Università
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Master's Degree in Science and Technology of Bio and Nanomaterials

Final Thesis

Organic Synthesis and Characterization (NMR and Mass Spectroscopy) of a β-Glucosyl-Cholesterol's equivalent for Parkinson's Disease research

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Ringraziamenti

Abstract

The aim of this research consists in the organic synthesis and characterization of a synthetic equivalent of β-glucosyl-cholesterol bearing a terminal alkyne functionality to ascertain, in collaboration with a research group of biologists from University of Padova, the possible involvement of this class of derivatives in the Parkinson's disease's aetiology. The target molecule, thanks to the presence of a reactive acetylenic moiety is suitable to further conjugation by "Click Chemistry" with azido functionalized solid supports or fluorophores. The synthetic approach consisted into overall 12 synthetic steps to produce the 3 synthetic synthons (glucosyl, cholesteryl and acetylenic units) requested for the preparation of the final molecule. The synthesis of the three synthons has been almost completed and largely optimized and each intermediate has been thoroughly characterized by NMR spectroscopy and high-resolution mass spectrometry. In the near future the final target molecule will be obtained through final coupling methods

Table of Contents

1. Introduction

1.1 Parkinson's disease

Parkinson's disease (PD) is considered to be the second of the most common chronic neurodegenerative diseases.[1] Its symptomatology includes rest tremors, rigidity, slow movements and imbalance.

PD affects 1% of the population above the age of 65 but its manifestation increases exponentially with aging. Although there exist treatments for the symptoms there is no regenerative therapies due to the lack of knowledge regarding its etiology.

The neurodegenerative process can occur due to a number of genetic and environmental factors, their identification and mechanisms of action are of great interest for the scientific community.[2]

One of the most important factors that affect the neuronal degeneration typical of neurodegenerative diseases is the protein aggregation and the subsequent accumulation inside the neuronal cell. This process is caused by the misfolding of one or more types of proteins generally due to mutations on the genes involved in their expression, making them prone to aggregation. For example, several studies have found that, in all known forms of PD, the common cause for neuron's degeneration is, in fact, protein aggregation. This affects the dopaminergic neurons of the substantia nigra pars compacta (SNpc) of the brain; and is mainly caused by mutations in the genes expressing α-synuclein, parkin and ubiquitin C-terminal hydrolase L1 (UCHL1); all of these proteins can be found in the Lewi bodies typical of sporadic PD. Particularly, α-synuclein is thought to be an adjuvant factor in the transportation of vesicles that contains dopamine and, in its mutated form, can create pores in the lipid membrane, thus causing a loss of the neurotransmitter in the cytoplasm.[3]

GBA1 is another gene that has recently been investigated for its predominant involvement in synucleinopathies like PD. Furthermore, it has been discovered that some mutations of this gene are responsible for the intracellular accumulation of sterylglucosides which seems to play an important role in the neuron's degenerative process.

1.1.1 The Role of Cholesteryl-Glucoside in Parkinson's Disease Etiopathology

GBA1 is a genetic sequence that codes for β-glucocerebrosidase (GCase). This gene has recently gained a lot of interest due to the demonstration that homo- and etrozygous mutations in the sequence lead to the expression of a low-activity GCase, involved in the development of synucleinopathies like PD. It has also been shown that low activity GCase plays a role in the accumulation of endogenous sterylglycosides that may be neurotoxic for dopaminergic neurons. Sterylglycosides are molecules consisting by β-glycosidic linkage between the hemiacetal carbon of a monosaccharide and the 3'-hydroxyl group of a steroid unit. It has been demonstrated that GCase acts as a reversable glucosyltranferase in the production of β-cholesteryl glucoside (GlcChol) as a secondary activity (see Figure 1). There exist several evidences showing that in non-pathological conditions GCase's hydrolytic activity promotes the degradation of GlcChol; suggesting that mutated GCase could be involved in GlcChol accumulation.

Evidences suggest that the neurotoxicity caused by this accumulation are related to the alteration of the cell respiration and mitochondrial production of reactive oxygen species (ROS) even though the mechanisms are still a mystery. The accumulation of both the cholesterol derivative and ROS can in fact lead to a high level of oxysterols that contributes to the pathophysiology of PD; thus suggesting that GlcChol could be exploited as a biomarker or as a therapeutic target.[4]

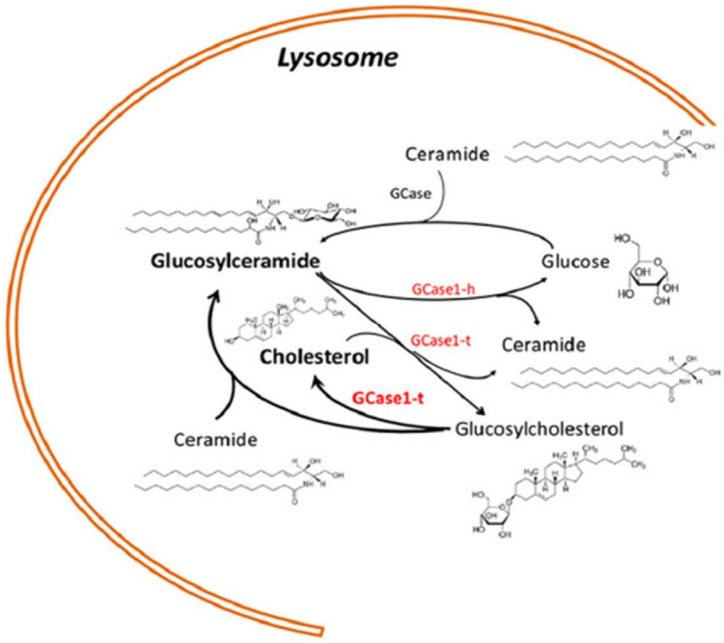
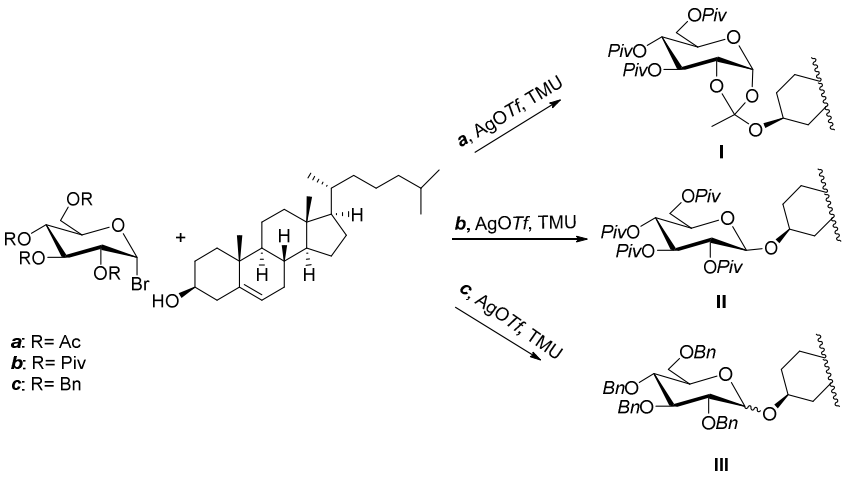


Figure 1: Simplified representation of GCase activities inside the lysosome. Enzyme hydrolase activity is identified by terminal "h" and transferase activity by terminal "t".

Other confirmations coming from studies regarding the sterylglucoside's content and composition variety in mammalian's brain suggest the involvement of steryglucosides in the incidence of PD and similar pathologies. Sitosteryl-β-glucoside is a particularly interesting example due to its involvement in a severe syndrome that affects the inhabitants of the Guam archipelago. This molecule is present in high concentrations inside cycad seeds (*Cycas Cirilnalis)* that are part of the diet of the population of Guam. The consumption of these seeds causes a sitosteryl-β-glucoside accumulation that apparently triggers a pathology known as ALS-PDC whose symptoms includes sclerosis and/or parkinsonism/dementia complex. Studies carried out in rats have shown that the administration of synthetic sitosteryl-β-glucoside causes a progressive parkinsonism proving its neurotoxic properties.[5]

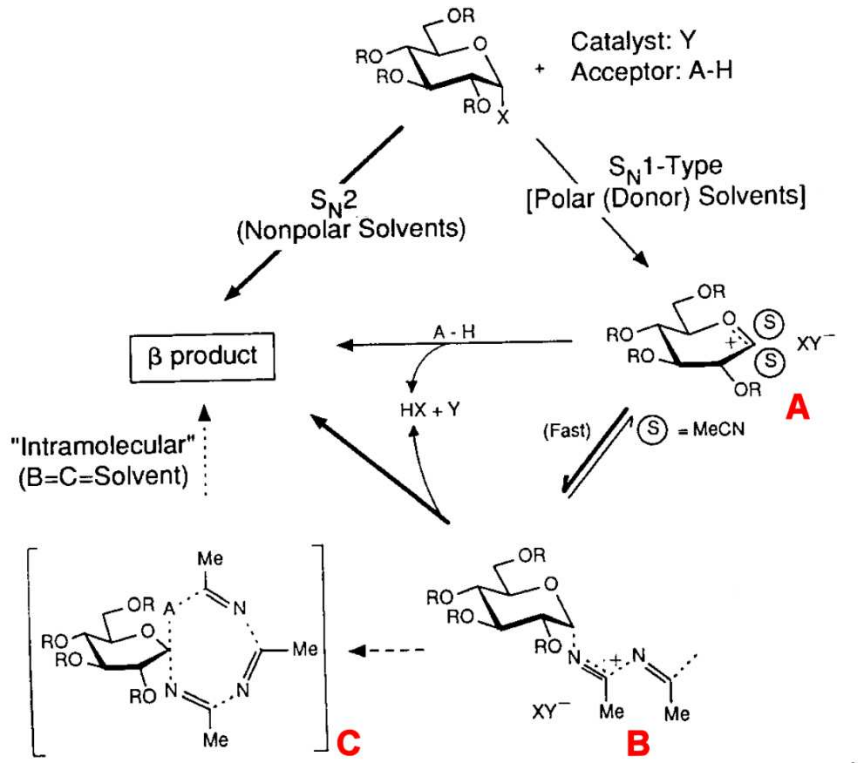
1.2 Synthesis of b-Cholesteryl Glucosides

Since the discovery of Koenigs-Knorr method,[6] that exploit the reaction between glycosyl halide and alcohol to produce a glycoside in the presence of silver salts, much progress has been made to obtain stereoselective β-derivatives of sterols. It is known that to obtain this result the glycosidic donor requires alcoholic protections able to also act as neighbouring participating groups. An example of these protector groups are acetyls moieties. However, as described by Presser et al. in 2006[7] working with this kind of protecting group, the orthoester **I** has been revealed to be the only product obtained, as described in Scheme 1. In contrast, using a more sterically hindered group R= Piv have proven that glycosylation of the same aglycone with **b** resulted exclusively in the formation of the β-D-glucoside **II** in good yield. When glycosylation was performed with R = Bn, the cholesteryl 2,3,4,6-tetra-O-benzyl-D-glucopyranoside **III** was obtained in 67% yield as a mixture of α- and β-anomers (α:β = 3:2).



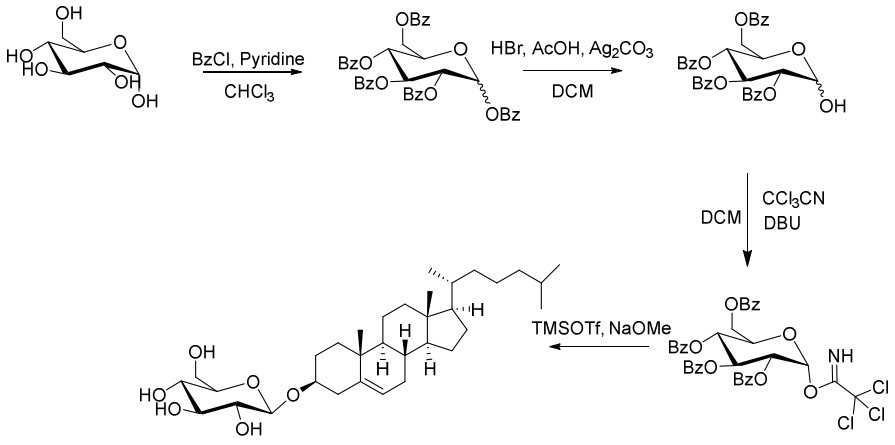
Scheme 1: Glycosylation of cholesterol using hindered glycosyl donors.

The β stereoselectivity can be achieved using different leaving groups (LG) in place of halide on the anomeric carbon; for example the trichloroacetimidate. The role of this LG in stereoselectivity is describe in Scheme 2.[8] Two mechanisms are possible: S_N1 or S_N2 depending on the solvent nature. In both cases small quantities of Lewis Acid (TMSOTf) is required to catalyse the reaction. Using polar donor solvents such as acetonitrile, S_N1 mechanism is preferred with the formation of carbocation A, that evolves into intermediate B that becomes the target of the solvent action to form a reactive α-nitrilium-nitrile-conjugate. The α-nitrilium-nitrile-conjugate reacts with acceptor A-H in β-face to give the intermediate C and the corresponding product that reflect the same stereochemistry. Starting from sugars with tricholoacetimidate as leaving group on the anomeric carbon, in α or β configurations, and using polar (donor) solvents, only glycosides β have been detected. This result support the S_N1 nature of the mechanism. Instead if non-polar solvents such DCM are employed, S_N2 reaction is preferred.



Scheme 2: Schematic representation of SN1 and SN2 mechanisms depending on solvent nature

Trichloroacetimidate approach via S_N2 reaction, has been used in glycosylation of cholesterol by Deng *et al* in 1999,[9] as describe in scheme 3 below.

