UNIVERSITÀ DEGLI STUDI DEL PIEMONTE ORIENTALE
"AMEDEO AVOGADRO"

DIPARTIMENTO DI SCIENZE DEL FARMACO

Dottorato di Ricerca in Chemistry and Biology

XXXI cycle

PENTACYCLIC TRITERPENIC ACIDS AS MODULATORS OF TRANSCRIPTION FACTORS: OLD SCAFFOLDS WITH A "*BRIGHT*" FUTURE?

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Percorso del file

ai miei Nonni,
ai miei angeli

"una nave è al sicuro nel porto,
ma questo non è il posto
per cui le navi sono fatte"

William G.T Shedd

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~ PREFACE ~

"Mankind is continually screening low-molecular-weight compounds from a plethora of synthetic and natural sources in the search for molecules with novel or superior pharmaceutical or biological activities. Various bioprospecting, synthetic and biotech strategies to produce and diversify natural products are being exploited to provide new pipelines for bioactive molecules, e.g. for use as drugs or agrochemicals. Plants are a potential rich source of such molecules. However, because of their extreme diversity and complex chemistry, plant metabolism is still under-explored. Consequently, the full potential of plant-derived, low-molecular weight, bioactive compounds is still largely untapped". [1]

This PhD program was funded by a European project called TriForC (Triterpenes for Commercialization), whose aim is the discovery of new triterpenic compounds marketable in medicinal and agrochemical fields. The consortium involves different research groups with complementary skills in the field of chemistry, genomics and pharmacology, flanked by companies able to transfer academic discoveries to commercial reality.

The final purpose of our research group, as a part of this project, was the identification of new bioactive chemical entities, based on triterpenic acids extracted from edible sources.

Terpenes are a large family of secondary metabolites mainly produced by plants with different functions both in vegetables and mammalians. They are part of the defensive strategy of plants against microorganism and insects, and they play other important ecological roles such as pollinator attractants and allelopathic agents. In mammalians, apart from their contribution to the stabilization of membranes and to the regulation of some enzymatic reactions, they show an interesting biological profile in the treatment of different pathologies. The members of this large class of natural products have structures that can be traced back to isoprene, following the so called "isoprenic rule" deduced and described by the Nobel laureate Leopold Ruzicka (*Figure 1*). They are classified according to the number of carbon atoms of the structure: hemiterpenes (C-5), monoterpenes (C-10), sesquiterpenes (C-15), diterpenes (C-20), triterpenes (C-30), tetraterpenes (C-40).[2]

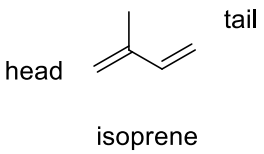


Figure 1*: isoprenic unit.*

Nowadays it's known that the precursor of terpenes is not isoprene itself, but two isoprenic units, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), mainly originating from mevalonic acid (MVA) pathway. IPP and DMAPP are linked together into head-tail fashion furnishing geranyl diphosphate (GPP, C-10) and, after an iterative phase, farnesyl diphosphate (FPP, C-15) and geranylgeranyl pyrophosphate (GGPP) are obtained. FPP and GGPP homo-dimerization, through head-head interaction, provides squalene C-30 (precursor of triterpenes and steroids) and phytoene C-40 (precursor of carotenoids and tetraterpenes) (*Figure 2*).[2]

IPP and DMAPP are synthesized by the mevalonate pathway in eukaryotes, but in eucobacteria they are produced by following methylerythritol phosphate (MEP) biogenetic pathway.[2]

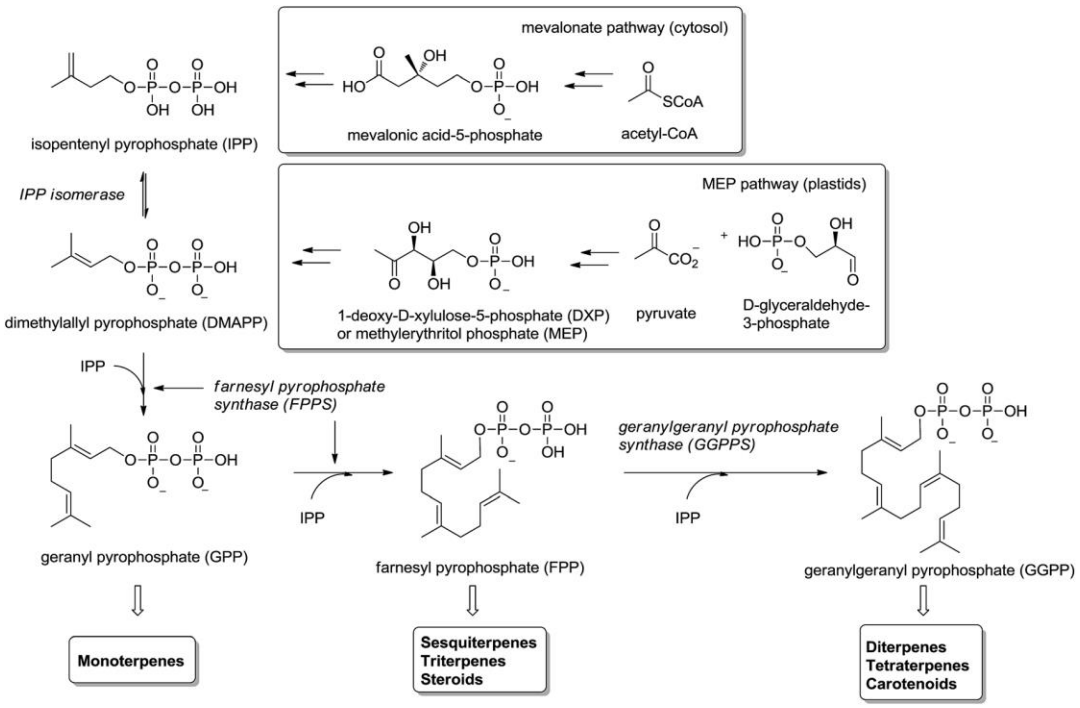


Figure 2: *biosynthesis of isoprenoids: mevalonate pathway and MEP pathway.*

Pentacyclic triterpenic acids (PCTTAs) are members of the terpene family containing six isoprene units and they are synthesized by the oxidative cyclization of squalene (formed by condensation of two farnesyl PP molecules) following a similar biogenetic pattern used for the synthesis of steroids (*Figure 3*).[2]

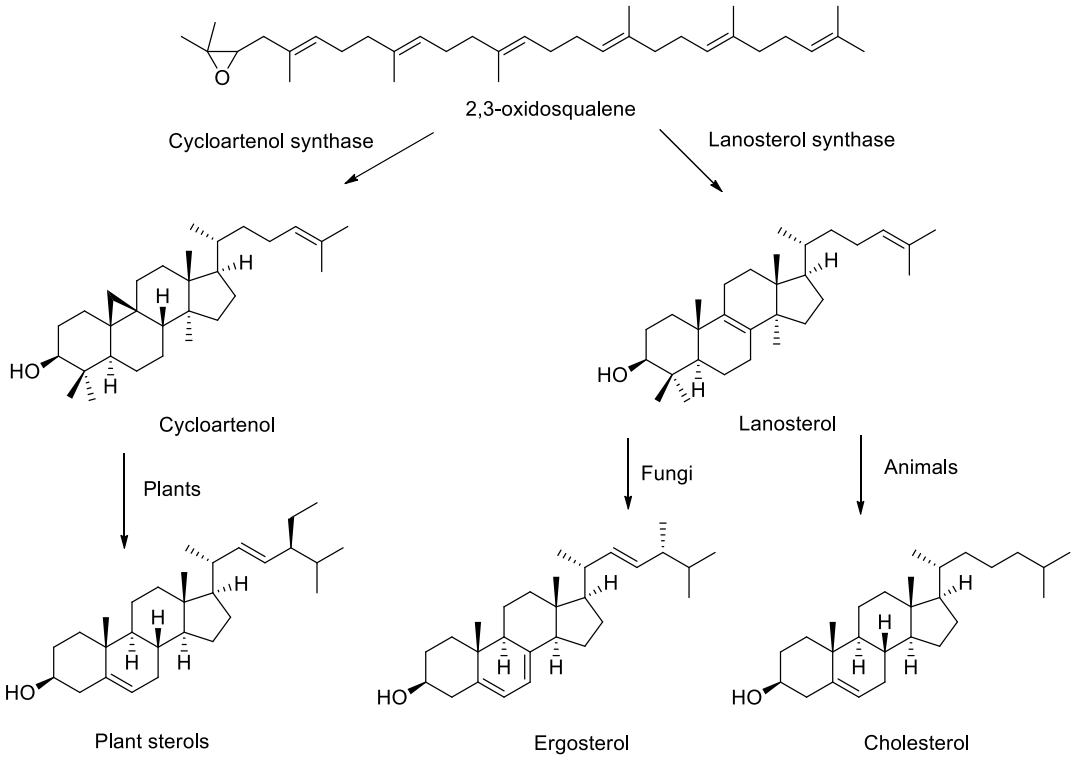
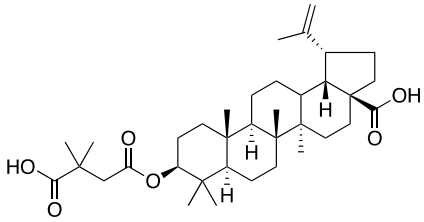


***Figure 3**: cyclization of 2,3-oxidosqualene forms either lanosterol or cycloartenol via a series of enzymatic cyclizations leading to sterols in plants, fungi and animals.*[3]

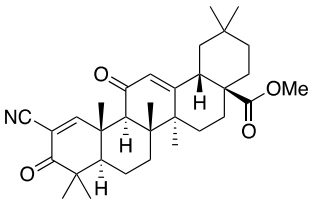
More than 20000 triterpenes are known in nature, many of them occurring in their free form (sapogenins), whilst others being glycosides (saponins). Triterpenes can be divided into acyclic, mono-, bi-, tri-, tetra- and pentacyclic triterpenes (PTs).

In the plant kingdom there are hundreds of naturally occurring PTs which are classified into three main categories based on their structural skeleton: oleanane type (like oleanolic acid, maslinic acid, hederagenin, β-amyrin, glycyrrhizic acid), lupane type (such as lupeol, betulinic acid and betulin) and ursane type (like ursolic acid, α-amyrin).[4]

Natural PCTTAs can target many macromolecular end-points with low-to moderate affinity[5] and their multi-target biological profile have not gone unnoticed in the biomedical community, spurring drug discovery campaigns aimed at improving the activity of PCTTAs leads or, alternatively, using their rigid scaffold to recognize specific protein surfaces. Clear examples of the pharmacological potential of this class of secondary metabolites are represented by the anti-viral agent bevirimat (BMS-955176) **1**[5] and the ultrapotent antioxidant inflammation modulator bardoxolone methyl **2**[6], both included in advanced clinic trials.



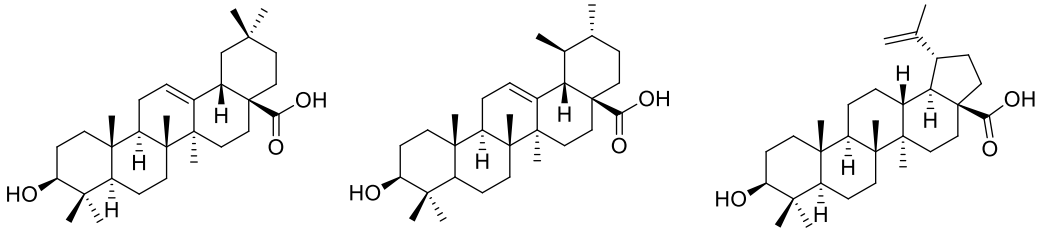
1



2

Although these two molecules represent an excellent result, the research around the PCTTAs turns out to be still lively.

In this context, for this PhD project we have decided to explore in more depth the pharmacological space of three natural triterpenic acids easily obtainable from both edible sources and urban waste such as oleanolic, ursolic and betulinic acid (*Figure 4*).



oleanolic acid ursolic acid betulinic acid

Figure 4: *pentacyclic triterpenoid acids modified in this thesis.*

PHARMACOLOGICAL ACTIVITY OF PENTACYCLIC TRITERPENOID ACIDS

A wide range of pharmacological activities has been described for PCTTAs:

- *ANTIDIABETIC PROPERTIES*: PCTTAs reduce glucose level in blood through inhibition of α-amylase and β-glucosidase. They stimulate glucose uptake and glycogen synthesis via AMPK-GSK- 3β pathway and they stimulate also insulin biosynthesis by increasing its secretion and sensitivity through the inhibition of Protein Tyrosine Phosphatase 1B (PTP1B) and the resulting increase in PI3K/ Akt. [4],[7]
- *HYPOLIPIDEMIC and ANTI-OBESITY ACTIVITIES:* PCTTAs have been shown to decrease the total level of cholesterol, the concentration of triglycerides, the body weight and abdominal fat accumulation by increasing plasma levels of insulin and leptin; PCTTAs lead to reduction of intestinal cholesterol absorption and they also inhibits pancreatic lipase reducing the absorption of lipids in small intestine and increasing the mobilization of fats through lipolysis in adipose tissues.[4],[7]
- *ANTI-INFLAMMATORY PROPERTIES:* scientific studies have shown triterpenoids to be potential anti-inflammatory agents, thanks to the downregulation of NF-kB, inhibition of phospholipase A_2 (involved in the inflammation process) and repression of COX-2 and its product PGE_2. These properties could be an interesting alternative in treatment of the metabolic syndrome. [8]
- *ANTITUMOR ACTIVITY:* it is one of the most widely studied aspects of these compounds' pharmacology. It seems clear that the main mechanisms involved are the stimulation of apoptosis, inhibition of NF-kB and STAT3 and the antioxidant effect.[8]

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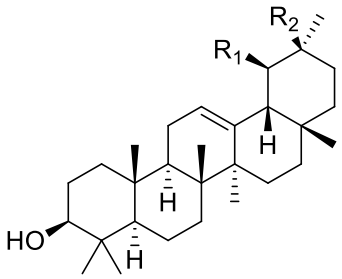
Chapter 1

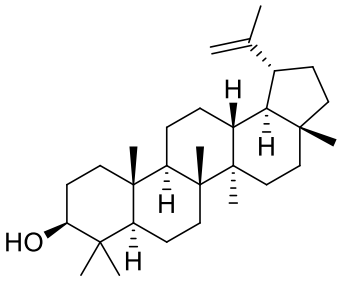
DEOXYGENATION OF URSOLIC, OLEANOLIC AND BETULINIC ACID TO THEIR CORRESPONDING C-28 METHYL DERIVATIVES (α-AMYRIN, β-AMYRIN, LUPEOL)

1.1 INTRODUCTION

α-amyrins (**1**), β-amyrins (**2**) and lupeol (**3**) are widespread in plant kingdom and represent the archetypal pentacyclic triterpenoid alcohols of ursane (α-amyrin), oleanane (β-amyrin) and lupane (lupeol) series.



1: R_1 = Me, R_2 = H
2: R_1 = H, R_2 = Me



3

Amyrins and lupeol have a similar pharmacological profile as antifungal, antimicrobial and anti-inflammatory agents and they exhibit an interesting antioxidant effect. [1],[2]

Interest in these compounds was spurred by the discovery that amyrins show sub-nanomolar activity in animal assays on the endocannabinoid system. This surprising finding was next shown to result from the inhibition of a series of esterases (α,β-hydrolases and to a lesser extent, monoacylglycerol lipase (MAGL)) involved in the hydrolytic degradation of the endocannabinoid 2-arachidonoyl glycerol (2AG) and not from the direct activation of cannabinoid receptors (CBs).[3] The resulting potentiation of endocannabinoid signaling might underline the anti-inflammatory and antinociceptive activity of amyrins.[4]

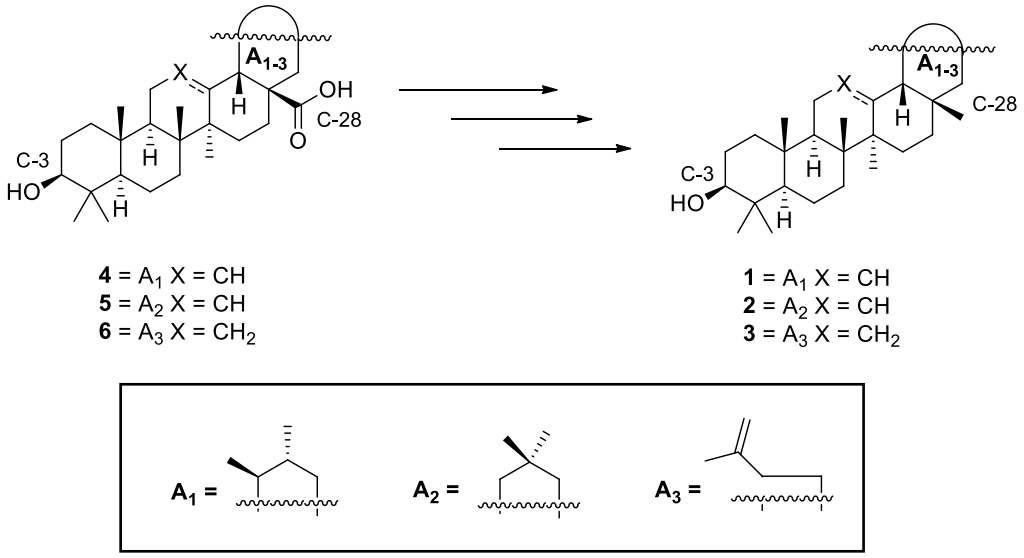
A recent study of Mannowetz *et al.*[5] shows that lupeol can act as contraceptive compound, due to the occupation of the progesterone binding site of ABHD2, a serine hydrolase expressed in spermatozoa that degradates 2-AG to arachidonic acid and glycerol. The inhibition of 2-AG degradation prevents CatSper activation by progesterone via a competitive antagonist-type mechanism.

