eq), $PdCl_2(PPh_3)_2$ (40 mg, 0.06 mmol, 0.05 eq), cyclopropylacetylene (290 µL, 3.42 mmol, 3 eq) and triethylamine (1.6 mL , 11.4 mmol, 10 eq) in dry DMF (10 mL) at 50°C were employed. Purification by flash chromatography (Hexane/EtOAc, 17:1) gave compound **135 e** as a light brown oil (166.8 mg, yield 73%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.80 (d, *J* = 0.6 Hz, 1 H), 7.45 (d, *J* = 7.9 Hz, 1 H), 7.33 (ddd, *J* = 7.9, 1.7, 0.6 Hz, 1 H), 2.43 (s, 3 H), 1.58 – 1.48 (m, 1 H), 0.99 – 0.87 (m, 4 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 149.8, 138.5, 134.4, 133.4, 124.7, 116.4, 101.5, 71.2, 21.1, 9.1 (2C), 0.6.

4-Nitro-2-(phenylethynyl)aniline 146 a:



2-Iodo-4-nitroaniline **145 a** (200 mg, 0.75 mmol), CuI (7.14 mg, 0.04 mmol), $PdCl_2(PPh_3)_2$ (26.7 mg, 0.04 mmol), phenylacetylene (100 µL, 0.91 mmol) and triethylamine (0.5 mL, 3.75 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 4:1) gave compound **146 a** as a pale yellow solid (168.3 mg, yield 95%). ^{1}H NMR (400 MHz, $CDCl_3$):δ = 8.33 (d, *J* = 2.6 Hz, 1 H), 8.07 (dd, *J* = 9.0, 2.6 Hz, 1 H), 7.59 – 7.53 (m, 2 H), 7.45 – 7.38 (m, 3 H), 6.73 (d, *J* = 9.0 Hz, 1 H), 5.02 (bs, 2 H).[241]

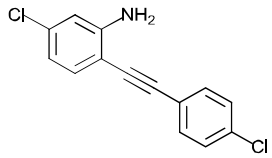
4-Cyano-2-(phenylethynyl)aniline 146 b:



4-Amino-3-iodobenzonitrile **145 b** (200 mg, 0.82 mmol) CuI (7.8 mg, 0.04 mmol), $PdCl_2(PPh_3)_2$ (28.8 mg, 0.04 mmol), phenylacetylene (108 µL, 0.98 mmol) and triethylamine (0.56 mL, 4.10 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 5:1) gave compound **146 b** as a brown oil (157.9 mg, yield 88%). ^{1}H

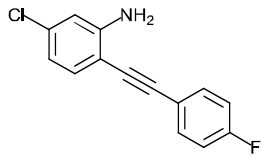
NMR (400 MHz, $CDCl_3$): δ = 7.66 (d, *J* = 1.9 Hz, 1 H), 7.57 – 7.53 (m, 2 H), 7.42 – 7.38 (m, 4 H), 6.74 (d, *J* = 8.5 Hz, 1 H), 4.81 (bs, 2 H).[241]

5-Chloro-2-[(4-chlorophenyl)ethynyl]aniline 158:



To a solution of 1-chloro-4-iodobenzene (283 mg, 1.19 mmol) in dry THF (10 mL) under N_2 atmosphere, CuI (11.3 mg, 0.06 mmol), $PdCl_2(PPh_3)_2$ (41 mg, 0.06 mmol), (trimethylsilyl)acetylene (502 µL, 3.56 mmol) and triethylamine (0.8 mL, 5.93 mmol) were added and the resulting mixture was heated at 70°C for 1h. After 1 h, solvent was removed under reduced pressure. Crude material was filtrated through a short silica plug and dissolved in a solution of KOH (133 mg, 2.37 mmol) in MeOH (7 mL). The resulting mixture was stirred at 23°C for 1h. After 1h, this mixture was added to a solution of 5-chloro-2-iodoaniline **129** (200 mg, 0.79 mmol), CuI (7.5 mg, 0.04 mmol), $PdCl_2(PPh_3)_2$ (28 mg, 0.04 mmol) in dry THF (10 mL). Then triethylamine (0.5 mL, 3.95 mmol) was added and stirring was continued at 70°C for 2h. After that, the reaction mixture was cooled to 23°C and diluted with water (15 mL) and extracted with EtOAc (2 × 15 mL). The combined organic phases were washed with brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure to obtain a dark solid which was purified by flash chromatography (Hexane/EtOAc, 5:1) to give compound **158** as a light brown solid (165.6 mg, yield 80%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.48 – 7.44 (m, 2 H), 7.37 – 7.33 (m, 2 H), 7.28 (d, *J* = 8.2 Hz, 1 H), 6.75 (d, *J* = 1.9 Hz, 1 H), 6.71 (dd, *J* = 8.2, 2.0 Hz, 1 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 148.7, 135.6, 134.5, 133.2, 132.7 (2C), 128.8 (2C), 121.5, 118.3, 114.2, 106.1, 94.3, 86.0.

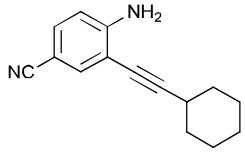
5-Chloro-2-[(4-fluorophenyl)ethynyl]aniline 161:



5-Chloro-2-iodoaniline **129** (200 mg, 0.79 mmol), CuI (7.5 mg, 0.04 mmol), $PdCl_2(PPh_3)_2$ (27.7 mg, 0.04 mmol), 4-fluorophenylacetylene (180 µL, 1.58 mmol) and triethylamine (0.5 mL , 4 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 8:1) gave compound **161** as an orange solid (167 mg, yield 86%). ^{1}H NMR (400 MHz,

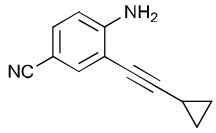
$CDCl_3$): δ = 7.54 – 7.49 (m, 2 H), 7.28 (d, *J* = 8.2 Hz, 1 H), 7.11 – 7.04 (m, 2 H), 6.75 (d, *J* = 1.9 Hz, 1 H), 6.71 (dd, *J* = 8.2, 2.0 Hz, 1 H), 4.35 (bs, 2 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ =162.6 (d, J_{CF} = 250.1 Hz), 161.3, 148.6, 135.4, 133.4 (d, J_{CF} = 8.2 Hz), 133.1, 119.1, 119.0, 118.2, 115.76 (d, J_{CF} = 22.2 Hz), 114.1, 106.3, 94.3, 84.6; -110.6 (dq, *J* = 8.6, 5.4 Hz); ^{19}F NMR (376 MHz, $CDCl_3$) δ = -110.53 – -110.61 (m).

4-Amino-3-(cyclohexylethynyl)benzonitrile 164 a:



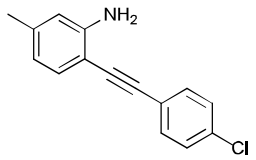
4-Amino-3-iodobenzonitrile **145 b** (100 mg, 0.41 mmol), CuI (4 mg, 0.02 mmol, 0.1 eq), $PdCl_2(PPh_3)_2$ (14.3 mg, 0.0 mmol), cyclohexylacetylene (107 µL, 0.82 mmol) and triethylamine (284 µL , 9.5 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 4:1) gave compound **164 a** as a pale yellow solid (86.3 mg, yield 93%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.53 (d, *J* = 1.9 Hz, 1 H), 7.32 (dd, *J* = 8.4, 1.9 Hz, 1 H), 6.68 (d, *J* = 8.5 Hz, 1 H), 4.69 (s, 2 H), 2.71 – 2.63 (m, 1 H), 1.96 – 1.87 (m, 2 H), 1.82 – 1.72 (m, 2 H), 1.62 – 1.49 (m, 3 H), 1.44 – 1.34 (m, 3 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 151.0, 136.1, 132.5, 119.6, 113.6, 109.2, 101.9, 99.8, 74.8, 32.7 (2C), 29.8, 25.8 (2C), 24.9.

4-Amino-3-(cyclopropylethynyl)benzonitrile 164 b:



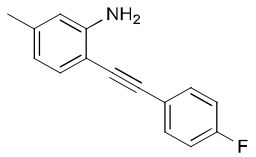
4-Amino-3-iodobenzonitrile **145 b** (100 mg, 0.41 mmol) CuI (4 mg, 0.02 mmol), $PdCl_2(PPh_3)_2$ (14.4 mg, 0.02 mmol), cyclopropylacetylene (104 µL, 1.23 mmol) and triethylamine (0.3 mL, 2.05 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 5:1) gave compound **164 b** (46.6 mg, yield 61%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.48 (d, *J* = 1.9 Hz, 1 H), 7.30 (dd, *J* = 8.5, 2.0 Hz, 1 H) , 6.66 (d, *J* = 8.5 Hz, 1 H), 4.72 (bs, 2 H), 1.55 – 1.46 (m, 1 H), 0.97 – 0.91 (m, 2 H), 0.86 – 0.81 (m, 2 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 151.3, 136.3, 132.5, 119.6, 113.7, 108.9, 100.8, 99.8, 69.9, 9.0 (2C), 0.2.

Synthesis of 2-[(4-chlorophenyl)ethynyl]-5-methylaniline 136 a:



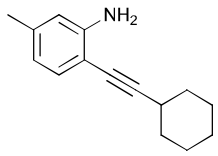
To a solution of compound **135 a** (80 mg, 0.29 mmol) in EtOH-AcOH (1:1, v/v, 6 mL) was added Zn (92.6 mg, 1.45 mmol) and the resulting reaction mixture was stirred at 23°C for 2h. The crude reaction mixture was neutralised with sat. $NaHCO_3$ (15 mL) and extracted with EtOAc (3 × 20 mL). The combined organic layers were dried over Na_2SO_4 and evaporated *in vacuo*. Purification by flash chromatography (hexane/CH_2Cl_2, 2:1) gave compound **136 a** as a yellow solid (56.6 mg, yield 79%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.49 – 7.44 (m, 2 H), 7.36 – 7.32 (m, 2 H), 7.28 (d, *J* = 8.0 Hz, 1 H), 6.60 – 6.56 (m, 2 H), 4.23 (bs, 2 H), 2.31 (s, 3 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 147.8, 140.4, 134.0, 132.6 (2C), 132.0, 128.7 (2C), 122.1, 119.3, 115.1, 104.8, 93.0, 87.2, 21.7.

Synthesis of 2-[(4-fluorophenyl)ethynyl]-5-methylaniline 136 b:



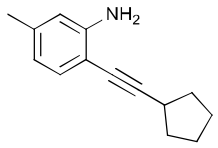
Compound **135 b** (162.1 mg, 0.64 mmol) in EtOH-AcOH (1:1, v/v, 10 mL) was added Zn (203 mg, 3.18 mmol) and the resulting reaction mixture was stirred at 23°C for 18h. The crude reaction mixture was neutralised with sat. $NaHCO_3$ (15 mL) and extracted with EtOAc (3 × 20 mL). The combined organic layers were dried over Na_2SO_4 and evaporated *in vacuo*. Purification by flash chromatography (Hexane/EtOAc, 8:1) gave compound **136 b** as a white solid (74.8 mg, yield 52%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.55 – 7.48 (m, 2 H), 7.28 – 7.26 (m, 1 H), 7.10 – 7.03 (m, 2 H), 6.58 (s, 1 H), 6.57 (d, *J* = 6.8 Hz, 1 H), 4.22 (bs, 2 H), 2.31 (s, 3 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ =162.4 (d, J_{CF} = 249.5 Hz), 147.8, 140.2, 133.3 (d, J_{CF} = 8.2 Hz) (2C) , 132.0, 119.67 (d, J_{CF} = 3.3 Hz), 119.2, 115.7 (d, J_{CF} = 22.2 Hz) (2C), 115.1, 105.0, 93.0, 85.8, 21.7; ^{19}F NMR (376 MHz, $CDCl_3$): δ -111.33 (tt, *J* = 8.7, 5.3 Hz).

Synthesis of 2-cyclohexyl-5-methylaniline 136 c:



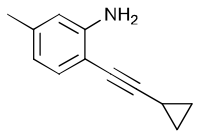
To a solution of compound **135 c** (125 mg, 0.52 mmol) in EtOH-AcOH (1:1, v/v, 4 mL) was added Zn (166 mg, 2.6 mmol) and the resulting reaction mixture was stirred at 23°C for 18h. The crude reaction mixture was neutralised with sat. $NaHCO_3$ (15 mL) and extracted with EtOAc (3 × 20 mL). The combined organic layers were dried over Na_2SO_4 and evaporated *in vacuo*. Purification by flash chromatography (hexane/CH_2Cl_2, 2:1) gave compound **136 c** (72.2 mg, yield 64%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.15 (d, *J* = 7.7 Hz, 1 H), 6.55 – 6.48 (m, 2 H), 4.11 (bs, 2 H), 2.70 – 2.62 (m, 1 H), 2.26 (s, 3 H), 1.94-1.90 (m, 2 H), 1.81-1.75 (m, 2 H), 1.62 – 1.51 (m, 3 H), 1.43-1.35 (m, 3 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 147.5, 138.9, 131.8, 118.9, 114.8, 106.2, 99.2, 77.0, 33.1 (2C), 29.9, 25.9 (2C), 24.9, 21.5.

Synthesis of 2-cyclopentyl-5-methylaniline 136 d:



To a solution of compound **135 d** (93 mg, 0.4 mmol) in EtOH (8 mL) were added $SnCl_2$ (380 mg, 2 mmol) and c.HCl (133 µL, 1.6 mmol). The resulting reaction mixture was heated at 50°C for 3h. The crude reaction mixture was cooled at 23°C and neutralised with sat. $NaHCO_3$ (15 mL) and extracted with EtOAc (3 × 20 mL). The combined organic layers were dried over Na_2SO_4 and evaporated in vacuo. Compound **136 d** was used in the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.14 (d, *J* = 7.7 Hz, 1 H), 6.54 – 6.47 (m, 2 H), 4.10 (bs, 2 H), 2.96 – 2.85 (m, 1 H), 2.26 (s, 3 H), 2.08 – 1.98 (m, 4 H), 1.82 – 1.58 (m, 6 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 147.4, 138.9, 131.7, 119.0, 114.8, 106.3, 99.3, 76.5, 34.3 (2C), 31.1, 25.0 (2C), 21.5.

Synthesis of 2-(cyclopropylethynyl)-5-methylaniline 136 e:

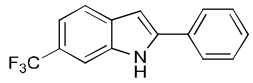


To a solution of compound **135 e** (166 mg, 0.82 mmol) in EtOH-AcOH (1:1, v/v, 10 mL), Fe (229 mg, 4.1 mmol) was added and the resulting mixture was stirred at 23°C for 18h. The crude reaction mixture was neutralised with sat. $NaHCO_3$ (50 mL) and extracted with EtOAc (3 × 30 mL). The combined organic extracts were dried over Na_2SO_4 and evaporated *in vacuo.* Purification by flash chromatography (Hexane/EtOAc, 10:1) gave compound **136 e** as a yellow oil (42.5 mg, yield 31 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.13 (d, *J* = 7.7 Hz, 1 H), 6.52 (s, 1 H), 6.49 (d, *J* = 7.8 Hz, 1 H), 4.11 (bs, 2 H), 2.26 (s, 3 H), 1.55 – 1.48 (m, 1 H), 0.93 – 0.86 (m, 2 H), 0.85 – 0.79 (m, 2 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 147.8, 139.0, 131.9, 118.9, 114.8, 105.9, 98.1, 72.2, 21.5, 8.8 (2C), 0.3.

General procedure for indole formation:

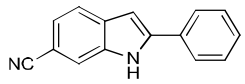
To a solution of aniline (0.46 mmol) in dry toluene (7 mL), $InBr_3$ (0.02 mmol) was added and the resulting mixture was heated at 120°C for 18 h. After cooling at 23°C, the crude reaction mixture was diluted with EtOAc (20 mL) and washed with water (3 × 15 mL), the combined organic extracts were washed with brine and dried over Na_2SO_4 and evaporated *in vacuo*. Purification by flash chromatography (silica gel) gave the desired product.

2-Phenyl-6-(trifluoromethyl)-1*H*-indole 127 a:



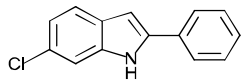
Compound **126 a** (120 mg, 0.46 mmol) and $InBr_3$ (8.15 mg, 0.02 mmol) were employed. Purification by flash chromatography (Hexane/CH_2Cl_2, 2:1) gave compound **127 a** as a pale yellow solid (95.4 mg, yield 80%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.55 (bs, 1 H), 7.74 – 7.69 (m, 4 H), 7.53 – 7.48 (m, 2 H), 7.43 – 7.36 (m, 2 H), 6.90 (dd, *J* = 2.1, 0.8 Hz, 1 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 140.6, 135.6, 131.6, 129.2 (3C), 128.5, 125.4 (2C), 125.2 (q, J_{CF} = 271.4 Hz), 124.2 (d, J_{CF} = 32.1 Hz), 120.9, 117.0 (q, J_{CF} = 3.4 Hz), 108.4 (q, J_{CF} = 4.4 Hz), 100.0; ^{19}F NMR (376 MHz, $CDCl_3$): δ = -60.86.

2-Phenyl-1*H*-indole-6-carbonitrile 127 b:



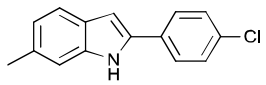
Compound **126 b** (222 mg, 1.02 mmol) and $InBr_3$ (18 mg, 0.05 mmol) were employed. Purification by flash chromatography (Hexane/CH_2Cl_2, 1:2) gave compound **127 b** as a white solid (106.4 mg, yield 48%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.69 (bs, 1 H), 7.77 – 7.75 (m, 1 H), 7.74 – 7.68 (m, 3 H), 7.54 – 7.49 (m, 2 H), 7.46 – 7.41 (m, 1 H), 7.38 (dd, *J* = 8.2, 1.4 Hz, 1 H), 6.91 (dd, *J* = 2.1, 0.9 Hz, 1 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 141.8, 135.5, 132.5, 131.1, 129.3 (2C), 128.9, 125.6 (2C), 123.4, 121.3, 120.7, 115.6, 104.4, 100.5.

6-Chloro-2-phenyl-1*H*-indole 131:



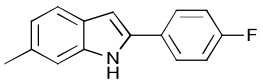
Compound **130** (170.3 mg, 0.75 mmol) and $InBr_3$ (13.2 mg, 0.04 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 9:1) gave compound **131** as a white solid (139.6 mg, yield 81%). ^{1}H NMR (400 MHz, CDCl3): δ = 8.35 (s, 1 H), 7.70 – 7.65 (m, 2 H), 7.55 (d, *J* = 8.0 Hz, 1 H), 7.48 (t, *J* = 7.6 Hz, 2 H), 7.42 (s, 1 H), 7.39 – 7.33 (m, 1 H), 7.12 (dd, *J* = 8.4, 1.8 Hz, 1 H), 6.82 (d, *J* = 1.3 Hz, 1 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 138.6, 137.1, 131.9, 129.1 (2C), 128.5, 128.0, 127.8, 125.2 (2C), 121.5, 121.0, 110.8, 99.9.

2-(4-Chlorophenyl)-6-methyl-1*H*-indole 137 a:



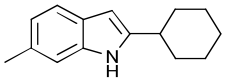
Compound **136 a** (45 mg, 0.19 mmol) and $InBr_3$ (3.3 mg, 0.01 mmol) were employed. Purification by flash chromatography (Hexane/CH_2Cl_2, 3:1) gave the desired product **137 a** (24.8 mg, yield 53%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.16 (s, 1 H), 7.58 (d, *J* = 8.4 Hz, 2 H), 7.53 (d, *J* = 8.1 Hz, 1 H), 7.42 (d, *J* = 8.5 Hz, 2 H), 7.20 (s, 1 H), 6.99 (dd, *J* = 8.0, 0.7 Hz, 1 H), 6.79 (d, *J* = 1.6 Hz, 1 H), 2.50 (s, 3 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 137.4, 136.0, 133.2, 132.6, 131.1, 129.2 (2C), 127.0, 126.1 (2C), 122.3, 120.4, 110.9, 100.3, 21.8.

2-(4-Fluorophenyl)-6-methyl-1*H*-indole 137 b:



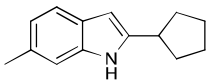
Compound **136 b** (74 mg, 0.33 mmol) and $InBr_3$ (5.8 mg, 0.02 mmol) were employed. Purification by flash chromatography (Hexane/CH_2Cl_2, 2:1) gave compound **137 b** as a white solid (52.2 mg, yield 70%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.13 (bs, 1 H), 7.66 – 7.59 (m, 2 H), 7.53 (d, *J* = 8.1 Hz, 1 H), 7.21 (s, *J* = 7.2 Hz, 1 H), 7.18 – 7.12 (m, 2 H), 6.99 (dd, *J* = 8.1, 0.9 Hz, 1 H), 6.73 (d, *J* = 1.5 Hz, 1 H), 2.50 (s, 3 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ =162.3 (d, J_{CF} = 247.3 Hz), 137.3, 136.04, 132.3, 128.9 (d, J_{CF} = 3.2 Hz), 127.1, 126.7 (d, J_{CF} = 8.0 Hz) (2C), 122.2, 120.3, 116.0 (d, J_{CF} = 21.7 Hz) (2C), 110.9, 99.8, 21.8; ^{19}F NMR (376 MHz, $CDCl_3$): δ -114.35 (tt, *J* = 8.6, 5.1 Hz).