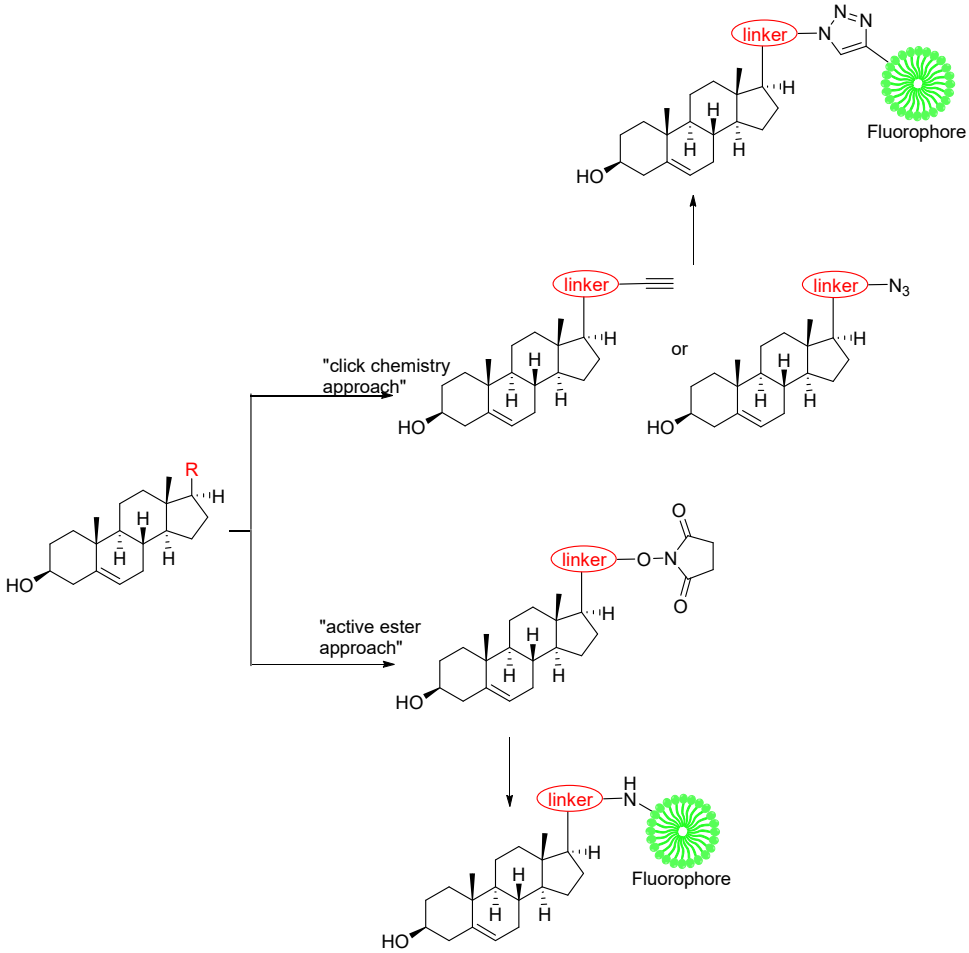


Scheme 3: Schematic synthesis of tricholoacetimidate glycosyl donor and subsequent glycosylatyion of cholesterol.

An important feature of this process is the lack of metal catalysts that could potentially react with other sites on the cholesterol leading to unwanted side products.

1.3 The armed 17 derivatives of Cholesterols

The investigation of biologically active steryl-glucosides (SG) can be carried out using different bio-imaging techniques. To perform this kind of techniques a precise modification of the cholesteryl skeleton is required, specifically in 17th carbon position. These modifications allow the molecule to be bound to fluorophores or other types of traceable moieties. In general, the preferred reaction in order to label SGs is the "click chemistry" thanks to its highly regio- and chemo-selectivity, as well as compatibility with biological media, and as a consequence of this, acetylenic or azide moieties are needed into SG's derivative's structure. Another strategy revolves around the transformation of the alkyl chain in 17th position into an active ester to form amide bonds. These general approaches are summarized in Scheme 4.



Scheme 4: Simplified schematic overall of some general approaches for the introduction of "Click Chemistry" suitable active linker on the 17th position of SG's structure.

Here we sum up, in a qualitative overview, the reaction sequences for the synthesis of cholesterol derivatives divided by the three main starting compounds: Pregnenolone, Dehydroepiandrosterone and Cholenic Acid; (see Figure 2).

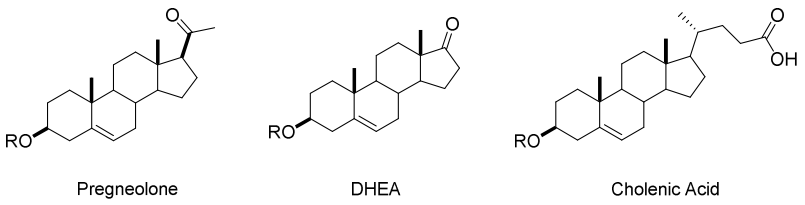
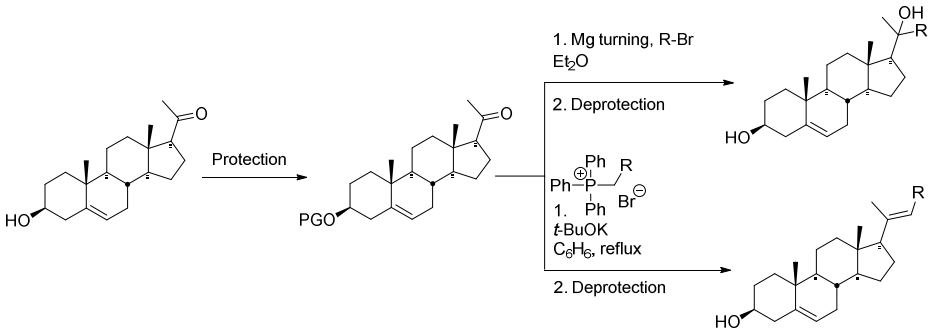


Figure 2: The three main starting compounds for the synthesis of cholesterol-derivatives

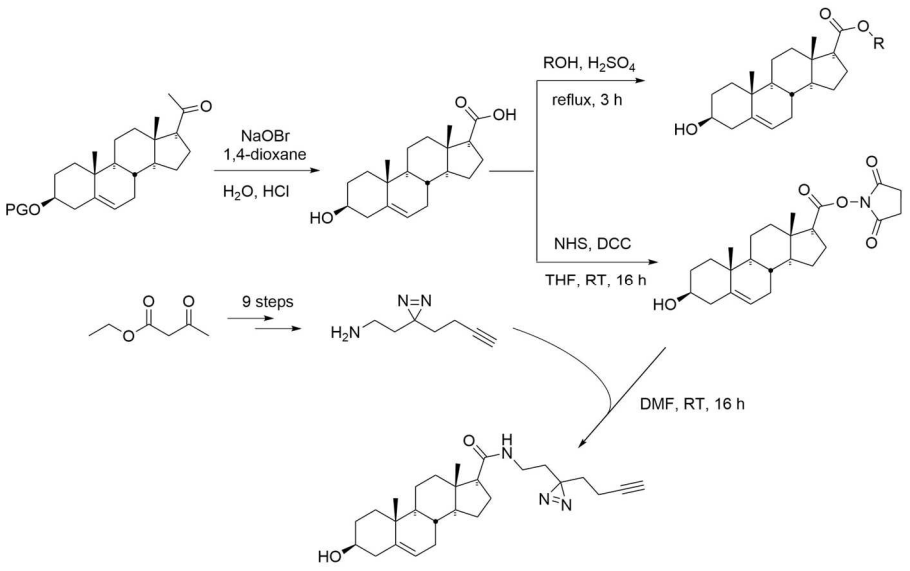
1.3.1 Pregnenolone as starting compound

Pregnenolone is an endogenous steroid involved in the biosynthesis of many steroid hormones such as androgens, estrogens, glucocorticoids and more. It is also classified as a neurosteroid and in 1950 it was used as a supplement to treat inflammatory processes. This molecule is suitable for the following modifications in 17th position as shown in Scheme 5



Scheme 5: Schematic synthesis for cholesterol derivatives using Pregnenolone as precursor and Greignard/Wittig reactions.

Both synthetic processes require a preliminary protection on the alcoholic function on the 3rd position of the molecule, using a protecting group (PG). Then, the active linker can be attached by Grignard reaction generating a new hydroxyl group (tertiary) or, alternatively using a Wittig reaction[10].

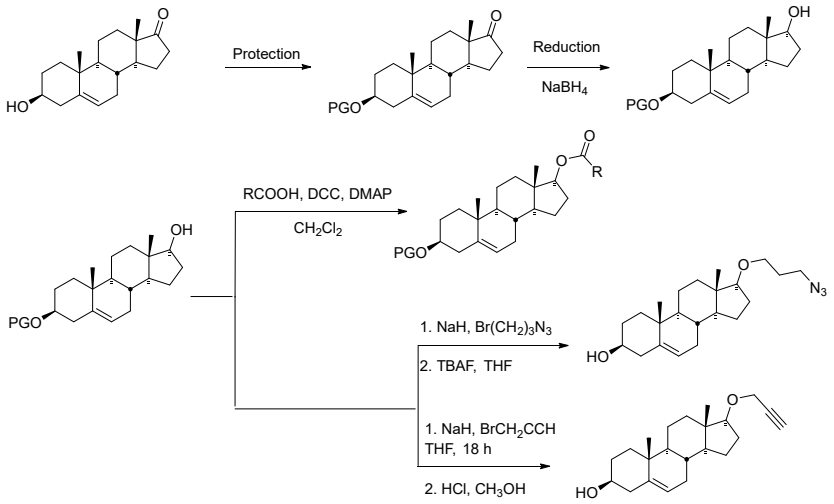


Scheme 6: schematic synthesis that allows to obtain an active ester through an haloform reaction starting from Pregnenolone.

A different strategy depicted in Scheme 6 involves the use of the protected Pregnenolone as precursor for an haloform reaction[11] whose product can be converted in two different products: a steryl-ester derivative, if the reaction is carried out using an alcohol[12], or an active ester, if the reaction is carried out with *N*-hydroxysuccinimide (NHS) and DCC. The last compound is very useful due to its reaction with a primary ammine containing an alkyne function, that can be further exploited for a *click-chemistry* reaction.[13]

1.3.2 Dehydroepiandrosterone as starting compound

Dehydroepiandrosterone (DHEA) or androstenolone is one of the most abundant circulating steroids in the human body. It is produced by the adrenal glands, brain and gonads and it is a very important precursor for the endogenous synthesis of sex steroids. DHEA have also a signaling function acting as a neurosteroid with the ability to interact with several cellular receptors, present in the nucleus and on the cell surface. Here we sum up 3 different schematic protocols to obtain useful derivatives of DHEA reported in Scheme 7

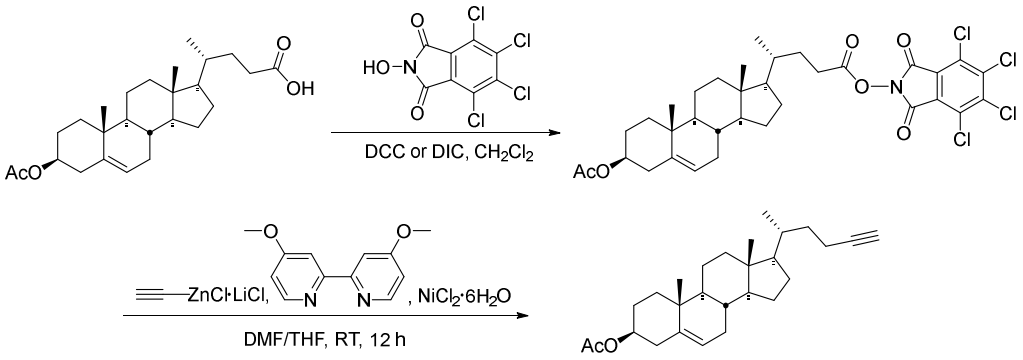


Scheme 7: Schematic synthesis of different cholesterol derivatives using DHEA as precursor.

Starting from a protected DHEA it is possible to reduce the 17th carbonyl group to create a hydroxyl function, after the protection of 3rd position. This can be exploited to produce an alkylsteryl ether by reaction with a carboxylic acid[14], or it can be used to obtain an azide/alkyn-containing sterol[15, 13].

1.3.3 Cholenic Acid as starting compound

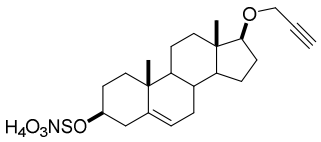
The last precursor is the cholenic acid, which is interesting due to its carboxylic function at the end of alkylic chain on the 17th carbon. This can be exploited for the generation of particular active ester that can be converted to acetylenic function by decarboxylative cross-coupling reaction with an alkynyl-zinc chloride in the presence of a catalyst.[16] This produce an alkynyl cholesteryl derivative similar to the one obtained from pregnenolone described before (see Scheme 5)



Scheme 8: Schematic synthesis for the alkynylation of Cholenic acid.