CatSper is a progesterone sensitive calcium channel that guides sperm to the ovulum and makes fecundation possible and it has recently been identified as possible target for the production of a male contraceptive.[5]

α and β amyrins are accumulated in significant amounts (> 10%) in various plants and plant materials such as leaves, bark and wood. Considerable amounts (up to g/kg) of these triterpenes are available in the resins of *Bursera* and *Protium* species of the *Burseraceae* family, but they occur as mixtures very difficult to separate by chromatography or by fractionate crystallization. [2],[6]

Lupeol, instead, is found in very low concentration in common fruit plants such as Olive fruit (3μg/g), Mango (180 μg/g), Aloe leaves (280 μg/g), elm plant (880 μg/g) etc.[7] The richest source is an expensive South-American ornamental plant (*Angelonia angustifolia* Benth), in whose roots it occurs in ca 1% concentration.[8] Furthermore, lupeol is obtained from side-cuts from the purification of betulin from *Betulla alba* L. bark,[9] a process detailed in various patents.[10]

On the other hand, ursolic acid (**4**), oleanolic acid (**5**) and betulinic acid (**6**), the C-28 oxidized analogues of, respectively, α-amyrin (**1**), β-amyrin (**2**) and lupeol (**3**), are easily available in high yield, low cost and excellent purity from agricultural waste (olive tree pruning for oleanolic acid)[11] or urban landscape waste (bark peels of plane trees for betulinic acid) and from leaves of *Ilex paraguariensis* St. Hil. (for ursolic acid).[12]



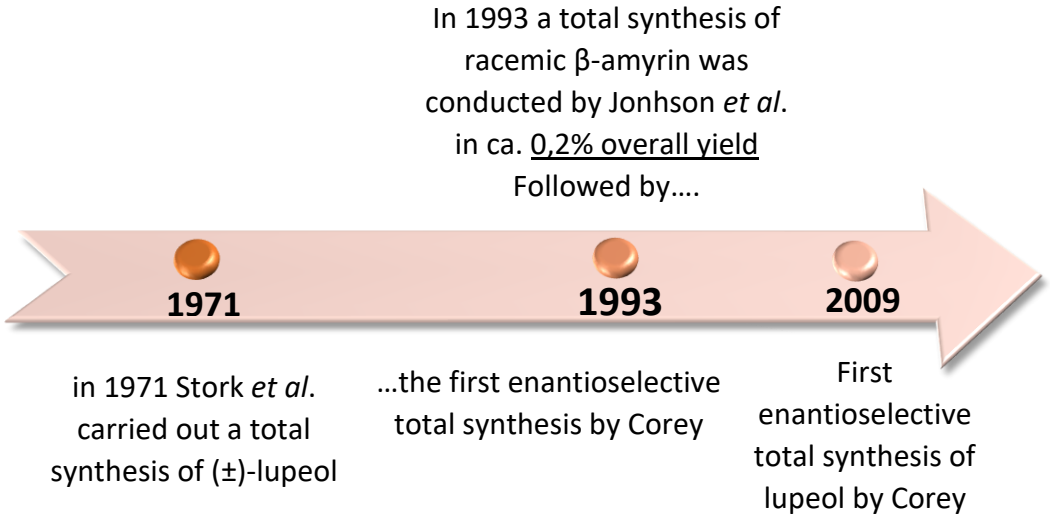
4 = A_1 X = CH
5 = A_2 X = CH
6 = A_3 X = CH_2

1 = A_1 X = CH
2 = A_2 X = CH
3 = A_3 X = CH_2

Using these semi-synthetic samples, their capacity to inhibit 2AG degradation by the α,β-hydrolase ABHD2 could be comparatively evaluated.

1.2 RATIONALE OF THE PROJECT

Despite lupeol and amyrins have interesting pharmacological profile, further studies are blocked or slowed down by the difficulties in obtaining these secondary metabolites in good amount and purity: they generally occur in complex mixture very difficult to separate by chromatography or by fractionate crystallization and the total synthesis of naturally occurring pantacyclic triterpenes is still a big challenge with extremely high cost and very low overall yield.[13],[14][15][16]



In this contest a semisynthetic approach which provides the C-28 deoxygenation of these three triterpenic acids, could offer a good alternative to afford large amounts of these compounds.

Most of the synthetic protocols in literature to deoxygenate a carboxyl to a methyl involve the nucleophilic displacement of the corresponding alcohol by a nucleophilic hydride source.

However, the neopentylic nature of the C-28 carboxylate and the resulting complete lack of reactivity of the corresponding alcohol in nucleophilic displacement reactions restricted the choice to the Wolff-Kishner deoxygenation.[6],[17]

The deoxygenation of C-28 triterpenoid carboxylates via a modified Wolff-Kishner reaction based on semicarbazones was reported in the classic structural studies of amyrins and lupeol from the 1930s. This strategy was based on extensive functional groups' manipulations:

a) protection of C-3 hydroxyl group as acetate
b) conversion of C-28 carboxylate into the corresponding acylchloride
c) Rosemund reaction and deoxygenation via the semicarbazone modification of the Wolff-Kishner reaction[18]

In view of the very low yield of the process and the impossibility to scale it up to gram scale, we have decided to design a new strategy for an easy access to these interesting and rare secondary metabolites.

1.3 RESULTS AND DISCUSSION

1.3.1 Chemistry

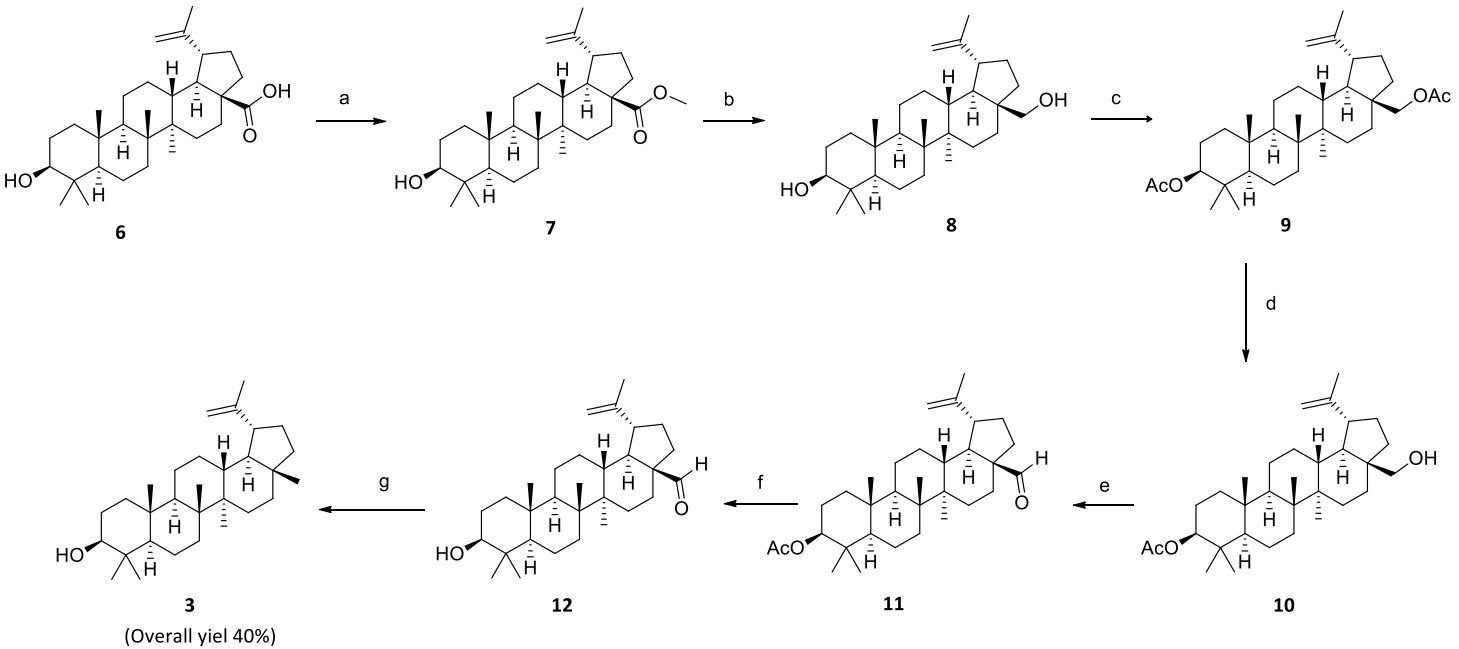
The first synthetic strategy that we have planned (*Scheme 1*) was a 7 steps process in which the first step was the conversion of the carboxylic moiety of betulinic acid into the more soluble methyl ester, that was easily reduced to the corresponding 3,28-dihydroxy derivative **8** by using $LiAlH_4$. Acetylation and selective deacetylation furnished the C-28 primary alcohol (**10**) that was readily oxidized with IBX to the corresponding aldehyde (**11**). The latter was deacetylated and the final reductive step was then carried out according to the Huang-Minlon modification of the Wolff Kishner reaction (heating in ethylene glycol with hydrazine and KOH), affording the expected C-28 methyl derivative **3**.

While the 6 previous steps furnished the expected products in good to excellent yields, the deoxygenation step gave a less than 40% yield.

Attempts to improve this critical step by using other variations of the original Wolff-Kishner protocol (Cram,[19] Myers[20]) or replacing hydrazine with tosylhydrazine and the base with $LiAlH_4$ (Caglioti modification[21]) did not improve the yield, while methylation, silylation as well as acetylation of the 3-hydroxyl group shut down completely the reaction under both the original Wolff- Kishner protocol and the Huang-Minlon modification.

This observation is a classic example of the *Gestalt* (shape)-effect that trouble the chemistry of triterpenoids and might be the reason why the old studies, that used acetylated substrates, resorted to the semicarbazide version of the reaction, that affords significant amounts of the corresponding alcohol.[18]

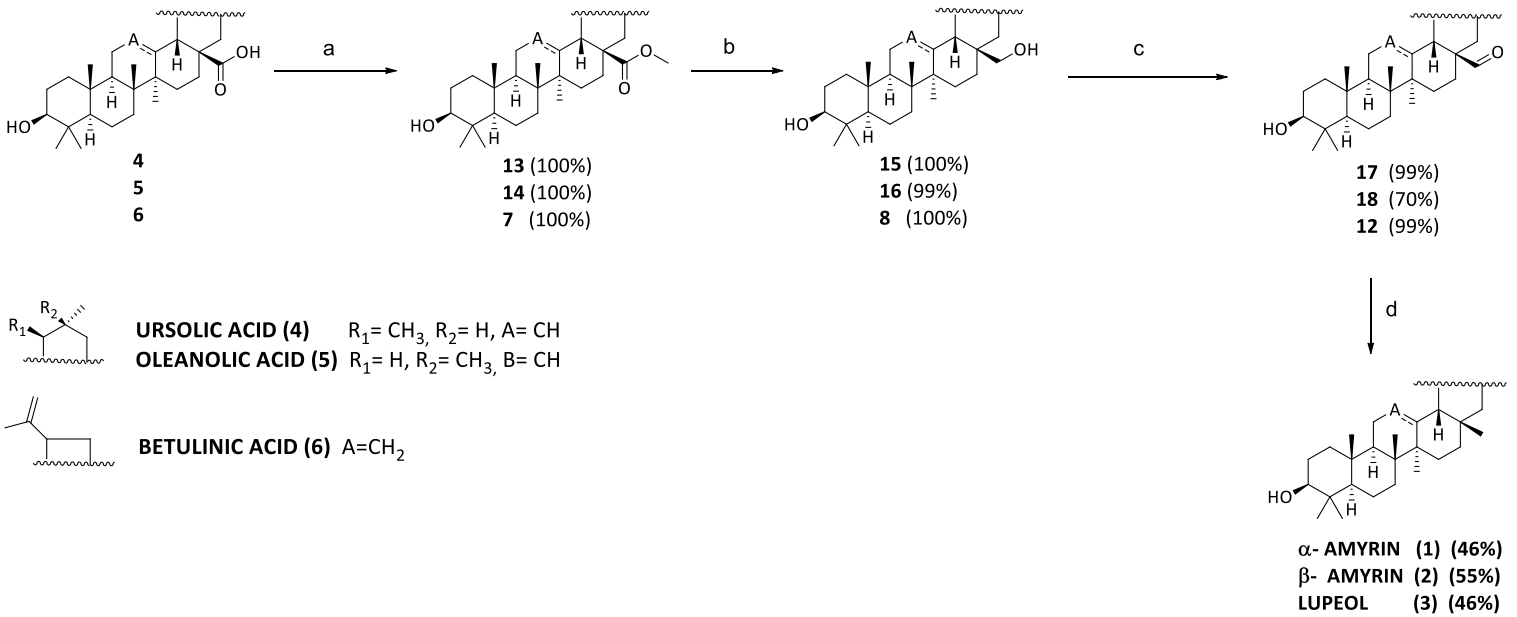
For the acetate these observations are even more surprising, since the 3-acetyl group does not survive to the basic conditions of the reaction.



***Scheme 1**: first synthetic pathway.* (a) Me_2SO_4, Na_2CO_3, DMF; (b) $LiAlH_4$, THF dry; (c) Ac_2O, DMAP, Pyridine; (d) $Al(O\text{-}i\text{-}Pr)_3$, i-PrOH; (e) IBX, DMSO; (f) NaOH, MeOH; (g) NH_2NH_2, KOH, ethylene glycol 200°C.

In order to optimize the strategy by removing the use of protecting groups and reducing the synthetic steps, 3,28-dihydroxy derivative **8** was chemoselectively oxidized to the corresponding C-28 aldehyde by using the TEMPO (2,2,6,6-tetramethylpiperidinyloxy)- NCS (N-chlorosuccinimide) protocol (*Scheme 2*). The aldehyde, obtained in high yield and complete chemoselectivity, underwent to the final deoxygenation furnishing the final product (**3**) in 50% yield.

This strategy was successfully applied to the synthesis of the three deoxygenated compounds (gram scale) with an overall yield of 32% for α-amyrin, 42% for β-amyrin and 40% for lupeol (*Scheme 2*).



Scheme 2. *Final optimized synthesis.* (a) Me_2SO_4, Na_2CO_3, DMF; (b) $LiAlH_4$, THF dry; (c) $NaHCO_3$, TBACl, TEMPO, NCS, DCM/H_2O dark; (d) NH_2NH_2, KOH, etylene glycol 200°C. TBACl= Tetrabutylammonium chloride.

Unfortunately, on september 2017 Dongyn Chen *et al*.[22] published a paper about the synthesis of the deoxygenated derivatives of oleanolic, ursolic and betulinic acid in 7 steps retracing on our first strategy in which the only difference was in the oxidation step where in place of the nontoxic IBX was used highly toxic PCC.

1.3.2 Biological Evaluation

The C-28 methyl triterpene alcohols **1, 2, 3** and all the intermediates were sent to our partner Prof. Jurg Gertsch (University of Bern, Switzerland) to be evaluated as possible inhinitors of the hydrolisis of 2-AG.

1.3.3 Conclusions

A new and protection-free semi-synthetic route to obtain amyrins and lupeol in 4 synthetic steps has been developed. The process is flexible and easily scalable to gram scale starting from easily accessible starting materials, furnishing the desired deoxygenated products in good overall yields.

1.4 EXPERIMENTAL SECTION

General Methods and Materials.

Commercially available reagents and solvents were purchased from Aldrich or Alfa-Aesar and were used without further purification. *N,N'*-Dimethylformamide (DMF) was dried over a neutral alumina pad and stored on 4 Å activated molecular sieves. Dichloromethane was dried by distillation from P_2O_5 and stored on 4 Å activated molecular sieves. Pyridine was dried over neutral alumina pad and stored on activated 4 Å molecular sieves under nitrogen. When needed, the reactions were performed in flame- or oven-dried glassware under a positive pressure of dry nitrogen. For spectroscopic characterization, a JEOL ECP 300 MHz spectrometer was used for ^{1}H and ^{13}C spectra. Chemical shifts are reported in parts per million (ppm) using the residual solvent peak as reference ($CHCl_3$ at δ 7.27). A Thermo Finningan LCQ-deca XP-plus equipped with an ESI source and an ion trap detector was employed for mass spectrometry. Flash column chromatography was performed on silica gel (Merck Kieselgel 60, 230−400 mesh ASTM). Thin-layer chromatography (TLC) was carried out on 5 × 20 cm plates with a layer thickness of 0.25 mm (Merck silica gel 60 F254). When necessary, $KMnO_4$ was used for visualization. All the intermediates are known and our data are in accordance to those published in literature.