2-Cyclohexyl-6-methyl-1*H*-indole 137 c:



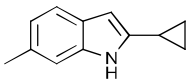
Compound **136 c** (70 mg, 0.33 mmol) and $InBr_3$ (5.8 mg, 0.02 mmol) were employed. Purification by flash chromatography (Hexane/CH_2Cl_2, 5:1) gave compound **137 c** as a white solid (25.8 mg, yield 36%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.64 (bs, 1 H), 7.33 (d, *J* = 8.0 Hz, 1 H), 6.99 (s, 1 H), 6.80 (t, *J* = 8.3 Hz, 1 H), 6.08 (d, *J* = 0.8 Hz, 1 H), 2.63 – 2.54 (m, 1 H), 2.36 (s, 3 H), 2.01 – 1.94 (m, 2 H), 1.80 – 1.72 (m, 2 H), 1.71 – 1.62 (m, 1 H), 1.45 – 1.26 (m, 4 H), 1.25 – 1.14 (m, 1 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 144.4, 136.0, 130.6, 126.4, 121.2, 119.5, 110.4, 97.2, 37.3, 33.0 (2C), 26.3 (2C), 26.2, 21.7.

2-Cyclopentyl-6-methyl-1*H*-indole 137 d:



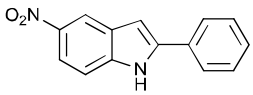
Compound **136 d** (60 mg, 0.30 mmol) and $InBr_3$ (5.3 mg, 0.02 mmol) were employed. Purification by flash chromatography (Hexane/CH_2Cl_2, 8.5:1.5) gave compound **137 d** as a white solid (16.2 mg, yield 26%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.76 (bs, 1 H), 7.43 (d, *J* = 8.0 Hz, 1 H), 7.11 (s, 1 H), 6.92 (d, *J* = 8.0 Hz, 1 H), 6.23 – 6.21 (m, 1 H), 3.22 – 3.15 (m, 1 H), 2.47 (s, 3 H), 2.18 – 2.08 (m, 2 H), 1.88 – 1.68 (m, 6 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 143.3, 136.3, 130.7, 126.4, 121.2, 119.4, 110.3, 97.8, 38.9, 32.8 (2C), 25.2 (2C), 21.7.

2-Cyclopropyl-6-methyl-1*H*-indole 137 e:



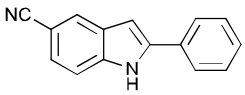
Compound **136 e** (79.5 mg, 0.46 mmol) and $InBr_3$ (8.15 mg, 0.02 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 15:1) gave compound **137 e** as a white solid (39.3 mg, yield 50%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.78 (bs, 1 H), 7.45 (d, *J* = 8.0 Hz, 1 H), 7.09 (d, *J* = 0.6 Hz, 1 H), 6.96 (dd, *J* = 8.0, 0.9 Hz, 1 H), 6.17 – 6.13 (m, 1 H), 2.51 (s, 3 H), 2.01 – 1.92 (m, 1 H), 1.03 – 0.96 (m, 2 H), 0.84 – 0.77 (m, 2 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 141.0, 136.3, 130.7, 126.5, 121.4, 119.4, 110.4, 97.5, 21.7, 8.9, 7.2 (2C).

5-Nitro-2-phenyl-1*H*-indole 147 a:



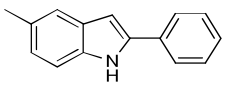
Compound **146 a** (170 mg, 0.71 mmol) and $InBr_3$ (12.6 mg, 0.04 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 5:1) gave compound **147 a** as a pale yellow solid (124.2 mg, yield 74%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.70 (bs, 1 H), 8.62 (d, *J* = 2.2 Hz, 1 H), 8.15 (dd, *J* = 8.9, 2.2 Hz, 1 H), 7.73 – 7.68 (m, 2 H), 7.55 – 7.40 (m, 4 H), 7.00 (dd, *J* = 2.1, 0.8 Hz, 1 H).[241]

2-Phenyl-5-cyanoindole 147 b:



Compound **146 b** (214.6 mg, 0.98 mmol), and $InBr_3$ (17.4 mg, 0.05 mmol) were employed. Compound **147 b** was used in the next step without further purifications. (197.4 mg, yield 93%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.70 (bs, 1 H), 8.00 (s, 1 H), 7.60 (d, *J* = 8 Hz, 2 H), 7.54 – 7.39 (m, 5 H), 6.90 (dd, *J* = 2.1, 0.7 Hz, 1 H).[241]

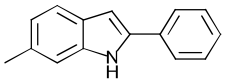
5-Methyl-2-phenyl-1*H*-indole 151:



To a solution of *p*-tolylhydrazine·HCl (500 mg, 3.15 mmol) in EtOH (12 mL), acetophenone (367 µL, 3.15 mmol) was added and the resulting mixture was stirred at

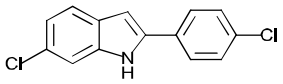
23°C for 18h. Then solvent was removed and the crude material dissolved in toluene (15 mL), $ZnBr_2$ (3.5 g, 15.8 mmol) was added and the reaction mixture was heated at 120°C for 5h. After cooling at 23°C, the crude reaction mixture was diluted with EtOAc (50 mL) and washed with water (3 × 20 mL), the combined organic extracts were washed with brine and dried over Na_2SO_4 and evaporated *in vacuo*. Purification by flash chromatography (Hexane/CH_2Cl_2, 2:1) gave compound **151** as a white solid (436 mg, yield 67%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.26 (bs, 1 H), 7.70 – 7.65 (m, 2 H), 7.49 – 7.43 (m, 3 H), 7.37 – 7.30 (m, 2 H), 7.05 (dd, *J* = 8.3, 1.2 Hz, 1 H), 6.78 (dd, *J* = 2.1, 0.9 Hz, 1 H), 2.48 (s, 3 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 138.0, 135.2, 132.5, 129.6, 129.5, 129.0 (2C), 127.6, 125.1 (2C), 124.0, 120.3, 110.6, 99.6, 21.5.

6-Methyl-2-phenyl-1*H*-indole 154:



To a solution of *m*-tolylhydrazine·HCl (4 g, 25.2 mmol) in EtOH (50 mL), acetophenone (3.2 mL, 27.2 mmol) was added and the resulting mixture was stirred at 23°C for 18h. Then solvent was removed and the crude material dissolved in toluene (40 mL), PPA was added and the reaction mixture was heated at 100°C for 18h. After cooling at r.t, aq. $NaHCO_3$ was added until pH = 7 then the reaction mixture was diluted with EtOAc (50 mL) and washed with water (3 × 20 mL) and brine (2 × 20 mL), the combined organic extracts were washed with brine and dried over Na_2SO_4 and evaporated *in vacuo*. Crystallization with hexane/EtOAc afforded compound **154** as a white solid (3.23 g, yield 62%).^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.22 (bs, 1 H), 7.69 – 7.65 (m, 2 H), 7.54 (d, *J* = 8.0 Hz, 1 H), 7.49 – 7.43 (m, 2 H), 7.36 – 7.31 (m, 1 H), 7.22 (s, 1 H), 6.98 (dd, *J* = 8.1, 0.9 Hz, 1 H), 6.82 – 6.79 (m, 1 H), 2.50 (s, 3 H).

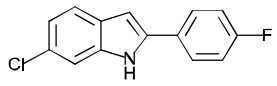
6-Chloro-2-(4-chlorophenyl)-1*H*-indole 159:



Compound **158** (100 mg, 0.38 mmol) and $InBr_3$ (6.8 mg, 0.02 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 8:1) gave compound **159** as a white solid (69.2 mg, yield 68%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.26 (s, 1 H), 7.59 – 7.52 (m, 3 H), 7.43 (d, *J* = 8.5 Hz, 2 H), 7.39 (s, 1 H), 7.13 (dd, *J* = 8.4, 1.7 Hz, 1 H), 6.79 (d, *J* =

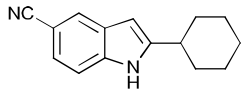
1.3 Hz, 1 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 137.4, 137.2, 133.8, 130.4, 129.3 (2 C), 128.4, 127.7, 126.3 (2C), 121.6, 121.3, 110.9, 100.4.

6-Chloro-2-(4-fluorophenyl)-1*H*-indole 162:



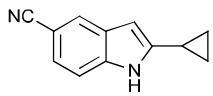
Compound **161** (160 mg, 0.65 mmol) and $InBr_3$ (11.5 mg, 0.03 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 10:1) gave compound **162** as a white solid (132.6 mg, yield 83%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.26 (s, 1 H), 7.66 – 7.60 (m, 2 H), 7.54 (d, *J* = 8.4 Hz, 1 H), 7.42 – 7.39 (m, 1 H), 7.21 – 7.14 (m, 2 H), 7.12 (dd, *J* = 8.4, 1.8 Hz, 1 H), 6.75 (dd, *J* = 2.1, 0.8 Hz, 1 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 162.6 (d, J_{CF} = 248.2 Hz), 137.8, 137.1, 128.3, 128.3 (d, J_{CF} = 3.2 Hz), 127.8, 126.9 (d, J_{CF} = 8.1 Hz) (2C), 121.5, 121.2, 116.2 (d, J_{CF} = 21.7 Hz) (2C), 110.8, 99.9; ^{19}F NMR (376 MHz, $CDCl_3$) δ = -113.35 (dq, *J* = 8.6, 5.4 Hz).

2-Cyclohexyl-1*H*-indole-5-carbonitrile 165 a:



Compound **164 a** (146 mg, 0.65 mmol) and $InBr_3$ (11.5 mg, 0.03 mmol) were employed. Purification by flash chromatography (Hexane/CH_2Cl_2, 2:1) gave compound **165 a** as a white solid (103.2 mg, yield 71%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.25 (bs, 1 H), 7.89 (s, 1 H), 7.37 (s, 2 H), 6.32 (d, *J* = 2.1 Hz, 1 H), 2.81 – 2.71 (m, 1 H), 2.15 – 2.07 (m, 2 H), 1.93 – 1.85 (m, 2 H), 1.84 – 1.75 (m, 1 H), 1.57 – 1.38 (m, 4 H), 1.38 – 1.25 (m, 1 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 147.5, 137.3, 128.5, 125.3, 124.2, 121.0, 111.1, 102.6, 98.4, 37.2 (2C), 32.7, 26.1 (2C), 26.0.

2-Cyclopropyl-1*H*-indole-5-carbonitrile 165 b:



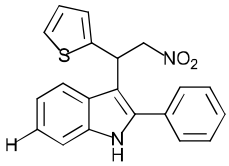
Compound **164 b** (107 mg, 0.60 mmol) and $InBr_3$ (10.6 mg, 0.03 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 4:1) gave compound **165 b** as a white solid (65.6 mg, yield 60%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.26 (s, 1 H), 7.85 (s, 1 H), 7.39 – 7.31 (m, 2 H), 6.23 (d, *J* = 2.1 Hz, 1 H), 2.04 – 1.95 (m, 1 H), 1.06 (dt, *J* = 6.4,

4.5 Hz, 2 H), 0.84 (dt, *J* = 6.6, 4.7 Hz, 2 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 144.3, 137.5, 128.6, 125.1, 124.2, 120.95, 110.9, 98.4, 8.8, 7.7 (2C).

General procedure for conjugate addition:

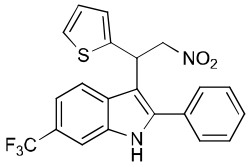
To a suspended solution of indole (0.19 mmol) and 2-(2-nitrovinyl) thiophene[225] (0.29 mmol) in H_2O-*t*BuOH (5:1, *v/v*, 2.4 mL) in a sealed microwave vessel was added $InBr_3$ (0.009 mmol). The resulting mixture was irradiated by microwave to 150°C for 15 min then it was cooled to 23°C prior to add another portion of $InBr_3$ (0.009 mmol) followed by irradiation with microwave to 150°C for further 15 min. After cooling to 23°C, the reaction mixture was diluted with water (5 mL) and extracted with CH_2Cl_2 (3 × 10 mL). The combined organic layers were dried over Na_2SO_4 and evaporated *in vacuo* to obtain a brown solid which was purified by flash chromatography (silica gel) to give the desired product.

3-[2-nitro-1-(thiophen-2-yl)ethyl]-2-phenyl-1*H*-indole 120:



2-Phenylindole (20 mg, 0.10 mmol) and 2-(2-nitrovinyl) thiophene[225] (24.2 mg, 0.16 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 9:1) gave compound **120** as a white solid (27.7 mg, yield 77%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.18 (s, 1 H), 7.58 – 7.41 (m, 7 H), 7.28 – 7.21 (m, 2 H), 7.15 (ddd, *J* = 8.1, 7.1, 1.0 Hz, 1 H), 6.99 – 6.95 (m, 2 H), 5.52 (t, *J* = 7.8 Hz, 1 H), 5.23 (dd, *J* = 12.6, 7.3 Hz, 1 H), 5.12 (dd, *J* = 12.5, 8.2 Hz, 1 H).

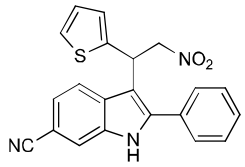
3-[2-Nitro-1-(thiophen-2-yl)ethyl]-2-phenyl-6-(trifluoromethyl)-1*H*-indole 128 a:



Compound **127 a** (50 mg, 0.19mmol), $InBr_3$ (2 × 3.4 mg, 0.018 mmol) and 2-(2-nitrovinyl) thiophene[225] (44.2 mg, 0.29 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 9:1) gave compound **128 a** as a pale yellow solid (57.9 mg, yield 74%). ^{1}H NMR (400 MHz, CDCl3): δ = 8.42 (s, 1 H), 7.71 (s, 1 H), 7.63 (d, *J* = 8.4 Hz, 1 H),

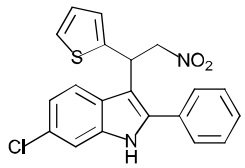
7.54 – 7.52 (m, 5 H), 7.39 (dd, *J* = 8.4, 1.1 Hz, 1 H), 7.25 (dd, *J* = 5.1, 1.0 Hz, 1 H), 6.99 (dd, *J* = 5.1, 3.6 Hz, 1 H), 6.95 (dt, *J* = 3.5, 1.2 Hz, 1 H), 5.55 – 5.50 (m, 1 H), 5.16 (ddd, *J* = 21.3, 12.5, 7.9 Hz, 2 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 143.1, 139.8, 134.9, 131.1, 129.4, 129.2 (2C), 128.8 (2C), 127.1, 125.1, 124.9 (q, J_{CF} = 271.9 Hz), 124.9, 124.7 (d, J_{CF} = 32.1 Hz), 124.5, 120.2, 117.1 (q, J_{CF} = 3.5 Hz), 109.9, 109.0 (q, J_{CF} = 4.3 Hz), 78.8, 36.7; ^{19}F NMR (376 MHz, $CDCl_3$): δ= -60.86.

3-[2-Nitro-1-(thiophen-2-yl)ethyl]-2-phenyl-1*H*-indole-6-carbonitrile 128 b:



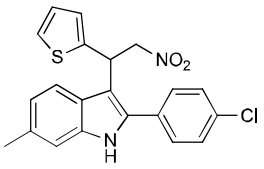
Compound **127 b** (50 mg, 0.23 mmol) $InBr_3$ (2 × 4 mg, 0.024 mmol) and 2-(2-nitrovinyl) thiophene[225] (53.5 mg, 0.35 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 8:1) gave compound **128 b** as a white solid (10 mg, yield 13%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.49 (bs, 1 H), 7.77 (s, 1 H), 7.60 (d, *J* = 8.4 Hz, 1 H), 7.54 (s, *J* = 7.6 Hz, 5 H), 7.39 (dd, *J* = 8.4, 1.2 Hz, 1 H), 7.26 (d, *J* = 5.1 Hz, 1 H), 7.01 – 6.97 (m, 1 H), 6.95 – 6.92 (m, 1 H), 5.55 – 5.48 (m, 1 H), 5.14 (ddd, *J* = 21.5, 12.5, 8.0 Hz, 2 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 142.7, 141.0, 134.8, 130.7, 129.7, 129.6, 129.3 (2C), 128.8 (2C), 127.2, 125.2, 124.9, 123.4, 120.5, 120.1, 116.1, 110.4, 105.2, 78.7, 36.5.

6-Chloro-3-[2-nitro-1-(thiophen-2-yl)ethyl]-2-phenyl-1*H*-indole 132:



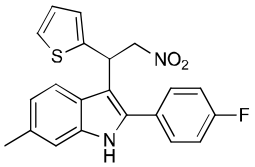
Compound **131** (50 mg, 0.22 mmol), $InBr_3$ (2 × 4 mg, 0.022 mmol) and 2-(2-nitrovinyl) thiophene[225] (51.2 mg, 0.33 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 9:1) gave compound **132** as a pale yellow solid (64.8mg, yield 77%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.18 (s, 1 H), 7.52 – 7.49 (m, 5 H), 7.44 (d, *J* = 8.6 Hz, 1 H), 7.41 (dd, *J* = 1.8, 0.4 Hz, 1 H), 7.24 (dd, *J* = 5.1, 1.0 Hz, 1 H), 7.13 – 7.10 (m, 1 H), 6.98 (dd, *J* = 5.1, 3.6 Hz, 1 H), 6.95 (dt, *J* = 3.6, 1.2 Hz, 1 H), 5.49 (ddd, *J* = 8.4, 7.0, 1.1 Hz, 1 H), 5.18 (dd, *J* = 12.4, 7.0 Hz, 1 H), 5.09 (dd, *J* = 12.4, 8.7 Hz, 1 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 143.3, 137.8, 136.4, 131.4, 129.1 (2C), 129.0, 128.7(2C), 128.5, 127.1, 125.1, 125.1, 124.8, 121.1, 120.7, 111.4, 109.6, 78.9, 36.7.

2-(4-Chlorophenyl)-6-methyl-3-[2-nitro-1-(thiophen-2-yl)ethyl]-1*H*-indole 138 a:



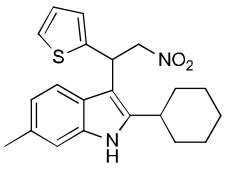
Compound **137 a** (23.2 mg, 0.10 mmol), $InBr_3$ (2 × 1.7 mg, 0.01 mmol) and 2-(2-nitrovinyl) thiophene[225] (22.3 mg, 0.14 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 9:1) gave compound **138 a** as a white solid (31.9 mg, yield 83%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.03 (s, 1 H), 7.49 – 7.41 (m, 5 H), 7.24 – 7.20 (m, 2 H), 7.02 – 6.94 (m, 3 H), 5.45 – 5.40 (m, 1 H), 5.16 (qd, *J* = 12.5, 7.8 Hz, 2 H), 2.49 (s, 3 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 143.5, 136.6, 135.0, 134.7, 132.9, 130.5, 129.9 (2C), 129.3 (2C), 127.0, 125.0, 124.9, 124.2, 122.3, 119.6, 111.4, 109.8, 79.0, 36.9, 21.7.

2-(4-Fluorophenyl)-6-methyl-3-[2-nitro-1-(thiophen-2-yl)ethyl]-1*H*-indole 138 b:



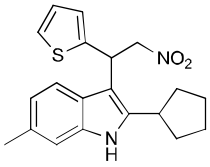
To a suspended solution of compound **137 b** (15 mg, 0.07 mmol) in H_2O-THF (6:1, *v/v*, 3.5 mL) was added $InBr_3$ (1.4 mg, 0.004 mmol) and 2-(2-nitrovinyl) thiophene[225] (16.2 mg, 0.11 mmol) and the resulting mixture was heated at 100°C for 3.5 h. After cooling to 23°C, the reaction mixture was diluted with water (5 mL) and extracted with CH_2Cl_2 (3 × 10 mL). The combined organic layers were dried over Na_2SO_4 and evaporated *in vacuo* and the crude material was purified by flash chromatography (Hexane/EtOAc, 9:1) to give compound **138 b** as a white solid (20.2 mg, yield 71%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.02 (s, 1 H), 7.48 (dd, *J* = 8.5, 5.4 Hz, 2 H), 7.43 (d, *J* = 8.2 Hz, 1 H), 7.24 – 7.16 (m, 4 H), 7.02 – 6.94 (m, 3 H), 5.41 (t, *J* = 7.8 Hz, 1 H), 5.16 (qd, *J* = 12.4, 7.8 Hz, 2 H), 2.49 (s, 3 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ =162.9 (d, J_{CF} = 249.0 Hz), 143.6, 136.5, 135.3, 132.8, 130.6 (d, J_{CF} = 8.4 Hz) (2C), 128.14 (d, J_{CF} = 3.4 Hz), 127.0, 124.9, 124.8, 124.2, 122.2, 119.6, 116.12 (d, J_{CF} = 21.7 Hz) (2C), 111.4, 109.5, 79.1, 36.9, 21.7; ^{19}F NMR (376 MHz, $CDCl_3$) δ = -112.37 (tt, *J* = 8.4, 5.2 Hz).