1.4 Modified cholesterols as probe for bio-imaging

Imaging in biological systems is a fundamental tool to investigate the mechanism of action and intra/extra cellular position of molecules of interest. A recent example worth of notice comes from the work of M. Chisari *et al*[13] relative to the cellular distribution of sulphated neuroactive steroids. These biomolecules perform a very important modulatory activity on the γ-amino-butyric acid receptors (GABARs) and the N-methyl-D-aspartate receptor (NMDARs). Specifically, pregnenolone sulfate (PREGS) acts as potentiator of the NMDARs and It has been shown that it positively influences memory and learning as well as reducing schizophrenia symptoms. To better understand the mechanisms of action and the cellular level of distribution, PREGS structure has been modified with different side chain alterations to afford retrospective click chemistry for visualization. The most relevant of these is the KK-169 reported in Figure 3



KK-169

Figure 3: Structure of Pregnenolone's derivative KK-169.

Cell cultures (*Xenopus laevis* oocytes) were incubated with the same amount of different PREGS analogues and fixed for click-chemistry labelling. After that, the fluorophore (AlexaFluor 488-azide) and the click reagent were added to afford retrospective *in situ* click chemistry.

Photomicrographs of cells incubated with and without KK-169 (Figure 4) showed the cellular distribution of the molecule. Furthermore, the evaluation of the receptor modulation proved that the side chain alterations retained the NMDAR potentiation of the molecule, thus proving the tolerability of the modifications.

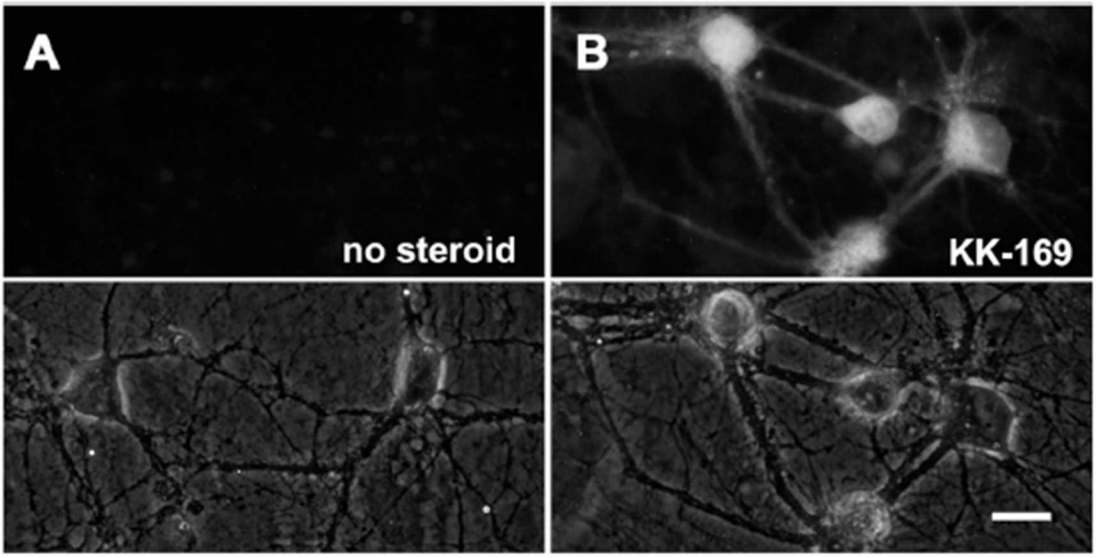


Figure 4: Photomicrographs of cells before and after incubation with labeled KK-169.

Another work worth of notice is the one reported by Lin and co-workers in 2016,[15] introducing a novel metabolic labelling of α-GC extracted by *H. Pylori.* This pathogen is auxotrophic for cholesterol which it converts in cholesteryl α-glucosides derivatives that are involved in the pathological process. The method consists in the labelling of activated αGC through click chemistry (see Figure 5) between an unsaturated fluorophore and a terminal azide function in the saturated chain of the metabolite; thus affording a femtomolar detection.

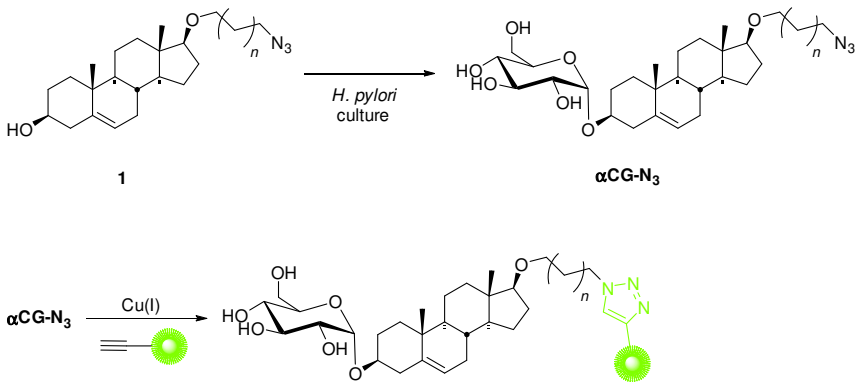


Figure 5: Schematic sequence for metabolic labeling of αGC derivatives from H. Pylory functionalized with a floorescent alkyne (green part).

2. Our contribution

The aim of this work is the synthesis of a β-gluchosyl cholesterol (**β–GluC**) derivative with a terminal acetylenic modification suitable for the conjugation with fluorophores/nanoparticles using click-chemistry; that will be subsequentially used to study glucosyl cholesterol's role and mechanisms in the ethiopathology of PD, similarly to what has been described in 1.4 paragraph. (See Figure 6)

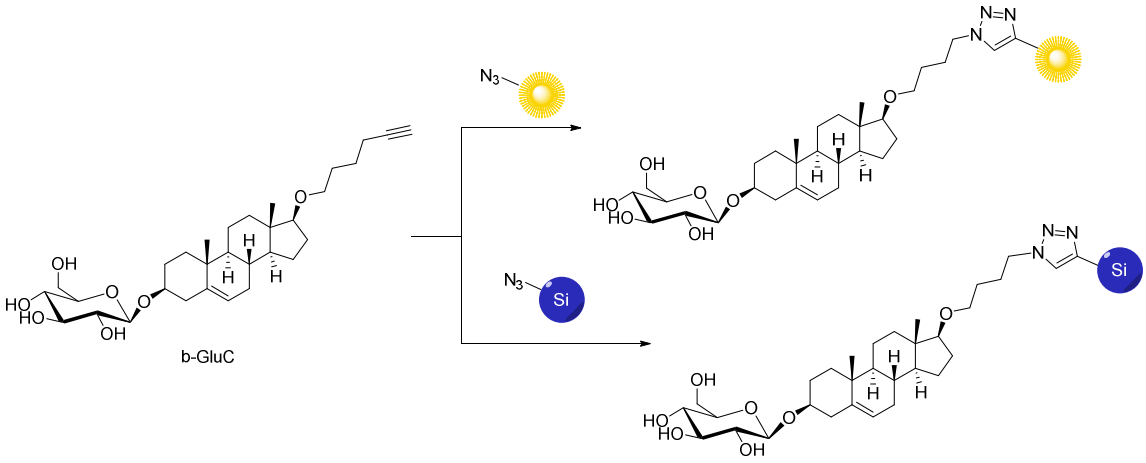
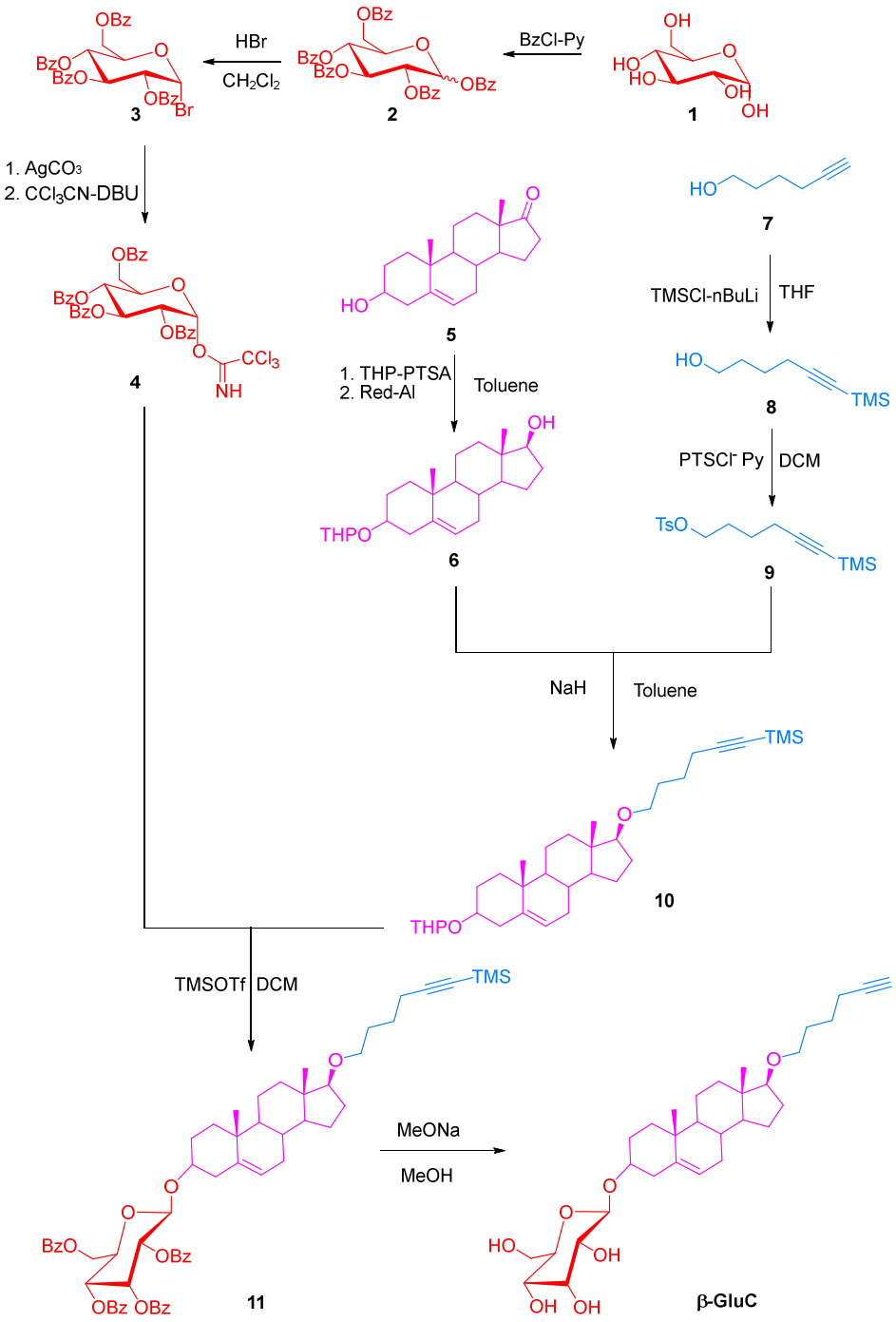


Figure 6: Schematic representation of the final product conjugated with either a fluorophore or a nanoparticle using "Click Chemistry"

The synthesis that we propose is schematized in Scheme 9 and described point to point as follows:

- α-D-glucose **1** has been protected by benzoyl groups in positions 1,2,3,4,6 and then converted into bromo-compound **3**. One-spot reactions transform **3** first into hydroxy group and then in 2,3,4,6-Tetra-O-benzoyl-α-D-glucopyranosyl-trichloroacetimidate **4**.
- 3β-hydroxy-5-epiandrosten-17β-one **5** was chosen as cholesteryl backbone and protected with tetrahydropyranyl group. The resulting compound was selectively reduced at the 17β carbonyl moiety to afford 3β-(2-Tetrahydropyranyloxy)-5-androsten-17β-ol **6**.
- 5-hexyn-1-ol **7** was protected with a trimethylsilyl group on position 6, in order to prevent side reaction in final coupling between colesteryl moiety and glucosyl portion. The -OH was converted in LG by tosylation to give the final active arm **9**

- A nucleophilic substitution has been carried out between compound **6** and **9** leading to 3β-(2-Tetrahydropyranyloxy)-5-androsten-17β-6-trimethylsilyl-hex-5-yne **10**
- Glycosilation of **10** using **4** and subsequent deprotection of TMS and Benzoyl locked moieties leads to the final 3β-glucosyl-(2-Tetrahydropyranyloxy)-5-androsten-17β-6-trimethylsilyl-hex-5-yne **11.**



Scheme 9: Reaction scheme for the synthesis of β–GluC

The choice of benzoyl protector (Bz) groups for the synthesis guaranteed the correct sterical orientation of the glucose moiety during the glycoside coupling; as well the cleavage of Bz required basic conditions compatible with others moieties present in the molecule (i.e acetylic and double bond). It is worth pointing out that the glycosidic coupling cannot be carried out using metallic catalysts (for example Silver) due to the potential interactions with the terminal acetylenic, we've then chosen to employ Deng *et al*. methodology[9] already described in introduction section, that uses the precursor **4** and Lewis acid TMSOTf.

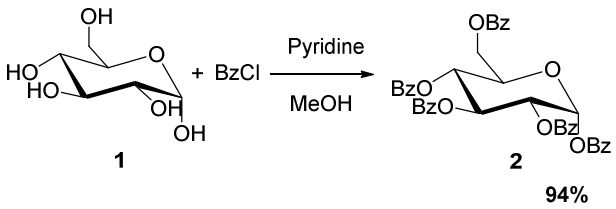
3. Results and discussion

3.1 Synthesis of Protected Glucoside Precursor

The synthesis of the benzoyl-protected glucoside moiety has been carried out taking inspiration from the procedure described by Deng *et al.*[9] already described in paragraph 1.2, as well the reactions sequence shown in scheme 9.