Betulinic and ursolic acid were easily obtained respectively from sycamore tree (*Platanus acerifolia L.*) outer bark that comes off unassisted each year in autumn and yerba mate (*Ilex paraguariensis*), through an efficient extraction processes. High pure oleanolic acid was commercially available in bulk (5 Kg).

EXTRACTION OF BETULINIC ACID

PROCEDURE: the milled bark was placed inside a PET bottle and acetone was added to about half of the container, leaving it to macerate overnight.
After 48 hours the vegetable matrix was filtered under vacuum on a sintered filter and the brown acetone extract was collected in a round bottom flask.
The acetonic extract was concentrated to half its volume and subsequently left at -18 ° C overnight favouring the precipitation of betulinic acid as pale-yellow crystals. Betulinic acid was filtered under vacuum on a sintered filter and washed with petroleum ether previously cooled to -18 ° C. The crude betulinic acid crystals were collected in a beaker and left in vacuum dryer to obtain a completely dry pale-yellow solid (yield ca 1-2%).

EXTRACTION OF URSOLIC ACID

For the extraction of ursolic acid we have used yerba mate (*Ilex paraguariensis*), sold in supermarkets.
The leaves of mate are rich of ursolic acid, but they contain also α and β-amyrines esterified with fatty acids. Therefore, it is necessary to separate first the amyrine esters (using petroleum ether) to extract the pure ursolic acid.

PROCEDURE: Mate leaves was placed inside a PET bottle that was half filled with petroleum ether. The vegetable material was left in extraction overnight, filtered under vacuum using a sintered filter to separate the petroleum extract from the

vegetable matrix. After evaporation of petroleum ether extract, we have obtained a brown residue rich in amyrin esters.

Then vegetable matrix was re extracted in acetone overnight, filtered under vacuum, concentrated for half of its volume and left at -18 ° C to allow the precipitation of ursolic acid.

Ursolic acid filtrate was filtered under vacuum and washed with the minimum amount of previously cooled petroleum ether (-18 ° C). Precipitated ursolic acid was collected from the filter, placed in a beaker and dried to obtain a green powder (yield ca 1-2%).

General procedure of esterification (13, 14, 7): to a stirred solution of triterpenic acid (1 eq/mol) in DMF (10 mL), sodium carbonate (5 eq/mol) and dimethylsulfate (5 eq/mol) were added. The reaction was stirred at room temperature overnight, then quenched with H_2SO_4 2N and extracted with a 3:1 mixture of petroleum ether and diethyl ether. The organic layers were dried over Na_2SO_4, filtered and evaporated to afford the methyl ester without further purification (quantitative yield).

General procedure of methyl esters reduction (15, 16, 8): to a cooled solution (0°C) of **13, 14, 7** (1 mol/eq) in dry THF (10mL), $LiAlH_4$ (3 eq/mol) was slowly added. The mixture was stirred at room temperature overnight, then quenched with H_2SO_4 2M and extracted with ethyl acetate. The organic layers were washed with $NaHCO_3$ sat. sol., dried over Na_2SO_4, filtered and evaporated to afford the corresponding 3,28-dihydroxy derivative (**15, 16, 8)** without further purification (quantitative yield).

General procedure of primary alcohol oxidation (17, 18, 12): to a stirred solution of 3,28-dihydroxy derivative (**15**, **16**, **8**) (1 mol/eq) in DCM (5 mL/0,23 mmol), water

(5 mL/0,23 mmol), potassium carbonate (250 mg/mmol substrate), sodium bicarbonate (1.5 g/mmol substrate) and tetra-butylammonium chloride (1 mol/eq) were added. The obtained biphasic solution was stirred for 10 minutes then N-chlorosuccinimide (6 mol/eq) and TEMPO (2 mol/eq) were sequentially added. The flask was covered with aluminium foil and the biphasic mixture was vigorously stirred at room temperature overnight. Then reaction was diluted with brine, extracted with DCM and the combined organic layers were dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude was purified over silica gel using Pe/EtOAc 9:1 for **17** (99%), Pe/EtOAc 95:5 for **18** (70%) and Pe/EtOAc 9:1 for **12** (99%).

General deoxygenation procedure (1, 2, 3): to a stirred solution of the formyl derivative (**17, 18, 12**) (1mol/eq) in ethylene glycol (20 mL/0.222 mmol), hydrazine monohydrate (43 eq/mol) and potassium hydroxide (60 eq/mol) were added. The reaction was heated at 200 °C overnight, then cooled at room temperature, quenched with H_2SO_4 2N and extracted with a 3:1 mixture of petroleum ether and diethyl ether. The organic layers were dried over Na_2SO_4, filtered and evaporated. The crude was purified over silica gel using Pe/EtOAc 95:5 for **1** (46%), Pe/EtOAc 95:5 for **2** (55%) and Pe/EtOAc 95:5 for **3** (46%).

Compound 1: white solid. ^{1}H NMR ($CDCl_3$, 300MHz) δ 5.13 (t, *J*= 3.6 Hz, 1H), 3.23 (dd, *J*= 5.3, 10.2 Hz, 1H), 2.06–1.78 (m, 4H), 1.68–0.72 (m, 19H), 1.07 (s, 3H), 1.01 (s, 3H), 1.00 (s, 3H), 0.96 (s, 3H), 0.91 (s,3H), 0.80 (s, 3H), 0.79 (s, 3H), 0.77 (s, 3H); ^{13}C NMR ($CDCl_3$, 75MHz) δ 139.6, 124.4, 79.1, 59.1, 55.2, 47.7, 42.1,41.5, 40.0, 39.7, 39.6, 38.8, 36.9, 33.8, 33.0, 31.3, 28.7, 28.1,27.3, 26.6, 23.4, 23.3, 21.4, 18.4, 17.5, 16.9, 15.7, 15.6.

Compound 2: white solid. ^{1}H NMR ($CDCl_3$, 300MHz) δ 5.19 (t, *J*= 2.1Hz, 1H), 3.22 (dd, *J*= 2.9, 6.8 Hz, 1H), 2.03–0.73 (m, 23H), 1.14 (s, 3H), 1.00 (s, 3H), 0.97 (s, 3H),

0.94 (s, 3H), 0.87 (s, 6H), 0.83 (s, 3H), 0.79 (s, 3H); ^{13}C NMR ($CDCl_3$, 75MHz) δ 145.2, 121.8, 79.0,55.2, 47.7, 47.3, 46.9, 41.8, 39.8, 38.8, 38.6, 37.2, 37.0, 34.8,33.3, 32.7, 32.5, 31.1, 28.4, 28.1, 27.3, 27.0, 26.2, 26.0, 23.7,23.5, 18.4, 16.8, 15.6, 15.5.

Compound 3: white solid. ^{1}H NMR ($CDCl_3$, 300MHz) δ 4.68 (s,1H), 4.57 (s, 1H), 3.18 (dd, *J*= 5.3, 10.8 Hz, 1H), 2.42–2.33 (m,1H), 1.99–1.85 (m, 1H), 1.68–0.67 (m, 23H), 1.68 (s, 3H), 1.03 (s,3H), 0.97 (s, 3H), 0.94 (s, 3H), 0.83 (s, 3H), 0.79 (s, 3H), 0.76 (s,3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 151.0, 109.3, 79.0, 55.3, 50.4, 48.3, 48.0, 43.0, 42.8, 40.8, 40.0, 38.8, 38.7, 38.0, 37.2, 35.6, 34.3, 29.8, 28.0, 27.4, 27.3, 25.1, 20.9, 19.3, 18.3, 18.0, 16.1, 16.0, 15.4, 14.5.

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Chapter 2

TRITERPENOID HYDROXAMATES AS HIF PROLYL HYDROLASE INHIBITORS

2.1 INTRODUCTION

Natural Pentacyclic Triterpenoid Acids (PCTTAs) can target many macromolecular end-points with low-to moderate affinity.[1] To improve potency, the most common strategy has been to implant functional groups on the lipophilic triterpenoid scaffold, as exemplified by the development of bardoxolone methyl from oleanolic acid (**3a,** *Scheme 1*), whose potency could be increased several orders of magnitude by the insertion of a cyanoacrylate Michael acceptor element on ring A.[2] Inspired by the success of this approach, we have attempted to replicate its "dock-and-bind" [3] strategy to discover new modulators of the hypoxia-inducible factors (HIFs).

HIF-1α and HIF-2α are transcription factors stabilized by a cellular low oxygen status (hypoxia).[4]

Hypoxia is most commonly associated with ischemic disease, vascular disease, chronic inflammation and may be lethal at arterial O_2 pressures below 50 mmHg.

Oxygen is the electron sink for oxidation of carbohydrates and fats and is the terminal electron acceptor in the electron transport chain of oxidative phosphorylation that aerobes use to produce ATP.[5]

It is known that all nucleated cells in the body are able to detect changes in O_2 levels and mount a physiological response when O_2 drops to pathophysiological levels.

The heterodimeric transcription factor HIF accumulates in response to hypoxia and binds to HRE (hypoxic response element) motif in DNA promotor region, controlling gene transcription.[5]

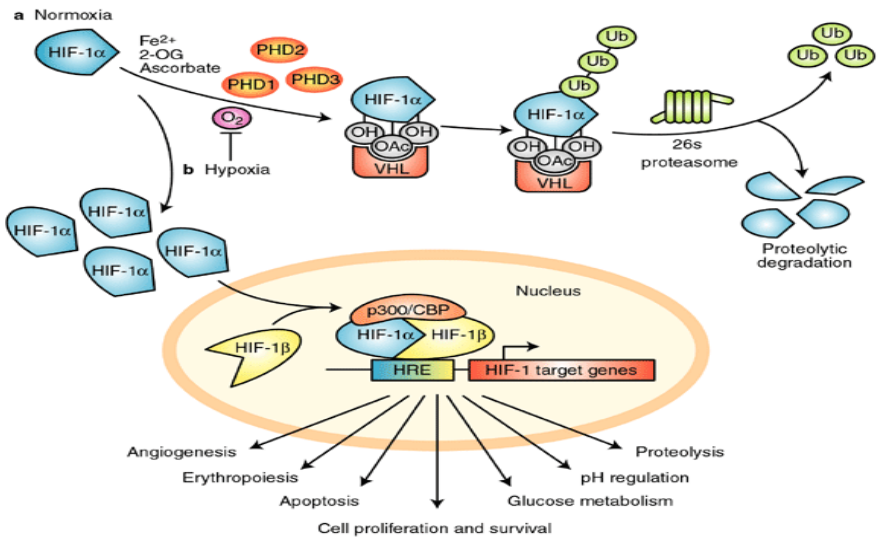


Figure 1: *HIF-1α regulation by proline hydroxylation*

HIF is found in cells in two forms: HIF-1 and HIF-2. They are consisting of HIF-β subunits and a HIF-1α or HIF-2α partner, sharing 48% sequence homology. Unlike HIF-β, HIF-α is sensitive to O_2 [5]: molecular oxygen controls the stability of HIF-1α and HIF-2α via HIF prolyl hydroxylases (PHDs), a class of iron-containing dioxygenases that, in the presence of molecular oxygen and 2-oxoglutarate, hydroxylate HIF-1α and HIF-2α, inducing their ubiquitination by an E3-ubiquitin ligase and degradation by the 26S proteasome.[4]

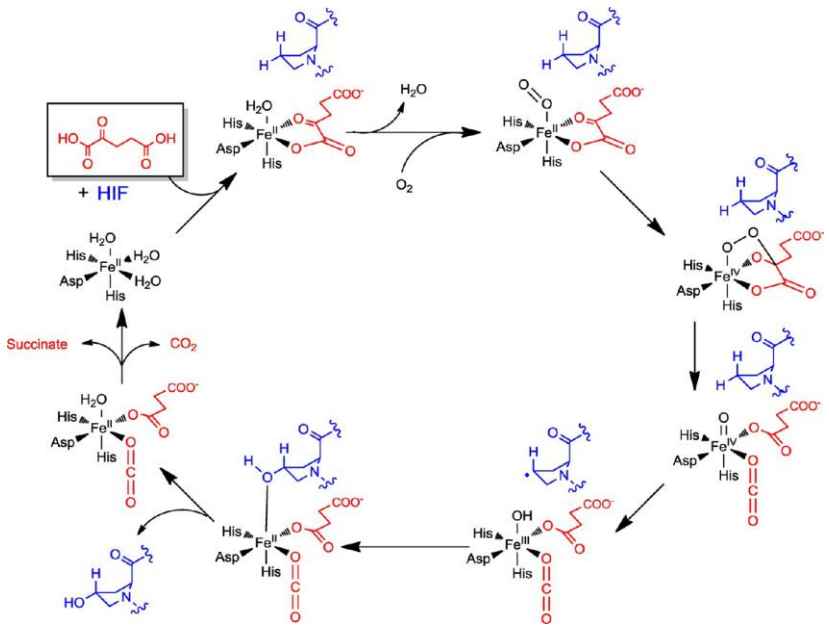


Figure 2: *catalytic cycle of PHD enzymes.*

Several lines of evidence suggest that the HIF-1α/HIF-2α stabilization induced by pharmacological inhibition of PHDs may be of clinical relevance for the treatment of ischemic, inflammatory conditions and neurological disorders.[4]

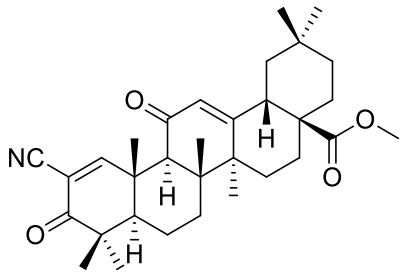
HIF-1α upregulates the vascular endothelial growth factor (VEGF) and erythropoietin (EPO), both showing neuroprotective activity in different animal models of disease including Huntington Disease (HD).[6] Efforts to discover small molecule PHDs inhibitors have led to FG-2216 (FibroGen), an orally bioavailable compound capable to increase the plasmatic levels of EPO,[7] later replaced by Roxadustat (FG-4592), currently in Phase III clinical trials for the treatment of renal anemia in patients with end-stage kidney disease.[8] GlaxoSmithKline, Bayer, Japan Tobbaco Inc. and Akebia Therapeutics are some of the major industrial players in the developing of oral PHDs inhibitors,[9] with Huntington Disease (HD) being a challenging additional pharmaceutical target for this class of compounds.[10]

HD is a fatal autosomal dominant and progressive neurodegenerative disease caused by a mutation in the Huntingtin gene (*Htt*). Mutated Htt protein leads to death and dysfunction of the GABAergic medium spiny striatal neurons, leading to severe neurological symptoms that included chorea, cognitive impairment, and changes in mood and personality.[11]

2.2 RATIONALE OF THE PROJECT

The HIF pathway is sensitive to chelators. Thus, the tri-hydroxamate siderophore desferrioxamine B (DFX) blocks PHD activity by forming a very stable hexadentate complex with ferric iron,[12] and other hydroxamates have also been shown to target PHDs and activate the HIF pathway due to their iron-chelating properties.[13]

Since the polycyclic lipophilic scaffold of triterpenoids seems remarkably versatile in terms of protein surface recognition,[1] we wondered if, by introducing a chelating element, the “dock and bind” strategy of bardoxolone methyl (**2**) could be replicated in another biological setting, combining shape affinity associated to the lipophilic scaffold with the specific binding properties of the chelating element. The hydroxamates of some triterpenoid acids have been described.[14,15]



2

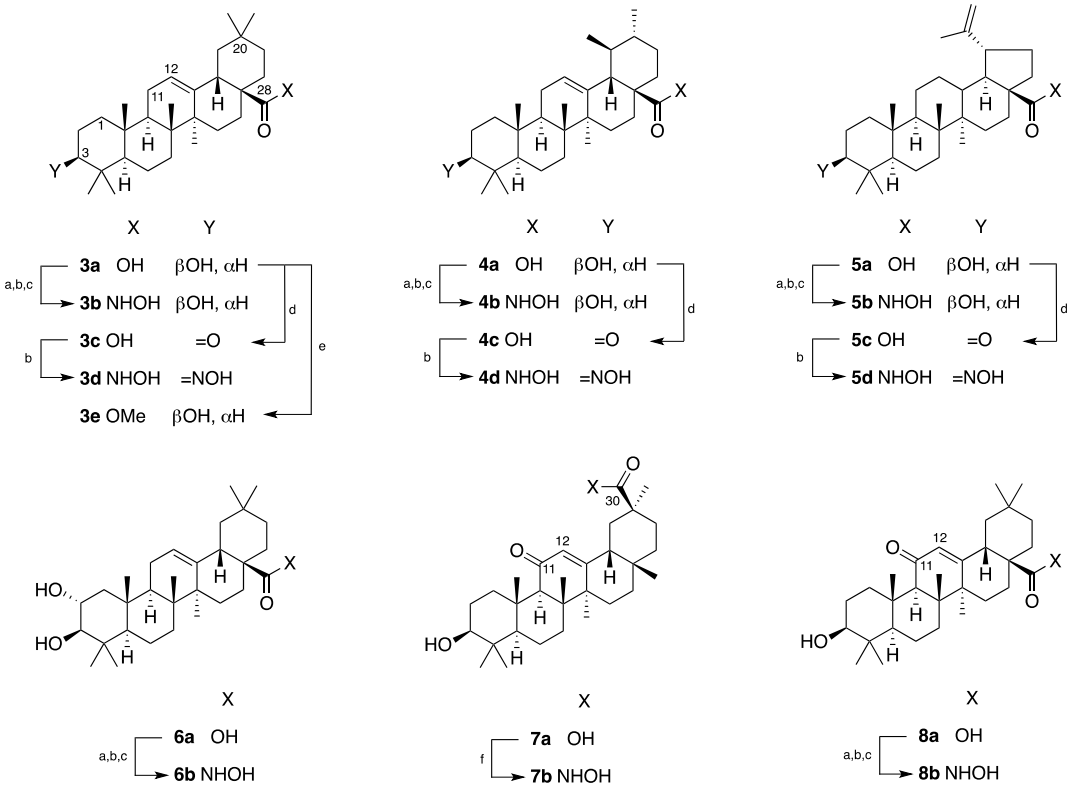
2.3 RESULTS AND DISCUSSION

2.3.1 Chemistry

Five PCTTAs were selected as starting material based on their multigram availability by isolation [ursolic acid (**4a**), betulinic acid (**5a**), maslinic acid (**6a**)], purchase [oleanolic acid (**3a**), glycyrrhetinic acid (**7a**)], or semi-synthesis (the oleanolic/glycyrrhetinic acid hybrid **8a**).[16]

Hydroxyamidation was realized, after protection of the 3-hydroxyl as acetate, by activation as chloride (**3a-6a**, **8a**) or mixed phosphonic anhydride (**7a**),[17] followed by reaction with hydroxylammonium chloride in pyridine and deprotection, affording the hydroxamate alcohols **3b-8b** (*Scheme 1*).