2-Cyclohexyl-6-methyl-3-[2-nitro-1-(thiophen-2-yl)ethyl]-1*H*-indole 138 c:



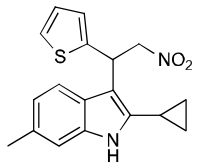
Compound **137 c** (25 mg, 0.12 mmol), $InBr_3$ (2 × 2.2 mg, 0.012 mmol) and 2-(2-nitrovinyl) thiophene[225] (28 mg, 0.18 mmol) were employed. Purification by flash chromatography (Hexane/CH_2Cl_2, 2:1) gave compound **138 c** as a white solid (31.7 mg, yield 75%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.84 (s, 1 H), 7.24 (d, *J* = 8.1 Hz, 1 H), 7.19 (dd, *J* = 4.9, 1.1 Hz, 1 H), 7.13 (s, 1 H), 6.96 – 6.92 (m, 2 H), 6.88 (d, *J* = 8.1 Hz, 1 H), 5.43 – 5.37 (m, 1 H), 5.25 (dd, *J* = 12.3, 6.8 Hz, 1 H), 5.10 (dd, *J* = 12.3, 8.7 Hz, 1 H), 2.92 – 1.86 (m, 1 H), 2.44 (s, 3 H), 1.92 – 1.80 (m, 5 H), 1.53 – 1.42 (m, 4 H), 1.36 – 1.25 (m, 2 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 143.9, 141.5, 135.8, 131.3, 126.8, 124.7, 124.3, 123.9, 121.4, 118.7, 110.9, 106.9, 79.2, 36.4, 35.7, 33.9, 32.9, 26.6, 26.5, 26.0, 21.6.

2-Cyclopentyl-6-methyl-3-[2-nitro-1-(thiophen-2-yl)ethyl]-1*H*-indole 138 d:



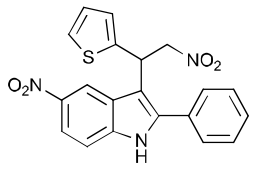
Compound **137 d** (16 mg, 0.08 mmol), InBr3 (2 × 1.4 mg, 0.008 mmol) and 2-(2-nitrovinyl) thiophene[225] (18.6 mg, 0.12 mmol) were employed. Purification by flash chromatography (Hexane/CH_2Cl_2, 2:1) gave compound **138 d** as a white solid (23.3 mg, yield 88%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.81 (s, 1 H), 7.25 (d, *J* = 8.1 Hz, 1 H), 7.20 – 7.17 (m, 1 H), 7.13 (s, 1 H), 6.96 – 6.92 (m, 2 H), 6.89 (dd, *J* = 8.1, 0.7 Hz, 1 H), 5.44 – 5.39 (m, 1 H), 5.25 (dd, *J* = 12.3, 6.9 Hz, 1 H), 5.10 (dd, *J* = 12.3, 8.6 Hz, 1 H), 3.43 – 3.33 (m, 1 H), 2.44 (s, 3 H), 2.18 – 2.08 (m, 2 H), 1.93 – 1.73 (m, 4 H), 1.72 – 1.60 (m, 2 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 143.9, 139.9, 135.9, 131.3, 126.8, 124.6, 124.3, 124.1, 121.4, 118.6, 110.9, 108.1, 79.3, 36.8, 36.6, 33.9, 33.1, 25.7 (2C), 21.6.

2-Cyclopropyl-6-methyl-3-[2-nitro-1-(thiophen-2-yl)ethyl]-1*H*-indole 138 e:



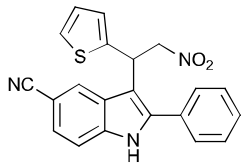
Compound **137 e** (37.4 mg, 0.22 mmol), $InBr_3$ (4 mg, 0.011 mmol) and 2-(2-nitrovinyl) thiophene[225] (51.2 mg, 0.33 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 9:1) gave compound **138 e** as a white solid (22.4, yield 32%).^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.69 (bs, 1 H), 7.27 (d, *J* = 9.3 Hz, 1 H), 7.21 – 7.17 (m, 1 H), 7.09 (s, 1 H), 6.96 – 6.92 (m, 2 H), 6.90 (dd, *J* = 8.1, 0.9 Hz, 1 H) , 5.64 – 5.58 (m, 1 H), 5.26 (dd, *J* = 12.3, 7.1 Hz, 1 H), 5.13 (dd, *J* = 12.3, 8.6 Hz, 1 H), 2.44 (s, 3 H), 2.11 – 2.02 (m, 1 H), 1.09 – 1.00 (m, 2 H), 0.83 – 0.75 (m, 2 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 143.7, 136.9, 135.3, 131.6, 126.8, 124.7, 124.4 (2C), 121.6, 118.5, 111.0, 110.1, 79.1, 36.6, 21.6, 7.2, 6.5, 5.9.

5-Nitro-3-[2-nitro-1-(thiophen-2-yl)ethyl]-2-phenyl-1*H*-indole 148 a:



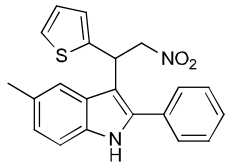
Compound **147 a** (44.2 mg, 0.19 mmol), $InBr_3$ (2 × 3.3 mg, 0.002 mmol) and 2-(2-nitrovinyl) thiophene[225] (43 mg, 0.28 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 4:1) gave compound **148 a** as a pale yellow solid (30 mg, yield 41%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.58 (bs, 1 H), 8.50 (d, *J* = 2.1 Hz, 1 H), 8.18 (dt, *J* = 6.5, 3.2 Hz, 1 H), 7.57 – 7.52 (m, 5 H), 7.48 (d, *J* = 8.9 Hz, 1 H), 7.27 (dd, *J* = 5.2, 1.1 Hz, 1 H), 7.01 (dd, *J* = 5.1, 3.6 Hz, 1 H), 6.97 (dt, *J* = 3.6, 1.2 Hz, 1 H), 5.53 (t, *J* = 7.9 Hz, 1 H), 5.22 – 5.16 (m, 2 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 142.3, 142.2, 140.4, 138.9, 130.6, 129.7, 129.3 (2C), 128.8 (2C), 127.3, 125.9, 125.3, 125.0, 118.4, 116.8, 111.6, 111.4, 78.7, 36.6.

3-[2-Nitro-1-(thiophen-2-yl)ethyl]-2-phenyl-1*H*-indole-5-carbonitrile 148 b:



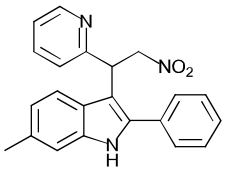
Compound **147 b** (50 mg, 0.23 mmol), $InBr_3$ (2 × 4.07 mg, 0.011 mmol) and 2-(2-nitrovinyl) thiophene[225] (53.5 mg, 0.34 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 7:3) gave compound **148 b** as a white solid (29.7 mg, yield 36%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.46 (s, 1 H), 7.85 (d, *J* = 0.8 Hz, 1 H), 7.57 – 7.50 (m, 5 H), 7.49 (d, *J* = 1.1 Hz, 2 H), 7.29 – 7.23 (m, 1 H), 7.01 (dt, *J* = 10.1, 5.1 Hz, 1 H), 6.94 (dt, *J* = 3.5, 1.2 Hz, 1 H), 5.50 (ddd, *J* = 9.1, 6.6, 1.0 Hz, 1 H), 5.14 (ddd, *J* = 21.7, 12.4, 8.0 Hz, 2 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 142.5, 139.5, 137.7, 130.7, 129.6, 129.3 (2C), 128.8 (2C), 127.3, 126.3, 125.6, 125.3, 125.2, 124.8, 120.4, 112.3, 110.2, 103.7, 78.7, 36.6.

5-Methyl-3-[2-nitro-1-(thiophen-2-yl)ethyl]-2-phenyl-1*H*-indole 152:



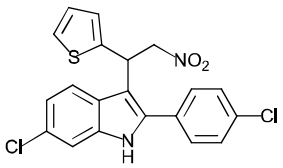
Compound **151** (39.3 mg, 0.19 mmol), $InBr_3$ (3.36 mg, 0.001 mmol) and 2-(2-nitrovinyl) thiophene[225] (44.5 mg, 0.29 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 9:1) gave compound **152** as a white solid (55.1 mg, yield 79%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.09 (bs, 1 H), 7.55 – 7.49 (m, 4 H), 7.49 – 7.45 (m, 1 H), 7.33 (s, 1 H), 7.31 (d, *J* = 8.3 Hz, 1 H), 7.25 – 7.22 (m, 1 H), 7.08 (dd, *J* = 8.1, 1.3 Hz, 1 H), 7.00 – 6.96 (m, 2 H), 5.50 (t, *J* = 7.7 Hz, 1 H), 5.23 (dd, *J* = 12.6, 7.4 Hz, 1 H), 5.10 (dd, *J* = 12.6, 8.1 Hz, 1 H), 2.46 (s, 3 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 143.9, 137.0, 134.4, 132.1, 129.6, 129.0 (2C), 128.7 (2C), 128.7, 127.0, 126.8, 124.9, 124.9, 124.3, 119.5, 111.1, 109.1, 79.2, 36.9, 21.7.

6-Methyl-3-[2-nitro-1-(pyridin-2-yl)ethyl]-2-phenyl-1*H*-indole 156:



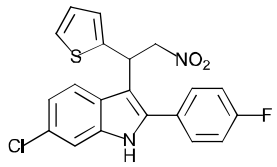
To a suspended solution of compound **154** (84 mg, 0.41 mmol) in H_2O-dioxane (1:3, *v/v*, 8 mL) was added TBAB (92 mg, 0.29 mmol) and 2-(2-nitrovinyl)pyridine[242] (34.2 mg, 0.23 mmol) and the resulting mixture was heated at 100°C for 18 h. After cooling to 23°C, the reaction mixture was diluted with water (10 mL) and extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were dried over Na_2SO_4 and evaporated *in vacuo* and the crude material was purified by flash chromatography (Hexane/ CH_2Cl_2, 1:2) to give compound **156** as a white solid (23 mg, yield 32%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.58 (dd, *J* = 4.9, 0.7 Hz, 1 H), 8.08 (s, 1 H), 7.60 – 7.40 (m, 6 H), 7.29 (d, *J* = 7.1 Hz, 1 H), 7.18 (s, 1 H), 7.16 – 7.05 (m, 2 H), 6.88 (dd, *J* = 8.2, 0.9 Hz, 1 H), 5.82 (dd, *J* = 13.4, 8.2 Hz, 1 H), 5.48 (t, *J* = 7.5 Hz, 1 H), 4.97 (dd, *J* = 13.4, 6.9 Hz, 1 H), 2.45 (s, 3 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 159.4, 148.5, 136.6, 136.5, 136.1, 132.6, 132.3, 129.1 (2C), 128.6 (2C), 128.6, 124.8, 123.4, 122.0, 121.8, 119.7, 111.1, 109.4, 76.8, 41.6, 21.7.

6-Chloro-2-(4-methylphenyl)-4-[2-nitro-1-(thiophen-2-yl)ethyl]-1*H*-indole 160:



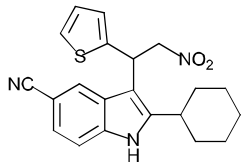
Compound **159** (28 mg, 0.11 mmol), $InBr_3$ (2 × 2 mg, 0.011 mmol) and 2-(2-nitrovinyl) thiophene[225] (25.6 mg, 0.17 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 8:1) gave compound **160** as a pale yellow solid (24.1 mg, yield 55%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.17 (s, 1 H), 7.51 – 7.47 (m, 2 H), 7.45 – 7.41 (m, 4 H), 7.25 (dd, *J* = 5.1, 0.8 Hz, 1 H), 7.12 (dd, *J* = 8.6, 1.8 Hz, 1 H), 7.00 – 6.97 (m, 1 H), 6.94 – 6.92 (m, 1 H), 5.45 – 5.38 (m, 1 H), 5.20 – 5.07 (m, 2 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 143.3, 137.8, 136.4, 131.4, 129.1 (2C), 129.0, 128.7 (2C), 128.5, 127.1, 125.1, 125.1, 124.8, 121.1, 120.7, 111.4, 109.6, 78.9, 36.7.

6-Chloro-2-(4-fluorophenyl)-3-[2-nitro-1-(thiophen-2-yl)ethyl]-1*H*-indole 163:



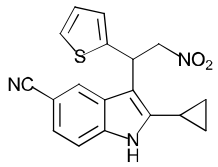
Compound **162** (40 mg, 0.16 mmol), $InBr_3$ (2 × 2.8 mg, 0.016 mmol) and 2-(2-nitrovinyl) thiophene[225] (37.2 mg, 0.24 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 9:1) gave compound **163** as a white solid (41.5 mg, yield 63%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.15 (s, 1 H), 7.50 – 7.46 (m, 2 H), 7.43 (d, *J* = 8.6 Hz, 1 H), 7.42 – 7.40 (m, 1 H), 7.25 (dd, *J* = 5.1, 0.9 Hz, 1 H), 7.23 – 7.18 (m, 2 H), 7.12 (dd, *J* = 8.5, 1.9 Hz, 1 H), 6.98 (dd, *J* = 5.1, 3.6 Hz, 1 H), 6.93 (dt, *J* = 3.6, 1.2 Hz, 1 H), 5.43 – 5.38 (m, 1 H), 5.19 – 5.08 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 163.1 (d, J_{CF} = 249.7 Hz), 143.0, 136.8, 136.4, 130.6 (d, J_{CF} = 8.2 Hz), 128.7, 127.5, 127.4, 127.1, 125.1, 124.9, 124.8, 121.2, 120.7, 116.3 (d, J_{CF} = 21.7 Hz) (2C), 111.4, 109.7, 78.8, 36.7; ^{19}F NMR (376 MHz, $CDCl_3$) δ = -111.56 (dq, *J* = 8.5, 5.1 Hz).

2-Cyclohexyl-4-[2-nitro-1-(thiophen-2-yl)ethyl]-1*H*-indole-5-carbonitrile 166 a:



Compound **165 a** (50 mg, 0.22 mmol), $InBr_3$ (2 × 4 mg, 0.022 mmol) and 2-(2-nitrovinyl) thiophene[225] (51.2 mg, 0.33 mmol) were employed. Purification by flash chromatography (Hexane/EtOAc, 4:1) gave compound **166 a** as a white solid (45.3 mg, yield 55%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.34 (bs, 1 H), 7.65 (d, *J* = 0.7 Hz, 1 H), 7.39 (d, *J* = 1.1 Hz, 2 H), 7.24 (dd, *J* = 5.1, 0.6 Hz, 1 H), 6.99 (dd, *J* = 5.1, 3.6 Hz, 1 H), 6.90 (dt, *J* = 3.6, 1.2 Hz, 1 H), 5.42 (dd, *J* = 9.4, 6.4 Hz, 1 H), 5.25 (dd, *J* = 12.2, 6.0 Hz, 1 H), 5.10 (dd, *J* = 12.2, 9.8 Hz, 1 H), 3.02 – 2.93 (m, 1 H), 1.98 – 1.82 (m, 5 H), 1.56 – 1.41 (m, 4 H), 1.36 – 1.25 (m, 1 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 145.0, 142.5, 137.3, 127.1, 126.0, 125.2, 124.7, 124.4, 124.3, 120.7, 111.8, 107.9, 102.9, 78.8, 36.1, 35.7, 33.9, 32.6, 26.5, 26.4, 25.9.

2-Cyclopropyl-4-[2-nitro-1-(thiophen-2-yl)ethyl]-1*H*-indole-5-carbonitrile 166 b:



Compound **165 b** (55.2 mg, 0.3 mmol), $InBr_3$ (5.3 mg, 0.015 mmol) and 2-(2-nitrovinyl) thiophene[225] (69 mg, 0.45 mmol) were employed. Only 15 min of microwave irradiation to 150°C were necessary. Purification by flash chromatography (Hexane/EtOAc, 2:1) gave compound **166 b** as a white solid (79.7 mg, yield 80%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.32 (bs, 1 H), 7.69 (s, 1 H), 7.39 – 7.32 (m, 2 H), 7.24 (d, *J* = 5.1 Hz, 1 H), 6.99 (dd, *J* = 5.1, 3.6 Hz, 1 H), 6.93 (dt, *J* = 3.5, 1.1 Hz, 1 H), 5.65 – 5.60 (m, 1 H), 5.26 (dd, *J* = 12.2, 6.3 Hz, 1 H), 5.15 (dd, *J* = 12.2, 9.7 Hz, 1 H), 2.14 – 2.05 (m, 1 H), 1.17 – 1.06 (m, 2 H), 0.88 – 0.82 (m, 2 H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 142.3, 140.8, 136.7, 127.1, 126.5, 125.1, 124.8, 124.5, 123.9, 120.7, 111.9, 110.8, 102.9, 78.7, 36.3, 7.2, 7.0, 6.4.

Biological methods:

Initial screening of candidate compounds was performed using an in vitro assay to determine functional characteristics.

The PathHunter™ β-Arrestin assay (from DiscoveRX, Fremont, USA) was performed as follows. HEK293 CB_1 β-arrestin cells were plated 48 hours before use and incubated at 37°C, 5% CO_2 in a humidified incubator. Test compounds were dissolved in dimethylsulfoxide (DMSO) and diluted in optimized cell culture media (OCC, as supplied by DiscoveRX) media to the required concentrations. 5 µL of test compound or vehicle solution was added to each well and incubated for 60 minutes at 37°C, 5% CO_2 in a humidified incubator. 5 µL of increasing concentrations of anandamide was added to each well followed by a 90 minute incubation at 37°C, 5% CO_2 in a humidified incubator. 55 µL of detection reagent (as supplied by DiscoveRX) was then added, followed by a further 90 minute incubation at room temperature in the dark. Chemiluminescence, reported in relative light units (RLU – a dimensionless value, standardised as a % of maximum stimulation with anandamide), was measured on a standard luminescence plate reader.

Data were plotted as % of maximal stimulation (E_{max}) caused by agonist versus the logarithm of concentration of agonist, in the presence or absence of a fixed concentration of test compound (modulator) or vehicle. For example, stimulation in the presence of

agonist alone will give a value for stimulation expressed in RLU, which can be expressed as a range from 0% (at lowest agonist concentration, e.g., 1 nM) to 100% (at the highest agonist concentration, e.g., 10 μM). Addition of a test compound which is a positive allosteric modulator at a fixed concentration (e.g., 100 nM) will give increased RLU values leading to an increase in the maximum stimulation elicited by the agonist, which can be expressed as a % of the control (agonist alone). The percentage increase above the E_{max} value will be represented for positive allosteric modulators.

All values are expressed as the mean, and variability is expressed as S.E.M or as 95% confidence limits.

6 Bibliography:

[1] H. C. Kolb, M. G. Finn, K. B. Sharpless, *Angew. Chemie Int. Ed.* **2001**, *40*, 2004–2021.

[2] H. C. Kolb, K. B. Sharpless, *Drug Discov. Today* **2003**, *8*, 1128–1137.

[3] R. Huisgen, G. Szeimies, L. Möbius, *Chem. Ber.* **1967**, *100*, 2494–2507.

[4] C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057–3064.

[5] V. V Rostovtsev, L. G. Green, V. V Fokin, K. B. Sharpless, *Angew. Chem. Int. Ed. Engl.* **2002**, *41*, 2596–9.

[6] J. E. Hein, V. V Fokin, *Chem. Soc. Rev.* **2010**, *39*, 1302–1315.

[7] M. Meldal, C. W. Tomøe, *Chem. Rev.* **2008**, *108*, 2952–3015.

[8] J. Hou, X. Liu, J. Shen, G. Zhao, P. G. Wang, *Expert Opin. Drug Discov.* **2012**, *7*, 489–501.

[9] T. R. Chan, R. Hilgraf, K. B. Sharpless, V. V Fokin, *Org. Lett.* **2004**, *6*, 2853–5.

[10] S. Bräse, C. Gil, K. Knepper, V. Zimmermann, *Angew. Chem. Int. Ed. Engl.* **2005**, *44*, 5188–240.

[11] P. Appukkuttan, W. Dehaen, V. V Fokin, E. Van der Eycken, *Org. Lett.* **2004**, *6*, 4223–5.

[12] B. T. Worrell, J. a. Malik, V. V. Fokin, *Science* **2013**, *340*, 457–460.

[13] C. O. Kappe, E. Van der Eycken, *Chem. Soc. Rev.* **2010**, *39*, 1280–1290.

[14] F. Fazio, M. C. Bryan, O. Blixt, J. C. Paulson, C.-H. Wong, *J. Am. Chem. Soc.* **2002**, *124*, 14397–14402.

[15] E.-H. Ryu, Y. Zhao, *Org. Lett.* **2005**, *7*, 1035–7.

[16] T. Posner, *Berichte der Dtsch. Chem. Gesellschaft* **1905**, *38*, 646–657.

[17] C. E. Hoyle, A. B. Lowe, C. N. Bowman, *Chem. Soc. Rev.* **2010**, *39*, 1355–1387.

[18] C. E. Hoyle, T. Y. Lee, T. Roper, *J. Polym. Sci. Part A Polym. Chem.* **2004**, *42*, 5301–5338.

[19] F. Dénès, M. Pichowicz, G. Povie, P. Renaud, *Chem. Rev.* **2014**, *114*, 2587–2693.

[20] C. Ferreri, A. Samadi, F. Sassatelli, L. Landi, C. Chatgilialoglu, *J. Am. Chem. Soc.* **2004**, *126*, 1063–1072.

[21] W. D. Cook, F. Chen, D. W. Pattison, P. Hopson, M. Beaujon, *Polym. Int.* **2007**, *56*, 1572–1579.

[22] L. M. Campos, K. L. Killops, R. Sakai, J. M. J. Paulusse, D. Damiron, E. Drockenmuller, B. W. Messmore, C. J. Hawker, *Macromolecules* **2008**, *41*, 7063–7070.

[23] M. Uygun, M. A. Tasdelen, Y. Yagci, *Macromol. Chem. Phys.* **2010**, *211*, 103–110.

[24] S. P. S. Koo, M. M. Stamenović, R. A. Prasath, A. J. Inglis, F. E. Du Prez, C. Barner-Kowollik, W. Van Camp, T. Junkers, *J. Polym. Sci. Part A Polym. Chem.* **2010**, *48*, 1699–1713.