3.1.1 Synthesis of 1,2,3,4,6-Penta-O-benzoyl-D-glucopyranose

The protection of the D-Glucose **1** has been carried out with nearly quantitative yield after extractive work-up without any further purification processes (see Scheme 10).[17]



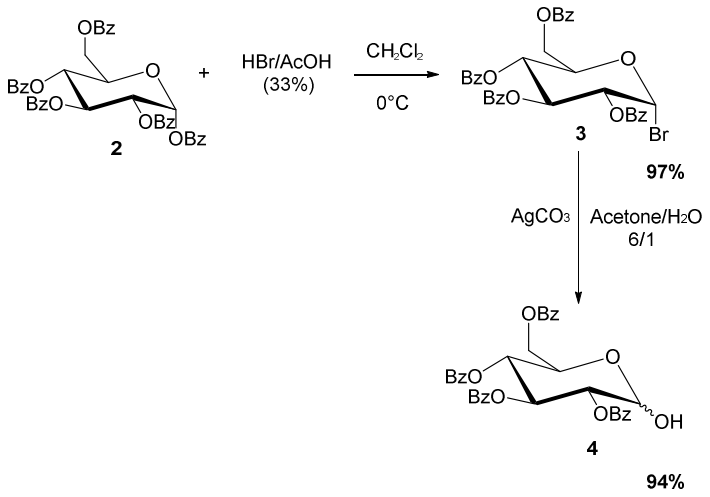
Scheme 10: Synthesis of 1,2,3,4,6-Penta-O-benzoyl-α-D-glucopyranose

The obtained 1,2,3,4,6-Penta-O-benzoyl-α-D-glucopyranose (**2**) presented itself as a white powder. The ^{1}H NMR spectrum analysis revealed the presence of an axial proton as doublet at 6.85 ppm, thus suggesting the presence of the α-anomer of product **2**, while β-anomer was not be detected.

3.1.2. Synthesis of Synthesis of 2,3,4,6-Tetra-O-benzoyl-1-hydroxy-D-glucopyranose

The introduction of hydroxyl group on the anomeric carbon is described on scheme 11. Specifically, the procedure required two consecutive reactions: (*i*) the first step is a site-specific halogenation on the anomeric carbon using HBr/AcOH.[17] This kind of reaction is totally stereoselective as revealed by the only doublet present in ^{1}H NMR at 6.87 with J = 4.0 Hz ascribed the to anomeric equatorial proton; (*ii*) After isolation of halogenated product **3** by simple extraction with DCM from reaction crud; the second step required the addition of Silver

Carbonate portion-wise in order to re-establish the alcoholic function on the anomeric carbon.[18]

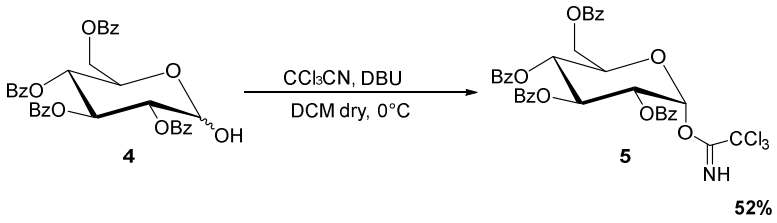


Scheme 11: Synthesis of 2,3,4,6-Tetra-O-benzoyl-1-hydroxy-D-glucopyranose

The silver salts formed during the reactions were turned away by simple filtration through celite pad to give **4** in 94% overall yield as white solid mixture of two anomers.

3.1.3. Synthesis of 2,3,4,6-Tetra-O-benzoyl-α-D-glucopyranosyl trichloroacetimidate

As described in Scheme 12 the final step of the synthesis of the glucoside precursor **5** required the use of trichloroacetonitrile to convert the -OH moiety into a LG trichloroacetimidate using DBU as non-nucleophilic base in anhydrous conditions.[18]



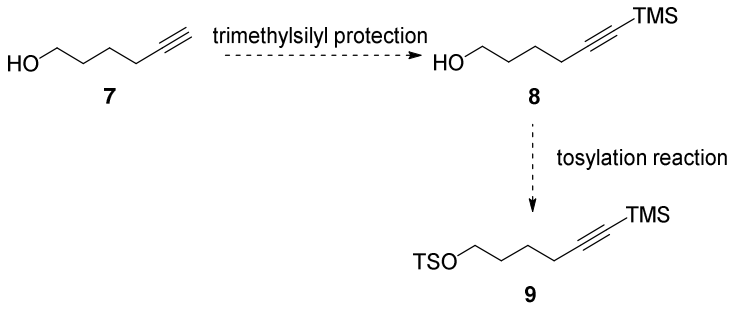
Scheme 12: Synthesis of 2,3,4,6-Tetra-O-benzoyl-α-D-glucopyranosyl trichloroacetimidate

Contrary to what is described in literature, the product **5** was obtained as pure α-anomer only after flash chromatography (FC) purification in a yield equal to 52% as detected by ^{1}H NMR by the precence of doublet at 6.84 ppm with *J* = 3.6 Hz, charateristic of anomeric equatorial proton.[19] Through this purification it was possible to isolate 17% of product β, identified by the anomeric axial proton as a doublet at 6.23 ppm *J* = 7.5 Hz.[20] Probably the partial lack of

stereoselectivity of the reaction is mostly due to the ineffective temperature control, based on a simple external ice bath. Better results in terms of stereoselectivity would have most likely been obtained by using a cryostatic bath with external circulation.

3.2 Synthesis of the alkynyl chain

The synthesis of the arm **9** revealed herself to be more challenging than expected, requiring the trial of different solvents and reaction conditions. The common strategy to obtain **9** is reported in sequence reactions depicted in scheme 13

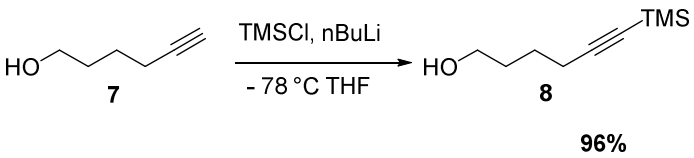


*Scheme 13: Reaction scheme for the synthesis of 1-tosyl-6-trimethylsilyl-hex-5-yne **9***

As previous described in Chapter 2, the first step was the protection of acetylenic moiety, and then the tosylation reaction.

3.2.1 Synthesis of 6-(Trimethylsilyl)hes-5-yn-1-ol

As shown in scheme 14 the synthesis of acetylenic **8** was carried out by action of the base *n*BuLi in THF at -78°C as described by P. Srihari et al reported in 2011.[21]

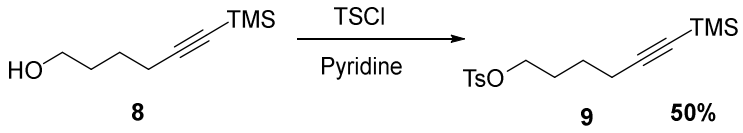


Scheme 14: Synthesis of 6-(Trimethylsilyl)-hes-5-yn-1-ol

The reaction was nearly quantitative, requiring only an extractive work-up with solvent. The identity of product **8** was confirmed by the presence of the α-acetylenic proton at 2.27 pmm as triplet (J = 6.6 Hz) and TMS group as singlet, resonanting at 0.15 ppm.

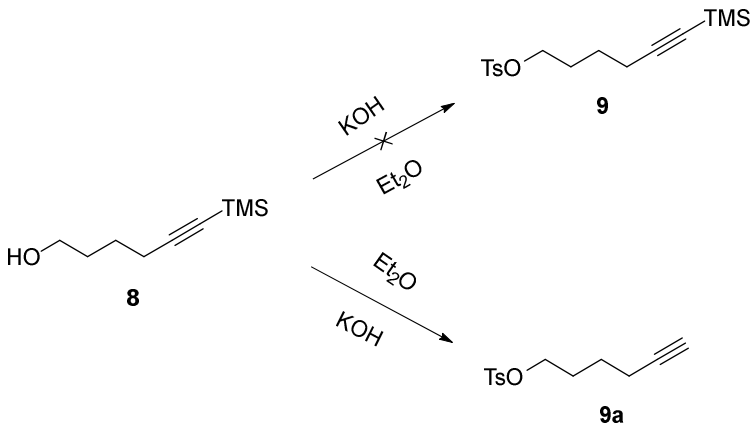
3.2.2. Synthesis of 1-tosyl-6-trimethylsilyl-hex-5-yne

In order to obtain the reactive alkynyl chain **9**, the procedure of Davison *et al*[22] has been adopted as first trial, in which pyridine is used both as solvent and HCl scavenger (see Scheme 15).



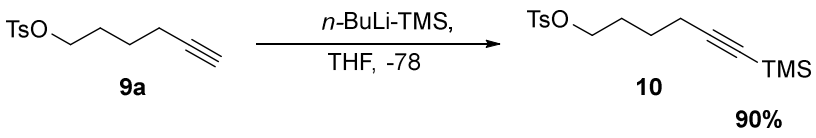
Scheme 15: First attempt in the synthesis of 1-tosyl-6-trimethylsilyl-hex-5-yne

Unfortunately, using the reaction conditions described in literature, the product **9** was obtained in poor yield. In fact, ^{1}H NMR analysis of crude reaction revealed the presence of a second triplet, resonating at 3.59 ppm, probably of chlorinated side-product, formed by the action of the nucleophile Cl^- on the newly formed tosyl **9**. These unsatisfactory results required the application of a different protocol to obtain molecule **8** as suggested by J. Bucher *et al*,[23] using KOH as scavenger agent in Et_2O. In this case ^{1}H-NMR analysis revealed the absence of the desired product **9**, and the presence in almost quantitative yield of the tosylated **9a** where, the TMS function has been lost (see scheme 16).



*Scheme 16: Schematic representation of the erroneous outcome of the synthesis of **9** using the J. Butcher et al strategy, leading to the production of **9a**.*

Since the quantity of the unwanted **9a** product was considerable, we decided to recover it by treating **9a** with *n*BuLi and TMSCl at low temperature as suggest by Sinha *et al.*[24] (see Scheme 17).



*Scheme 17: Synthesis of **10** starting from **9a***

The reaction yield was 90% and the product did not required further purification after the extraction work-up. This excellent result convinced us to re-think the original sequence described in scheme 9 in favour of that depicted in scheme 18, where the reaction order is inverted.

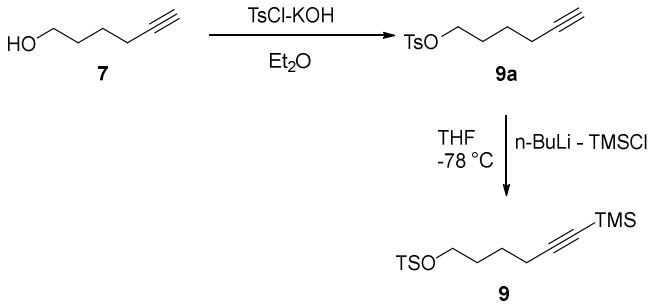
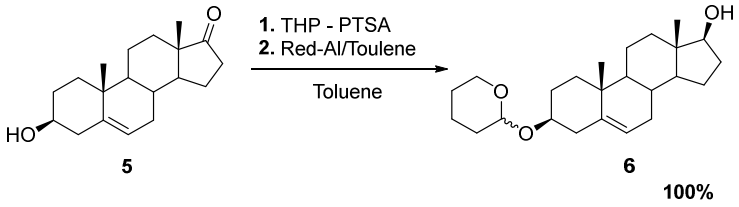


Figure 18: Schematic representation for the new strategy used to obtain product 9 from 7

The new sequence allowed us to produce **9** in multigram scale, without purification steps and avoiding the use of toxic solvent Pyridine.

3.3 Synthesis of Sterol Precursor

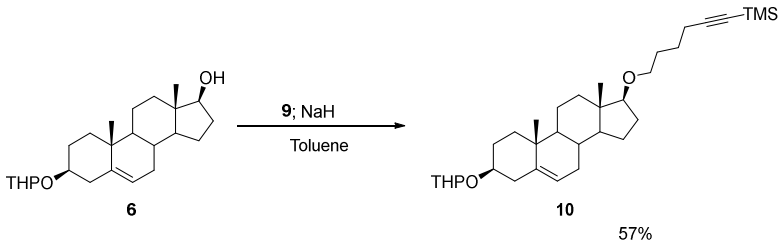
For the synthesis of the cholesterol backbone we chose to adapt the procedure described by Pouzar *et al.*[25] As sterol skeleton we have chosen the DHEA precuror **5**. The reaction is schematized in Scheme 19:



Scheme 19: Reaction scheme for the synthesis of 3Beta-(2-Tetrahydropyranyloxy)-5-androsten-17Beta-ol

This 2 steps procedure involves firstly the protection of position 2 with THP, using a catalytic quantity of PTSA. This intermediate is directly reduced by action of Red/Al agent in the same solvent to give **6** in quantitative yield. The ^{1}H NMR signals are consistent with those present in literature.[25]

3.4. Synthesis of modified Cholesterol with acetylenic linker

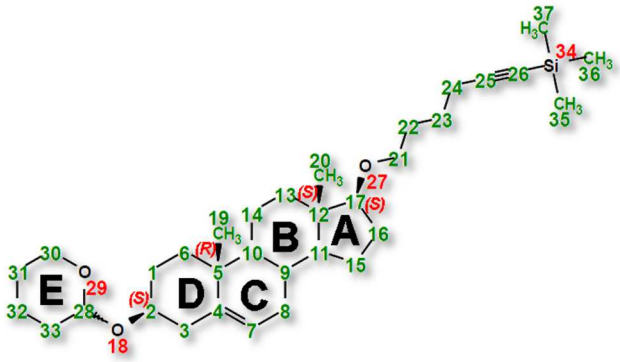


Scheme 20: reaction scheme for the synthesis of TMS-Hexyn-THP-DHEA

For the synthesis of TMS-Hexyne-THP-DHEA we adapted the protocol described by Jan M.H. et al[26, 13] (see Scheme 20). Specifically, we proceeded to create a nucleophilic site on the oxygen in position 17 of **6**, by reaction with NaH. This allowed us to perform a nucleophilic substitution in presence of **9** leading, after an extraction work-up and FC purification, to the desire product with a yield of 57%. The exhaustive NMR and MS analysis of **10** is describe in the next paragraph.