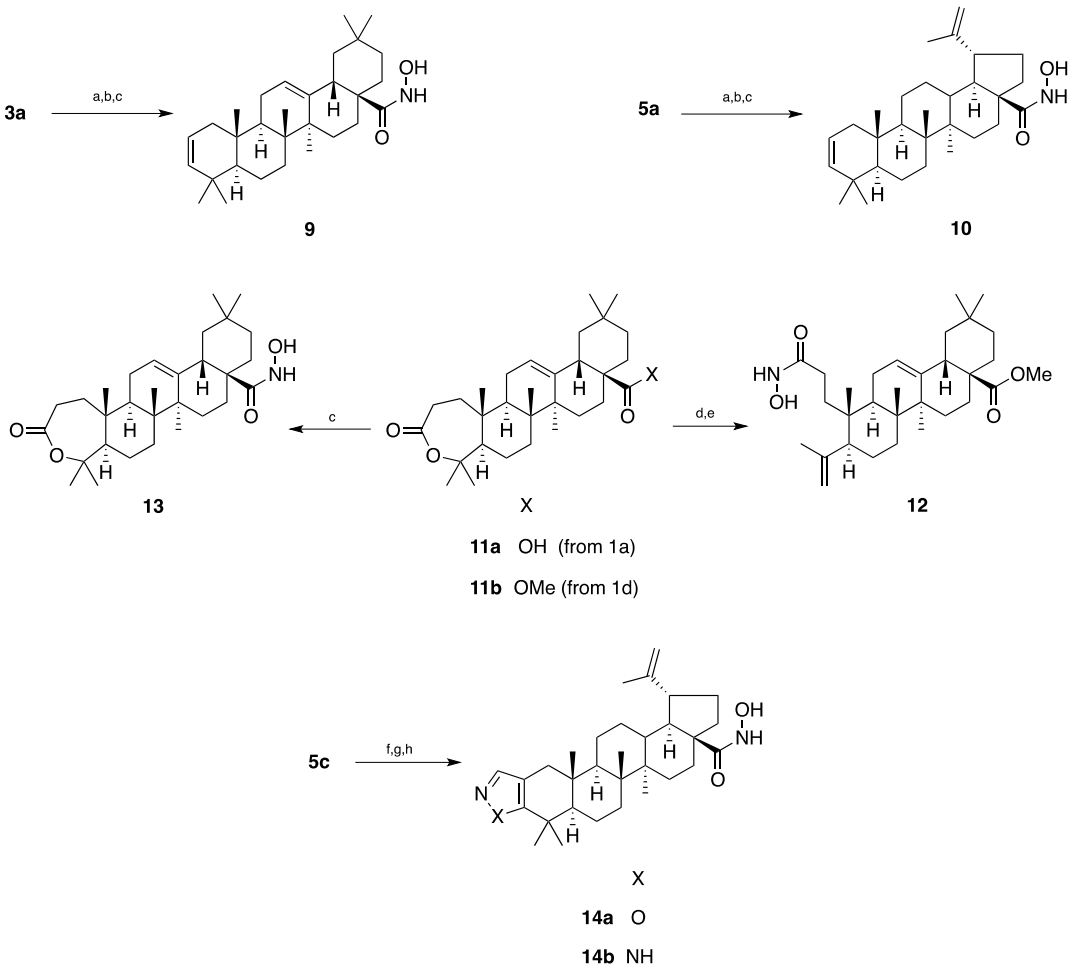
The effect of an additional hydroxylamine-derived functional group was evaluated in the 3-hydroxyimino-28-hydroxamates **3d-5d** achieved by two-fold hydroxyamination of the 3-dehydroderivatives of oleanolic-, ursolic- and betulinic acids (**3c-5c**, respectively).



Scheme 1: *synthesis of the first generation PCTTA hydroxamates and oxyminohydroxamates.* (a) Ac_2O, DMAP, pyridine (quantitative); (b) Oxalyl Chloride, DCM 40 °C then $NH_2OH.HCl$, Pyridine 40 °C (38-70%); (c) NaOH 4M, THF/MeOH 1:1 40 °C (50-60%); (d) Jones reagent, acetone/EtOAc 5:1 (nearly quantitative); (e) K_2CO_3, DMS, DMF (98%); (f) $NH_2OH.HCl$, TEA, T3P®, CH_3CN (64%). Ac_2O = Acetic anhydride, DMAP= 4-Dimethylaminopyridine, Jones reagent= Sulfuric acid-chromium trioxide mixture, DMS= Dimethylsulfate, DMF= Dimethylformamide, TEA= Triethylamine

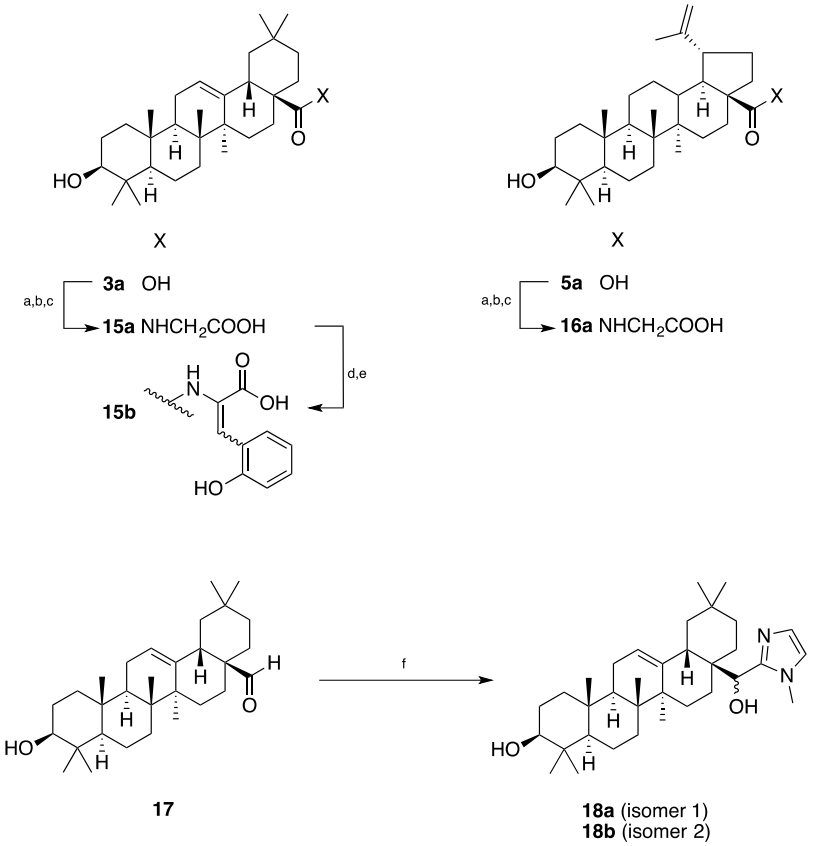
Further derivatives were obtained (*Scheme 2*) by modifications on ring A of oleanolic and betulinic acids, whose hydroxamates (**3b** and **5b**, respectively) had given the most interesting bioactivity (see biological part).

Thus, a double bond was generated on ring A by dehydration of the 3-hydroxyl group, affording, after hydroxyamidation, oleanane **9** and lupane **10**. Alternatively, Baeyer-Villiger expansion of ring A of the 3-dehydroderivative of oleanolic acid (**3a**) or its methyl ester (**3e**) afforded, respectively, the lactones **11a** and **11b**. The carboxylate **11a** was next directly hydroxyamidated to **12**, while the lactonized methyl ester **11b** underwent alkaline-promoted β-elimination to an A-seco derivative, next hydroxyamidated to **13**. Further changes on ring A were carried out on the 3-dehydroderivative of betulinic acid (**5c**), that, after formylation, was condensed with hydroxylamine or hydrazine to afford the heterocyclic 1,2-azoles **14a** and **14b**.



Scheme 2: *synthesis of ring A-modified PCTTA hydroxamates.* (a) TsCl, Pyridine; (b) NaOAc, DMF 120 °C (40-50 over two steps); (c) Oxalyl Chloride, DCM 40 °C then $NH_2OH.HCl$, Pyridine 40 °C (35-50%); (d) KOH, DCM/MeOH/H_2O 3:3:1 (20%); (e) Oxalyl Chloride, DCM 40 °C then $NH_2OH.HCl$, Pyridine 40 °C (35%); (f) Ethyl formate, NaOEt, toluene 50 °C for 19; (g) $NH_2OH.HCl$, EtOH/H_2O, 80 °C for **14a** or NH_2NH_2, EtOH/H_2O 80 °C for **14b**; (h) Oxalyl Chloride, DCM 40 °C then $NH_2OH.HCl$, Pyridine 40 °C (40-50%). TsCl= *p*-Toluenesulfonyl chloride, DMF= Dimethylformamide

To compare the biological activity, the hydroxamate group of oleanolic (**3b**) and betulinic (**5b**) hydroxamate hits was replaced by a series of different chelating elements [glycinamide, 2-hydroxybenzalglycinamide, (*N*-methyl-2-imidazolyl)hydroxymethyl], generating the analogues **15a**, **15b**, **16a**, **18a** and **18b.** (*Scheme 3*). These compounds were prepared from the starting acids by amidation (**15a** and **16a**) followed by *ortho*-hydroxybenzalization for the preparation of **15b**, or from the corresponding C-28 aldehydes by addition of 2-lithio *N*-methylimidazole (**18a** and **18b**).



Scheme 3**:** *synthesis of PCTTA bearing at C-28 a chelating group*. (a) Ac_2O, DMAP, pyridine (quantitative); (b) Oxalyl Chloride, $_2$ 40 °C then Glycine methyl ester hydrochloride, Pyridine 40 °C; (c) NaOH 4M, THF/MeOH 1:1 40 °C (50-60% over two steps); (d) Salicylaldehyde, NaOAc, Ac_2O 140 °C; (e) NaOH 4M, THF/MeOH 1:1 40 °C (38% over two steps); (f) 1-Methylimidazole, *n*-BuLi, THF -78 °C (40% cumulative yield). Ac_2O = Acetic anhydride, DMAP= 4-Dimethylaminopyridine

To better investigate the active site of the target protein, we have planned the synthesis of the biotinylated derivative of betulinic hydroxamate.

The biotinylated derivative **19** is composed by the hydroxamate triterpenic core of betulinic acid connected with biotin by an aminoacidic linker of 6 carbon atoms (*Figure 3*).

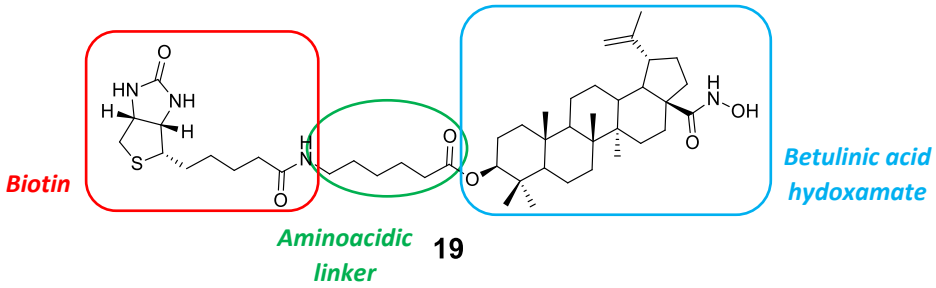
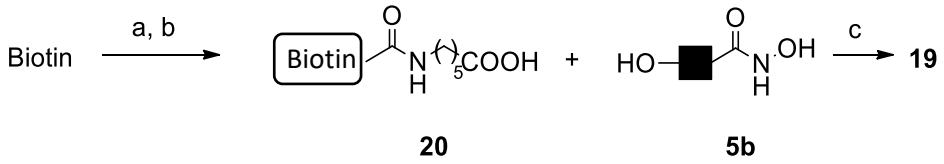


Figure 3 : *biotinilated derivative of the hydroxamic acid of betulinic acid.*

Despite biotinilation is widely used in medicinal chemistry, the synthesis of compound **19** was challenging: several strategies were considered and different chemical problems were solved.

Here below we report the evolution from the first to the last approach with the problems faced and the proposed solutions.

- *First synthetic strategy*



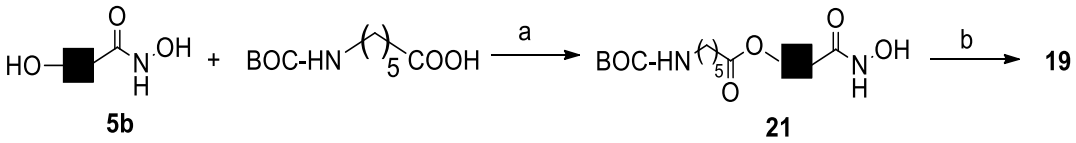
***Scheme 4**:* (a) isobutyl chloroformate, tri-*N*-butylamine, DMF; (b) amino hexanoic acid; (c) EDC, DMAP, TEA, DCM.

Problem: monitoring by TLC of the first step.

In this first approach we have decided to link biotin to the spacer at the early stages of the process and then proceeding with the esterification of the key intermediate **20** with compound **5b**. Unfortunately compound **20** couldn't be easily detected in TLC and it was very difficult to follow the reaction.

Solution: to link the amino acid to betulinic acid first. In this way we were able to follow the reaction by TLC.

- *Second synthetic strategy*



***Scheme 5:** (a) EDC, DMAP, TEA, DCM; (b) deprotection; (c) Biotin.*

Problem: formation of isocyanate during the esterification reaction by Lossen rearrangement (*Figure 4*).