[25] A. Dondoni, *Angew. Chem. Int. Ed. Engl.* **2008**, *47*, 8995–7.

[26] D. P. Nair, M. Podgórski, S. Chatani, T. Gong, W. Xi, C. R. Fenoli, C. N. Bowman, *Chem. Mater.* **2014**, *26*, 724–744.

[27] A. B. Lowe, *Polym. Chem.* **2010**, *1*, 17.

[28] J. W. Chan, C. E. Hoyle, A. B. Lowe, M. Bowman, *Macromolecules* **2010**, *43*, 6381–6388.

[29] E. P. Kohler, H. Potter, *J. Am. Chem. Soc.* **1935**, *57*, 1316–1321.

[30] A. Massi, D. Nanni, *Org. Biomol. Chem.* **2012**, *10*, 3791–807.

[31] B. D. Fairbanks, T. F. Scott, C. J. Kloxin, K. S. Anseth, C. N. Bowman, *Macromolecules* **2009**, *42*, 211–217.

[32] B. D. Fairbanks, E. A. Sims, K. S. Anseth, C. N. Bowman, *Macromolecules* **2010**, *43*, 4113–4119.

[33] A. B. Lowe, *Polymer (Guildf).* **2014**, *55*, 5517–5549.

[34] M. Lo Conte, S. Staderini, A. Marra, M. Sanchez-Navarro, B. G. Davis, A. Dondoni, *Chem. Commun. (Camb).* **2011**, *47*, 11086–8.

[35] M. Minozzi, A. Monesi, D. Nanni, P. Spagnolo, N. Marchetti, A. Massi, *J. Org. Chem.* **2011**, *76*, 450–9.

[36] M. Lo Conte, S. Pacifico, A. Chambery, A. Marra, A. Dondoni, *J. Org. Chem.* **2010**, *75*, 4644–7.

[37] M. Lo Conte, S. Staderini, A. Marra, M. Sanchez-Navarro, B. G. Davis, A. Dondoni, *Chem. Commun. (Camb).* **2011**, *47*, 11086–8.

[38] G. Barany, R. B. Merrifield, *J. Am. Chem. Soc.* **1977**, *99*, 7363–7365.

[39] R. K. Iha, K. L. Wooley, A. M. Nyström, D. J. Burke, M. J. Kade, C. J. Hawker, *Chem. Rev.* **2009**, *109*, 5620–5686.

[40] I. Javakhishvili, W. H. Binder, S. Tanner, S. Hvilsted, *Polym. Chem.* **2010**, *1*, 506.

[41] J. Budhathoki-Uprety, J. F. Reuther, B. M. Novak, *Macromolecules* **2012**, *45*, 8155–8165.

[42] K. Öberg, Y. Hed, I. J. Rahmn, J. Kelly, P. Löwenhielm, M. Malkoch, *Chem. Commun. (Camb).* **2013**, *49*, 6938–40.

[43] J. A. van Hensbergen, R. P. Burford, A. B. Lowe, *Polym. Chem.* **2014**, *5*, 5339.

[44] J. W. Chan, C. E. Hoyle, A. B. Lowe, *J. Am. Chem. Soc.* **2009**, *131*, 5751–3.

[45] E. M. Sletten, C. R. Bertozzi, *Acc. Chem. Res.* **2011**, *44*, 666–676.

[46] J. C. Jewett, C. R. Bertozzi, *Chem. Soc. Rev.* **2010**, *39*, 1272–1279.

[47] P. Ostrovskis, C. M. R. Volla, M. Turks, D. Markovic, *Curr. Org. Chem* **2013**, *17*, 610–640.

[48] C. Remzi Becer, R. Hoogenboom, U. S. Schubert, *Angew. Chemie - Int. Ed.* **2009**, *48*, 4900–4908.

[49] E. Virtanen, E. Kolehmainen, *European J. Org. Chem.* **2004**, *2004*, 3385–3399.

[50] S. Mukhopadhyay, U. Maitra, *Curr. Sci.* **2004**, *87*, 1666–1683.

[51] a. F. Hofmann, L. R. Hagey, *Cell. Mol. Life Sci.* **2008**, *65*, 2461–2483.

[52] K. V Kowdley, *Am. J. Med.* **2000**, *108*, 481–486.

[53] A. F. Hofmann, L. R. Hagey, *J. Lipid Res.* **2014**, *55*, 1553–1595.

[54] K. Mitamura, N. Hori, T. Iida, M. Suzuki, T. Shimizu, H. Nittono, K. Takaori, H. Takikawa, A. F. Hofmann, S. Ikegawa, *Steroids* **2011**, *76*, 1609–14.

[55] H.-U. Marschall, W. J. Griffiths, U. Götze, J. Zhang, H. Wietholtz, N. Busch, J. Sjövall, S. Matern, *Hepatology* **1994**, *20*, 845–853.

[56] M. J. Whiting, *Adv. Clin. Chem.* **1986**, *25*, 169–232.

[57] A. F. Hofmann, K. J. Mysels, *Colloids and Surfaces* **1987**, *30*, 145–173.

[58] D. W. Russell, K. D. R. Setchell, *Biochemistry* **1992**, *31*, 4737–4749.

[59] D. F. Jelinek, S. Andersson, C. A. Slaughter, D. W. Russell, *J. Biol. Chem.* **1990**, *265*, 8190–8197.

[60] A F. Hofmann, *Hepatology* **1984**, *4*, 4S–14S.

[61] P. A. Dawson, M. Hubbert, J. Haywood, A. L. Craddock, N. Zerangue, W. V Christian, N. Ballatori, *J. Biol. Chem.* **2005**, *280*, 6960–8.

[62] P. J. Meier, B. Stieger, *Annu. Rev. Physiol.* **2002**, *64*, 635–661.

[63] G. P. Van Berge-Henegouwen, A. F. Hofmann, *Eur. J. Clin. Invest.* **1983**, *13*, 433–437.

[64] J. M. Ridlon, D.-J. Kang, P. B. Hylemon, *J. Lipid Res.* **2006**, *47*, 241–59.

[65] J. Y. L. Chiang, *J. Hepatol.* **2004**, *40*, 539–51.

[66] C. Gruy-Kapral, K. H. Little, J. S. Fordtran, T. L. Meziere, L. R. Hagey, A. F. Hofmann, *Gastroenterology* **1999**, *116*, 15–21.

[67] G. Mazzella, N. Rizzo, F. Azzaroli, P. Simoni, L. Bovicelli, A. Miracolo, G. Simonazzi, A. Colecchia, G. Nigro, C. Mwangemi, D. Festi, E. Roda, *Hepatology* **2001**, *33*, 504–8.

[68] U. Leuschner, M. Leuschner, J. Sieratzki, W. Kurtz, K. Hübner, *Dig. Dis. Sci.* **1985**, *30*, 642–649.

[69] R. Sharma, a. Long, J. F. Gilmer, *Curr. Med. Chem.* **2011**, *18*, 4029–4052.

[70] A. Enhsen, W. Kramer, G. Wess, *Drug Discov. Today* **1998**, *3*, 409–418.

[71] D. B. Salunke, B. G. Hazra, V. S. Pore, *Curr. Med. Chem.* **2006**, *13*, 813–847.

[72] D. Vivian, J. E. Polli, *Int. J. Pharm.* **2014**, *475*, 597–604.

[73] R. Rais, S. Fletcher, J. E. Polli, *J. Pharm. Sci.* **2011**, *100*, 1184–95.

[74] S. Lee, K. Kim, T. S. Kumar, J. Lee, S. K. Kim, D. Y. Lee, Y. Lee, Y. Byun, *Bioconjug. Chem.* **2005**, *16*, 615–20.

[75] E. Sievänen, *Molecules* **2007**, *12*, 1859–1889.

[76] A. Dalpiaz, G. Paganetto, B. Pavan, M. Fogagnolo, A. Medici, S. Beggiato, D. Perrone, *Mol. Pharm.* **2012**, *9*, 957–968.

[77] M. J. Rogers, J. C. Crockett, F. P. Coxon, J. Mönkkönen, *Bone* **2011**, *49*, 34–41.

[78] R. Graham, G. Russell, *Phosphorus. Sulfur. Silicon Relat. Elem.* **1999**, *144*, 793–820.

[79] S. E. Papapoulos, J. O. Landman, O. L. M. Bijvoet, C. W. G. M. Löwik, R. Valkema, E. K. J. Pauwels, P. Vermeij, *Bone* **1992**, *13*, S41–S49.

[80] H. Fleish, W. F. Neuman, *Am J Physiol -- Leg. Content* **1961**, *200*, 1296–1300.

[81] H. Fleisch, R. G. G. Russell, F. Straumann, *Nature* **1966**, *212*, 901–903.

[82] H. A. Fleisch, R. G. G. Russell, S. Bisaz, R. C. Mühlbauer, D. A. Williams, *Eur. J. Clin. Invest.* **1970**, *1*, 12–18.

[83] H. Fleisch, R. Graham, G. Russell, M. D. Francis, *Science* **1969**, *165*, 1262–1264.

[84] R. G. Russell, M. J. Rogers, J. C. Frith, S. P. Luckman, F. P. Coxon, H. L. Benford, P. I. Croucher, C. Shipman, H. a Fleisch, *J. Bone Miner. Res.* **1999**, *14 Suppl 2*, 53–65.

[85] R. G. G. Russell, *Ann. N. Y. Acad. Sci.* **2006**, *1068*, 367–401.

[86] F. H. Ebetino, A.-M. L. Hogan, S. Sun, M. K. Tsoumpra, X. Duan, J. T. Triffitt, A. a Kwaasi, J. E. Dunford, B. L. Barnett, U. Oppermann, et al., *Bone* **2011**, *49*, 20–33.

[87] F. P. Coxon, K. Thompson, A. J. Roelofs, F. H. Ebetino, M. J. Rogers, *Bone* **2008**, *42*, 848–60.

[88] A. A. Reszka, G. a. Rodan, *Curr. Osteoporos. Rep.* **2003**, *1*, 45–52.

[89] P. P. Lehenkari, *Mol. Pharmacol.* **2002**, *61*, 1255–1262.

[90] D. Amin, S. a Cornell, S. K. Gustafson, S. J. Needle, J. W. Ullrich, G. E. Bilder, M. H. Perrone, *J. Lipid Res.* **1992**, *33*, 1657–1663.

[91] J. D. Bergstrom, R. G. Bostedor, P. J. Masarachia, A. A. Reszka, G. Rodan, *Arch. Biochem. Biophys.* **2000**, *373*, 231–41.

[92] E. van Beek, E. Pieterman, L. Cohen, C. Löwik, S. Papapoulos, *Biochem. Biophys. Res. Commun.* **1999**, *264*, 108–11.

[93] J. E. Dunford, K. Thompson, F. P. Coxon, S. P. Luckman, F. M. Hahn, C. D. Poulter, F. H. Ebetino, M. J. Rogers, *J. Pharmacol. Exp. Ther.* **2001**, *296*, 235–242.

[94] M. K. Dhar, A. Koul, S. Kaul, *N. Biotechnol.* **2013**, *30*, 114–123.

[95] F. P. Coxon, K. Thompson, M. J. Rogers, *Curr. Opin. Pharmacol.* **2006**, *6*, 307–12.

[96] J. E. Dunford, A. A. Kwaasi, M. J. Rogers, B. L. Barnett, F. H. Ebetino, R. G. G. Russell, U. Oppermann, K. L. Kavanagh, *J. Med. Chem.* **2008**, *51*, 2187–95.

[97] A. J. Roelofs, F. P. Coxon, F. H. Ebetino, M. W. Lundy, Z. J. Henneman, G. H. Nancollas, S. Sun, K. M. Blazewska, J. L. F. Bala, B. A. Kashemirov, et al., *J. Bone Miner. Res.* **2010**, *25*, 606–16.

[98] S. Sun, K. M. Błażewska, B. a. Kashemirov, A. J. Roelofs, F. P. Coxon, M. J. Rogers, F. H. Ebetino, M. J. McKenna, C. E. McKenna, *Phosphorus. Sulfur. Silicon Relat. Elem.* **2011**, *186*, 970–971.

[99] B. a. Kashemirov, J. L. F. Bala, X. Chen, F. H. Ebetino, Z. Xia, R. G. G. Russell, F. P. Coxon, A. J. Roelofs, M. J. Rogers, C. E. McKenna, *Bioconjug. Chem.* **2008**, *19*, 2308–2310.

[100] J. M. Sanders, Y. Song, J. M. W. Chan, Y. Zhang, S. Jennings, T. Kosztowski, S. Odeh, R. Flessner, C. Schwerdtfeger, E. Kotsikorou, et al., *J. Med. Chem.* **2005**, *48*, 2957–2963.

[101] A. J. Roelofs, C. a Stewart, S. Sun, K. M. Błażewska, B. a Kashemirov, C. E. McKenna, R. G. G. Russell, M. J. Rogers, M. W. Lundy, F. H. Ebetino, et al., *J. Bone Miner. Res.* **2012**, *27*, 835–47.

[102] J. A F. Vermeer, I. D. C. Jansen, M. Marthi, F. P. Coxon, C. E. McKenna, S. Sun, T. J. de Vries, V. Everts, *Bone* **2013**, *57*, 242–251.

[103] B. Abrahamsen, *Calcif. Tissue Int.* **2010**, *86*, 421–35.

[104] S. Bae, S. Sun, T. Aghaloo, J.-E. Oh, C. E. McKenna, M. K. Kang, K.-H. Shin, S. Tetradis, N.-H. Park, R. H. Kim, *Int. J. Mol. Med.* **2014**, *34*, 559–63.

[105] A. Ezra, G. Golomb, *Adv. Drug Deliv. Rev.* **2000**, *42*, 175–195.

[106] M. Fazil, S. Baboota, J. K. Sahni, Ameeduzzafar, J. Ali, *Drug Deliv.* **2015**, *22*, 1–9.

[107] J. W. Park, S. R. Hwang, O. C. Jeon, H. T. Moon, Y. Byun, *J. Pharm. Sci.* **2013**, *102*, 341–346.

[108] J. W. Park, Y. Byun, *Arch. Pharm. Res.* **2013**, *37*, 1560–1569.

[109] B. A. Tertov, A. V. Koblik, *Chem. Heterocycl. Compd.* **1969**, *3*, 751.

[110] W. J. Houlihan, Ed. , *Chemistry of Heterocyclic Compounds*, John Wiley & Sons, Inc., Hoboken, NJ, USA, **1971**.

[111] S. Clementi, P. Linda, G. Marino, *J. Chem. Soc. Chem. Commun.* **1972**, 427b.

[112] S. Lancianesi, A. Palmieri, M. Petrini, *Chem. Rev.* **2014**, *114*, 7108–49.

[113] J. George, B. V. S. Reddy, *Org. Biomol. Chem.* **2012**, *10*, 4731–8.

[114] A. Barakat, M. S. Islam, A. M. A. Al Majid, Z. A. Al-Othman, *Tetrahedron* **2013**, *69*, 5185–5192.

[115] G. R. Humphrey, J. T. Kuethe, *Chem. Rev.* **2006**, *106*, 2875–2911.

[116] R. Vicente, *Org. Biomol. Chem.* **2011**, *9*, 6469–80.

[117] G. W. Gribble, *J. Chem. Soc. Perkin Trans. 1* **2000**, 1045–1075.

[118] D. F. Taber, P. K. Tirunahari, *Tetrahedron* **2011**, *67*, 7195–7210.

[119] G. Bartoli, R. Dalpozzo, M. Nardi, *Chem. Soc. Rev.* **2014**, *43*, 4728–50.

[120] M. Inman, C. J. Moody, *Chem. Sci.* **2013**, 29–41.

[121] E. Fischer, O. Hess, *Berichte der Dtsch. Chem. Gesellschaft* **1884**, *17*, 559–568.

[122] E. Fischer, F. Jourdan, *Berichte der Dtsch. Chem. Gesellschaft* **1883**, *16*, 2241–2245.

[123] B. Robinson, *Chem. Rev.* **1963**, *63*, 373–401.

[124] S. M. Hutchins, K. T. Chapman, *Tetrahedron Lett.* **1996**, *37*, 4869–4872.

[125] S. Wagaw, B. H. Yang, S. L. Buchwald, *J. Am. Chem. Soc.* **1998**, *120*, 6621–6622.

[126] K. Matcha, A. P. Antonchick, *Angew. Chem. Int. Ed. Engl.* **2014**, *53*, 11960–4.

[127] Z. Wang, *Comprehensive Organic Name Reactions and Reagents*, John Wiley & Sons, Inc., Hoboken, NJ, USA, **2010**.

[128] S. Müller, M. J. Webber, B. List, *J. Am. Chem. Soc.* **2011**, *133*, 18534–18537.

[129] B. L. Li, D.-Q. Xu, A. G. Zhong, *J. Fluor. Chem.* **2012**, *144*, 45–50.

[130] S. Gore, S. Baskaran, B. König, *Org. Lett.* **2012**, *14*, 4568–4571.

[131] R. C. Larock, E. K. Yum, *J. Am. Chem. Soc.* **1991**, *113*, 6689–6690.

[132] R. C. Larock, E. K. Yum, M. D. Refvik, *J. Org. Chem.* **1998**, *63*, 7652–7662.

[133] M. Shen, G. Li, B. Z. Lu, A. Hossain, F. Roschangar, V. Farina, C. H. Senanayake, *Org. Lett.* **2004**, *6*, 4129–32.

[134] O. Leogane, H. Lebel, *Angew. Chem. Int. Ed. Engl.* **2008**, *47*, 350–2.

[135] I. Nakamura, T. Nemoto, N. Shiraiwa, M. Terada, *Org. Lett.* **2009**, *11*, 1055–8.

[136] D. Zhang, K. Y. Eul, Z. Liu, R. C. Larock, *Org. Lett.* **2005**, *7*, 4963–4966.

[137] S. Cacchi, G. Fabrizi, *Chem. Rev.* **2005**, *105*, 2873–2920.

[138] T. Guo, F. Huang, L. Yu, Z. Yu, *Tetrahedron Lett.* **2015**, *56*, 296–302.

[139] B. E. Evans, K. E. Rittle, M. G. Bock, R. M. DiPardo, R. M. Freidinger, W. L. Whitter, G. F. Lundell, D. F. Veber, P. S. Anderson, *J. Med. Chem.* **1988**, *31*, 2235–2246.

[140] L. Costantino, D. Barlocco, *Curr. Med. Chem.* **2006**, *13*, 65–85.

[141] H. Johansson, T. B. Jørgensen, D. E. Gloriam, H. Bräuner-Osborne, D. S. Pedersen, *RSC Adv.* **2013**, *3*, 945.

[142] M. El-Sayed, R. Verpoorte, *Phytochem. Rev.* **2007**, *6*, 277–305.

[143] Y. Ban, Y. Murakami, Y. Iwasawa, M. Tsuchiya, N. Takano, *Med. Res. Rev.* **1988**, *8*, 231–308.

[144] N. K. Kaushik, N. Kaushik, P. Attri, N. Kumar, C. H. Kim, A. K. Verma, E. H. Choi, *Molecules* **2013**, *18*, 6620–6662.

[145] Y. Gaoni, R. Mechoulam, *J. Am. Chem. Soc.* **1964**, *86*, 1646–1647.

[146] R. Mechoulam, L. O. Hanuš, R. Pertwee, A. C. Howlett, *Nat. Rev. Neurosci.* **2014**, *15*, 757–764.

[147] P. Zogopoulos, I. Vasileiou, E. Patsouris, S. E. Theocharis, *Fundam. Clin. Pharmacol.* **2013**, *27*, 64–80.

[148] P. Pacher, S. Bátkai, G. Kunos, *Pharmacol. Rev.* **2006**, *58*, 389–462.

[149] A C. Howlett, F. Barth, T. I. Bonner, G. Cabral, P. Casellas, W. a Devane, C. C. Felder, M. Herkenham, K. Mackie, B. R. Martin, et al., *Pharmacol. Rev.* **2002**, *54*, 161–202.

[150] F. R. Kreitzer, N. Stella, *Pharmacol. Ther.* **2009**, *122*, 83–96.

[151] C. M. Henstridge, *Pharmacology* **2012**, *89*, 179–87.

[152] E. Ryberg, N. Larsson, S. Sjögren, S. Hjorth, N.-O. Hermansson, J. Leonova, T. Elebring, K. Nilsson, T. Drmota, P. J. Greasley, *Br. J. Pharmacol.* **2007**, *152*, 1092–101.

[153] M. P. Davis, *Expert Opin. Investig. Drugs* **2014**, 1–18.

[154] F. Fezza, M. Bari, R. Florio, E. Talamonti, M. Feole, M. Maccarrone, *Molecules* **2014**, *19*, 17078–17106.

[155] B. E. Alger, *Prog. Neurobiol.* **2002**, *68*, 247–286.

[156] P. C. Schmid, P. V Reddy, V. Natarajan, H. H. Schmid, *J. Biol. Chem.* **1983**, *258*, 9302–9306.

[157] T. Sugiura, S. Kishimoto, S. Oka, M. Gokoh, *Prog. Lipid Res.* **2006**, *45*, 405–46.

[158] C. J. Hillard, W. S. Edgemond, A. Jarrahian, W. B. Campbell, *J. Neurochem.* **2002**, *69*, 631–638.

[159] M. D. Jhaveri, D. Richardson, V. Chapman, *Br. J. Pharmacol.* **2007**, *152*, 624–32.

[160] G. Pryce, C. Visintin, S. V. Ramagopalan, S. Al-Izki, L. E. De Faveri, R. a. Nuamah, C. a. Mein, A. Montpetit, A. J. Hardcastle, G. Kooij, et al., *FASEB J.* **2014**, *28*, 117–130.