3.5 Complete ^{1}H-^{13}C NMR Spectroscopy Signal Assignment and MS analysis of 10

In NMR analysis, cholesteryl systems can be defined as "*Everest Mountain*" for signals attribution, because all protons and carbons are different, than theoretically all assignable. This characteristic is due to the chirality of the molecule. The presence of four stereo-centres in position 17, 12, 5 and 2 as shown Figure 7, produces several diastereotopic situations in several protons with huge geminal coupling. This asymmetry causes complex ^{1}H NMR spectra that are difficult to interpret, and no-useful as starting point in signals attribution.



10

*Figure 7: Molecular atom-numbered structure of **10**.*

It is important to underline that the molecule **10** is present as mixture of diastereomers thanks to presence of the new stereo-centre **28** generated through the protection of DHEA with THP. The presence of two molecules instead of one, will be more evident in carbon spectra analysis where the doubling of the signals can be observed.

First, the starting point of our analysis was the exact chemical shift protons attribution of **2**, **17**, **21** and **30** using HSQC spectra (Figure 8, portion A). This approach is justified for ^{1}H NMR complexity, as above mentioned. The correct attribution of these protons allowed to assign H_{28} *via* dipolar interactions in NOESY experiment (Figure8, portion B).

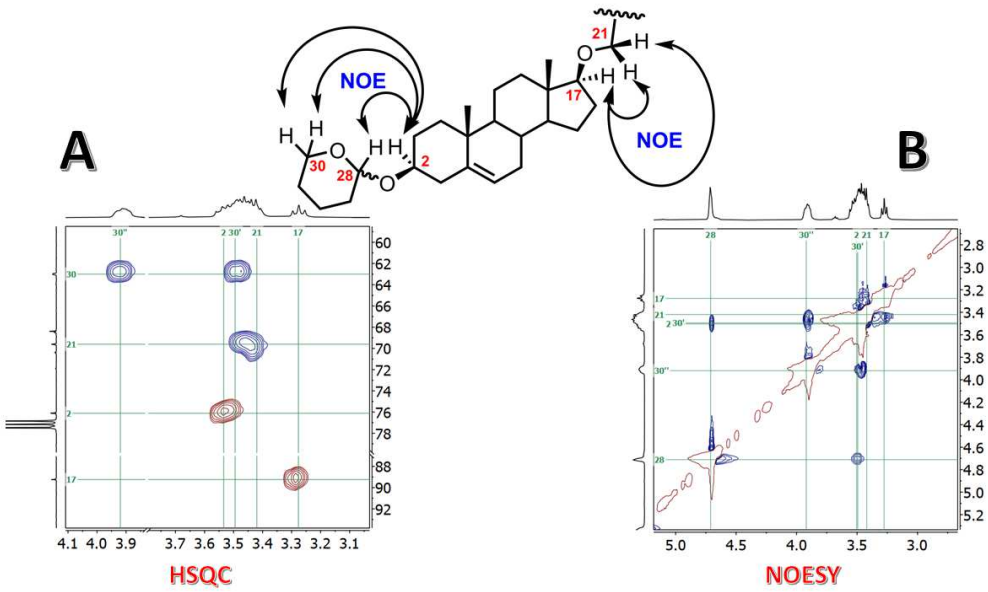


*Figure 8: NMR 2D of **10**. (A) Spectral region showing a portion of HSQC spectra for atoms at positions 2, 17,21 and 20. (B) Spectral region showing the dipolar interaction between cholesterol skeleton and alkenyl chain and THP protecting group*.

The position of H_{28} and H_{30} allow to identify the other protons present in THP ring E *via* dipolar interactions as shown in NOESY portions A and B in Figure 9, and scalar interactions in portion C, same figure, in COSY experiment.

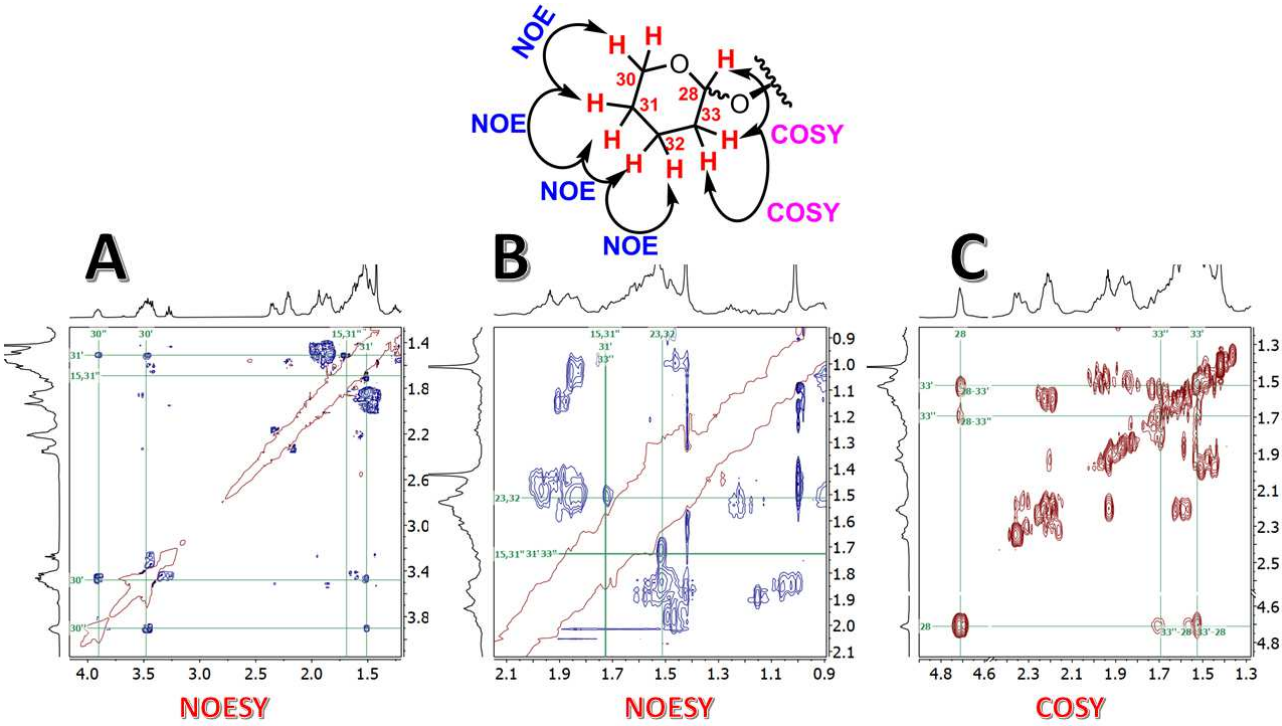


*Figure 9: NMR 2D **A** and **B** Portion of NOESY spectra and related dipolar interaction of **10**; C portion scalar interaction between H_{28} and H_{33}*

Thus, using an existing ^{13}C attribution carried out by us for a similar structure,[1] using the HSCQ spectra (portion A Figure 10) all of protons present in rings A, B, C and D in cholesterol skeleton has been assigned. The remaining quaternary carbons signals C_5, C_6, C_{12}, C_{25} and C_{26} can be easily identified by long range interactions in heteronuclear HMBC spectra (portion B and C, Figure 10).

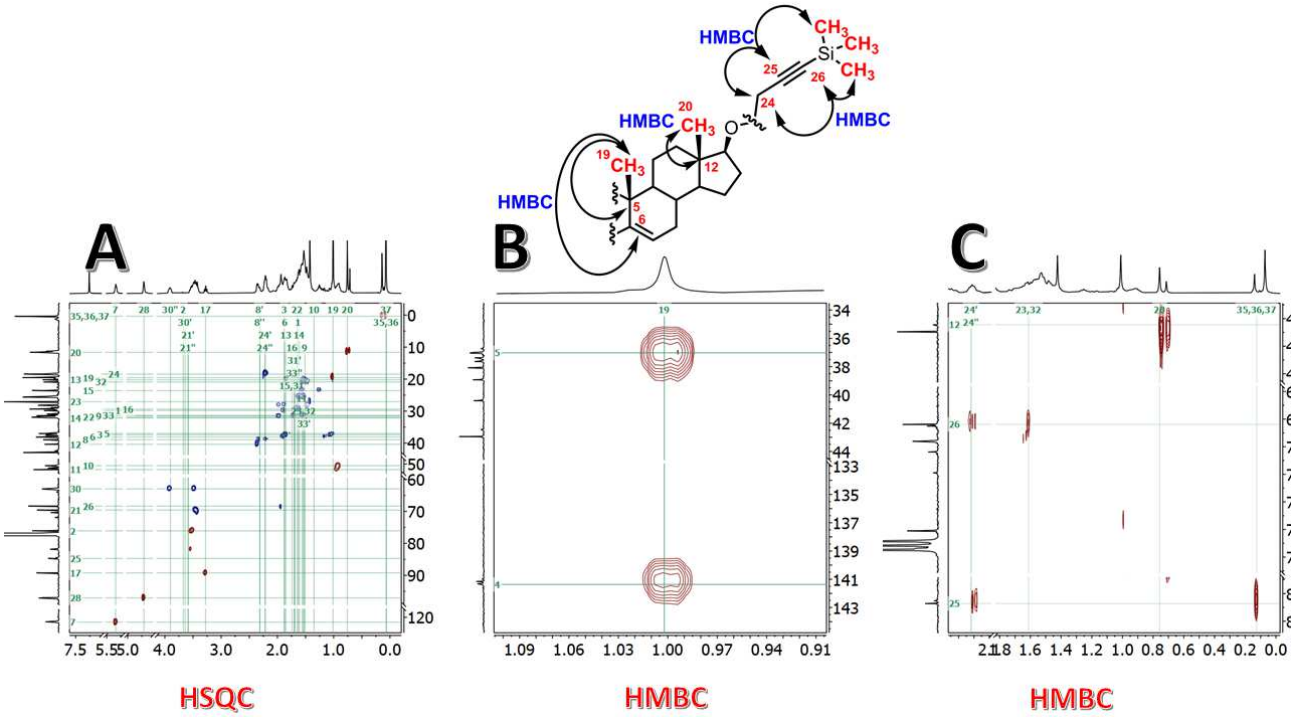


*Figure 10: NMR 2D: Portion A HSQC spectrum of **10**; Portions B and C HMBC spectra of **10**.*

All these observations are summarized clearly in Figure 11 through the complete mono-dimensional proton assignment for **10**.

[1] Lunardon, S. *"Total Synthesis of Functionalized Steryl α-D-Glucoside Derivative Linked to Parkinson's Disease"*, **2020**, *Master's Degree Thesis*

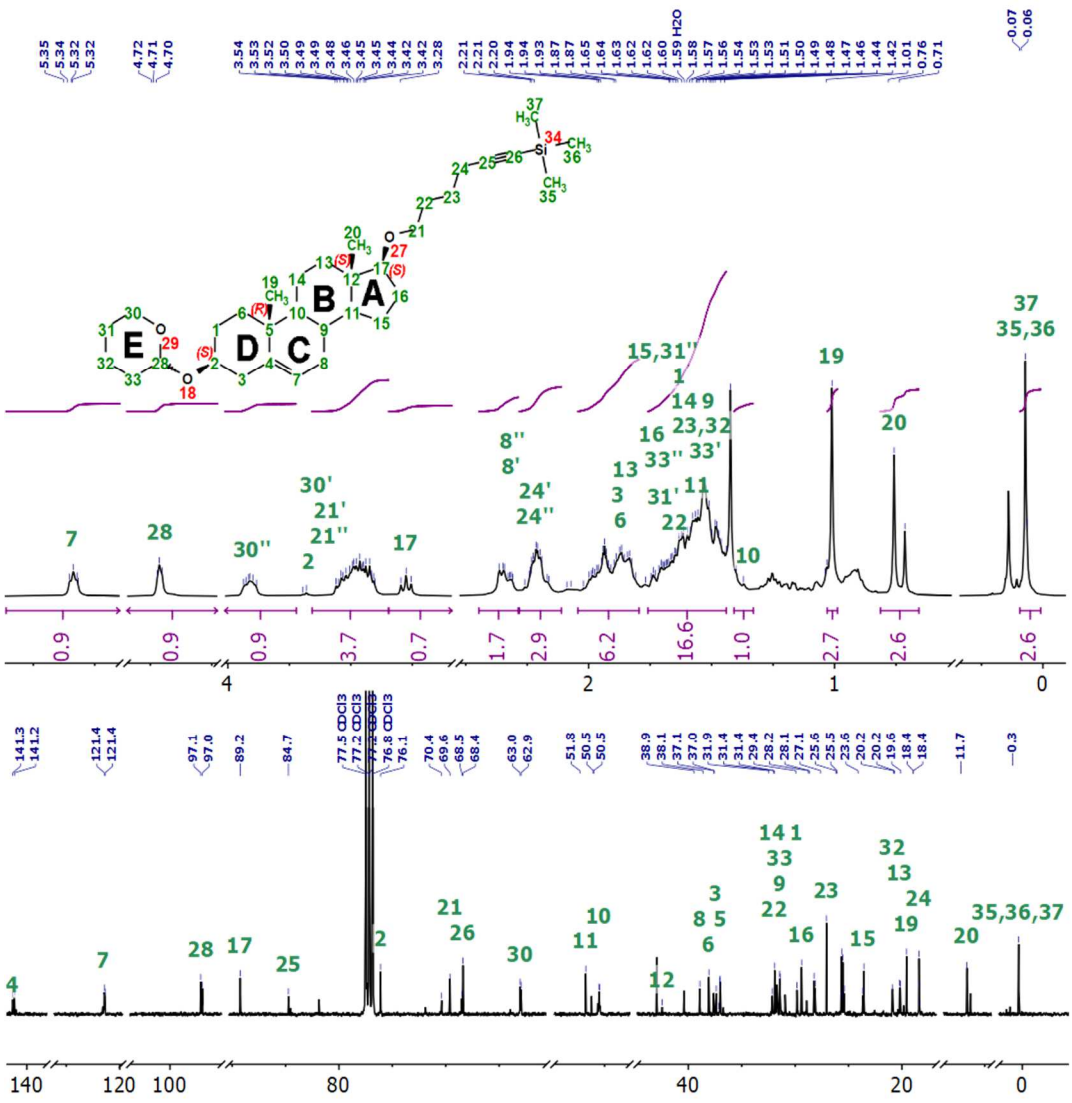


Figure 11 ^{1}H (top) and ^{13}C (bottom) NMR spectra of **10**.