In case of the second approach we have tried to link the spacer to compound **5b** following a classical esterification process with the use of carbodiimides, without considering that hydroxamic acids can easily undergo to Lossen rearrangement to give the corresponding isocyanate **21.**

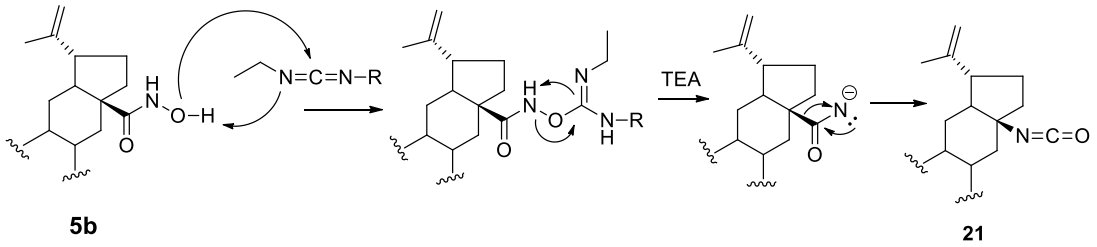
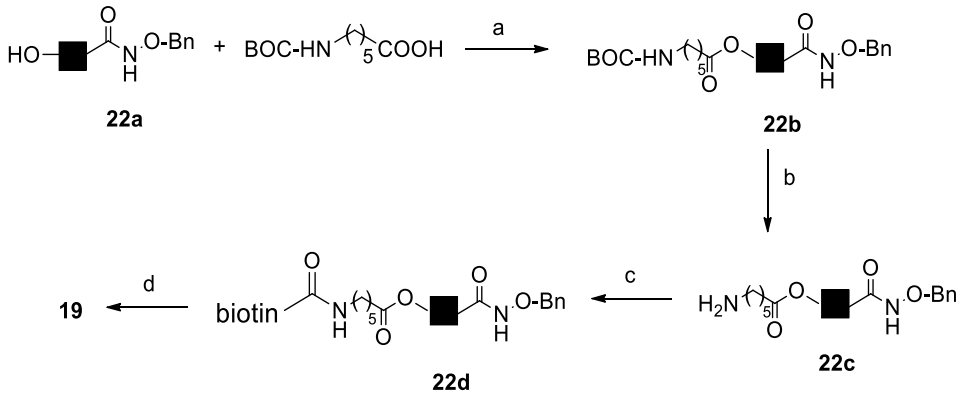


***Figure 4**: Lossen rearrangement during the esterification reaction.*

Solution: protection of the hydroxamate group with benzyl group to avoid the formation of isocyanate.

- *<u>Third synthetic strategy</u>*



***Scheme 6:** (a) Benzoyl chloride, DMAP, TEA, THF dry; (b) TFA, DCM; (c) Biotin, EDC, HOBt, DCM; (d) H_2, Pd/C, MeOH/EtOAc*

Problem: acid catalysed E ring expansion.

To overcome the problem of Lossen rearrangement we have prepared the *O*-benzylhydroxamate **22a** that was esterified with the *N*-BOC-protected amino acid giving the key intermediate **22b**. The deprotection of the amino group was done following the procedure published by Milan Urban *et al*. [18]

^{1}H-NMR of the product **22c** shows the presence of a single proton singlet at 3.99 ppm, whilst the olefinic protons of betulinic acid are missing (*Figure 7*). Despite the protection of the carboxylic moiety at C-28, the strong acid medium of the reaction (TFA) catalyzes the ring expansion followed by the formation of trifluoromethyl ester at position 19 (*Figure 5*).

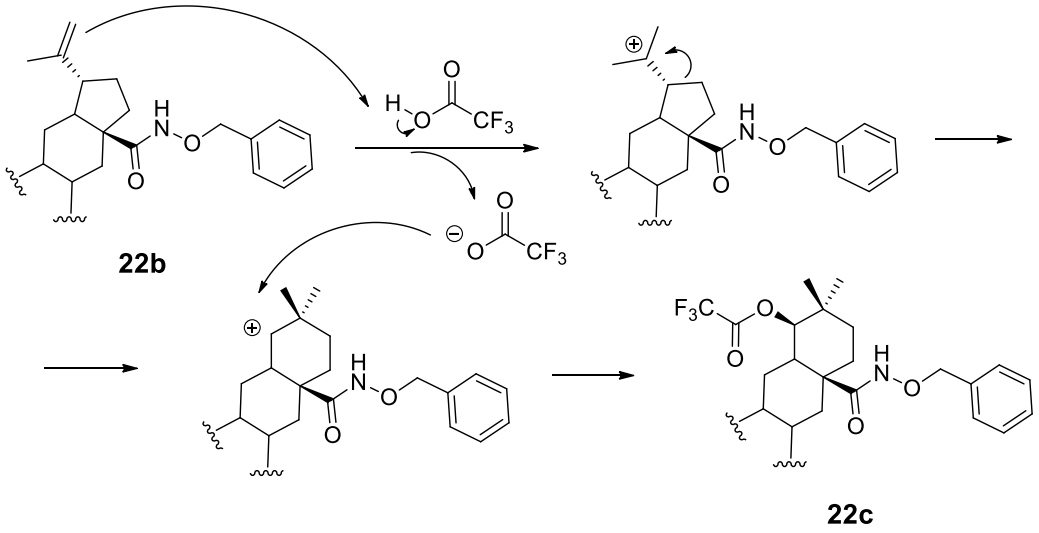


Figure 5*: reaction of TFA with betulinic derivative with consequent formation of six membered ring.*

The ^{1}H-NMR of compound **23** reported by Milan Urban,[18] also shown the same anomalies: the missing of the olefinic protons of betulinic acid, and the presence of a singlet at 3.99 ppm, showing a misinterpretation of the NMR data (*Figure 6*).

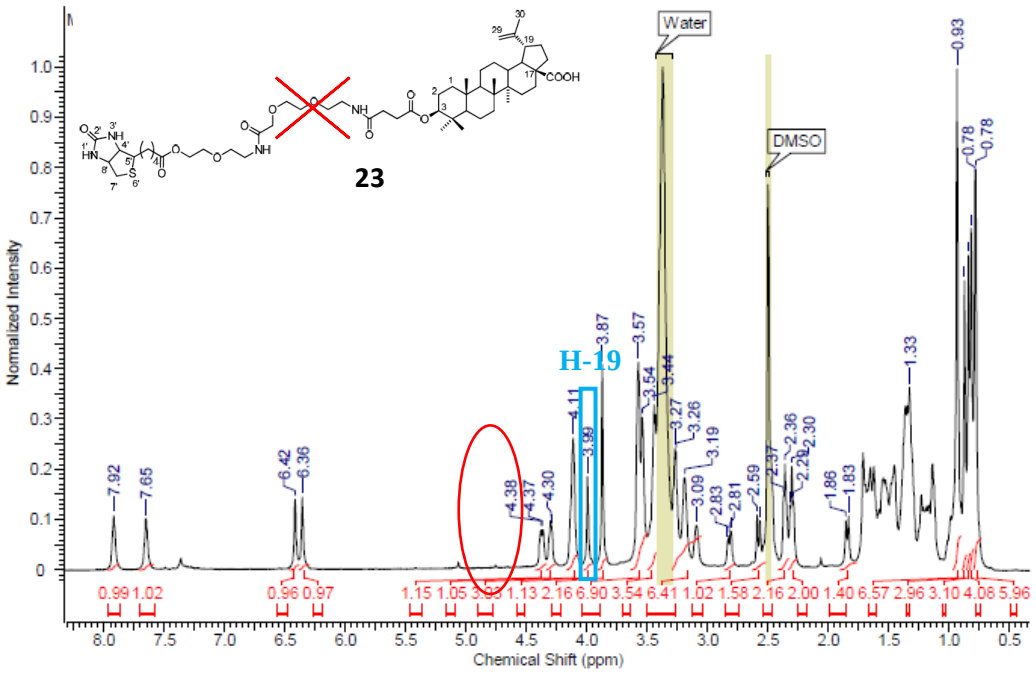


Figure 6: *^{1}H-NMR spectra of the wrong structure of the biotinylated betulinic acid synthesized by Milan Urban et al. The spectra shows no signals of the hydrogens of betulinic acid double bond (at 4,5 ppm), but there is the signal at 3,99 ppm of the hydrogen in position C-19, which reveals the transposition and the formation of the six membered ring.* [18]

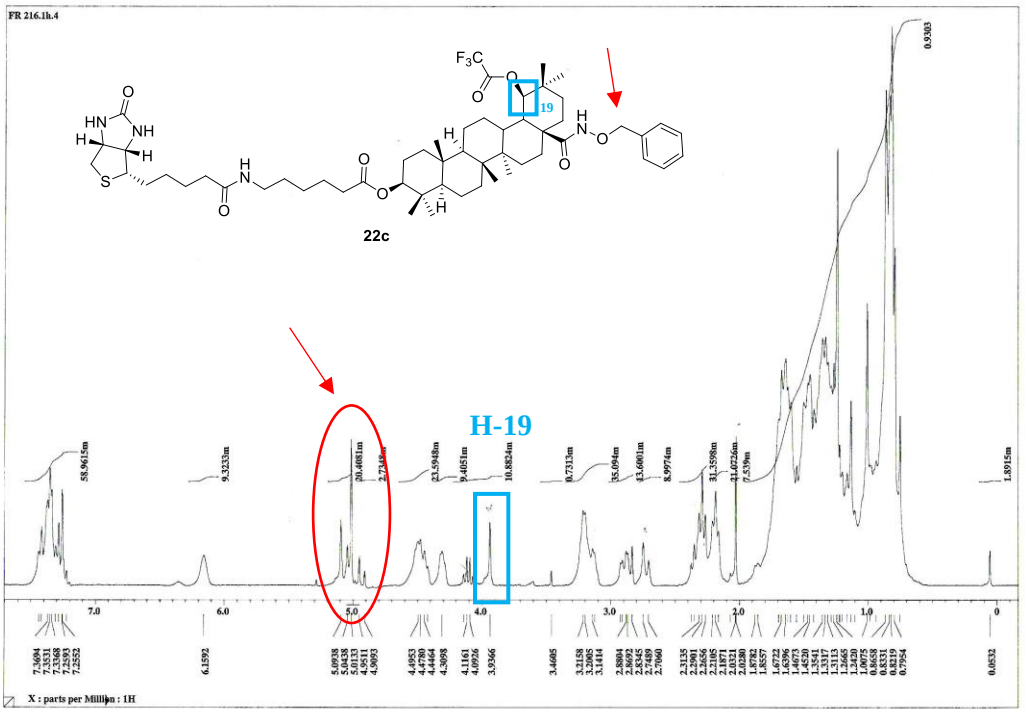
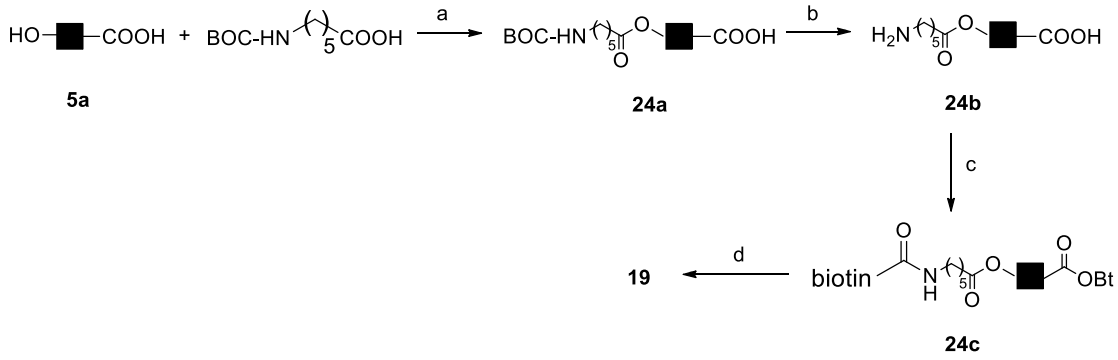


Figure 7: *^{1}H-NMR spectra of compound **22c**, in which there is the same signal at 3.99 ppm present in **Figure 6**.*

Solution: to avoid E ring expansion we have decided to conduct the reaction by using the Lewis acid BF_3 instead of the Bronsted acid CF_3COOH.

- *Fourth synthetic strategy*

In view of the problems faced in the previous approaches, we have rethinked completely the synthetic startegy by starting from betulinic acid **5a**, that was esterified at position 3 with the *N*-BOC-protected amino acid giving compound **24a,** that was deprotected with BF_3*Et_2O to furnish the intermediate **24b**. The latter was condensed with biotin leading the activated ester **24c**, that by treatment with hydroxylamine in pyridine furnished the desired hydroxamate **19**.

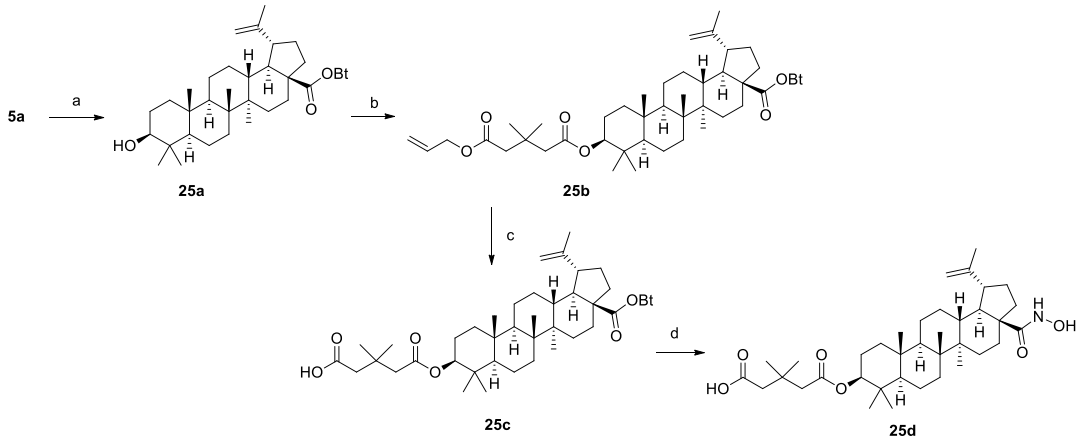


Scheme 7: (a) Benzoyl chloride, DMAP, TEA, THF dry; (b) BF_3OEt_2, DCM; (c) Biotin, EDC, HOBt, DCM; (d) NH_2OH*HCl, Pyridine dry.

This strategy still had some problems related to Yamaguchi reaction that did not lead to the total consumption of the starting material, making the product purification very challenging.

In order to improve the solubility of our lead compound **5b** for further *in-vivo* experiments**,** we have planned the synthesis of a pro-drug (**25d**) with the insertion in position 3 of a 3,3-dimethylglutarate moiety (*Scheme 8*).

To reduce the use of protecting groups we have planned a synthetic strategy in which the carboxylic acid at C-28 was at the same time protected and activated by the synthesis of the hydroxybenzotriazole ester **25a**. This key intermediate was esterified with 5-(allyloxy)-3,3-dimethyl-5-oxopentanoic acid through modified Yamaguchi reaction affording compound **25b** that was deprotected and converted into the corresponding hydroxamate **25d** for treatment with hydroxylamine in pyridine.



Scheme 8*: synthesis of the prodrug of* ***5b****.* (a) HOBt, EDC, DCM (quantitative); (b) 5-(allyloxy)-3,3-dimethyl-5-oxopentanoic acid, Benzoyl chloride, TEA, DMAP, THF dry (quantitative); (c) $Pd(OAc)_2$, morpholine, PPh_3, THF 40°C (60%), (d) NH_2OH*HCl, pyridine 80°C (50%).

2.3.2 Biological Evaluation

Effect of PCTT hydroxamates on PHD activity

HIF-1α transactivation assays were performed in NIH-3T3-EPO-Luc cells. The EPO-Hypoxia Response Element (HRE)-luciferase reporter plasmid contains three copies of the HRE consensus sequence from the promoter of the erythropoietin gene fused to the luciferase gene and is therefore is a useful surrogated marker for the screening compounds for the induction of HIF-1α/HIF-2α stabilization.[19] None of the starting PCTTAs (**3a**-**8a**) showed hypoxia mimetic activity, while their hydroxamates, with the notable exception of the one of glycyrrhethinic acid (**7b**), were, to a various extent, all significantly active. Conversely, no activity was observed in PCTTA bearing at C-28 chelating groups different from the hydroxamate (**15a**, **15b**, **16a**, **19a** and **19b**). While emphasizing the relevance of the hydroxamate moiety for activity, these observations also highlight the relevance of its location on the triterpenoid backbone, since the C-30 hydroxamate **7b** was inactive, while its corresponding C-28 hydroxamate **8b** was significantly active. Within the C-28 hydroxamates, activity was then fine-tuned by changes in connectivity and functionalization of the triterpenoid backbone. Thus, the lupane hydroxamate **5b** was more potent than its oleanane (**3b**, **6b**) and ursane (**4b**) analogues, while oxymation of the C-3 dehydroderivatives **3d-e** was accompanied by a decrease of activity. Comparison of the activity of the hydroxamates of oleanolic (**3b**) and maslinic (**6b**) derivatives, showed that introduction of a further hydroxyl on ring A was moderately beneficial, while fusion of ring A with a heterocyclic ring or dehydration of the 3-hydroxyl were not critical. Overall, changes on ring A did not significantly affect potency. On the other hand, its expansion in **12** was associated to a decrease of activity, while a certain activity was also observed in the A-*seco*-C-3 hydroxamate **13**.

Taken together, these observations show that, within the PCTTA scaffold, the presence of a hydroxamate group and its location are both critical for PHD targeting, since activity was observed when this group was located at C-28, but not at C-30, while no activity was observed when the C-28 hydroxamate was replaced by other chelating groups.