[161] J. M. Adam, J. K. Clark, K. Davies, K. Everett, R. Fields, S. Francis, F. Jeremiah, T. Kiyoi, M. Maidment, A. Morrison, et al., *Bioorg. Med. Chem. Lett.* **2012**, *22*, 2932–7.

[162] D. R. Janero, L. Lindsley, V. K. Vemuri, A. Makriyannis, *Expert Opin. Drug Discov.* **2011**, *6*, 995–1025.

[163] C.-P. Chang, C.-H. Wu, J.-S. Song, M.-C. Chou, Y.-C. Wong, Y. Lin, T.-K. Yeh, A. a Sadani, M.-H. Ou, K.-H. Chen, et al., *J. Med. Chem.* **2013**, *56*, 9920–33.

[164] R. Guidetti, P. C. Astles, A. J. Sanderson, S. P. Hollinshead, M. P. Johnson, M. G. Chambers, *Bioorg. Med. Chem. Lett.* **2014**, *24*, 5572–5.

[165] G. C. Hsieh, M. Pai, P. Chandran, B. A. Hooker, C. Z. Zhu, A. K. Salyers, E. J. Wensink, C. Zhan, W. A. Carroll, M. J. Dart, et al., *Br. J. Pharmacol.* **2011**, *162*, 428–40.

[166] S. Nicolussi, J. M. Viveros-Paredes, M. S. Gachet, M. Rau, M. E. Flores-Soto, M. Blunder, J. Gertsch, *Pharmacol. Res.* **2014**, *80*, 52–65.

[167] F. Nasirinezhad, S. Jergova, J. P. Pearson, J. Sagen, *Neuropharmacology* **2014**, in Press.

[168] M. R. Price, G. L. Baillie, A. Thomas, L. a Stevenson, M. Easson, R. Goodwin, A. McLean, L. McIntosh, G. Goodwin, G. Walker, et al., *Mol. Pharmacol.* **2005**, *68*, 1484–95.

[169] K. H. Ahn, M. M. Mahmoud, D. a. Kendall, *J. Biol. Chem.* **2012**, *287*, 12070–12082.

[170] T. F. Gamage, B. M. Ignatowska-Jankowska, J. L. Wiley, M. Abdelrahman, L. Trembleau, I. R. Greig, G. A. Thakur, R. Tichkule, J. Poklis, R. A. Ross, et al., *Behav. Pharmacol.* **2014**, *25*, 182–5.

[171] D. M. Shore, G. L. Baillie, D. H. Hurst, F. Navas, H. H. Seltzman, J. P. Marcu, M. E. Abood, R. a Ross, P. H. Reggio, *J. Biol. Chem.* **2014**, *289*, 5828–45.

[172] G. A. Thakur, P. M. Kulkarni, PCT, WO 2013/103967 A1.

[173] F. Piscitelli, A. Ligresti, G. La Regina, A. Coluccia, L. Morera, M. Allarà, E. Novellino, V. Di Marzo, R. Silvestri, *J. Med. Chem.* **2012**, *55*, 5627–31.

[174] D. Verzele, S. Figaroli, A. Madder, *Molecules* **2011**, *16*, 10168–86.

[175] U. Maitra, *Org. Biomol. Chem.* **2008**, *6*, 657.

[176] J. Tamminen, E. Kolehmainen, *Molecules* **2001**, *6*, 21–46.

[177] Y. Li, J. R. Dias, *Chem. Rev.* **1997**, *97*, 283–304.

[178] R. Sharma, F. Majer, V. K. Peta, J. Wang, R. Keaveney, D. Kelleher, A. Long, J. F. Gilmer, *Bioorg. Med. Chem.* **2010**, *18*, 6886–95.

[179] N. Deuk Kim, *Curr. Cancer Drug Targets* **2015**, *6*.

[180] L. Mrózek, L. Dvořáková, Z. Mandelová, L. Rárová, A. Řezáčová, L. Plaček, R. Opatřilová, J. Dohnal, O. Paleta, V. Král, et al., *Steroids* **2011**, *76*, 1082–97.

[181] L. Coufalová, L. Mrózek, L. Rárová, L. Plaček, R. Opatřilová, J. Dohnal, K. Král'ová, O. Paleta, V. Král, P. Drašar, et al., *Steroids* **2013**, *78*, 435–53.

[182] V. D. Bock, H. Hiemstra, J. H. Van Maarseveen, *European J. Org. Chem.* **2006**, *2006*, 51–68.

[183] D. Wu, S. Ji, Y. Wu, Y. Ju, Y. Zhao, *Bioorganic Med. Chem. Lett.* **2007**, *17*, 2983–2986.

[184] L. Ma, M. Melegari, M. Colombini, J. T. Davis, *J. Am. Chem. Soc.* **2008**, *130*, 2938–9.

[185] T. Robak, *Curr. Cancer Drug Targets* **2015**, *5*.

[186] T. Robak, A. Korycka, E. Lech-Maranda, P. Robak, *Molecules* **2009**, *14*, 1183–226.

[187] C. M. Galmarini, J. R. Mackey, C. Dumontet, *Lancet Oncol.* **2002**, *3*, 415–424.

[188] D. Genini, S. Adachi, Q. Chao, D. W. Rose, C. J. Carrera, H. B. Cottam, D. A. Carson, L. M. Leoni, *Blood* **2000**, *96*, 3537–43.

[189] E. Van Den Neste, S. Cardoen, F. Offner, F. Bontemps, *Int. J. Oncol.* **2005**, *27*, 1113–1124.

[190] B. Dayal, K. Rao, G. Salen, *Steroids* **1995**, *60*, 453–457.

[191] Z. Łotowski, D. Guzmański, *Monatshefte fur Chemie* **2006**, *137*, 117–124.

[192] L. J. Lawless, A. G. Blackburn, A. J. Ayling, M. N. Pérez-Payán, A. P. Davis, *J. Chem. Soc. Perkin Trans. 1* **2001**, 1329–1341.

[193] N. G. Aher, V. S. Pore, S. P. Patil, *Tetrahedron* **2007**, *63*, 12927–12934.

[194] G. Sagi, L. Otvos, S. Ikeda, G. Andrei, R. Snoeck, E. De Clercq, *J. Med. Chem.* **1994**, *37*, 1307–1311.

[195] K. Sonogashira, Y. Tohda, N. Hagihara, *Tetrahedron Lett.* **1975**, *16*, 4467–4470.

[196] F. Denizot, R. Lang, *J. Immunol. Methods* **1986**, *89*, 271–277.

[197] D. Brossard, L. El Kihel, M. Clément, W. Sebbahi, M. Khalid, C. Roussakis, S. Rault, *Eur. J. Med. Chem.* **2010**, *45*, 2912–8.

[198] O. Bortolini, G. Fantin, M. Fogagnolo, S. Rossetti, L. Maiuolo, G. Di Pompo, S. Avnet, D. Granchi, *Eur. J. Med. Chem.* **2012**, *52*, 221–9.

[199] D. Perrone, O. Bortolini, M. Fogagnolo, E. Marchesi, L. Mari, C. Massarenti, M. L. Navacchia, F. Sforza, K. Varani, M. L. Capobianco, *New J. Chem.* **2013**, *37*, 3559.

[200] E. J. Corey, S. G. Pyne, W. Su, *Tetrahedron Lett.* **1983**, *24*, 4883–4886.

[201] C. R. Degenhardt, D. C. Burdsall, *J. Org. Chem.* **1986**, *51*, 3488–3490.

[202] M. Weinhart, D. Gröger, S. Enders, J. Dernedde, R. Haag, *Biomacromolecules* **2011**, *12*, 2502–11.

[203] P. C. Bulman Page, J. P. . Moore, I. Mansfield, M. J. McKenzie, W. B. Bowler, J. a Gallagher, *Tetrahedron* **2001**, *57*, 1837–1847.

[204] H. Skarpos, S. N. Osipov, D. V Vorob'eva, I. L. Odinets, E. Lork, G.-V. Röschenthaler, *Org. Biomol. Chem.* **2007**, *5*, 2361–7.

[205] G. V. Reddy, H. K. Jacobs, A. S. Gopalan, R. E. Barrans, M. L. Dietz, D. C. Stepinski, A. W. Herlinger, *Synth. Commun.* **2004**, *34*, 331–344.

[206] G. Weber, *Biochem. J.* **1952**, *51*, 155–67.

[207] L. Li, J. Li, A. Kulkarni, S. Liu, *J. Mater. Chem. B* **2013**, *1*, 571.

[208] I. Javakhishvili, W. H. Binder, S. Tanner, S. Hvilsted, *Polym. Chem.* **2010**, *1*, 506.

[209] B. Iskin, G. Yilmaz, Y. Yagci, *J. Polym. Sci. Part A Polym. Chem.* **2011**, *49*, 2417–2422.

[210] A. S. Goldmann, A. Walther, L. Nebhani, R. Joso, D. Ernst, K. Loos, C. Barner-Kowollik, L. Barner, A. H. E. Müller, *Macromolecules* **2009**, *42*, 3707–3714.

[211] P. Antoni, M. J. Robb, L. Campos, M. Montanez, A. Hult, E. Malmström, M. Malkoch, C. J. Hawker, *Macromolecules* **2010**, *43*, 6625–6631.

[212] D. Simoni, N. Gebbia, F. P. Invidiata, M. Eleopra, P. Marchetti, R. Rondanin, R. Baruchello, S. Provera, C. Marchioro, M. Tolomeo, et al., *J. Med. Chem.* **2008**, *51*, 6800–7.

[213] C. E. McKenna, M. T. Higa, N. H. Cheung, M.-C. McKenna, *Tetrahedron Lett.* **1977**, *18*, 155–158.

[214] S. H. Szajnman, V. S. Rosso, L. Malayil, A. Smith, S. N. J. Moreno, R. Docampo, J. B. Rodriguez, *Org. Biomol. Chem.* **2012**, *10*, 1424–33.

[215] R. J. Barney, B. M. Wasko, A. Dudakovic, R. J. Hohl, D. F. Wiemer, *Bioorg. Med. Chem.* **2010**, *18*, 7212–20.

[216] X. Zhou, S. V Hartman, E. J. Born, J. P. Smits, S. a Holstein, D. F. Wiemer, *Bioorg. Med. Chem. Lett.* **2013**, *23*, 764–6.

[217] I. T. Horváth, J. Rábai, *Science* **1994**, *266*, 72–5.

[218] J. Gladysz, D. P. Curran, *Tetrahedron* **2002**, *58*, 3823–3825.

[219] A. Studer, *Science* **1997**, *275*, 823–826.

[220] J. Inanaga, K. Hirata, H. Saeki, T. Katsuki, M. Yamaguchi, *Bull. Chem. Soc. Jpn.* **1979**, *52*, 1989–1993.

[221] A. A. Malik, C. M. Sharts, *J. Fluor. Chem.* **1987**, *34*, 395–408.

[222] H. Uludag, *Curr. Pharm. Des.* **2002**, *8*, 1929–1944.

[223] M. De Rosa, A. Soriente, *Tetrahedron* **2010**, *66*, 2981–2986.

[224] C.-W. Kuo, C.-C. Wang, H.-L. Fang, B. R. Raju, V. Kavala, P. M. Habib, C.-F. Yao, *Molecules* **2009**, *14*, 3952–63.

[225] S. Jalal, S. Sarkar, K. Bera, S. Maiti, U. Jana, *European J. Org. Chem.* **2013**, *2013*, 4823–4828.

[226] A. P. Terent'ev, E. V. Vinogradova, V. P. Chetverikov, V. S. Lenenko, *Chem. Heterocycl. Compd.* **1972**, *5*, 196–198.

[227] K. C. Joshi, V. N. Pathak, P. Chand, *J. F. Prakt. Chemie* **1978**, 701–704.

[228] T. Sandmeyer, *Berichte der Dtsch. Chem. Gesellschaft* **1884**, *17*, 2650–2653.

[229] W. B. Austin, N. Bilow, W. J. Kelleghan, K. S. Y. Lau, *J. Org. Chem.* **1981**, *46*, 2280–2286.

[230] M. Bandini, P. Melchiorre, A. Melloni, A. Umani-Ronchi, *Synthesis (Stuttg).* **2002**, *2002*, 1110–1114.

[231] R. Severin, J. Reimer, S. Doye, *J. Org. Chem.* **2010**, *75*, 3518–3521.

[232] Y. Kondo, F. Shiga, N. Murata, T. Sakamoto, H. Yamanaka, *Tetrahedron* **1994**, *50*, 11803–11812.

[233] J. Itoh, K. Fuchibe, T. Akiyama, *Angew. Chemie* **2008**, *120*, 4080–4082.

[234] P. M. Habib, V. Kavala, C. W. Kuo, M. J. Raihan, C. F. Yao, *Tetrahedron* **2010**, *66*, 7050–7056.

[235] A. Nomland, I. D. Hills, *Tetrahedron Lett.* **2008**, *49*, 5511–5514.

[236] J. D. Violin, R. J. Lefkowitz, *Trends Pharmacol. Sci.* **2007**, *28*, 416–422.

[237] D. McGuinness, A. Malikzay, R. Visconti, K. Lin, M. Bayne, F. Monsma, C. a Lunn, *J. Biomol. Screen. Off. J. Soc. Biomol. Screen.* **2009**, *14*, 49–58.

[238] A. Wetzel, F. Gagosz, *Angew. Chemie - Int. Ed.* **2011**, *50*, 7354–7358.

[239] I. Sapountzis, H. Dube, R. Lewis, N. Gommermann, P. Knochel, *J. Org. Chem.* **2005**, *70*, 2445–2454.

[240] K. Hiroya, S. Itoh, T. Sakamoto, *J. Org. Chem.* **2004**, *69*, 1126–1136.

[241] N. Sakai, K. Annaka, A. Fujita, A. Sato, T. Konakahara, *J. Org. Chem.* **2008**, *73*, 4160–4165.

[242] S. Maity, T. Naveen, U. Sharma, D. Maiti, *Org. Lett.* **2013**, *15*, 3384–7.

NJC

RSC Publishing

PAPER

Synthesis and *in vitro* cytotoxicity of deoxyadenosine–bile acid conjugates linked with 1,2,3-triazole

Cite this: *New J. Chem.*, 2013, **37**, 3559

Daniela Perrone,*[a] Olga Bortolini,[a] Marco Fogagnolo,[a] Elena Marchesi,[b] Lara Mari,[a] Chiara Massarenti,[a] Maria Luisa Navacchia,*[c] Fabio Sforza,[b] Katia Varani[b] and Massimo Luigi Capobianco[c]

Received (in Montpellier, France) 14th May 2013,
Accepted 23rd August 2013

DOI: 10.1039/c3nj00513e

www.rsc.org/njc

We report herein the synthesis and biological evaluation of novel deoxynucleoside–bile acid conjugates linked through a 1,2,3-triazole ring. The conjugates were synthesized *via* Cu(I) mediated 1,3-dipolar cycloaddition reaction ('click' chemistry) of 3-azidobile acid derivatives and terminal alkyne moieties linked to the C-8 position of deoxyadenosine. All novel molecules were evaluated *in vitro* for their anti-proliferative activity against four human cell lines (*i.e.*, leukemic T Jurkat and K562; colon carcinoma HCT116; and ovarian cancer A2780) and their cytotoxicity toward human fibroblast cells. Several conjugates exhibited strong anti-proliferative activity against human leukemia T cells. The best cytotoxicity was observed for **HdA-CDC** on both leukemia cell lines with IC_{50} up to 8.51 μM. The apoptotic activity of several conjugates was also established.

Introduction

Among the variety of molecular scaffolds employed as building blocks for the design and synthesis of novel conjugates, bile acids and their derivatives have received a great deal of attention in many fields.[1–6] Bile acids are a class of biogenetic compounds involved in different biological processes.[7] The interest in this class of natural compounds is explained by the peculiar combination of features such as rigidity, chirality, amphiphilicity and modularity, together with their availability and low cost.

Moreover, lipophilicity, another well-known feature of bile acid structure, is believed to be correlated with induction of cytotoxicity and apoptosis in several human cancer cells.[8–11] Synthetic derivatives of hydrophobic bile acids, especially chenodeoxycholic acid and ursodeoxycholic acid conjugates, significantly inhibit cell growth and induce apoptosis in various human cancer cells, including breast carcinoma,[12] leukemic T cells,[13] prostate cancer cells,[14] stomach cancer cells,[15] colon cancer,[16] cervical carcinoma,[17] glioblastoma multiforme,[18] and hepatocellular carcinoma.[19]

The literature also describes a vast amount of bile acid conjugates with improved pharmacological profiles in terms of bioavailability and biostability, which demonstrate the further potential of modified bile acids to act as hybrid molecules and prodrug moieties in drug discovery.[3,20]

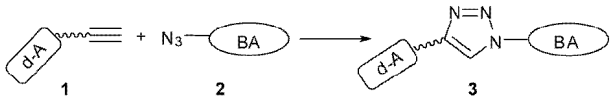
We recently synthesized a new prodrug obtained by the conjugation of zidovudine (AZT) with ursodeoxycholic acid, aimed at obviating the poor ability of AZT to permeate in the CNS or intracellular compartments.[21] At the same time, Bortolini *et al.* reported the efficient synthesis of a new class of bile acid-based hydroxyl-bisphosphonates with the aim of improving the bioavailability of bone disorder drugs.[22]

The expanding number of applications employing bile acid templates has stimulated the development of new conjugates with enhanced properties, obtained using specific and in some cases orthogonal reactions. The copper(I)-catalyzed Huisgen–Sharpless–Meldal 1,3-dipolar cycloaddition between alkynes and azides (CuAAC or 'click' chemistry) to form 1,4-disubstituted 1,2,3-triazole linkers is a well-established reaction for the preparation of bioconjugates.[23–25] 1,2,3-Triazole moieties are attractive linkers, because they are stable under typical physiological conditions and form hydrogen bonds, which can be suitable for solubility enhancement and for binding of biomolecular targets.[26–28]

Few examples of bile acid–nucleoside conjugates have been reported in the literature,[21,29–32] and to the best of our knowledge there are no synthetic approaches with 1,2,3-triazoles as connecting units, nor about the cytotoxic evaluation of bile acid–nucleoside conjugates as new potential anticancer drugs.

Purine nucleoside analogues are a pharmacologically interesting class of compounds with a long history for treatment of various viral and tumor diseases.[33–35] Particularly, several

[a] *Università degli Studi di Ferrara, Dipartimento di Scienze Chimiche e Farmaceutiche, via L. Borsari, 46, 44121 Ferrara, Italy. E-mail: daniela.perrone@unife.it*
[b] *Università degli Studi di Ferrara, Dipartimento di Scienze Mediche, corso Giovecca, 203, 44121 Ferrara, Italy*
[c] *ISOF-CNR, via P. Gobetti, 101, 40129, Bologna, Italy. E-mail: marialuisa.navacchia@isof.cnr.it*



Scheme 1 d-A = deoxyadenosine derivative, BA = bile acid part.

deoxyadenosine nucleoside analogues represent a group of cytotoxic agents with high activity in lymphoid and myeloid malignancies.[36,37]

With all this in mind, and in continuation with our recent work on labelled deoxyadenosines,[38,39] we considered of interest the synthesis and biological evaluation of bile acid–deoxyadenosine conjugates **3** (Scheme 1). They were prepared by the 'click' ligation between a terminal C≡C bond of a deoxyadenosine derivative **1** and the azide moiety of the bile acid part **2**. As a result, twelve novel bile acid-based conjugates **3** were synthesized. Their *in vitro* anti-proliferative activity against four human cell lines (*i.e.*, leukemic T Jurkat and K562; colon carcinoma HCT116; and ovarian cancer A2780) and cytotoxicity toward human fibroblast cells were evaluated.

Results and discussion

Chemistry

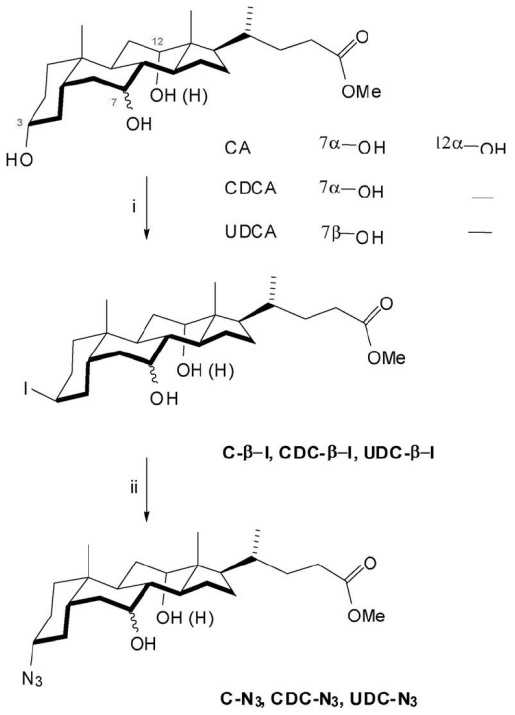
In our approach to synthesize these new conjugates using 'click' chemistry, three bile acids with different hydrophobic nature were converted into their 3α-azido derivatives. As outlined in Scheme 2, azido derivatives **C-N$_3$**, **CDC-N$_3$**, and **UDC-N$_3$** were prepared in two steps starting from the methyl esters[40] of commercially available cholic acid (CA), chenodeoxycholic acid (CDCA), and ursodeoxycholic acid (UDCA), respectively. Introduction of 3α-azides was accomplished through a double nucleophilic displacement, *i.e.*, with retention of configuration, according to a literature procedure,[41] with small modifications. Thus, 3-iodides **C-β-I**, **CDC-β-I**, and **UDC-β-I** were generated upon treatment of the corresponding unprotected bile acid methyl esters with I_2 and Ph_3P in the presence of imidazole and 1,3-dioxolane.