It is possible to appreciate the diastereotopic splitting of several signals in ^{13}C NMR spectra, as described in the previous paragraph, induced by the chirality of the cholesteryl structure. Further identity confirmation of **10** has been obtained with ESI-MS analysis by direct infusion of sample. The molecular mass (LRMS m/z: $[M + Ag]^+$ calcd for $C_{33}H_{54}AgO_3Si^+$ 633.2; found 633.6) and relative isotopic pattern is in accordance to NMR analysis (Figure 12). In order to ionize **10**, a solution of Silver Nitrate has been used (10 µg/mL in $AgNO_3$ in MeOH).

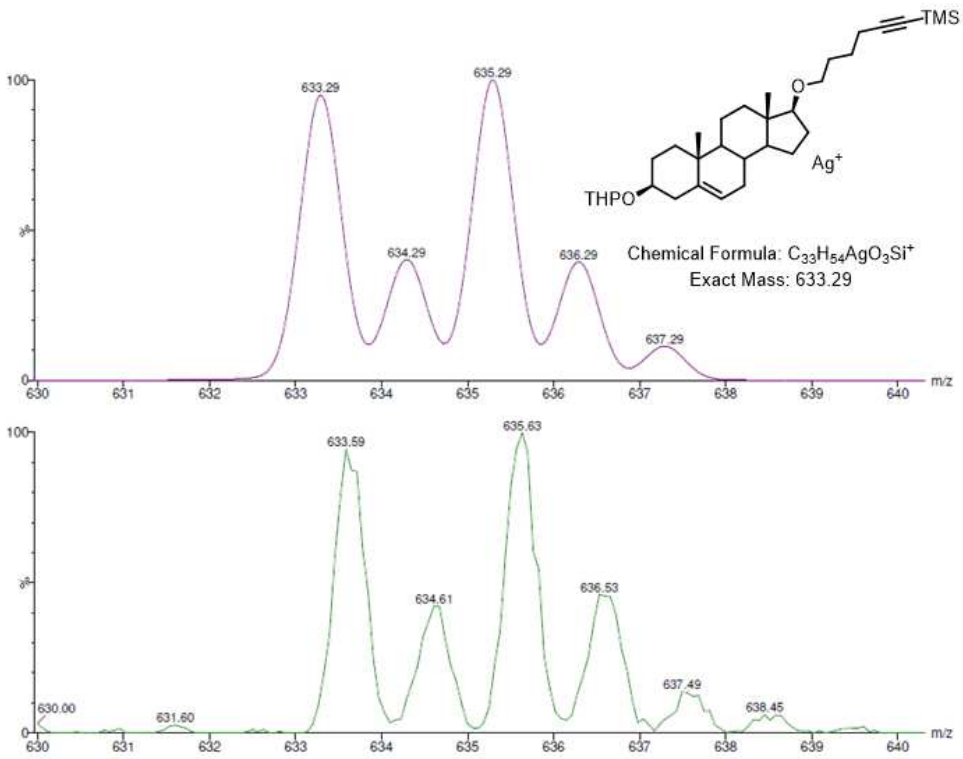


Figure 12: ESI-MS of adduct [M+Ag⁺] for **10**.

6. Conclusions and future perspectives

The first part of this project was dedicated to the development of a synthetic strategy that would allow us to obtain a glucoside derivative suitable for a regio-and stereo-selective glycosylation of a cholesterol derivative. To achieve this goal, we employed a procedure that revolved around the protection of the reactive moieties of glucose and 3-3-hydroxy-5-androsten-17-one. For the glucoside precursor, protector and leaving groups were carefully selected for the reasons explained in chapters 1.2 and 2.

The protection of the cholesterol precursor **6** was carried out quite easily and allowed us to obtain a large amount of it for future developments of the project.

The protection of the acetylenic linker **9** required several trials and errors to synthetize properly, but the new procedure discovered during this thesis allowed to obtain this molecule in high yield, and avoid the using toxic solvent Pyridine.

The nucleophilic substitution allowed us to obtain enough quantity of product **10** to exhaustively characterize it using NMR and mass spectroscopies. The low yield that affects this synthesis could be improved with further optimization of the reaction's conditions.

The new obtained product was characterized using 1D and 2D ^{1}H, ^{13}C NMR spectroscopy and ESI-MS spectroscopy.

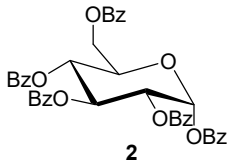
Future development of this project will revolve around the completion of the synthetic path leading to the molecule **11** and its coupling with different probes, exploiting its "Click Chemistry" prone terminal. These studies could strengthen our understanding in the role played by glucosyl-cholesterols inside our bodies and their connection with PD; leading to a potential drug-target or biological marker useful for the treatment of this disease.

5. Experimental Section

5.1 General Methods

Reagents and solvents with high purity degree purchased by the providers were used as given. Otherwise, they were purified following the procedures reported in literature. Anhydrous solvents were prepared by adding activated 4 Å molecular sieves to the solvent under inert atmosphere. Molecular sieves were activated shortly before the use by continuous heating under *vacuum*. The reactions were followed with TLC Polygram® Sil G/UV_{254}, 0.25 mm thickness. Flash chromatography was performed with silica gel Merk 60 (230-400 mesh) following procedures reported in literature. ^{1}H NMR, ^{13}C NMR proton decoupled, and 2D spectra were recorded with Bruker Avance 300 and Ascend 400 spectrometers, working at 300-400 and 75-100 MHz respectively. Resonance frequencies are referred to tetramethylsilane, chemical shifts are expressed in ppm with reference to the deuterated solvent residual peak. Multiplicity is addressed as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), m (multiplet), and so on. IR spectra were recorded with a Perkin Elmer Spectrum One spectrophotometer. Mass spectrometric measurements were performed using a Waters ZQ Spectrometer for low resolution analysis. The compounds studied were dissolved in methanol with a concentration of 5×10^{-3} M. They were injected into the ESI source by direct infusion with a syringe pump integrated in the mass spectrometer. Mass spectra were acquired in positive-polarity mode.

1,2,3,4,6-Penta-O-benzoyl-α-D-glucopyranose

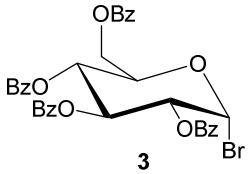


2

A solution of *D*-Glucose (10.0 g, 55.5 mmol) and anhydrous pyridine (120 mL) was created, and cooled to 0 °C. Than benzoyl chloride (42 mL 362 mmol) was poured slowly, portion wise, and the reaction was allowed to reach room temperature overnight under stirring. At this point MeOH (50 mL) was added to the mixture and then stirred for 15 min, diluted with CH_2Cl_2, and washed with 1M HCl, water, brine. The obtained mixture was dried using $MgSO_4$ and the remaining solvent was allowed to evaporate under reduced pressure. 2,3,4,6-Penta-O-benzoyl-α-D-glucopyranose **2** (36.4 g, 94%) was obtained as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.17 (dd, J = 8.3, 1.4 Hz, 2H, Ar-H)), 8.03 (dd, J = 8.3, 1.4 Hz, 2H, Ar-H), 7.95 (dd, J = 8.3, 1.4 Hz, 2H, Ar-H), 7.92 – 7.83 (m, 4H, Ar-H), 7.69 – 7.65 (m, 1H, Ar-H), 7.58 – 7.27 (m, 14H, Ar-H), 6.85 (d, J = 3.7 Hz, 1H, H-1), 6.32 (t, J = 10.0 Hz, 1H, H-3), 5.86 (t, J = 9.8 Hz, 1H, H-4), 5.68 (dd, J = 10.3, 3.8 Hz, 1H, H-2), 4.65 – 4.58 (m, 2H, H-5, H-6a), 4.52 – 4.45 (m, 1H, H-6b).

2,3,4,6-Tetra-O-benzoyl-α-D-glucopyranosyl bromide



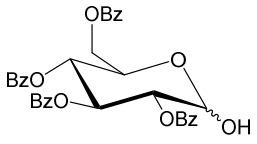
3

A solution of 1,2,3,4,6-Penta-O-benzoyl-α-D-glucopyranose **2** (15 g, 22.7 mmol) and CH_2Cl_2 (190 mL) was prepared and cooled to 0 °C. While being stirred HBr (33% in AcOH, 45 mL) was added and the reaction was allowed to reach room temperature overnight. The reaction mixture cooled using ice-water and stirred for 15 mins, allowing the layers to separate. The aqueous layer was extracted using CH_2Cl_2. The remaining organic layer was washed with $NaHCO_3$ (satd.) and brine, then dried using $MgSO_4$. The solvent was evaporated under reduced

pressure, to give 2,3,4,6-Tetra-O-benzoyl-α-D-glucopyranosyl bromide **3** (13.63 g, 97%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 – 8.04 (m, 2H, Ar-H), 8.02 – 7.98 (m, 2H, Ar-H), 7.98 – 7.93 (m, 2H, Ar-H), 7.90 – 7.85 (m, 2H, Ar- H), 7.61 – 7.49 (m, 2H, Ar-H), 7.48 – 7.34 (m, 8H, Ar-H), 7.34 – 7.27 (m, 2H, Ar-H), 6.87 (d, J = 4.0 Hz, 1H, H-1), 6.27 (t, J = 9.8 Hz, 1H, H-3), 5.83 (t, J = 10.0 Hz, 1H, H-4), 5.33 (dd, J = 10.0, 4.0 Hz, 1H, H-2), 4.78 – 4.71 (m, 1H, H-5), 4.67 (dd, J = 12.5, 2.7 Hz, 1H, H-6a), 4.52 (dd, J = 12.5, 4.5 Hz, 1H, H-6b).

2,3,4,6-Tetra-O-benzoyl-D-glucopyranose

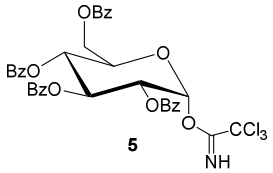


4

2,3,4,6-Tetra-O-benzoyl-α-D-glucopyranosyl bromide **3** (5g 7.59 mmol) was dissolved in a solution of acetone/H_2O (6:1 35 ml) and Ag_2CO_3 (35 ml) was added. The mixture was than stirred for 4 hours and then filtered through a celite pad. The solvent was removed under reduced pressure giving 2,3,4,6-Tetra-O-benzoyl-D-glucopyranose **4** (4.23 g 94%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11- 8.02 (overlapping signals, 4H, Ar-H), 8.00-7.96 (overlapping signals, 4H, Ar-H), 7.96-7.92 (overlapping signals, 2H, Ar-H), 7.92-7.84 (overlapping signals, 4H, Ar-H), 7.59-7.46 (overlapping signals, 4H, Ar-H), 7.45-7.33 (overlapping signals, 10H, Ar-H), 7.32-7.26 (overlapping signals, 2H, Ar-H), 6.25 (t, J = 10.0 Hz, 1H, H-3α), 5.97 (t, J = 9.7 Hz, 1H, H-3β), 5.77 (d, J = 3.6 Hz, 1H, H-1α), 5.73 (t, J = 9.8 Hz, 2H, H-4α), 5.37-5.34 (m, 1H, H-2β), 5.32 (dd, J = 10.2, 3.6 Hz, 1H, H-2α), 5.07 (d, J = 8.0 Hz, 1H, H-1β), 4.72-4.62 (overlapping signals, 3H, H-5α, H-6aα and H-6aβ), 4.51 (dd, J = 12.2, 5.1 Hz, 1H, H-6bβ), 4.46 (dd, J = 12.0, 4.3 Hz, 1H, H-6bα), 4.20 (ddd, J = 10.1, 5.1, 3.0 Hz, 1H, H-5β), 3.14 (br s, 1H, OH), 1.57 (br s, 1H, OH).