We have next investigated the biological translation of the acid-to-hydroxamate maneuver on other biological targets of PCTTA. These compounds are remarkably pleiotropic agents, targeting and/or modulating a series of transcription factors that include NF-κB (Nuclear factor-kappa B), STAT3 (Signal transducer and activator of transcription 3), Nrf2 [NFE2L2 or Nuclear factor (erythroid-derived 2)-like 2], and the bile receptor GPBAR1.[20-22] Evaluation of the hydroxamates against these targets, showed a detrimental effect on bioactivity, with only some residual Nrf2- and TGR5-activity found in the hydroxamate of ursolic acid, and complete loss of activity toward NF-κB or STAT3 pathways (*Table 1*).

Table 1: *Activity of PCTTA hydroxamates on various transcription factors.*

Comp.	IC_{50} NF-κB (μM)	IC_{50} STAT3 (μM)	EC_{50} NRF2 (μM)	IC_{50} NRF2 (μM)	EC_{50} TGR5 (μM)
1a-8a	- (>50)	- (>50)	- (>50)	- (>50)	18.90
3b	- (>50)	- (>50)	- (>50)	- (>50)	- (>50)
3d	- (>50)	- (>50)	23.93	- (>50)	- (>50)
8b	- (>50)	- (>50)	10.04	- (>50)	- (>50)
9	- (>50)	- (>50)	- (>50)	- (>50)	- (>50)
12	- (>50)	- (>50)	9.02	- (>50)	22.15
13	- (>50)	- (>50)	- (>50)	- (>50)	- (>50)
5b	- (>50)	- (>50)	- (>50)	- (>50)	17.50
5d	- (>50)	- (>50)	- (>50)	- (>50)	9.62
10	- (>50)	- (>50)	- (>50)	- (>50)	- (>50)
14a	- (>50)	- (>50)	- (>50)	- (>50)	- (>50)
14b	- (>50)	- (>50)	38.36	- (>50)	11.46
4b	- (>50)	- (>50)	- (>50)	- (>50)	5.91
4d	- (>50)	- (>50)	- (>50)	- (>50)	- (>50)
6b	- (>50)	- (>50)	- (>50)	- (>50)	10.19
7b	- (>50)	- (>50)	- (>50)	- (>50)	- (>50)

The carboxylate-to-hydroxamate transformation has therefore a dramatic modulating activity on the biological profile of PCTTAs, focusing their blurred biological profile on the HIF pathway (*Table 2*). Based on considerations of potency and target selectivity, the hydroxamate of betulinic acid (**5b**) was selected for further studies.

Table 2: *effect of triterpenoid hydroxamates on HIF pathway activation.*

Comp.	Efficacy HIF-1α (IRA coefficient) [a]	Potency EC_{50} HIF-1α (μM)
1a-8a	-	(>50)
3b	0,39	16.37
3d	0,43	3.83
8b	0,15	11.39
9	0,16	5.00
12	1,19	5,84
13	2,2	7,53
5b	0,36	4.81
5d	0,31	6.82
10	0,48	3.24
14a	0,55	2.58
14b	0,34	2.41
4b	0,17	7.69
4d	0,1	8.93
6b	0,1	7.10
7b	-	(>50)

[a]EC50 and IRA (Intrinsic relative activity) values were determined relative to 150 μM deferoxamine (DFX) using the following equation: IRA coefficient = $(EC_{50\text{-}DFX} \times E_{max}) / (EC_{50} \times E_{max\text{-}DFX})$, where EC_{50} and E_{max} denote EC_{50} and E_{max} of the agonist, and $EC_{50\text{-}DFX}$ and $E_{max\text{-}DFX}$ denote EC_{50} and E_{max} values of the standard agonist DFX.

The effect of **5b** on the expression of HIF-1α at the protein level was investigated in 293T cells. This compound stabilized HIF-1α protein without affecting the steady state levels of the PHDs analysed (*Figure 8A*). In kinetic experiments, its stabilizing activity on HIF-1α was significantly detectable after 15 min, peaked at 60 min and was maintained after 3 h of stimulation (*Figure 8B*). Next, the induction of HIF-1α in cells cultured under hypoxic conditions was investigated in 293T cells, subjecting them to hypoxia (1% O_2) in the presence or the absence of **5b**. The levels of HIF-1α stabilization were similar to those observed under hypoxia conditions, and the combination of both stimuli produced no significant change (*Figure 8C*). In accordance with these findings, the PHD2 levels were almost unaffected in response to both conditions (*Figure 8C*). These observations suggest that the hypoxia mimetic activity of **5b** is associated to inhibition of prolyl hydrolase functional activity, as further shown by a close correlation between the inhibition of HIF-1α hydroxylation and HIF-1α stabilization in cells pre-incubated with the proteasome inhibitor MG132 (*Figure 8D*).

The nature of the interaction with the HIF pathway was evaluated by assessing the induction of EPO-Luc activity in wash-out experiments where **5b** was removed from the cell culture by washing the cells with PBS after 1 h of treatment, and the EPO-Luc activity was then measured after further 5 hours. Activity was greatly reduced 5 h after removal of **5b** from the cell medium, as expected for a reversible inhibitor (*Figure 8E*). Remarkably, competition assays demonstrated that betulinic acid (**5a**) (50 μM) inhibited the hypoxia-mimetic activity of its hydroxamate derivative **5b** (*Figure 8F*), suggesting a "dock and bind" mechanism, where the triterpenoid scaffold is involved in target recognition, presumably by shape complementarity associated to its lipophilic core, and the hydroxamate reinforces it with polar interaction and/or chelation, Thus, docking experiments using PHD2 (4BQW from

Protein Data Bank) revealed that both **5a** and **5b** bind to the catalytic domain of PHD2 with similar intermolecular energy (data not shown).

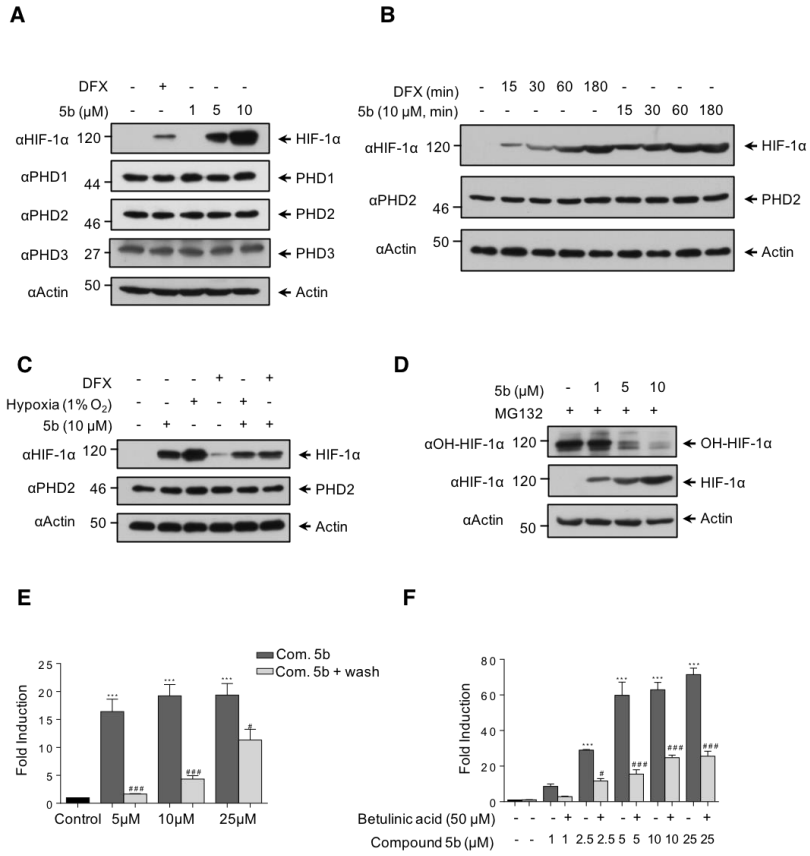


Figure 8: 5b mediates HIF-1α stabilization. *(A) HEK-293T cells were stimulated with either **5b**, at the indicated concentrations, of DFX (100 μM) for 3 h and the expression of HIF-1α, PHD1, PHD2 and PHD3 analysed by western blots. (B) Time-course induction of HIF-1α stabilization. (C) Effect of **5b** in the presence or the absence of low oxygen (1% O_2) on HIF-1α stabilization. (D) HEK-293T cells were treated with **5b** for 6 hours in the presence of MG132 and the levels of hydroxylated HIF-1α and HIF-1α were determined by immunoblot. (E) NIH-3T3-EPO-Luc cells were pre-treated with **5b** for 1 hour and then washed or not with PBS and incubated in complete medium for 6 hours. ***p<0.001 3a treated cells (no wash) vs untreated cells; # p<0.05, ### p<0.001 3a treated cells wash vs no wash cells (one-way ANOVA followed Tukey´s test). (F) NIH-3T3-EPO-Luc cells were pre-incubated with betulinic acid (**5a**) (50 μM) for 30 min and then treated with **5b** at the indicated concentrations for 6 h. Luciferase activity was measured in the cell lysates and fold induction relative to untreated cells is shown. ***p<0.001 3a treated cells vs untreated cells; # p<0.05, ### p<0.001 3a treated cells wash vs 3a + BA treated cells (one-way ANOVA followed Tukey´s test). Data represent the mean ± SD (n=3).*

To further identify which PHD is targeted by **5b,** HEK293T cells were transfected with HA tagged PHD1, PHD2 and PHD3 plasmids, and next treated with **5b** and DMOG (positive control). After treatments, PHDs were immunoprecipitated and the prolyl hydrolase activity was measured by the capacity to hydroxylate GST-HIF-1α. **5b** was found to clearly inhibit PHD1 and PHD2 activities, with only a marginal effect of PHD3 (*Figure 9*). Overall, our data suggest that **5b** binds the catalytic domain PHDs, but it does not mimic the activity of the iron chelator DFX in *in vitro* assays of PDH2 activity (data not shown), suggesting a different mechanism of inhibition. Thus, PHDs may undergo post-translational modifications affecting their activity, with phosphorylation at serine 125 by P70S6K for PHD2, and at serine 130 of PHD1 by cyclin-dependent kinases 2, -4, and -6.[23,24] It is therefore possible that triterpenoid hydroxamates could modulate these processes.

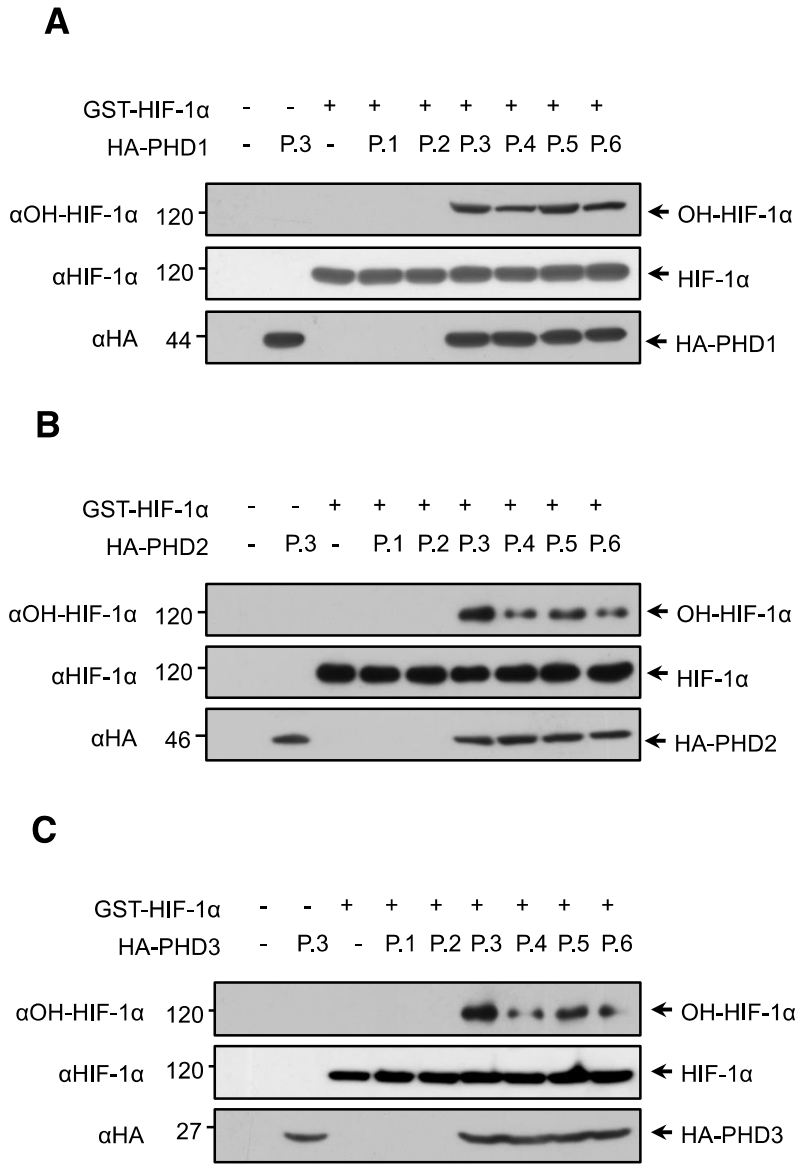


Figure 9: Effect of 5b on PDHs activity *HEK-293T cells were transfected with HA-PHD1 (A), HA-PHD2 (B) or HA-PHD3 (C) as indicated. After 24 hours of transfection, cells were treated as following: P1: non transfected cells; P2: cells were transfected with PHDs and immunoprecipitated with IgG-HA; P3: cells were transfected with PHDs and immunoprecipitated with αHA; P4: cells transfected with PHDs, stimulated with* ***5b*** *(10 uM) and immunoprecipitated with αHA; P5: cells transfected with PHDs, stimulated with* ***5b*** *(10 uM) and immunoprecipitated with αHA; P6: cells transfected with PHDs, stimulated with DMOG (1 mM) and immunoprecipitated with αHA. HIF Prolyl hydroxylase activity was measured using GST-HIF-1α protein and the levels of hydroxylated HIF-1α, HIF-1α and PHDs were analysed by immunoblot (n=3).*

Effect of 5b on HIF-dependent gene expression, angiogenesis and neuroprotection in vitro.

There is growing evidence that HIF-1α regulates the expression of a plethora of genes products have neuroprotective activity and mimic hypoxia preconditioning. [25-27] Thus, HIF-1α activates several proangiogenic genes, including vascular endothelial growth factor (VEGF-A) and fibroblast growth factor-2, mainly produced by vascular endothelial cells. These trophic factors are thought to maintain brain homeostasis within the context of the neurovascular unit. [28,29]

Accordingly, low doses of $VEGF_{165}$ are neuroprotective in in vitro and in vivo models of Huntington Disease (HD) caused by overexpression of mutated huntingtin. [30] Moreover, HIF PHDs inhibitors protected striatal cells bearing a mutated form of the huntingtin protein against mitochondrial toxin-induced cytotoxicity. [31]

These considerations prompted us to investigate the effect of **5b** on the expression of HIF-dependent genes. To this purpose, HBMEC were treated with **5b** for 12 hours, and the expression of 83 genes involved in the hypoxia pathway was then analysed by qPCR (*Figure 10A*). Fold up- or down-regulation was calculated for each gene, and those whose expression was upregulated more than 10-fold were identified. Several genes involved in neuroprotection such as Angiopoietin-like 4 (*Angptl4*), *N*-myc down-regulated gene 1(*Nrdg1*), erythropoietin (*epo*) and solute carrier family 2-member 1 (*slc2a1*) were upregulated in cells treated with **5b**. Angptl4 modulates brain blood barrier (BBB) dysfunction in ischemic stroke and is neuroprotective,[32] and several reports have demonstrated that EPO is also neuroprotective in different model of CNS diseases. [33-35]

On the other hand, Nrdg1 plays a role on oligodendrocytes survival and the gene is represses in the white matter of post-mortem Multiple Sclerosis patients. [35] The

gene *slc2a1* codifies for the glucose transporter-1 protein (Glut-1) that plays a key role in neuroprotection and brain microvasculature homeostasis. [36]

In addition, **5b** clearly induced the expression of VEFG (*Figure 10B*) and EPO (*Figure 5D*) in a concentration-dependent manner, enhancing their plasmatic levels *in vivo* (*Figure 10E*). Since it also induced the expression of VEGFA, we were interested in studying the potential activity in angiogenesis and compare its effects with those induced by the proangiogenic growth factors VEGF and FGF. Thus, in HUVEC co-cultured in a monolayer of primary fibroblasts, both rhVEGFA (10 ng/ml) and **5b** (1 μM) clearly induced an increase in the network of endothelial tubes (*Figure 10C*), suggesting that **5b** can induce angiogenesis in an autocrine manner after secretion of VEFG.