Although the conversion of bile acid methyl ester substrates was almost quantitative in all the cases, the lengthy separation of 3β-iodides from small quantities of not well-recognized by-products lowered the isolated yields ranging from 55 to 67%.

However, the nucleophilic displacement with iodide was selective for the less hindered 3α-OH of CA and CDCA esters, as previously reported[1,42,43] for the introduction of methanesulfonic acid as a nucleophile, under Mitsunobu conditions. In contrast, in the case of UDCA methyl ester, the formation of small amounts (less than 10%) of a diiodide derivative was pointed out according to the increased reactivity of the equatorial 7β-OH, and the further lowering of isolated yield (55%). The 3β-iodides were used for back displacement with NaN_3 in DMF to give the desired 3α-azides **C-N$_3$**, **CDC-N$_3$** and **UDC-N$_3$** in 80–94% isolated yields (Scheme 2).

For the 'click' conjugation, a series of three deoxyadenosines bearing a different alkynylic substituent on the C-8 position was selected (Fig. 1). The thiopurine **SdA-A** was prepared by coupling of 8-bromodeoxyadenosine with hexyne thiol, as described in our recent work.[38,39] The synthesis of the 8-substituted adenosine **HdA-A** was carried out by a palladium catalyzed cross-coupling reaction starting from commercially available 8-bromoadenosine as reported for the synthesis of compound **dA-A**.[44] For details see the Experimental section. Lastly, in order to further explore the requirements for bile acid-based conjugate cytotoxicity we decided to add propargyl alcohol (**HM-A**, Fig. 1) to the series of terminal alkynes.

Having the corresponding sets of 3-azidobile acids and 8-alkynylated deoxyadenosines in hand, our next aim was to



Scheme 2 *Reagents and conditions*: (i) I_2, PPh_3, imidazole, 1,3-dioxolane, 25 °C, 30 min, 55–67%; (ii) NaN_3, DMF, 25 °C, 6 h, 80–94%.

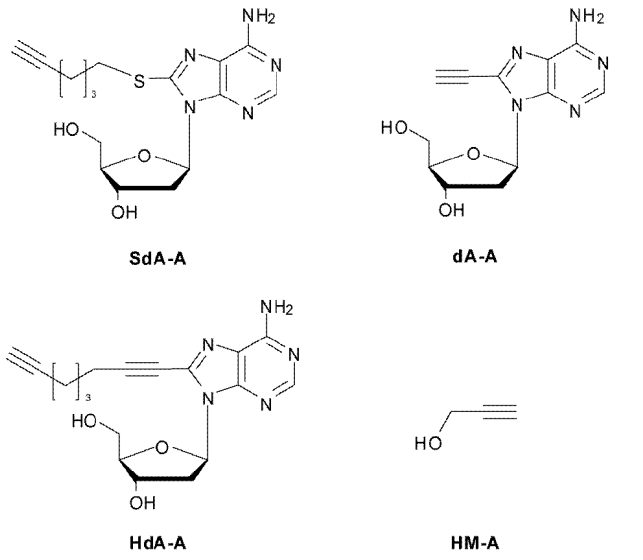


Fig. 1 Alkynes used for click reactions.

synthesize target bile acid–deoxyadenosine conjugates linked with a 1,2,3-triazole moiety. Azide **CDC-N_3** and terminal alkyne **SdA-A** were selected to explore the feasibility of the 'click' chemistry for the construction of novel conjugates.

The cycloaddition reaction was attempted under commonly used conditions: the *t*BuOH–H_2O mixture in the presence of the $CuSO_4$ catalyst and sodium ascorbate.[25] At first, using 0.04 molar equiv. of the catalyst and 2 molar excess of sodium ascorbate, we observed small amounts of conversion compound, even after 72 h at room temperature (by TLC and ^{1}H-NMR analysis). An analogous experiment with CuI and DIPEA[24] did not improve the conversion. We obtained the best result using 0.4 molar equiv. of $CuSO_4{\cdot}5H_2O$ relative to the alkyne, in the THF–*t*BuOH–H_2O mixture (Scheme 3). THF was the most effective co-solvent tested to dissolve lipophilic azide **CDC-N_3** in combination with water and *t*BuOH. The conversion was complete after 18 h and allowed the synthesis of the desired conjugate **SdA-CDC** in 70% yield after chromatographic purification. We carried out the same protocol to perform 'click' conjugation of all terminal alkynes shown in Fig. 1 with azidobile acid derivatives shown in Scheme 2. All reactions proceeded to completion with isolated yields of conjugates ranging from 68 to 85% as summarized in Table 1.

Despite being satisfied with our results, we evaluated to investigate 'click' reaction under microwave conditions aimed at improving the yields of conjugate compounds. The CuAAC reaction of **SdA-A** with **CDC-N_3** under microwave irradiation at 80 °C for 30 min with a 1 : 1.5 : 0.4 : 2 molar ratio of alkyne, azide, $CuSO_4{\cdot}5H_2O$, and sodium ascorbate in the THF–*t*BuOH–H_2O mixture (1.5 : 1 : 1, v/v) proceeded with complete conversion, nevertheless affording conjugate **SdA-CDC** in 73% isolated yield. Other optimization attempts failed, so only a rate enhancement of microwave assisted conjugation reaction was observed. All conjugated compounds were fully characterized by ^{1}H, ^{13}C NMR, IR spectra as well as by mass analysis.

Biological evaluation

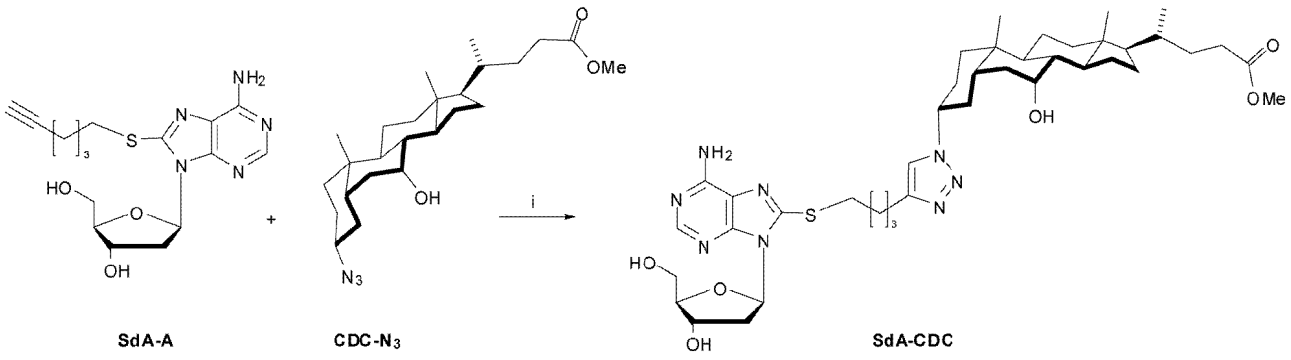
The conjugated compounds reported in Table 1, as well as the free alkynyl deoxyadenosines **dA-A**, **HdA-A**, and **SdA-A** shown in Fig. 1, were evaluated for their cytotoxic activity against human cells of different histopathological origin: two leukemia cell lines (Jurkat and K562), a colon cancer cell line (HCT116), an ovarian cancer cell line (A2780) and, as a control, normal human skin fibroblast cells. Cisplatin served as a reference compound.

Table 1 Isolated yields (%) of conjugate compounds

	Azide		
Alkyne	**C-N_3**	**CDC-N_3**	**UDC-N_3**
SdA-A	**SdA-C** (68%)	**SdA-CDC** (70%)	**SdA-UDC** (73%)
HdA-A	**HdA-C** (75%)	**HdA-CDC** (80%)	**HdA-UDC** (75%)
dA-A	**dA-C** (70%)	**dA-CDC** (75%)	**dA-UDC** (68%)
HM-A	**HM-C** (85%)	**HM-CDC** (80%)	**HM-UDC** (83%)

The cytotoxicity was evaluated using MTT assay,[45] as described in the Experimental section. Cell growth inhibition was determined at concentrations of 200, 100, 50, and 10 μM for all compounds after 72 h. The estimated IC_{50} values in comparison with that for cisplatin are shown in Table 2. Data for all the conjugated compounds against the K562 line as the mean of three independent experiments are graphically represented in Fig. 2. Similar results were obtained for the Jurkat cell line (data not shown).

No cytotoxicity was observed with **dA**–, **HdA**– and **SdA**–bile acid conjugates on HCT116 and A2780 cell lines up to 200 μM concentration. In contrast, some of these conjugates showed a significant concentration-dependent anti-proliferative effect on both leukemia cell lines. The highest activity was obtained with **CDC**-derivatives. Particularly, the best cytotoxicity was observed for **HdA-CDC** on Jurkat and K562 cell lines with IC_{50} values of 10.47 and 8.51 μM respectively. These values were quite close to the cisplatin ones, whereas **HdA-CDC** was not toxic against healthy fibroblast cells. **SdA-CDC** also displayed a substantial activity with IC_{50} 16.76–22.05 μM. Otherwise, **UDC**-based conjugates showed good cytotoxicity only on Jurkat line. A poor anti-proliferative effect was observed on the leukemia cell lines with compounds **dA-C**, **HdA-C**, and **SdA-C** including a cholic acid scaffold. On the other hand, the lipophilicity (log *P*) of all bile acid conjugates was predicted from structures using the MarvinSketch program (Table 2). The data in Table 2 show that compounds **HdA/SdA-CDC** and **HdA/SdA-UDC** have the highest lipophilicity, suggesting that a relationship between bile acid lipophilicity and cytotoxic activity of these conjugates can be assumed. This is consistent with several studies on bile acid derivatives, recently reported by other groups.[8,11,46]



Scheme 3 *Reagents and conditions*: $CuSO_4{\cdot}5H_2O$, sodium ascorbate, THF–*t*BuOH–H_2O (1.5 : 1 : 1), 25 °C, 18 h, 70%; or microwave 80 °C, 30 min, 73%.

Table 2 Cytotoxic activity of bile acid-based conjugates and alkynyl deoxyadenosines **dA-A**, **HdA-A**, and **SdA-A** on human cancer cell lines and human fibroblast cells[a]

Compound	log P^b	K562 IC_{50} (μM)	Jurkat IC_{50} (μM)	HCT116 IC_{50} (μM)	A2780 IC_{50} (μM)	Human fibroblast IC_{50} (μM)
dA-C	2.97	141.79 ± 2.49	168.97 ± 1.99	>200	>200	>200
HdA-C	4.77	131.90 ± 9.29	86.18 ± 7.32	>200	>200	>200
SdA-C	4.62	172.36 + 9.60	35.86 + 11.60	>200	>200	>200
HM-C	2.35	51.06 ± 4.24	77.80 ± 0.54	96.93 ± 9.00	84.47 ± 3.90	128.79 ± 19.09
dA-CDC	4.28	23.25 ± 4.32	21.88 ± 1.16	146.32 ± 7.34	>200	155.2 ± 3.15
HdA-CDC	6.08	8.51 ± 4.05	10.47 ± 2.64	>200	>200	>200
SdA-CDC	5.94	22.05 ± 0.61	16.76 ± 3.06	>200	>200	>200
HM-CDC	3.66	38.84 ± 2.50	51.43 ± 7.57	75.49 ± 11.70	69.88 ± 13.85	79.01 ± 3.90
dA-UDC	4.28	35.65 ± 2.23	36.17 ± 1.51	>200	>200	36.36 ± 1.21
HdA-UDC	6.08	102.35 ± 2.05	24.57 ± 2.31	>200	>200	>200
SdA-UDC	5.94	125.33 ± 26.87	23.93 ± 0.98	>200	>200	>200
HM-UDC	3.66	47.23 + 0.52	36.44 + 18.07	65.37 + 26.02	84.61 + 1.39	80.18 + 1.47
dA-A	0.19	>200	>200	>200	>200	>200
HdA-A	2.24	156.63 + 14.76	123.48 + 19.09	112.58 + 5.75	107.01 + 1.27	>200
SdA-A	2.09	174.87 ± 15.06	183.35 ± 17.06	110.69 ± 3.44	131.67 ± 1.94	>200
Cisplatin[c]		5.35 ± 1.01	2.21 ± 1.51	8.47 ± 1.20	0.95 ± 0.35	25.38 ± 3.51

[a] IC_{50} values were determined from the dose–response curves using MTT assay. Results are expressed as the mean of three independent experiments + SD. [b] log*P* was determined using the MarvinSketch program. [c] Used as a reference compound.

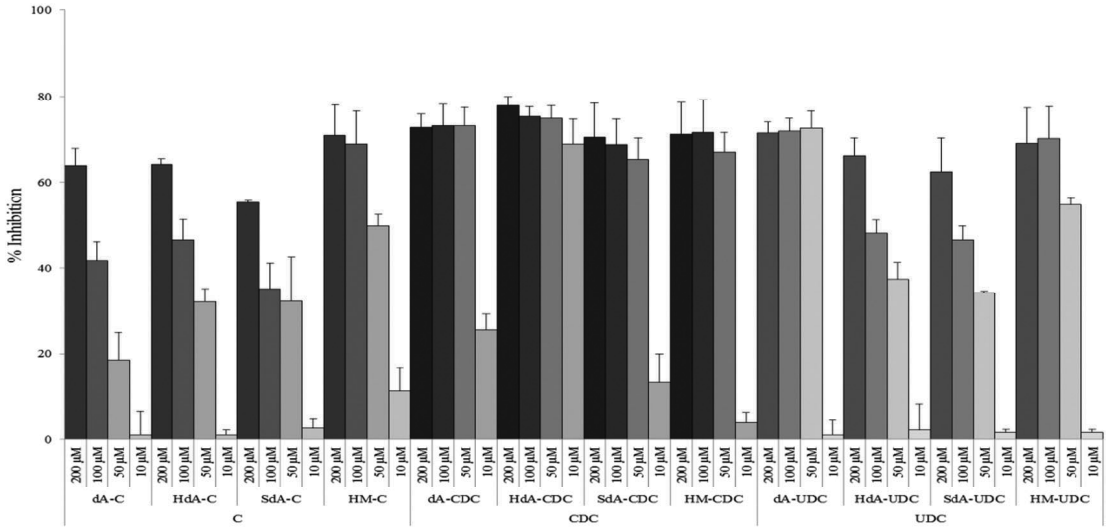


Fig. 2 Anti-proliferative activity of conjugated compounds against the K562 cell line. Mean of three independent experiments ± SD.

Furthermore, it is worth noting that, in the test on human fibroblast cells, no significant cytotoxic effect was observed for **dA**–, **HdA**– and **SdA**–bile acid conjugates, with compound **dA-UDC** as the only exception (IC_{50} 36.36 μM). As far as **dA-A**, **HdA-A**, and **SdA-A** are concerned, no considerable cytotoxic activity was detected in all lines tested (IC_{50} ranging from 107.01 to >200 μM). Finally, it can be seen that **HM**–bile acid conjugates caused cell death at a quite similar concentration in all cell lines tested including healthy fibroblast cells, suggesting that these conjugates showed a non-structure-specific cytotoxic mechanism.

Taken together these results, the following general structure-cytotoxicity observations can be made: (i) the activity of conjugated compounds was suppressed when the steroid moiety possessed the hydroxyl group in the C-12 position (cholic acid structure); (ii) comparing **CDC**-based with **UDC**-based conjugates the activity was enhanced with the 7-OH group in the alpha stereochemistry; (iii) the substitution of a sulphur with an alkynyl moiety increased the cytotoxic activity against the K562 cell line; (iv) comparing **HM**-bile acid with deoxyadenosine–bile acid compounds, it can be concluded that the conjugation to a C-8 alkynylated deoxyadenosine derivative played a crucial role in the selectivity of the cytotoxic process.

Following the results obtained, we decided to analyze the mechanism of K562 cell death. For this purpose, we investigated whether the anti-proliferative effects of **HdA/SdA-CDC** and **HM-CDC** were due to apoptosis. As shown by the annexin V test (Fig. 3), the percentage of dead cells significantly increased

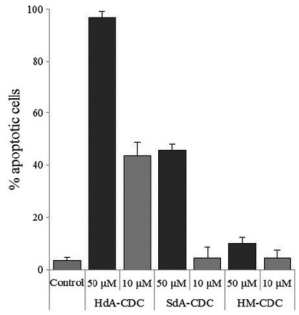


Fig. 3 Percentage of apoptotic K562 cells determined after 24 h treatment with **HdA-CDC**, **SdA-CDC** and **HM-CDC** (50–10 μM) by annexin V staining.

after 24 h treatment with 50 μM of **HdA-CDC** and **SdA-CDC**, but this effect was not present with **HM-CDC**. The percentage of specific apoptosis was found to be 96% for **HdA-CDC** and 46% for **SdA-CDC**. These data are in accordance with IC_{50} values. The low percentage of specific apoptosis of **HM-CDC** (9%) suggested that this conjugate induced K562 cell death through a necrosis process.

Further studies to investigate the mechanism by which conjugates **HdA/SdA-CDC** induced apoptosis in the K562 line are ongoing.

Conclusions

In conclusion, twelve novel bile acid-based conjugates were synthesized by using a 'click' chemistry approach. Their *in vitro* anti-proliferative activity on four human cell lines and cytotoxicity toward human fibroblast cells were evaluated. Some of them strongly and selectively inhibited cell proliferation and induced apoptosis in cultured human leukemic K562 cells. The best activity shown by **CDC**-based derivatives seemed to be correlated to the lipophilicity and to the 7α-OH group orientation. Furthermore, except **dA-UDC** and **HM**-bile acid series, all new conjugates did not show any significant cytotoxicity to the human fibroblast cells. Therefore, these derivatives constitute a starting lot of candidate drugs toward the development of promising chemical entities that, including a nucleoside-bile acid moiety in their structure, specifically target human cancer cell lines by triggering an apoptotic process.

Experimental

Chemistry

General. Reactions were monitored by TLC on pre-coated Silica Gel plates (thickness 0.25 mm, Merck), and phosphomolybdic acid solution was used as the spray reagent to visualize the steroids. Flash column chromatography was performed on silica gel 60 (230–400 mesh). The microwave (MW) irradiation was performed using a Biotage Initiator apparatus. Optimization experiments were performed in the ''single-run'' mode, *i.e.*, by manual filling of reaction vials and by specifying the irradiation time and maximum temperature. Melting points were determined using a capillary apparatus. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded for DMSO-d_6 solution, unless otherwise specified, in 5 mm tubes at room temperature, using a Varian Mercury Plus 400 spectrometer. Chemical shifts are given in δ values relative to TMS (tetramethylsilane) as internal standard. ESI-HRMS were acquired on an Agilent Dual ESI Q TOF 6520, in positive-ion mode, using methanol. HPLC-MS analysis was performed on an Agilent 1100 HPLC system and an Esquire 3000 Plus Bruker mass spectrometer only for compound **HdA-A**. IR spectra were recorded on a Perkin-Elmer Spectrum 100 FT-IR spectrometer. Cholic, chenodeoxycholic, and ursodeoxycholic bile acids are commercially available compounds used without further purification. The corresponding esters were prepared according to a literature procedure.[40]

General procedures for the synthesis of 3-β-iodides. To a solution of bile acid ester (12.3 mmol) in 1,3-dioxolane (100 ml), PPh_3 (4.8 g, 18.5 mmol) and imidazole (2.5 g, 36.9 mmol) were added. After 5 minute stirring, I_2 (4.7 g, 18.5 mmol) was added portionwise and the solution was stirred for 30 minutes at room temperature. The reaction mixture was poured into water containing a few drops of 30% H_2O_2 and extracted with AcOEt. The organic layer was washed with aq. $Na_2S_2O_5$ and dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography on silica gel using cyclohexane–EtOAc.

Methyl 3β-iodo-7α,12α-dihydroxy-5β-cholan-24-oate ***(C-β-I).*** Cyclohexane–EtOAc (2 : 1); white amorphous solid, yield 67%; ^{1}H-NMR ($CDCl_3$): δ = 4.98–4.97 (m, 1H), 3.99–3.97 (m, 1H), 3.84–3.83 (m, 1H), 3.66 (s, 3H), 0.99 (s, 3H), 0.98–0.96 (d, J = 6.25 Hz, 3H), 0.69 (s, 3H); ^{13}C-NMR ($CDCl_3$): δ = 174.7, 72.9, 68.2, 51.5, 47.3, 46.5, 42.0, 40.5, 39.8, 39.5, 38.5, 35.6, 35.1, 33.9, 32.6, 31.0, 30.9, 30.8, 28.3, 27.8, 27.4, 23.1, 22.7, 17.3, 12.5; HRMS of $[M + H]^+$ ions: calculated for $C_{25}H_{42}IO_4$ 533.2122, found 533.2131.

Methyl 3β-iodo-7α-hydroxy-5β-cholan-24-oate ***(CDC-β-I).*** Cyclohexane–EtOAc (7 : 1); white amorphous solid, yield 65%; ^{1}H-NMR ($CDCl_3$): δ = 4.99–4.96 (m, 1H), 3.84–3.82 (m, 1H), 3.66 (s, 3H), 1.00 (s, 3H), 0.92–0.90 (d, J = 6.44 Hz, 3H), 0.65 (s, 3H); ^{13}C-NMR ($CDCl_3$): δ = 174.7, 68.4, 55.8, 51.5, 50.5, 42.7, 40.7, 39.9, 39.6, 39.3, 38.6, 36.1, 35.4, 34.1, 33.9, 32.7, 31.1, 31.0, 30.9, 28.1, 23.7, 22.9, 20.6, 18.3, 11.8; HRMS of $[M + K]^+$ ions: calculated for $C_{25}H_{41}IO_3K$ 555.1732, found 555.1733.