2,3,4,6-Tetra-*O*-benzoyl-α-D-glucopyranosyl trichloroacetimidate



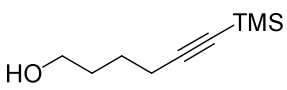
5

A solution of 2,3,4,6-Tetra-O-benzoyl-D-glucopyranose **4** (4.23 g, 7.9 mmol) was prepared using anhydrous CH_2Cl_2 (25 mL) as solvent. After being cooled to 0°C, Trichloroacetonitrile (5.5 mL, 54 mmol) and DBU (1.35 mL, 9 mmol) were both added dropwise and the obtained solution was stirred at 0°C for 2.5 h. The solvent was partially evaporated under reduced pressure and the remaining mixture was subjected to FC (cyclohexane-EtOAc 4:1) to give 2,3,4,6-Tetra-*O*-benzoyl-α-D-glucopyranosyl trichloroacetimidate **5** as a white solid (0.91g 52%). Rf 0.40 (cyclohexane-EtOAc 4:1);

^{1}H NMR (400 MHz, $CDCl_3$): α- anomer: δ = 4.48 (dd, J = 5.7, 12.9 Hz, 1 H, 6-H), 4.61-4.66 (m, 2 H, 5-H, 6-H), 5.61 (dd, J = 3.8, 10.0 Hz, 1 H, 2-H), 5.81 (t, J = 10.0 Hz, 1 H, 4-H), 6.27 (t, J = 10.0 Hz, 1 H, 3-H), 6.83 (d, J = 3.8 Hz, 1 H, 1-H), 7.24-7.58 (m, 12 H, ArH), 7.84-8.03 (m, 8 H, ArH), 8.62 (s, 1 H, NH); β-anomer: δ = 4.38 (ddd, J = 3.5, 4.8, 9.5 Hz, 1 H, 5-H), 4.54 (dd, J = 4.8, 11.9 Hz, 1 H, 6-H), 4.68 (dd, J = 3.5, 11.9 Hz, 1 H, 6-H), 5.82 (dd, J = 7.5, 9.0 Hz, 1 H, 2-H), 5.82 (dd, J = 9.0, 9.5 Hz, 1 H, 4-H), 5.97 (t, J = 9.0 Hz, 1 H, 3-H), 6.23 (d, J = 7.5 Hz, 1 H, 1-H), 7.24-7.56 (m, 12 H, ArH), 7.85-8.04 (m, 8 H, ArH), 8.70 (s, 1 H, NH) ppm.

^{13}C NMR (101 MHz, $CDCl_3$): α-anomer: δ = 62.3 (C-6), 68.5 (C-4), 70.0 (C-3), 70.5 (2 s, 2 C, C-2, C-5), 92.8 (C-1), 127.96, 127.99, 128.05, 128.17, 128.23, 128.5, 129.2, 129.3, 129.4, 129.5, 132.7, 132.9, 133.2, 160.0 (C-NH), 164.7 (C=O), 164.9 (C=O), 165.1 (C=O), 165.5 (C=O); β-anomer: δ = 62.6 (C-6), 68.9 (C-4), 70.5 (C-2), 72.4 (C-3), 72.8 (C-5), 95.6 (C-1), 127.96, 128.03, 128.05, 128.3, 128.6, 129.2, 129.37, 129.42, 129.44, 132.7, 133.0, 133.1, 160.5 (C-NH), 164.3 (C=O), 164.6 (C=O), 165.1 (C=O), 165.6 (C=O)

6-(trimethylsilyl)-hex-5-yn-1-ol

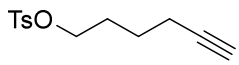


8

A solution of 5-hexyn-1-ol (3.37 mL, 30 mmol) and THF (52 mL) was cooled at -78 °C and put under stirring. Butyllithium (41 ml, 66.05 mmol, 10 M in hexanes) was added dropwise and, after 1 hour of stirring, chlorotrimethylsilane (8.4 mL, 66.05 mmol) was added while keeping the temperature at -78 °C. 1 hour later, the obtained solution was warmed to 0 °C. Aqueous 1M HCl (51 mL) was added dropwise and the stirring continued for other 30 minutes at room temperature. Extraction with diethyl ether (2 x 10 mL) was performed and, after the washing of the organic layer with water (30 mL) and brine (10 mL), the resulting solution was dried over anhydrous $MgSO_4$, filtered and the solvent was evaporated under vacuum obtaining 6-(trimethylsilyl)hex-5-yn-1-ol (**8**) as a colourless oil (4.93 g, 96%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.73 – 3.62 (m, 2H, CH2OH), 2.27 (t, J = 6.8 Hz, 2H, C≡CCH2), 1.80 – 1.52 (m, 4H, CH2CH2), 1.13 (br s, 1H, OH), 0.14 (s, 9H, TMS); 13C NMR (101 MHz, CDCl3) δ 107.3, 84.9, 62.6, 32.0, 25.0, 19.8, 0.3.

Hex-5-yn-1-yl 4-methylbenzenesulfonate

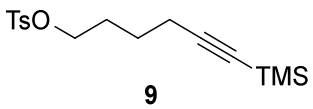


9a

A solution of *p*-toluensulfonyl chloride (1.16 g, 6.08 mmol) and 5-hexyn-1-ol (0.56 ml, 6.41 mmol) in diethyl ether (8.3 mL) was prepared and stirred at 0 °C under inert atmosphere, potassium hydroxide (1.57 g, 28.021 mmol) was added, and the mixture stirred at 0 °C under inert atmosphere for 19 hours. Cold water (20 mL) was added and the layers separated. The aqueous phase was extracted with diethyl ether thrice, the combined organic phases dried over $MgSO_4$, filtered and concentrated under *vacuum* to afford the product **9a** as clear colorless oil (0.67g, 43%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.78 (d, *J* = 8.3 Hz, 1H), 7.34 (d, *J* = 8.0 Hz, 2H), 4.05 (t, *J* = 6.2 Hz, 2H), 2.45 (s, 3H), 2.16 (td, *J* = 6.9, 2.6 Hz, 2H), 1.92 (t, *J* = 2.6 Hz, 1H), 1.84 – 1.70 (m, 2H), 1.63 – 1.47 (m, 2H). Further spectroscopic data (^{13}C, MS, IR) are in accordance with the literature.[23]

6-(trimethylsilyl)-hex-5-yn-1-yl 4-methylbenzenesulfonate

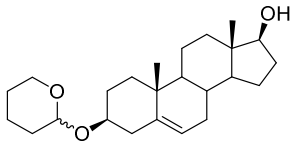


9

A solution of **9a** (0.67 g, 2.65 mmol) in dry THF (4.75 mL) was prepared and n-BuLi (1.6 M in hexane, 1.85 mL) was slowly poured dropwise at –75°C. The resulting mixture was put under stirring for 1 hour, then trimethylsilyl chloride (TMS-Cl) (0.5 mL) was added and the stirring continued for another 1.5 hours at the previous temperature. The reaction mixture was allowed to attain room temperature and after 4 hours it was quenched with 2% aq. Na_2CO_3 solution. Then an extraction with with diethyl ether (3 × 10 mL) was performed and the resulting mixture was concentrated under vacuum. After purification by FC (PE/EtOAc, 15:1) product **9** (0.74 g, 90%) was obtained as a pale-yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.82 – 7.76 (m, 2H), 7.38 – 7.32 (m, 2H), 4.05 (t, *J* = 6.3 Hz, 2H), 2.45 (s, 3H), 2.19 (t, *J* = 7.0 Hz, 2H), 1.76 (tt, *J* = 8.1, 6.1 Hz, 2H), 1.57 – 1.53 (m, 2H), 0.13 (s, 9H).

3b-(2-Tetrahydropyranyloxy)-5-androsten-17b-ol



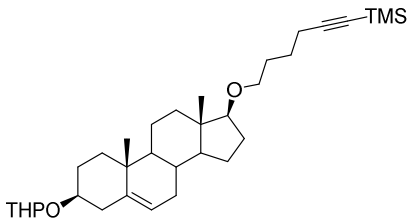
6

Dihydropyran (5.5 ml, 60 mmol) was poured dropwise into a solution of 3-3-hydroxy-5-androsten-17-one (5 g, 17 mmol), p-toluenesulfonic acid monohydrate (30 mg, 0.17 mmol) and toluene (200 ml). The obtained solution was put under stirring for 3.5 hours, then the mixture was cooled to 0°C and a solution 3.5M of bis(2-methoxyethoxy) aluminium hydride in benzene (11ml) was added . The solution was put under inert atmosphere reflux while stirring for 30 minutes, the ice bath was than removed and the mixture was allowed to reach the room temperature. After 30 minutes the mixture was heated to 110°C and for 1 hour and was again cooled to room temperature overnight. The excess hydride was decomposed with moist ether

and the remaining solution was washed with water and sodium tartrate (18.40 g, 80 mmol). The compound **6** (7.05 g 100%) was obtained after crystallization with methanol and water and presented itself as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 5.45 – 5.23 (td, J=5.4, 1.6, 1H), 4.73 – 4.48 (t, J=3.9, 1H), 3.98 – 3.70 (m, 1H), 3.63 – 3.52 (t, J=8.5, 1H), 3.50 – 3.35 (m, 2H), 2.45 – 2.21 (m, 2H), 2.21 – 1.99 (m, 1H), 2.04 – 1.92 (ddd, J=13.4, 7.3, 3.7, 1H), 1.97 – 1.81 (m, 1H), 1.84 – 1.65 (m, 4H), 1.70 – 1.56 (m, 1H), 1.60 – 1.35 (m, 7H), 1.40 – 1.30 (m, 1H), 1.34 – 1.12 (m, 2H), 1.05 – 0.92 (s, 4H), 1.11 – 0.72 (m, 5H), 0.72 – 0.62 (s, 3H).

3b-(2-Tetrahydropyranyloxy)-5-androsten-17b-(hex-1-yn-1-yl)-trimethylsilane



10

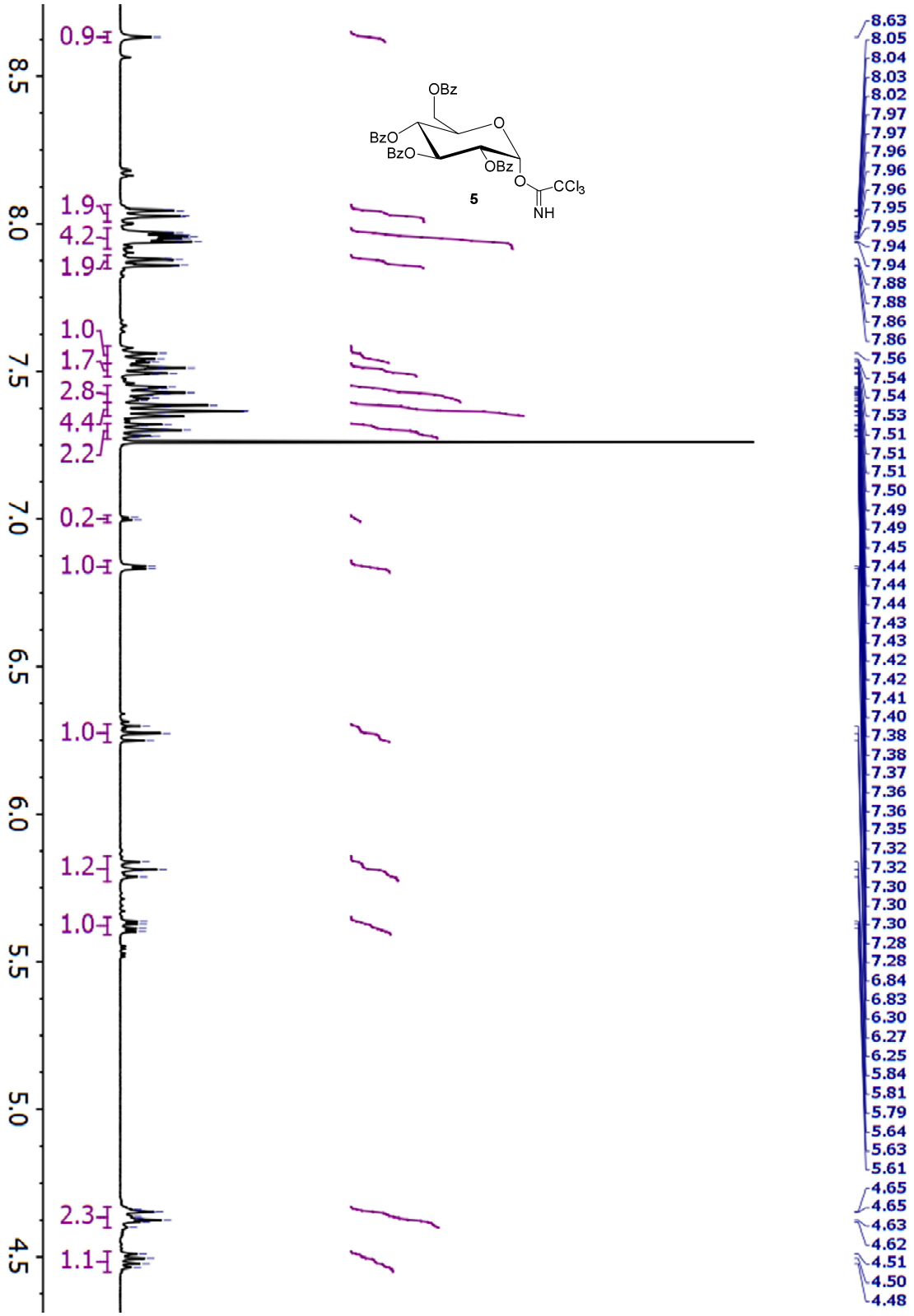
Sodium hydride (330 mg, 8.48 mmol) was putted under inert atmosphere and dissolved in 4 mL of anhydrous toluene, compound **6** (317 mg, 8.48 mmol) was also putted under inert atmosphere and dissolved in 7 mL of anhydrous toluene. This solution was added to the first one under stirring. The resulting mixture was stirred at room temperature under argon for 24 hours. Then, compound **10** (870 mg, 2.8 mmol) was dissolved in 4 mL of anhydrous toluene and added to the mixture. After being stirred under Ar at 130°C (reflux) for 30 h. The reaction was then quenched by addition of sat. aq. sol. NH_4Cl (12 mL) under vigorous stirring, the product was extracted from the mixture using ethyl acetate, the organic phases washed with Brine 2, dried over magnesium sulfate and concentrated under vacuum giving the product **10** (260 mg 57%). ESI-MS m/z: $[M + Ag]^+$ calcd for $C_{33}H_{54}AgO_3Si^+$ 633.2; found 633.6.