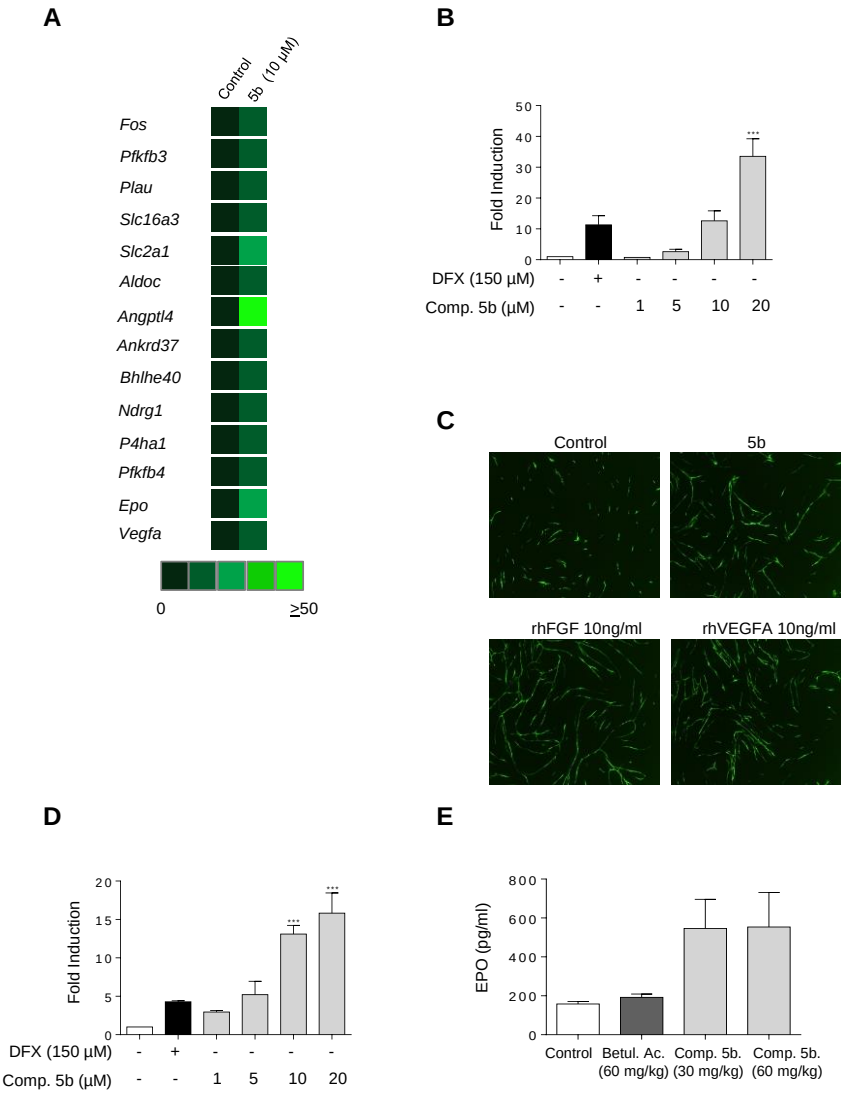


Figure 10: compound 5b induces the expression of HIF-dependent genes. *(A) Human brain microvascular endothelial cells were stimulated with* ***5b*** *(10 µM) for 12 h and the expression of genes involved in the human hypoxia signalling pathway determined by PCR array. Heat maps shows the significantly upregulated (green) genes in 3a-treated cells compared with control. (B-D) The mRNA expression levels of VEGFA and EPO genes respectively were quantified by qPCR in HBMEC cells. Data represent the mean ± SD (n=3). ***p<0.001 3a treated cells vs untreated cells (one-way ANOVA followed Dunnett´s test). (C) PrimeKit co-cultures were seeded on Day 0 and the indicated concentration of rhVEGFA, rhFGF or 3a was added on Day 2. Representative images from the experiment are shown. Magnification 4x. (E) EPO levels were determined in plasma from C57BL/6 treated with betulinic acid (****5a****) (60 mg/kg) or Compound* ***5b*** *(30 and 60 mg/kg) for 4 hours. Plasma levels were increased after the treatment with* ***5b****. Results are expressed as mean ± SEM (n= 6 animals per group).*

The effect of HIF pathway activation by **5b** was next evaluated in response to mitochondrial dysfunction in neurons induced by the selective complex II inhibitor 3-nitropropionic acid (3-NP). To this purpose, immortalized striatal neurons expressing normal huntingtin protein (*STHdh*^{Q7}) and striatal neurons expressing a mutant huntingtin protein associated with juvenile onset HD (*STHdh*^{Q111}) [37] were used. As expected, 3-NP was strongly cytotoxic in *STHdh*^{Q111} cells compared to *STHdh*^{Q7} cells, and treatment with **5b** protected both type of cells from 3-NP-induced cell death (*Figure 11).*

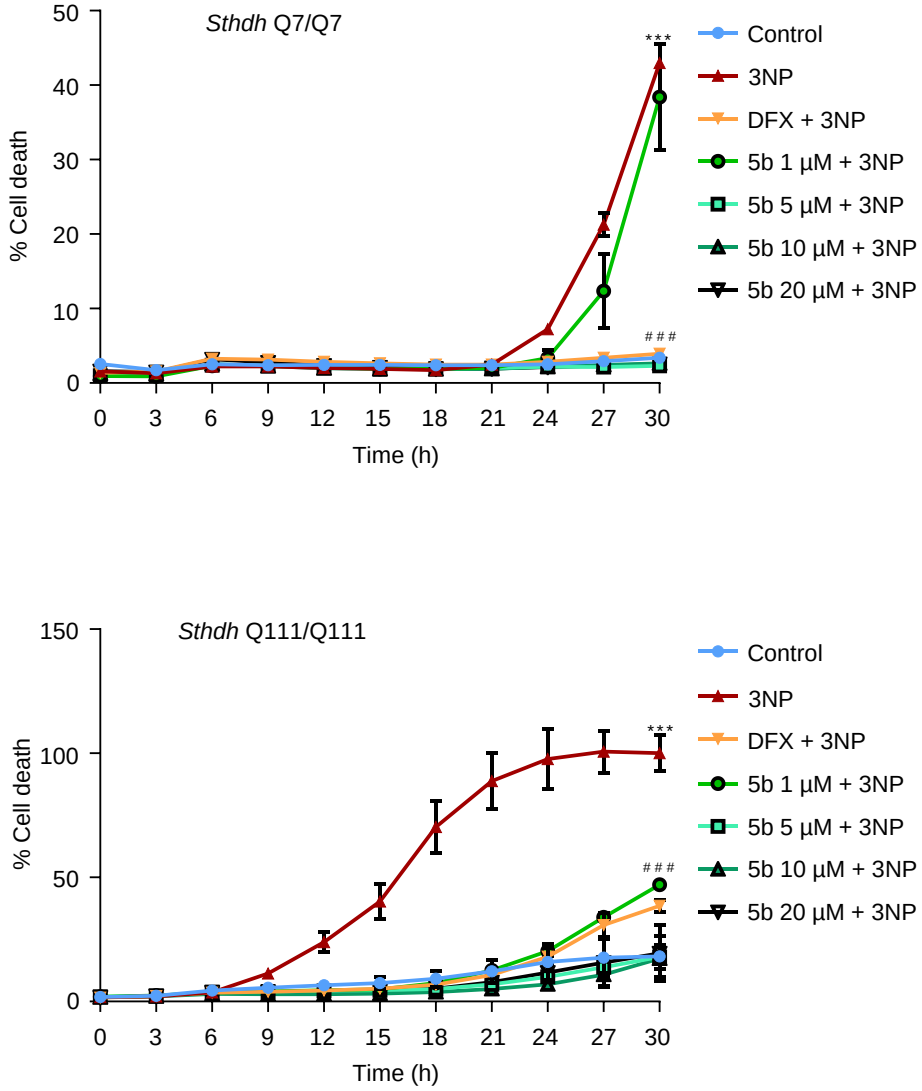


Figure 11: compound 5b prevents 3NP-induced cytotoxicity in striatal cells harboring wild type and mutant huntingtin. *STHdh^{Q7} and STHdh^{Q111} cells were pre-incubated with the indicated concentrations of **5b** for 6 hours and stimulated with 3NP for 30 h. Percentage of neuronal death was determined by YOYO-1 and referred to vehicle-treated neurons. Data represent the mean ± SD (n=3). ***p<0.001 3NP treated cells vs untreated cells; ### p <0.001 3NP+**5b** treated cells vs 3-NP treated cells (one-way ANOVA followed Tukey´s test).*

Neuroprotective effect of 5b in a murine model of neuronal striatal degeneration. In order to confirm the neuroprotective action of **5b** *in vivo,* a model of Huntington`s disease based on 3-NP administration was used. Treatment with 3-NP results in several alterations, including neurological and histological changes characteristic of some aspects of HD pathology.

Compared to the control animals treated with vehicle, the 3-NP-treated mice exhibited high scores in hind limb clasping, locomotor activity, hind limb dystonia and kyphosis (*Figure 12A*). Treatment with **5b** improved the clinical symptoms of 3NP-lesioned mice by improving locomotor activity hind limb clasping, dystonia and kyphosis. We have next investigated the impact of **5b** in striatal degeneration and atrophy. The administration of 3-NP reduced the number of neurons in the striatum, as determined by Nissl staining (*Figure 12B*).

In addition, **5b**-mediated neuroprotection was associated with reduced 3-NP-induced microgliosis and astrogliosis as determined by Iba1 and GFAP immunohistochemistry (*Figure 12C*). We have also analysed the expression of specific pro-inflammatory markers. In 3-NP-lesioned mice, an upregulation of mRNA levels of inflammatory markers such as COX-2, iNOS, IL-1 and IL-6 was observed, fully inhibited by treatment with **5b** (*Figures 12D-G*). Betulinic acid (**5a**), a compound unable to affect the HIF signalling, only showed a marginal efficacy in animals intoxicated with 3-NP, highlighting the role of this pathway in the activity of **5b**.

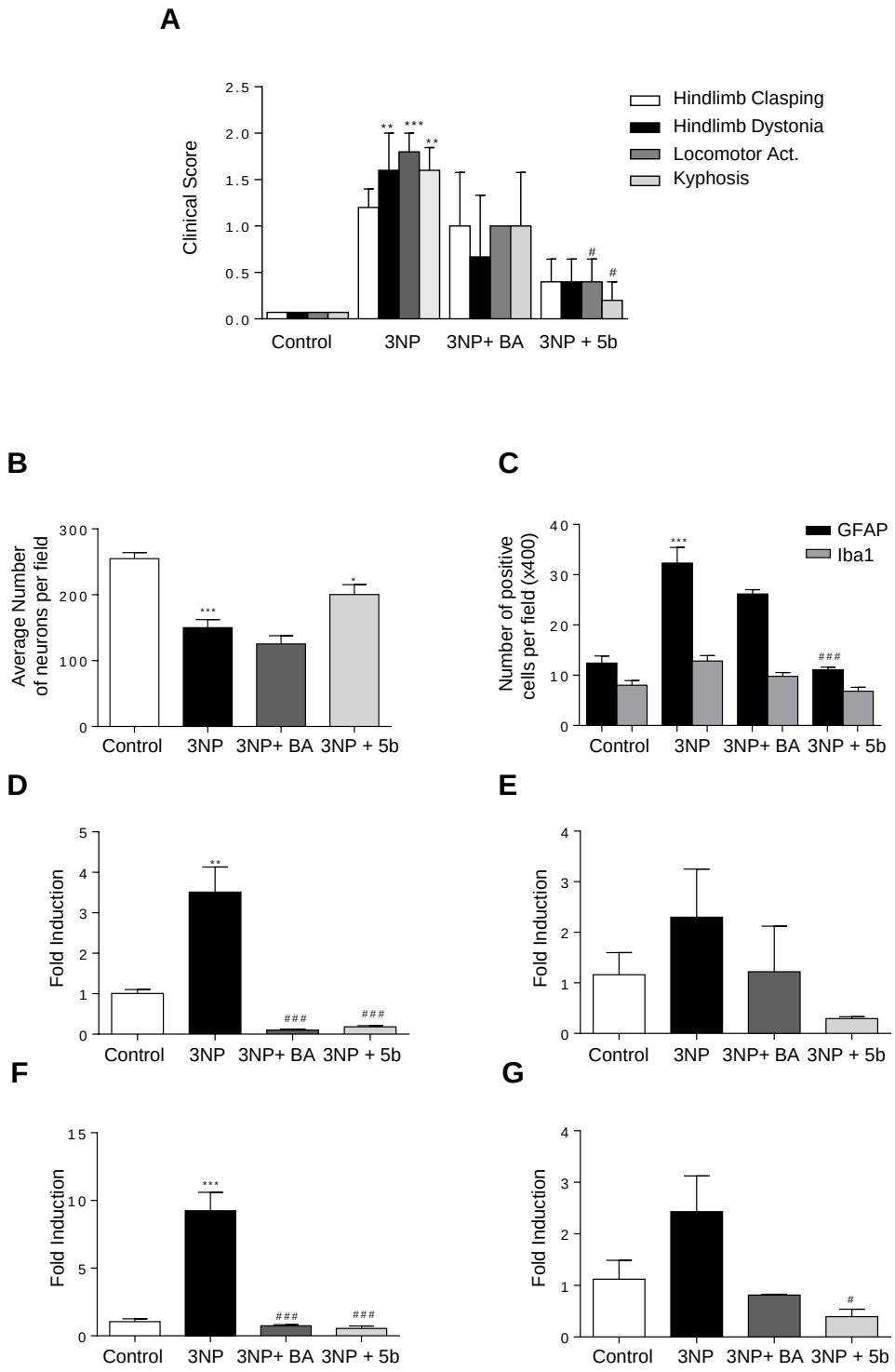

A
Clinical Score
2.5
2.0
1.5
1.0
0.5
0.0
Hindlimb Clasping
Hindlimb Dystonia
Locomotor Act.
Kyphosis
Control
3NP
3NP+ BA
3NP + 5b
B
Average Number of neurons per field
300
200
100
0
C
Number of positive cells per field (x400)
40
30
20
10
GFAP
Iba1
D
Fold Induction
E
F
G

Figure 12: compound 5b is neuroprotective in 3NP-intoxicated mice. *(A) Behavioural score was determined 12 hours after 3NP intoxication. Mice were treated with betulinic acid (BA) (**5a**) (30 mg/kg) or **5b** (30 mg/kg) as indicated. Hind limb clasping, general locomotor activity, hind limb dystonia and kyphosis were rated from 0 to 2 based on severity, with score 0 indicating normal function, and 2 indicating the most severe affected function. Values are expressed as means ± SEM (n = 6). (B-C) Quantification of Nissl staining, Iba-1 and glial fibrillary acidic protein (GFAP) immunostained sections through the different group performed on coronal striatal brain sections from the same mice groups. Quantification of the different markers was performed with Image J software. Total average number of neurons, microglia (Iba1+) and astrocytes (GFAP+) is shown. Values are expressed as mean ± SEM (n = 6). Gene expression of inflammatory markers including (D) interleukin (IL)-6, (E) interleukin (IL)-1β, (F) inducible nitric oxide synthase (iNOS) and (G) cyclooxygenase (COX)-2 was significantly downregulated in 3NP + compound 3a-treated mice compared with 3NP mice. Values are expressed as means ± SEM (n=6) animals per group. **p < 0.01, ***p < 0.001 3NP vs. Vehicle; #p < 0.05, ###p < 0.001 vs. 3NP+3a vs 3NP (one-way ANOVA followed Tukey´s test).*

2.3.3 Conclusions

Many compounds have shown promises for HIF-targeted therapies, but HIF-1 associated neurotoxicity and/or cytotoxicity have, in most cases, hindered their advancement to *in vivo* studies, with a resulting shortage of pre-clinically validated leads.[8]

We have provided evidence that PCTTA hydroxamates are a class of novel, selective and druggable modulators of HIF-1 signalling. Their mechanism of activity transcends simple chelation, since replacement of the carboxylate with other chelating elements was not associated to activity, while a definite spatial relationship between the hydroxamate moiety and the triterpenoid core was critical for activity. By combining data from *in vitro* and *in vivo* experiments, the hydroxamate of betulinic acid (**5b**) was selected for *in vivo* studies, validating at preclinical level its HIF-targeted therapeutic potential and qualifying it for further development as a neuroprotective agent.

2.4 EXPERIMENTAL SECTION

General Methods and Materials.

Commercially available reagents and solvents were purchased from Aldrich or Alfa-Aesar and were used without further purification. *N,N'*-Dimethylformamide (DMF) was dried over neutral alumina pad and stored on 4 Å activated molecular sieves. Dichloromethane was dried by distillation from P_2O_5 and stored on 4 Å activated molecular sieves. Pyridine was dried over neutral alumina pad and stored on activated 4 Å molecular sieves under nitrogen. When needed, the reactions were performed in flame- or oven-dried glassware under a positive pressure of dry nitrogen. For spectroscopic characterization, a JEOL ECP 300 MHz spectrometer was used for ^{1}H and ^{13}C spectra, and a Thermo Finningan LCQ-deca XP-plus equipped with an ESI source and an ion trap detector for mass spectrometry. Chemical shifts are reported in parts per million (ppm) using the residual solvent peak as reference ($CHCl_3$ at δ 7.27). Flash column chromatography was performed on silica gel (Merck Kieselgel 60, 230−400 mesh ASTM). Thin layer chromatography (TLC) was carried out on 5 × 20 cm plates with a layer thickness of 0.25 mm (Merck silica gel 60 F254). When necessary, $KMnO_4$ was used for visualization. Purity of tested compounds was established by elemental analysis. Elemental analysis (C, H, N) of the target compounds are within ±0.4% of the calculated values, confirming ≥95% purity. Compounds **3b**,[17] **4b**,[17] **5b**[16] and **7b**,[13] **11a**[38] and **11b,**[38] as well as **15a**,[39] **16a**[40] and **17** [41] have previously been reported.