Methyl 3β-iodo-7β-hydroxy-5β-cholan-24-oate ***(UDC-β-I).*** Cyclohexane–EtOAc (5 : 1); white amorphous solid, yield 55%; ^{1}H-NMR ($CDCl_3$): δ = 4.94–4.92 (m, 1H), 3.66 (s, 3H), 3.49–3.43 (m, 1H), 1.04 (s, 3H), 0.92–0.90 (d, J = 6.44 Hz), 0.67 (s, 3H); ^{13}C-NMR ($CDCl_3$): δ = 174.7, 71.9, 55.7, 54.9, 51.5, 43.7, 40.5, 40.1, 39.6, 37.9, 37.7, 36.1, 35.2, 35.1, 32.4, 31.0, 30.9, 28.6, 26.9, 23.7, 21.1, 18.4, 12.1; HRMS of $[M + K]^+$ ions: calculated for $C_{25}H_{41}IO_3K$ 555.1732, found 555.1736.

General procedures for the synthesis of 3-α-azides. Iodo-derivate bile ester (1.94 mmol) was dissolved in DMF (10 ml) and NaN_3 (378.4 mg, 5.8 mmol) was added. The reaction mixture was stirred at room temperature for 6 h and then poured into water (8 ml) and extracted twice with a mixture of Et_2O (12 ml)

and EtOAc (3 ml). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* to give azido-compounds: **C-N$_3$**, **CDC-N$_3$** and **UDC-N$_3$**.

*Methyl 3α-azido-7α,12α-dihydroxy-5β-cholan-24-oate (**C-N$_3$**).* White solid, yield: 80%; mp 108–110 °C (lit.[43] 107–109 °C); HRMS of $[M + Na]^+$ ions: calculated for $C_{25}H_{41}N_3O_4Na$ 470.2995, found 470.2997. IR, ^{1}H-NMR and ^{13}C-NMR spectroscopic data were in agreement with lit.[43]

*Methyl 3α-azido-7α-hydroxy-5β-cholan-24-oate (**CDC-N$_3$**).* Oil which solidified on standing, yield 87%; mp 87–89 °C; IR: ν (cm $^{-1}$) 3518 (O–H), 2947–2868 (C–H), 2089 (N_3), 1720 (C═O); ^{1}H-NMR ($CDCl_3$): δ = 3.85–3.80 (m, 1H), 3.64 (s, 3H), 3.17–3.09 (m, 1H), 0.90 (overl., 6H), 0.64 (s, 1H); ^{13}C-NMR ($CDCl_3$): δ = 174.7, 68.2, 61.3, 55.7, 51.5, 50.3, 42.6, 41.8, 39.6, 39.5, 39.3, 35.5, 35.4, 35.3, 35.1, 34.4, 32.7, 30.9, 28.1, 26.8, 23.7, 22.8, 20.5, 18.2, 11.7; HRMS of $[M + Na]^+$ ions: calculated for $C_{25}H_{41}N_3O_3Na$ 454.3046, found 454.3051.

*Methyl 3α-azido-7β-hydroxy-5β-cholan-24-oate (**UDC-N$_3$**).* Amorphous white solid, yield 94%; IR: ν (cm $^{-1}$) 3526–3315 (O–H), 2947–2867 (C–H), 2092 (N_3), 1736 (C═O); ^{1}H-NMR ($CDCl_3$): δ = 3.64 (s, 3H), 3.58–3.52 (m, 1H), 3.30–3.21 (m, 1H), 0.94 (s, 3H), 0.90 (d, J = 6.44 Hz, 3H), 0.65 (s, 3H); ^{13}C-NMR ($CDCl_3$): δ = 174.7, 71.1, 60.8, 55.6, 54.8, 51.5, 43.7, 43.6, 42.7, 40.0, 39.1, 36.6, 35.2, 35.1, 34.1, 33.4, 31.0, 31.0, 28.6, 26.8, 26.6, 23.4, 21.1, 18.4, 12.1; HRMS of $[M + Na]^+$ ions: calculated for $C_{25}H_{41}N_3O_3Na$ 454.3046, found 454.3001.

*8-(1,7-Octadynyl)-2′-deoxyadenosine (**HdA-A**).* Alkyne was prepared as reported in the literature for **dA-A**.[44] The reaction was monitored by HPLC-MS analysis using a Zorbax C8 column (4.6 × 150 mm, 5 μm) (linear gradient water–CH_3CN at a 0.5 ml min^{-1} flow rate, detection at λ 260 nm, R_t: 10.9 min). **HdA-A** was obtained in 80% yield and no chromatographic purification was necessary. An analytical sample was obtained after flash chromatography using CH_2Cl_2 : MeOH 9/1 as an eluent: colourless syrup; ^{1}H NMR: δ = 8.18 (s, 1H), 6.39 (dd, 1H, J = 6.4 Hz), 5.36 (bs, 1H, ex with D_2O), 5.30 (d, 1H, J = 6.4 Hz, ex with D_2O), 4.50–4.43 (m, 1H), 3.91–3.85 (m, 1H), 3.50–3.62 (m, 1H), 3.56–3.42 (m, 1H), 3.15–3.05 (m, 1H), 2.77 (t, 1H, J = 2.8 Hz), 2.71 (d, 1H, J = 0.4 Hz), 2.63–2.58 (m, 2H), 2.31–2.13 (m, 6H), 1.70–1.47 (m, 6H); ^{13}C NMR: δ = 156.4, 153.4, 149.5, 132.1, 129.6, 129.4, 88.6, 86.0, 85.0, 72.6, 66.0, 62.6, 40.2, 38.2, 27.7, 27.2, 18.8, 17.8; MS (ESI, ES+) m/z: 378 (M + 23), 356 (M + 1).

General procedures for the "click" reaction

Method A (room temperature conditions). To a solution of the appropriate alkyne **dA-A**, **HdA-A**, **SdA-A**, or **MO-A** (0.03 mmol) in 1.4 ml of a 1 : 1 : 1.5 mixture of H_2O–*tert*-BuOH–THF (v/v), sodium ascorbate (0.06 mmol) and $CuSO_4 \cdot 5H_2O$ (0.012 mmol) were added. The solution was stirred at room temperature, then the appropriate azide **C$_3$-N$_3$**, **CDC-N$_3$**, or **UDC-N$_3$** (0.045 mmol) was added and the resulting mixture was stirred overnight, then concentrated to dryness. The crude compound was purified by flash chromatography on silica gel using EtOAc–MeOH 9 : 1 and Et_3N (0.5%) as an eluent.

Method B (microwave conditions). To a solution of the appropriate alkyne **dA-A**, **HdA-A**, **SdA-A**, or **MO-A** (0.03 mmol) in 1.4 ml of a 1 : 1 : 1.5 mixture of H_2O–*tert*-BuOH–THF (v/v), sodium ascorbate (0.06 mmol), $CuSO_4 \cdot 5H_2O$ (0.012 mmol), and the appropriate azide **C$_3$-N$_3$**, **CDC-N$_3$**, or **UDC-N$_3$** (0.045 mmol) was added. The resulting mixture was premixed for 30 s, then heated in a sealed glass tube in a Biotage Initiator microwave apparatus at 80 °C for 30 min. After cooling at room temperature, the solvents were removed *in vacuo* and the crude material was purified by flash chromatography on silica gel using EtOAc–MeOH 9 : 1 and Et_3N (0.5%) as an eluent.

*Conjugate **SdA-C**.* Colourless syrup, yield 68%; IR: ν (cm $^{-1}$) 3329 (O–H), 2933–2867 (C–H), 1732 (C═O), 1640–1572 (C═C, C═N); ^{1}H-NMR: δ = 8.03 (s, 1H), 7.72 (s, 1H), 7.23 (br s, 2H), 6.23–6.20 (m, 1H), 5.45 (br s, 1H), 5.31 (br s, 1H), 4.42 (br s, 1H), 4.23–4.03 (m, 3H), 3.88–3.86 (m, 1H), 3.78 (br s, 1H), 3.66–3.61 (m, 2H), 3.55 (s, 3H), 3.51–3.45 (m, 1H), 3.13–3.06 (m, 1H), 2.77–2.61 (m, 3H), 2.34–0.94 (m, 30H), 0.87 (s, 3H), 0.91 (d, J = 6.44 Hz, 3H), 0.58 (s, 3H); ^{13}C-NMR: δ = 173.8, 154.4, 151.2, 150.5, 148.1, 146.0, 119.7, 119.6, 88.3, 84.8, 71.3, 71.1, 66.1, 62.3, 60.1, 51.2, 48.6, 46.1, 45.8, 41.7, 41.4, 37.4, 37.4, 36.9, 35.4, 35.0, 34.5, 31.8, 30.7, 30.4, 28.4, 28.4, 28.0, 27.2, 26.2, 24.5, 22.7, 22.5, 16.9, 12.3; HRMS of $[M + H]^+$ ions: calculated for $C_{41}H_{63}N_8O_7S$ 811.4540, found 811.4530.

*Conjugate **HdA-C**.* Colourless syrup, yield 75%; IR: ν (cm $^{-1}$) 3326 (O–H), 2933–2868 (C–H), 2238 (C≡C), 1731 (C═O), 1646–1572 (C═C, C═N); ^{1}H-NMR: δ = 8.11 (s, 1H), 7.76 (s, 1H), 7.52 (br s, 2H), 6.42–6.38 (m, 1H), 5.42–5.39 (m, 1H), 5.33–5.32 (d, J = 3.91 Hz, 1H), 4.48–4.44 (m, 1H), 4.23–4.17 (m, 1H), 4.14 (d, J = 3.71, 1H), 4.12 (d, J = 3.32 Hz, 1H), 3.89–3.87 (m, 1H), 3.77 (br s, 1H), 3.67–3.60 (m, 2H), 3.55 (s, 3H), 3.51–3.45 (m, 1H), 3.13–3.07 (m, 1H), 2.78–2.59 (m, 5H), 2.34–0.93 (m, 28H), 0.90 (d, J = 6.25 Hz, 3H), 0.87 (s, 3H), 0.58 (s, 3H); ^{13}C-NMR: δ = 173.8, 156.0, 155.9, 153.0, 148.3, 146.0, 133.4, 119.8, 119.1, 97.5, 88.3, 85.2, 71.4, 71.0, 70.3, 66.1, 62.3, 60.1, 51.2, 46.1, 45.8, 41.8, 41.4, 39.4, 37.6, 37.0, 35.4, 35.0, 34.5, 30.7, 30.4, 28.5, 28.3, 28.2, 27.2, 27.1, 26.2, 24.5, 22.7, 22.5, 18.3, 16.9, 12.3; HRMS of $[M + H]^+$ ions: calculated for $C_{43}H_{63}N_8O_7$ 803.4820, found 803.4805.

*Conjugate **dA-C**.* Colourless syrup, yield 70%; IR: ν (cm $^{-1}$) 3330 (O–H), 2937–2867 (C–H), 1730 (C═O), 1644–1582 (C═C, C═N); ^{1}H-NMR: δ = 8.58 (s, 1H), 8.11 (s, 1H), 7.47 (br s, 2H), 7.18–7.14 (m, 1H), 5.77–5.74 (m, 1H), 5.26 (d, J = 3.71 Hz, 1H), 4.50–4.44 (br s, 1H), 4.20 (d, J = 3.51 Hz, 1H), 4.17 (d, J = 3.91 Hz, 1H), 3.91–3.86 (m, 1H), 3.81–3.76 (m, 1H), 3.65–3.61 (m, 2H), 3.55 (s, 3H), 3.54–3.47 (m, 1H), 3.16–3.08 (m, 1H), 2.90–2.80 (m, 1H), 2.36–0.96 (m, 25H), 0.92–0.90 (m, 6H), 0.59 (s, 3H); ^{13}C-NMR: δ = 173.8, 156.1, 152.1, 149.8, 141.4, 138.3, 124.3, 119.5, 88.4, 85.9, 71.6, 71.1, 66.1, 62.4, 60.6, 51.2, 46.1, 45.8, 45.5, 41.6, 41.5, 38.2, 36.7, 35.2, 35.0, 34.4, 34.3, 30.7, 30.5, 28.4, 27.3, 27.2, 26.2, 22.7, 22.4, 17.0, 12.3; HRMS of $[M + H]^+$ ions: calculated for $C_{37}H_{55}N_8O_7$ 723.4194, found 723.4177.

*Conjugate **HM-C.*** Light yellow syrup, yield 85%; IR: ν (cm ^{1}) 3388 (O–H), 2938–2869 (C–H), 1734 (C═O); ^{1}H-NMR: δ = 7.85 (s, 1H), 4.48 (s, 2H), 4.31–4.21 (m, 1H), 4.16–4.12 (br s, 2H), 3.77 (br s, 1H), 3.61 (s, 1H), 3.55 (s, 3H), 2.80–2.70 (m, 1H), 2.34–0.94 (m, 24H), 0.91 (d, J = 6.44 Hz, 3H), 0.88 (s, 3H), 0.58 (s, 3H); ^{13}C-NMR: δ = 173.4, 147.2, 120.1, 70.7, 65.7, 59.7, 54.8, 50.8, 45.7, 45.4, 41.3, 41.0, 39.0, 36.6, 35.0, 34.6, 34.1, 34.1, 30.3, 30.1, 28.0, 27.0, 26.8, 25.8, 22.3, 22.1, 16.5, 11.9; HRMS of $[M + H]^+$ ions: calculated for $C_{28}H_{46}N_3O_5$ 504.3437, found 504.3434.

*Conjugate **SdA-CDC.*** Colourless syrup, yield 73%; IR: ν (cm^{-1}) 3324 (O–H), 2929–2865 (C–H), 1733 (C═O), 1639–1573 (C═C, C═N); ^{1}H-NMR: δ = 8.03 (s, 1H), 7.81 (s, 1H), 7.22 (br s, 2H), 6.23–6.19 (m, 1H), 5.45–5.42 (m, 1H), 5.31–5.30 (d, J = 4.10 Hz, 1H), 4.44–4.41 (m, 1H), 4.24–4.16 (m, 2H), 3.88–3.85 (m, 1H), 3.66–3.61 (m, 2H), 3.55 (s, 3H), 3.51–3.45 (m, 1H), 3.13–3.06 (m, 1H), 2.73–2.61 (m, 3H), 2.34–0.95 (m, 32H), 0.90 (s, 3H), 0.86 (d, J = 6.44 Hz, 3H), 0.60 (s, 3H); ^{13}C-NMR: δ = 174.2, 154.9, 151.60, 150.9, 148.5, 145.8, 129.5, 128.5, 88.7, 85.3, 71.8, 66.5, 62.7, 60.5, 55.9, 51.6, 50.4, 42.4, 42.1, 39.6, 39.5, 37.8, 37.6, 35.9, 35.3, 35.3, 34.8, 32.7, 32.3, 31.1, 30.8, 28.9, 28.4, 28.2, 27.7, 24.9, 23.5, 23.1, 20.7, 18.6, 12.1; HRMS of $[M + H]^+$ ions: calculated for $C_{41}H_{63}N_8O_6S$ 795.4586, found 795.4581.

*Conjugate **HdA-CDC.*** Colourless syrup, yield 80%; IR: ν (cm ^{1}) 3327 (O–H), 2930–2865 (C–H), 2238 (C≡C), 1732 (C═O), 1645–1572 (C═C, C═N); ^{1}H-NMR: δ = 8.13 (br s, 1H), 7.86 (s, 1H), 7.52 (br s, 2H), 6.42–6.38 (m, 1H), 5.41–5.37 (m, 1H), 5.33 (d, J = 3.91 Hz, 1H), 4.46 (br s, 1H), 4.26–4.16 (m, 2H), 3.89–3.86 (m, 1H), 3.67–3.61 (m, 2H), 3.55 (s, 3H), 3.51–3.44 (m, 1H), 3.14–3.07 (m, 1H), 2.74–2.59 (m, 5H), 2.35–2.13 (m, 3H), 1.91–0.95 (m, 27H), 0.90 (s, 3H), 0.86 (d, J = 6.44 Hz, 3H), 0.60 (s, 3H); ^{13}C-NMR: δ = 174.2, 157.8, 156.4, 153.4, 148.7, 142.8, 133.9, 120.4, 120.2, 98.0, 88.8, 85.6, 71.8, 70.8, 66.5, 62.7, 60.5, 55.9, 51.6, 50.4, 42.4, 42.1, 39.6, 38.0, 37.7, 35.9, 35.3, 35.3, 34.9, 32.7, 31.1, 30.8, 28.7, 28.2, 27.6, 27.5, 25.0, 23.5, 23.1, 20.7, 18.8, 18.6, 12.1; HRMS of $[M + H]^+$ ions: calculated for $C_{43}H_{63}N_8O_6$ 787.4865, found 787.4864.

*Conjugate **dA-CDC.*** Light yellow syrup, yield 75%; IR: ν (cm ^{1}) 3326 (O–H), 2931–2867 (C–H), 1732 (C═O), 1643–1583 (C═C, C═N); ^{1}H-NMR: δ = 8.70 (s, 1H), 8.12 (s, 1H), 7.44 (br s, 2H), 7.13–7.09 (m, 1H), 5.76–5.74 (m, 1H), 5.26 (br s, 1H), 4.52–4.44 (m, 2H), 4.22 (br s, 1H), 3.90–3.87 (m, 1H), 3.71–3.62 (m, 2H), 3.55 (s, 3H), 3.54–3.48 (m, 1H), 3.16–3.09 (m, 1H), 2.87–2.77 (m, 1H), 2.35–0.95 (m, 26H), 0.93 (s, 3H), 0.87 (d, J = 6.44, 3H), 0.61 (s, 3H); ^{13}C-NMR: 173.8, 156.1, 152.1, 149.7, 141.5, 138.1, 124.7, 119.4, 88.4, 85.9, 71.6, 66.1, 62.4, 60.7, 55.5, 51.2, 50.0, 42.1, 42.0, 41.6, 40.4, 38.2, 37.0, 35.3, 34.9, 34.8, 34.4, 32.2, 30.6, 30.4, 27.7, 27.1, 23.1, 22.6, 20.3, 18.2, 11.7; HRMS of $[M + H]^+$ ions: calculated for $C_{37}H_{55}N_8O_6$ 707.4239, found 707.4240.

*Conjugate **HM-CDC.*** Light yellow syrup, yield 80%, IR: ν (cm ^{1}) 3356 (O–H), 2927–2867 (C–H), 1735 (C═O). ^{1}H-NMR: δ = 7.94 (s, 1H), 5.12 (t, J = 5.50 Hz, 1H), 4.48 (d, J = 5.66 Hz, 2H), 4.33–4.23 (m, 1H), 4.18 (d, J = 3.32 Hz, 1H) 3.62 (br s, 1H), 3.55 (s, 3H), 2.77–2.67 (m, 1H), 2.35–0.93 (m, 25H), 0.91 (s, 3H), 0.87 (d, J = 6.44, 3H), 0.60 (s, 3H); ^{13}C-NMR: δ = 173.8, 147.5, 120.7, 69.8, 66.1, 60.0, 55.4, 55.1, 51.2, 49.9, 41.9, 41.6, 37.2, 35.4, 34.9, 34.8, 34.4, 32.2, 30.6, 30.4, 29.0, 27.7, 27.2, 23.1, 22.6, 20.3, 18.2, 11.7; HRMS of $[M + H]^+$ ions: calculated for $C_{28}H_{46}N_3O_4$ 488.3483, found 488.3502.

*Conjugate **SdA-UDC.*** Colourless syrup, yield 73%; IR: ν (cm ^{1}) 3322 (O–H), 2930–2862 (C–H), 1732 (C═O), 1638–1572 (C═C, C═N); ^{1}H-NMR: δ = 8.03 (s, 1H), 7.94 (s, 1H), 7.21 (br s, 2H), 6.23–6.20 (m, 1H), 5.45–5.42 (m, 1H), 5.31 (d, J = 4.10 Hz, 1H), 4.42 (br s, 1H), 4.38–4.30 (m, 1H), 3.90–3.85 (m, 2H), 3.66–3.61 (m, 1H), 3.55 (s, 3H), 3.54–3.45 (m, 1H), 3.32–3.27 (m, 1H), 3.13–3.06 (m, 1H), 2.64–2.61 (m, 2H), 2.34–0.97 (m, 33H), 0.92 (s, 3H), 0.86 (d, J = 6.44 Hz, 3H), 0.61 (s, 3H); ^{13}C-NMR: δ = 173.8, 154.4, 151.2, 150.5, 148.1, 146.0, 119.8, 119.6, 88.3, 84.8, 71.3, 66.1, 62.3, 60.1, 55.5, 51.2, 49.9, 41.9, 41.7, 40.1, 39.9, 37.4, 37.2, 35.4, 34.9, 34.8, 34.4, 32.2, 31.8, 30.7, 30.4, 28.4, 28.0, 27.7, 27.2, 24.5, 23.1, 22.6, 20.3, 18.1, 11.6; HRMS of $[M + H]^+$ ions: calculated for $C_{41}H_{63}N_8O_6S$ 795.4586, found 795.4581.