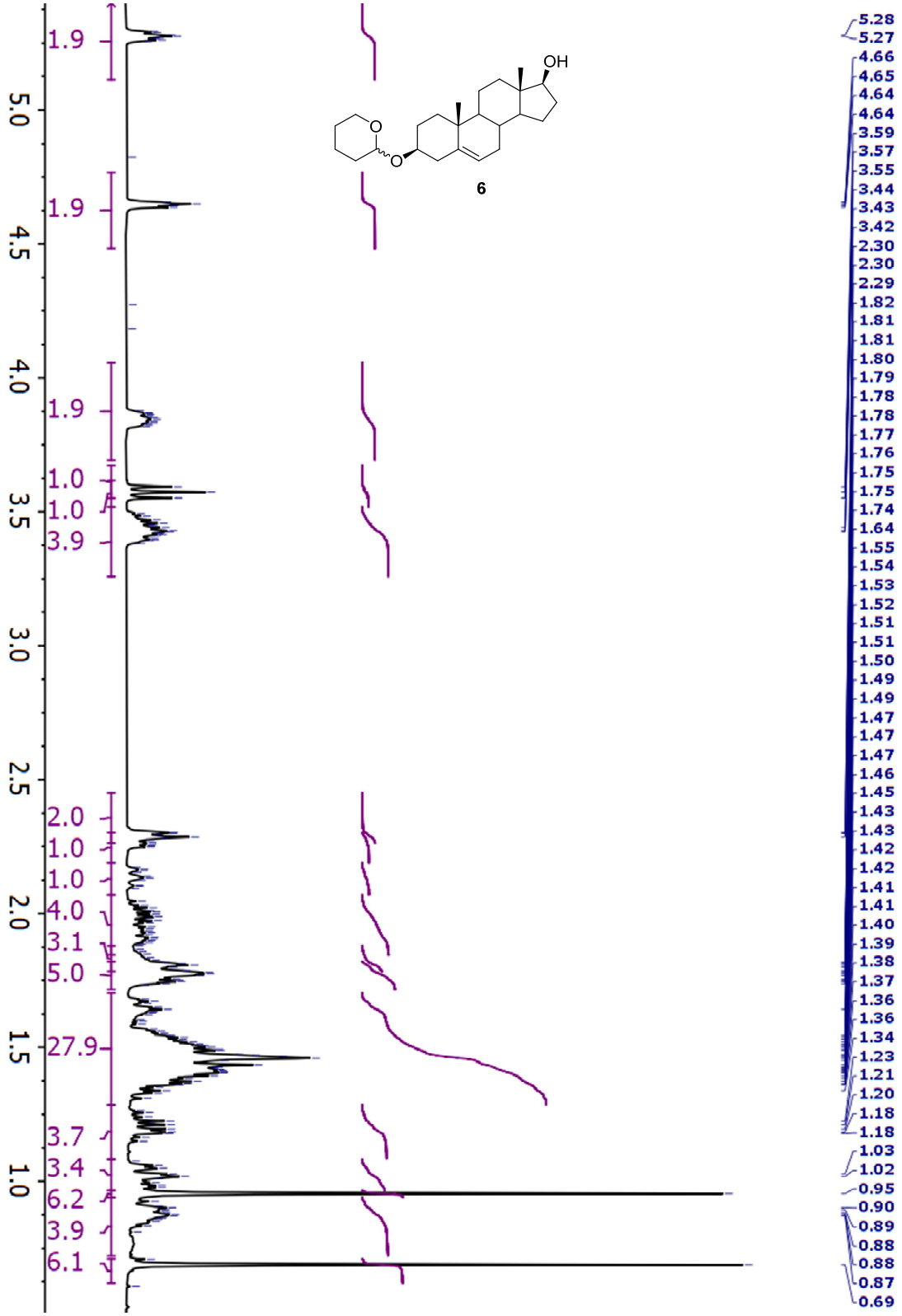
^{1}H NMR (400 MHz, $CDCl_3$) δ = 5.61 – 5.06 (t, *J*=6.0, 1H), 4.96 – 4.47 (t, *J*=4.1, 1H), 4.27 – 3.72 (dq, *J*=11.1, 6.0, 4.9, 1H), 3.66 – 3.35 (m, 4H), 3.35 – 3.06 (t, *J*=8.3, 1H), 2.45 – 2.29 (m, 2H),

2.28 – 2.11 (m, 2H), 2.04 – 1.80 (m, 6H), 1.76 – 1.44 (m, 17H), 1.41 – 1.33 (d, *J*=11.5, 1H), 1.03 – 0.99 (s, 3H), 0.81 – 0.66 (d, *J*=17.6, 3H), 0.09 – 0.01 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ = 141.3 (d), 121.4 (d), 97.0 (m), 89.2, 84.7, 76.1, 69.6, 68.4, 63.0 (m), 51.8, 50.5 (d), 42.5, 38.9, 38.1, 37.4, 37.0 (d), 32.2, 31.9, 31.4, 31.3, 29.8.73, 29.4, 27.1, 23.6(d), 20.9, 20.2 (d), 19.6, 18.4 (d), 11.7, 0.3.

6. ^{1}H NMR Spectra of Significant Compounds

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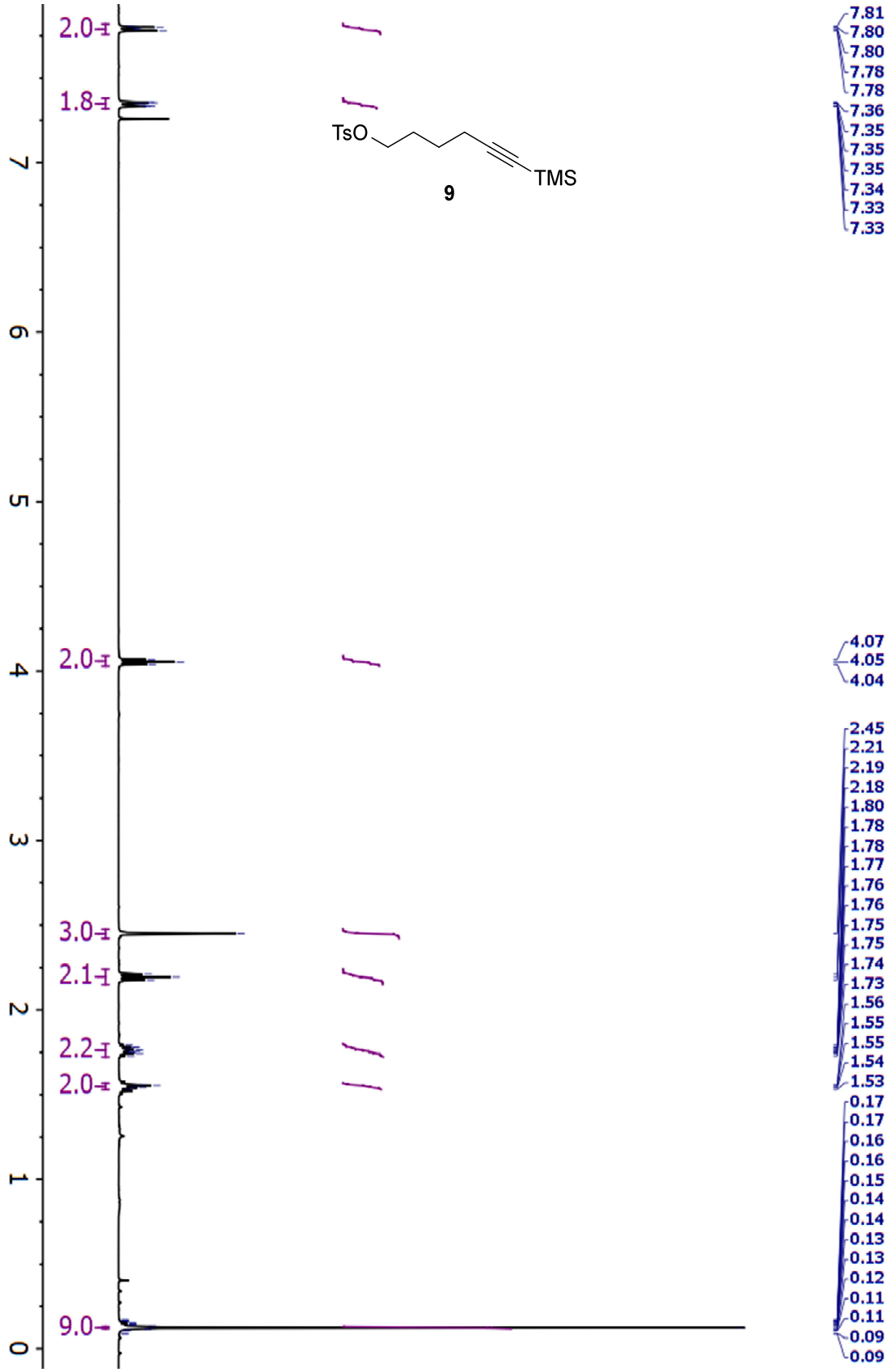
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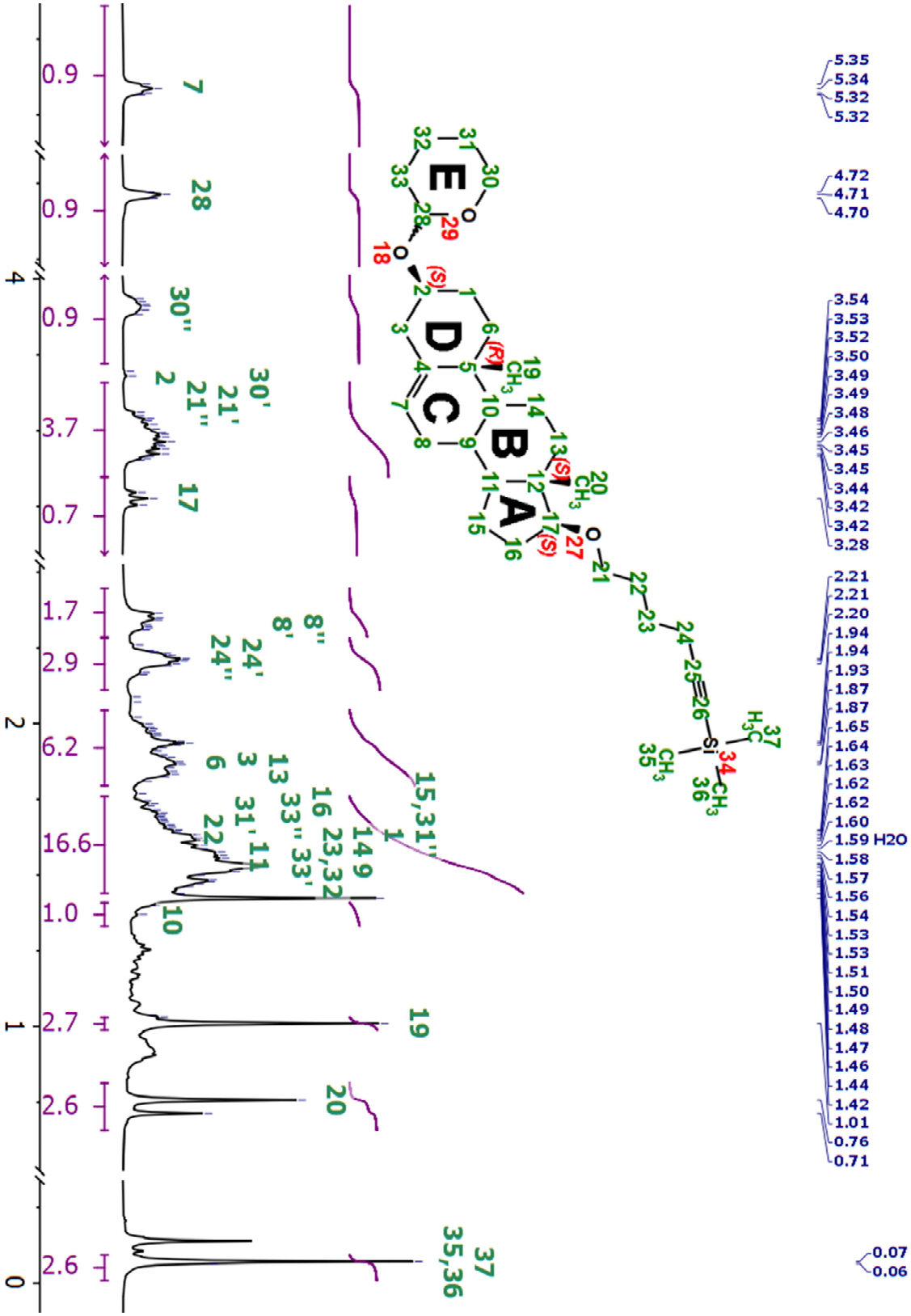
E
D
C
B
A
H2O

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