*Conjugate **HdA-UDC.*** Colourless syrup, yield 75%; IR: ν (cm ^{1}) 3331 (O–H), 2931–2865 (C–H), 2238 (C≡C), 1732 (C═O), 1645–1572 (C═C, C═N); ^{1}H-NMR: δ = 8.11 (s, 1H), 7.99 (s, 1H), 7.51 (br s, 2H), 6.42–6.38 (m, 1H), 6.54–6.37 (m, 1H), 5.32 (d, J = 3.91 Hz, 1H), 4.47–4.44 (m, 1H), 4.41–4.33 (m, 1H), 3.90–3.86 (m, 2H), 3.67–3.61 (m, 1H), 3.55 (s, 3H), 3.51–3.45 (m, 1H), 3.35–3.29 (m, 1H), 3.14–3.07 (m, 1H), 2.67–2.59 (m, 4H), 2.34–0.99 (m, 31H), 0.92 (s, 3H), 0.86 (d, J = 6.44 Hz, 3H), 0.60 (s, 3H); ^{13}C-NMR: δ = 173.8, 157.0, 155.9, 153.0, 148.3, 146.2, 133.4, 119.9, 119.1, 97.5, 88.3, 85.2, 71.4, 70.4, 69.1, 62.3, 59.6, 55.3, 54.6, 51.2, 43.1, 42.6, 39.5, 38.4, 37.6, 37.3, 35.0, 34.8, 34.2, 33.8, 30.7, 30.3, 28.2, 28.1, 27.7, 27.1, 26.7, 24.6, 23.2, 20.9, 18.3, 18.3, 12.0; HRMS of $[M + H]^+$ ions: calculated for $C_{43}H_{63}N_8O_6$ 787.4865, found 787.4851.

*Conjugate **dA-UDC.*** Colourless syrup, yield 68%; IR: ν (cm ^{1}) 3341 (O–H), 2932–2868 (C–H), 1730 (C═O), 1642–1583 (C═C, C═N); ^{1}H-NMR: δ = 8.94 (s, 1H), 8.12 (s, 1H), 7.41 (br s, 2H), 7.13–7.09 (m, 1H), 5.78–5.75 (m, 1H), 5.27 (d, J = 3.71, 1H), 4.66–4.58 (m, 1H), 4.47 (br s, 1H), 3.92–3.88 (m, 2H), 3.71–3.66 (m, 1H), 3.55 (s, 3H), 3.54–3.47 (m, 1H), 3.39–3.33 (m, 1H), 3.13–3.09 (m, 1H), 2.35–0.97 (m, 27H), 0.95 (s, 3H), 0.87 (d, J = 6.44, 3H), 0.62 (s, 3H); ^{13}C-NMR: δ = 173.8, 156.1, 152.1, 149.7, 141.5, 138.3, 124.9, 119.5, 88.5, 85.9, 71.6, 69.0, 62.4, 60.3, 55.2, 54.8, 51.2, 45.7, 43.1, 42.5, 39.6, 38.2, 38.1, 37.3, 35.0, 34.8, 33.9, 33.8, 30.7, 30.4, 28.2, 27.6, 26.7, 23.1, 21.0, 18.3, 12.0; HRMS of $[M + H]^+$ ions: calculated for $C_{37}H_{55}N_8O_6$ 707.4239, found 707.4232.

*Conjugate **HM-UDC.*** Colourless syrup, yield 83%; IR: ν (cm ^{1}) 3324 (O–H), 2931–2865 (C–H), 1732 (C═O), 1638–1572 (C═C, C═N); ^{1}H-NMR: δ = 8.11 (s, 1H), 5.13 (br s, 1H), 4.48 (s, 2H), 4.46–4.37 (m, 1H), 3.90 (br s, 1H), 3.55 (s, 3H), 3.32 (br s, 1H), 2.34–0.98 (m, 26H), 0.93 (s, 3H), 0.86 (d, J = 6.44 Hz, 3H), 0.61 (s, 3H); ^{13}C-NMR: δ = 174.2, 148.1, 121.3, 69.5, 60.0,

55.7, 55.6, 55.1, 51.7, 43.5, 43.0, 39.9, 38.7, 37.7, 35.5, 35.2, 34.7, 34.2, 31.2, 30.8, 29.4, 28.6, 28.1, 27.1, 23.6, 21.4, 18.8, 12.4; HRMS of $[M + H]^+$ ions: calculated for $C_{28}H_{46}N_3O_4$ 488.3483, found 488.3482.

Biological assay

Cell lines and culture

Leukemia cell lines Jurkat and K562, the colon cancer cell line HCT116 and the ovarian cancer cell line A2780 were obtained from ATCC (Manassas, VA) and maintained in RPMI 1640, supplemented with 10% fetal bovine serum (FBS), penicillin (100 U mL $^{-1}$), streptomycin (100 U mL $^{-1}$) and glutamine (2 mM); human fibroblast cells were maintained in DMEM, supplemented with 10% newborn bovine serum, penicillin (100 U mL $^{-1}$), streptomycin (100 U mL $^{-1}$) and glutamine (2 mM); the pH of the medium was 7.2 and incubation was performed at 37 °C in a 5% CO_2 atmosphere. Cells were routinely passaged every three days at 70% of confluence; for the adherent cell lines 0.05% trypsin-EDTA (Lonza) was used. The antiproliferative activity of compounds was tested with MTT assay.[45]

MTT assay (evaluation of anti-proliferative activity)

The MTT test was used to study the anti-proliferative activity of the compounds. The cells were seeded in triplicate in 96-well trays at a density of 25×10^3 in 50 μl of AIM-V medium for cancer cells lines and 5×10^3 in 50 μl of DMEM medium for human fibroblasts. Stock solutions (20 mM) of each compound were made in DMSO and diluted in AIM-V or DMEM medium depending on the cell type to give final concentrations of 10, 50, 100 and 200 μM. Untreated cells were placed in every plate as a negative control. The cells were exposed to the compounds, in 100 μl total volume, for 72 hours, and then 25 ml of a 3-(4,5-dimethylthiozol-2-yl)2,5-diphenyltetrazolium bromide solution (MTT) (12 mM) were added to each well and plates were incubated at 37 °C and 5% CO_2. After two hours, 100 μl of lysing buffer (50% ddH_2O, 50% DMF + 20% SDS, pH 4.7) were added to solubilize the MTT violet colored formazane crystals. After additional 24 hours the solution absorbance, proportional to the number of live cells, was measured using a spectrophotometer at 570 nm and converted into % of growth inhibition.[47] The results are expressed as the mean of three independent ± SD against five different cell lines.

Annexin V staining (evaluation of apoptosis percentage)

The percentage of apoptotic cells was assessed using the annexin V assay (Clontech, USA). Propidium iodide (PI) was used to avoid necrotic cell detection (annexin−/PI+). The drug-induced apoptotic rate (annexin+/PI− and annexin V+/PI+) was compared with the apoptosis in the absence of the drugs used as control (spontaneous apoptosis).[48] K562 cells were cultured in RPMI + 10% FBS in a 6-well plate for 24 h in the presence of compounds HdA-CDC, SdA-CDC and HM-CDC at concentrations of 50 and 10 μM. Cells were washed once with saline buffer (PBS) and resuspended in 250 μl of Binding Buffer 1X containing 100 ng of FITC-labeled annexin V and in the control sample, 500 ng of PI. Incubation with annexin V for 15 min on ice in the dark was directly followed by flow cytometric analysis of the cells with a FACScan (Becton Dickinson, San Jose, Ca, USA) at 488 nm and quantified using the Cell Quest Pro software (Becton Dickinson, San Jose, Ca, USA).

Acknowledgements

We thank Dr T. Bernardi and Dr E. Bianchini, Department of Chemical and Pharmaceutical Sciences, University of Ferrara, for running HRMS experiments and for technical assistance.

References

1 D. Verzele, S. Figaroli and A. Madder, *Molecules*, 2011, **16**, 10168–10186.
2 Nonappa and U. Maitra, *Org. Biomol. Chem.*, 2008, **6**, 657–669.
3 D. B. Salunke, B. G. Hazra and V. S. Pore, *Curr. Med. Chem.*, 2006, **13**, 813–847.
4 E. Virtanen and E. Kolehmainen, *Eur. J. Org. Chem.*, 2004, 3385–3399.
5 J. Tamminen and E. Kolehmainen, *Molecules*, 2001, **6**, 21–46.
6 Y. Li and J. R. Dias, *Chem. Rev.*, 1997, **97**, 283–304.
7 B.-S. Chu, A. P. Gunning, G. T. Rich, M. J. Ridout, R. M. Faulks, M. S. J. Wickham, V. J. Morris and P. J. Wilde, *Langmuir*, 2010, **26**, 9782–9793.
8 R. Sharma, F. Majer, V. K. Peta, J. Wanga, R. Keaveney, D. Kelleher, A. Long and J. F. Gilmer, *Bioorg. Med. Chem.*, 2010, **18**, 6886–6895.
9 N. M. Kim, E. Im, Y. H. Yoo and Y. H. Choi, *Curr. Cancer Drug Targets*, 2006, **6**, 681–689.
10 L. Mrózek, L. Dvořáková, Z. Mandelová, L. Rárová, A. Řezáčová, L. Plaček, R. Opatřilová, J. Dohnal, O. Paleta, V. Král, P. Drašar and J. Jampílek, *Steroids*, 2011, **76**, 1082–1097.
11 L. Coufalová, L. Mrózek, L. Rárová, L. Plaček, R. Opatřilová, J. Dohnal, K. Král'ová, O. Paleta, V. Král, P. Drašar and J. Jampílek, *Steroids*, 2013, **78**, 435–453.
12 E. O. Im, Y. H. Choi, K. J. Paik, H. Suh, Y. Jin, K. W. Kim, Y. H. Yoo and N. D. Kim, *Cancer Lett.*, 2001, **163**, 83–93.
13 Y. H. Choi, E. O. Im, H. Suh, Y. Jin, W. H. Lee, Y. H. Yoo, K. W. Kim and N. D. Kim, *Int. J. Oncol.*, 2001, **18**, 979–984.
14 Y. H. Choi, E. O. Im, H. Suh, Y. Jin, Y. H. Yoo and N. D. Kim, *Cancer Lett.*, 2003, **199**, 157–167.
15 J. H. Jeong, J.-S. Park, B. Moon, M. C. Kim, J.-K. Kim, S. Lee, H. Suh, N. D. Kim, J.-M. Kim, Y. C. Park and Y. H. Yoo, *Ann. N. Y. Acad. Sci.*, 2003, **1010**, 171–177.
16 S. E. Park, H. J. Choi, S. B. Yee, H. Y. Chung, H. Suh, Y. H. Choi, Y. H. Yoo and N. D. Kim, *Int. J. Oncol.*, 2004, **25**, 231–236.
17 E. Im, S. H. Choi, H. Suh, Y. H. Choi, Y. H. Yoo and N. D. Kim, *Cancer Lett.*, 2005, **229**, 49–57.

18 S.-B. Yee, W. J. Yeo, B. S. Park, J. Y. Kim, S. J. Baek, Y. C. Kim, S. Y. Seo, S. H. Lee, J. H. Kim, H. Suh, N. D. Kim, Y. J. Lim and Y. H. Yoo, *Int. J. Oncol.*, 2005, **27**, 653–659.
19 S. E. Park, S. W. Lee, M. A. Hossain, M. Y. Kim, M. N. Kim, E. Y. Ahn, Y. C. Park, H. Suh, G. Y. Kim, Y. H. Choi and N. D. Kim, *Cancer Lett.*, 2008, **270**, 77–86.
20 E. Sievänen, *Molecules*, 2007, **12**, 1859–1889.
21 A. Dalpiaz, G. Paganetto, B. Pavan, M. Fogagnolo, A. Medici, S. Beggiato and D. Perrone, *Mol. Pharmaceutics*, 2012, **9**, 957–968.
22 O. Bortolini, G. Fantin, M. Fogagnolo, S. Rossetti, L. Maiuolo, G. D. Pompo, S. Avnet and D. Granchi, *Eur. J. Med. Chem.*, 2012, **52**, 221–229.
23 R. Huisgen, *Angew. Chem., Int. Ed. Engl.*, 1963, **2**, 565–632.
24 C. W. Tornøe, C. Christensen and M. Meldal, *J. Org. Chem.*, 2002, **67**, 3057–3064.
25 V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2002, **41**, 2596–2599.
26 H. C. Kolb and K. B. Sharpless, *Drug Discovery Today*, 2003, **8**, 1128–1137.
27 D. K. Dalvie, A. S. Kalgutkar, S. C. Khojasteh-Bakht, R. S. Obach and J. P. O'Donnell, *Chem. Res. Toxicol.*, 2002, **15**, 269–299.
28 W. S. Horne, M. K. Yadav, C. D. Stout and M. R. Ghadiri, *J. Am. Chem. Soc.*, 2004, **126**, 15366–15367.
29 D. Wu, S. Ji, Y. Wu, Y. Jua and Y. Zhao, *Bioorg. Med. Chem. Lett.*, 2007, **17**, 2983–2986.
30 L. Ma, M. Melegari, M. Colombini and J. T. Davis, *J. Am. Chem. Soc.*, 2008, **130**, 2938–2939.
31 S. Ikonen, H. M. -Cahova, R. Pohl, M. Sanda and M. Hocek, *Org. Biomol. Chem.*, 2010, **8**, 1194–1201.
32 D.-q. Chen, X. Wang, L. Chen, J.-x. He, Z.-h. Miao and J.-k. Shen, *Acta Pharmacol. Sin.*, 2011, **32**, 664–672.
33 T. Robak, A. Korycka, M. Kasznicki, A. Wrzesien-Kus, P. Smolewski, A. Korycka, M. Kasznicki, A. Wrzesien-Kus and P. Smolewski, *Curr. Cancer Drug Targets*, 2005, **5**, 421–444.
34 T. Robak, A. Korycka, E. L. Maranda and P. Robak, *Molecules*, 2009, **14**, 1183–1226.
35 C. M. Galmarini, J. R. Mackey and C. Dumontet, *Lancet Oncol.*, 2002, **3**, 415–424.
36 D. Genini, S. Adachi, Q. Chao, D. W. Rose, C. J. Carrera, H. B. Cottam, D. A. Carson and L. M. Leoni, *Blood*, 2000, **96**, 3537–3543.
37 E. V. D. Neste, S. Cardoen, F. Offner and F. Bontemps, *Int. J. Oncol.*, 2005, **27**, 1113–1124.
38 M. L. Capobianco and M. L. Navacchia, *PCT Int. Appl.*, WO 2012164484 A1, 2012.
39 M. L. Capobianco, M. L. Navacchia, E. Marchesi and D. Perrone, *Bioconjugate Chem.*, 2013, **24**, 1398–1407.
40 B. Dayal, K. Raob and G. Salen, *Steroids*, 1995, **60**, 453–457.
41 Z. Lotowski and D. Guzmanski, *Monatsh. Chem.*, 2006, **137**, 117–124.
42 L. J. Lawless, A. G. Blackburn, A. J. Ayling, M. N. Pérez-Payán and A. P. Davis, *J. Chem. Soc., Perkin Trans. 1*, 2001, 1329–1341.
43 N. G. Aher, V. S. Pore and S. P. Patil, *Tetrahedron*, 2007, **63**, 12927–12934.
44 G. Sàgi, L. Otvos, S. Ikeda, G. Andrei, R. Snoeck and E. D. Clercq, *J. Med. Chem.*, 1994, **37**, 1307–1311.
45 F. Denizot and R. Lang, *J. Immunol. Methods*, 1986, **89**, 271–277.
46 D. Brossard, L. E. Kihel, M. Clément, W. Sebbahi, M. Khalid, C. Roussakis and S. Rault, *Eur. J. Med. Chem.*, 2010, **45**, 2912–2918.
47 O. Bortolini, A. D. Nino, T. Eliseo, R. Gavioli, L. Maiuolo, B. Russo and F. Sforza, *Bioorg. Med. Chem.*, 2010, **18**, 6970–6976.
48 G. Koopman, C. P. Reutelingsperger, G. A. Kuijten, R. M. Keehnen, S. T. Pals and M. H. v. Oers, *Blood*, 1994, **804**, 1415–1420.

Desidero ringraziare la Prof.ssa Olga Bortolini, relatore di questa tesi, per la grande disponibilità e cortesia dimostratemi.

Desidero ringraziare il Prof. Alessandro Massi e la Dott.ssa Daniela Perrone per la guida e l'aiuto durante questi anni di dottorato e durante la stesura di questa Tesi.

Ringrazio il Prof. Matteo Zanda per l'opportunità datami di lavorare nel proprio gruppo di ricerca ad Aberdeen, ed avermi dato l'occasione di fare una meravigliosa esperienza all'estero.

Ringrazio i miei colleghi dottoranti ed in particolare il Dott. Roberto Greco che ha condiviso con me questo periodo di scrittura della Tesi.

Desidero ringraziare la Dott.ssa Tatiana Bernardi e la Dott.ssa Erika Marzola per la grande cura nell'esecuzione degli spettri di massa delle molecole sintetizzate, il Dott. Paolo Formaglio per l'esecuzione degli spettri NMR. Ringrazio inoltre il Dott. Marco Carmosino per la disponibilità e la gentilezza dimostratemi e la Dott. Nikla Baricordi per aver reso tutto più "bello"!

Un ringraziamento speciale alla mia famiglia per avermi sempre sostenuto in tutte le mie decisioni permettendomi di raggiungere questo traguardo.

Un ultimo ringraziamento agli amici italiani e scozzesi, per essermi stati vicini sia nei momenti difficili, sia nei momenti felici, troppi da menzionare uno ad uno ma insostituibili!

UNIVERSITÀ DEGLI STUDI DI FERRARA
- EX LABORE FRUCTUS -

Sezioni

Dottorati di ricerca

Il tuo indirizzo e-mail

msschr@unife.it

Oggetto:

Dichiarazione di conformità della tesi di Dottorato

Io sottoscritto Dott. (Cognome e Nome)

Massarenti Chiara

Nato a:

Codigoro

Provincia:

Ferrara

Il giorno:

23/12/1987

Avendo frequentato il Dottorato di Ricerca in:

Scienze Chimiche

Ciclo di Dottorato

27

Titolo della tesi:

Bile acid and indole scaffolds for the synthesis of new biologically relevant compounds

Titolo della tesi (traduzione):

Scaffold biliare ed indolico per la sintesi di nuovi composti biologicamente rilevanti

Tutore: Prof. (Cognome e Nome)

Bortolini Olga

Settore Scientifico Disciplinare (S.S.D.)

CHIM/06

Parole chiave della tesi (max 10):

Click chemistry, bile acids, acidi biliari, bisphosphonates, bis-fosfonati, indole scaffold, scaffold indolico, endocannabinoid system, sistema endocannabinoide

Consapevole, dichiara

CONSAPEVOLE: (1) del fatto che in caso di dichiarazioni mendaci, oltre alle sanzioni previste dal codice penale e dalle Leggi speciali per l'ipotesi di falsità in atti ed uso di atti falsi, decade fin dall'inizio e senza necessità di alcuna formalità dai benefici conseguenti al provvedimento emanato sulla base di tali dichiarazioni; (2) dell'obbligo per l'Università di provvedere al deposito di legge delle tesi di dottorato al fine di assicurarne la conservazione e la consultabilità da parte di terzi; (3) della procedura adottata dall'Università di Ferrara ove si richiede che la tesi sia consegnata dal dottorando in 2 copie di cui una in formato cartaceo e una in formato pdf non modificabile su idonei supporti (CD-ROM, DVD) secondo le istruzioni pubblicate sul sito: http://www.unife.it/studenti/dottorato alla voce ESAME FINALE – disposizioni e modulistica; (4) del fatto che l'Università, sulla base dei dati

forniti, archivierà e renderà consultabile in rete il testo completo della tesi di dottorato di cui alla presente dichiarazione attraverso l'Archivio istituzionale ad accesso aperto "EPRINTS.unife.it" oltre che attraverso i Cataloghi delle Biblioteche Nazionali Centrali di Roma e Firenze; DICHIARO SOTTO LA MIA RESPONSABILITA': (1) che la copia della tesi depositata presso l'Università di Ferrara in formato cartaceo è del tutto identica a quella presentata in formato elettronico (CD-ROM, DVD), a quelle da inviare ai Commissari di esame finale e alla copia che produrrò in seduta d'esame finale. Di conseguenza va esclusa qualsiasi responsabilità dell'Ateneo stesso per quanto riguarda eventuali errori, imprecisioni o omissioni nei contenuti della tesi; (2) di prendere atto che la tesi in formato cartaceo è l'unica alla quale farà riferimento l'Università per rilasciare, a mia richiesta, la dichiarazione di conformità di eventuali copie; (3) che il contenuto e l'organizzazione della tesi è opera originale da me realizzata e non compromette in alcun modo i diritti di terzi, ivi compresi quelli relativi alla sicurezza dei dati personali; che pertanto l'Università è in ogni caso esente da responsabilità di qualsivoglia natura civile, amministrativa o penale e sarà da me tenuta indenne da qualsiasi richiesta o rivendicazione da parte di terzi; (4) che la tesi di dottorato non è il risultato di attività rientranti nella normativa sulla proprietà industriale, non è stata prodotta nell'ambito di progetti finanziati da soggetti pubblici o privati con vincoli alla divulgazione dei risultati, non è oggetto di eventuali registrazioni di tipo brevettale o di tutela. PER ACCETTAZIONE DI QUANTO SOPRA RIPORTATO

Firma del dottorando

Ferrara, li ___26/03/2015_____ (data) Firma del Dottorando

_____

Firma del Tutore

Visto: Il Tutore Si approva Firma del Tutore _____