Triterpenoid hydroxamates:

Protocol A (via chlorides): synthesis of **5b** as exemplificative: a) Acetylation: to an ice-cold solution of betulinic acid **5a** (1 gr, 2.19 mmol; 1 mol. equiv.) in dry pyridine (10 mL) acetic anhydride (413 µL, 4.38 mmol, 2 mol. equiv.) and DMAP (27 mg, 0.1 mol. equiv.) were sequentially added. The reaction was stirred at room temperature for 3 h quenched with methanol, diluted with 2 *N* H_2SO_4, and extracted with EtOAc. The organic phases were washed with brine, dried over Na_2SO_4, and evaporated under vacuum to give crude acetylbetulinic acid, used for the next step without further purification. b) Hydroxyamidation: to an ice-cold solution of crude acetylbetulinic acid (1.09 gr, 2.19 mmol, 1 mol. equiv.) in dry DCM (15 mL), oxalyl chloride (1.13 mL, 13.14 mmol, 6 mol. equiv.) was added dropwise, and the mixture was heated at 40°C for 1.5 hours. The solvent was then removed under vacuum, the residue dissolved in dry pyridine, and hydroxylammonium chloride (913 mg, 13.14 mmol, 6 mol. equiv.) was added. The reaction was heated at 40°C for 3 hours, quenched with 2*N* H_2SO_4 and extracted with EtOAc. The combined organic phases were washed with brine, dried over Na_2SO_4 and evaporated under vacuum. The crude reaction product was further purified over silica gel (PE/EtOAc 7:3) to afford 667 mg (60%) of residue. c) deprotection: to a solution of acetylbetulinylhydroxamate (667 mg, 1.31 mmol) in THF/MeOH 1:1 (8 mL), 4*N* NaOH (16,4 mL, 65,5 mmol; 50 mol. equiv.) was added. The mixture was heated at 40°C overnight, quenched with 2*N* H_2SO_4 and extracted with EtOAc. The organic phases were washed with brine, dried over Na_2SO_4 and evaporated. The crude product was purified over silica gel (PE/EtOAc 5:5) affording 330 mg (55%) of **5b** as an off-white powder. For physical and spectroscopic data, see ref. 16. The data for the new compounds are as follows:

(2α,3β) 2,3-Dihydroxy-*N*-hydroxy-olean-12-en-28-amide (6b) : off-white solid (45%). IR (KBr) cm-1: 2939, 2867, 1662, 1450, 1031, 883. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.44 (brt, 1H), 3.72-3.61 (m, 1H), 2.99 (d, *J* = 9.5 Hz, 1H), 2.45 (d, *J* = 12.2 Hz, 1H), 1.15 (s, 3H), 1.02 (s, 3H), 0.98 (s, 3H), 0.90 (s, 3H), 0.87 (s, 3H), 0.82 (s, 3H), 0.78 (s, 3H); ^{13}C NMR (75 MHz, CDCl3): δ 176.7, 167.1, 144.9, 123.8, 78.4, 76.6, 55.0, 47.3, 46.2, 46.1, 45.6, 41.2, 41.0, 39.0, 38.7, 37.8, 33.6, 32.6, 32.4, 32.3, 31.4, 28.5, 27.4, 27.3, 23.2, 23.1, 22.8, 18.6, 16.3, 16.5, 16.1. MS (ESI) *m/z* 488 (M + H)+. Anal. Calcd for $C_{30}H_{49}NO_4$: C, 73.88; H, 10.13; N, 2.87. Found: C, 73.99; H, 10.15; N, 2.94.

(3β)-3-Hydroxy-11-oxo-olean-12-en-*N*-hydroxy-28-amide (8b): pale yellow solid (38%). IR (KBr) cm-1: 2944, 2865, 1651, 1464, 1209, 1039, 994, 733. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.67 (s, 1H), 3.20 (t, J = 6.1 Hz, 1H), 2.74 (d, *J* = 12.2 Hz, 2H), 2.32 (s, 1H), 2.10-2.02 (m, 1H), 1.18 (s, 3H), 0.96 (s, 3H), 0.94 (s, 3H), 0.90 (s, 9H), 0.77 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 200.2, 174.6, 167.9, 128.0, 78.8, 62.1, 55.0, 45.2, 44.7, 43.6, 40.9, 39.2, 37.3, 33.7, 32.8, 32.7, 32.1, 30.7, 29.7, 28.1, 27.4, 27.3, 23.7, 23.4, 23.3, 19.0, 17.5, 16.2, 15.6, 14.2. MS (ESI) *m/z* 486 $(M + H)^+$. Anal. Calcd for $C_{30}H_{47}NO_4$: C, 74.19; H, 9.75; N, 2.88. Found: C, 74.23; H, 9.80; N, 2.93.

2. Protocol B (via mixed phosphoric anhydride): synthesis of **7b**: to a stirred solution of PPAA (50% in EtOAc, 146 μL, 0.264 mmol, 1.2 mol. equiv.) in acetonitrile (3 mL) triethylamine (126 μL, 0.88 mmol, 4 mol. equiv.) and glycyrrhetic acid (100 mg, 0.22 mmol, 1 mol. equiv.) were sequentially added. After stirring 30 min at room temperature, hydroxylammonium chloride (31 mg, 0.44 mmol, 2 mol. equiv.) was added and stirring was continued overnight at room temperature. The reaction was then worked up by dilution with EtOAc and washing with brine. The organic phases were dried over Na_2SO_4, evaporated and the residue was purified over silica gel (PE/EtOAc 5:5) affording 69 mg (64%) **7b** [13] as a white powder.

Triterpenoid oxyminohydroxamates. Synthesis of **3d** as exemplificative: a) To a stirred solution of oleanolic acid **3a** (500 mg, 1.1 mmol, 1 mol. equiv.) in EtOAc/acetone 1:1 (7 mL), Jones reagent was added dropwise until the reaction remined orange. The mixture was diluted with brine, extracted with EtOAc and the combined organic phases were dried over Na_2SO_4 and evaporated. The residue was purified silica gel (PE/EtOAc, 8:2), affording 474 mg (95%) of dehydrooleanolic acid [42]. b) Oxyminohydroxylation: to an ice-cold solution of dehydrooleanolic acid (474 mg, 1.04 mmol, 1 mol. equiv.) in dry DCM (7 mL), oxalyl chloride (353 μL, 6.24 mmol, 6 mol. equiv.) was added dropwise and the mixture was heated at 40°C for 1.5 hours. The solvent was then removed in vacuum, the residue dissolved in dry pyridine and hydroxylammonium chloride (430 mg, 6.24 mmol, 6 mol. equiv.) was added. The reaction was heated at 40°C for 3 hours, quenched with 2*N* H_2SO_4 and extracted with EtOAc. The combined organic phases were washed with brine, dried over Na_2SO_4, and evaporated under vacuum. The crude reaction product was further purified over silica gel (PE/EtOAc 5:5) to afford 353 mg (70%) **3d** as an off-white solid. IR (KBr) cm-1: 2944, 2859, 1701, 1674, 1632, 1463, 1389, 1364, 924. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.45 (brt, 1H), 3.13-3.08 (m, 1H), 2.46 (m, 1H), 1.98 (m, 3H), 1.13 (s, 3H), 1.07 (s, 6H), 1.05 (s, 3H), 0.86 (s, 6H), 0.82 (s, 3H) (only readily peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 176.7, 167.5, 144.9, 123.8, 55.7, 47.1, 46.2, 45.5, 42.0, 40.8, 40.3, 39.4, 38.4, 37.0, 33.9, 32.9, 31.9, 30.7, 29.7, 29.2, 27.2, 25.7, 25.5, 23.7, 23.5, 23.4, 19.0, 17.3, 16.7, 14.9. MS (ESI) *m/z* 485 $(M + H)^+$. Anal. Calcd for $C_{30}H_{48}N_2O_3$: C, 74.34; H, 9.98; N, 5.78. Found: C, 74.35; H, 10.01; N, 5.81.

3-Hydroxyimino-*N*-hydroxy-urs-12-en-28-amide (4d): pale yellow solid (70%). IR (KBr) cm-1: 2956, 2833, 1729, 1630, 1423, 1390, 1374, 982, 949. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.43 (brt, 1H), 3.07 (m, 1H), 2.12 (m, 1H), 1.24 (s, 3H), 1.15 (s, 3H), 1.08

(s, 3H), 1.06 (s, 3H), 1.03 (s, 3H), 0.94 (s, 3H), 0.81 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 177.3, 167.7, 140.6, 126.5, 55.7, 52.1, 47.0, 42.5, 40.2, 39.6, 39.4, 39.0, 38.5, 37.0, 36.7, 32.2, 30.6, 29.7, 27.7, 27.4, 24.8, 23.5, 23.4, 23.3, 21.1, 19.0, 17.3, 17.2, 16.8, 15.1. MS (ESI) *m/z* 485 $(M + H)^+$. Anal. Calcd for $C_{30}H_{48}N_2O_3$: C, 74.34; H, 9.98; N, 5.78. Found: C, 74.40; H, 10.06; N, 5.84.

3-Hydroxyimino-*N*-hydroxy-lup-20(29)-en-28-amide (5d): off-white solid (65%). IR (KBr) cm-1: 2966, 2831, 1751, 1715, 1665, 1453, 1449, 1034, 1007, 984, 866. ^{1}H NMR (300 MHz, $CO(CD_3)_2$): δ = 4.72 (s, 1H), 4.59 (s, 1H), 3.04-3.00 (m, 2H), 2.32 (s, 1H), 1.68 (s, 3H), 1.24 (s, 6H), 1.22 (s, 3H), 1.12 (s, 3H), 0.97 (s, 3H), 0.96 (s, 3H), 0.91 (s, 3H) (only readily peaks are reported); ^{13}C NMR (75 MHz, $CO(CD_3)_2$) δ = 176.7, 164.1, 150.5, 109.6, 56.0, 55.5, 55.3, 50.2, 47.1, 42.5, 40.8, 40.2, 38.7, 38.2, 37.9, 37.2, 34.0, 33.3, 30.8, 29.4, 27.4, 25.6, 22.9, 21.5, 21.2, 19.4, 19.1, 16.1, 15.8, 14.6. MS (ESI) *m/z* 485 $(M + H)^+$. Anal. Calcd for $C_{30}H_{48}N_2O_3$: C, 74.34; H, 9.98; N, 5.78. Found: C, 74.27; H, 9.94; N, 5.71.

Synthesis of Δ^2-hydroxamates: Synthesis of **9** as exemplificative: to a stirred solution of oleanolic acid **3a** (500 mg, 1.1 mmol, 1 mol. equiv.) in dry pyridine (9 mL), *p*-toluenesulfonyl chloride (735 mg, 3.8 mmol, 3.8 mol. equiv.) was added. The solution was stirred at room temperature for 24 h under nitrogen atmosphere, diluted with water and then extracted with CH_2Cl_2. The organic phases were washed with saturated $KHSO_4$ solution, dried over Na_2SO_4 and concentrated under reduced pressure. The crude was diluted in DMF (6 mL), sodium acetate (315 mg, 2.3 mmol) was added and the mixture was heated at 120 °C for 24 h under nitrogen atmosphere. The mixture was diluted with brine and extracted with DCM. The organic phases were dried over Na_2SO_4 and evaporated. The residue was purified over silica gel (PE/EtOAc, 9:1), affording 221 mg (42%) of Δ^2-oleanolic acid [42], next diluted in dry CH_2Cl_2 (4 mL) and cooled at 0 °C. Oxalyl chloride (249 μL, 2.9 mmol,

6 mol. equiv.) was then added dropwise and the mixture was heated at 40°C for 1.5 hours. The solvent was then removed in vacuum, the residue dissolved in dry pyridine, and hydroxylammonium chloride (201 mg, 2.9 mmol, 6 mol. equiv.) was added. The reaction was heated at 40°C for 3 hours, quenched with 2*N* H_2SO_4 and extracted with EtOAc. The organic phases were washed with brine, dried over Na_2SO_4 and evaporated under vacuum. The crude reaction product was purified over silica gel (PE/EtOAc, 7:3) affording 104 mg (48%) **9** as yellowish oil. IR (KBr) cm-1: 2949, 2868, 1632, 1461, 1387, 1362, 910, 731. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.43-5.32 (m, 3H), 2.44 (d, *J* = 11.3 Hz, 1H), 1.14 (s, 3H), 0,97 (s, 6H), 0,87 (s, 12H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 176.4, 144.5, 138.0, 124.1, 121.3, 51.9, 46.4, 46.1, 45.5, 42.1, 41.0, 40.7, 39.5, 36.1, 34.5, 34.0, 33.0, 31.9, 31.8, 31.6, 30.7, 27.2, 25.9, 25.7, 23.8, 23.5, 22.9, 19.6, 16.3, 15.6. MS (ESI) *m/z* 454 $(M + H)^+$. Anal. Calcd for $C_{30}H_{47}NO_2$: C, 79.42; H, 10.44; N, 3.09. Found: C, 79.37; H, 10.40; N, 3.06.

Lupa-2,20(29)-dien-*N*-hydroxy-28-amide (10): yellowish powder (54%). IR (KBr) cm-1: 2936, 2868, 1717, 1643, 1448, 1374, 881, 731. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.38-5.28 (m, 2H), 4.73 (s, 1H), 4.59 (s, 1H), 3.03 (t, *J* = 9 Hz, 1H), 2.37 (t, *J* = 12.1 Hz, 1H), 1.63 (s, 3H), 1.21 (s, 3H), 0,93 (s, 3H), 0,89 (s, 3H), 0,82 (s, 3H), 0,81 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 175.0, 150.5, 137.9, 121.6, 109.6, 54.3, 52.1, 50.4, 49.2, 42.3, 40.8, 38.4, 37.9, 36.4, 34.6, 33.5, 32.8, 31.7, 30.9, 30.8, 29.7, 29.3, 25.6, 22.6, 19.5, 16.4, 15.8, 14.6, 14.5, 14.3. MS (ESI) *m/z* 454 $(M + H)^+$. Anal. Calcd for $C_{30}H_{47}NO_2$: C, 79.42; H, 10.44; N, 3.09. Found: C, 79.48; H, 10.47; N, 3.13.

Triterpenoid hydroxamates from the products of Bayer-Villiger fragmentation (11a,11b):

Olean-12-en-*N*-hydroxy-28-amide-3-oic acid ε-lactone (13): prepared from **11a** [38] according to the hydroxyamidation protocol A: white solid (35%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.45 (brt, 1H), 2.63 (t, *J* = 5.4 Hz, 2H), 2.47 (brdd, *J* = 9.4 Hz, 1H), 2.05 (m, 2 H), 1.52 (s, 3H), 1.46 (s, 3H), 1.29 (s, 3H), 1.16 (s, 3H), 0.90 (*s*, 3H), 0.88 (*s*, 6H), 0.83 (s, 3H) (only readily peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 176.5, 175.4, 144.6, 123.6, 86.4, 55.2, 47.2, 46.1, 45.5, 42.2, 40.8, 40.0, 39.4, 37.4, 33.9, 32.96, 32.91, 31.99, 31.92, 30.7, 27.1, 25.5, 25.4, 23.9, 23.6, 23.4, 22.6, 20.8, 16.5, 16.4. MS (ESI) *m/z* 486 $(M + H)^+$. Anal. Calcd for $C_{30}H_{47}NO_4$: C, 74.19; H, 9.75; N, 2.88. Found: C, 74.06; H, 9.68; N, 2.81.

A-*seco*-4,12-dien-olean-*N*-hydroxy-3-amide-28-methyl ester (12): prepared from **11b** [38] after KOH hydrolysis and hydroxyamidation according to Protocol A. White solid (20%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.29 (brt, 1H), 4.85 (s, 1H), 4.66 (s, 1H), 3.62 (s, 3H), 2.74 (brdd, *J* = 9.7 Hz, 1H), 1.71 (s, 3H), 1.10 (s, 3H), 0.90 (s, 6H), 0.87 (s, 6H), 0.74 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 178.4, 171.9, 147.5, 143.7, 122.2, 113.7, 60.54, 51.6, 50.8, 46.8, 45.8, 42.2, 41.4, 39.3, 39.1, 38.1, 33.9, 33.1, 32.4, 31.4, 30.7, 27.7, 25.8, 24.4, 23.6, 21.1, 20.8, 19.4, 18.4, 16.9, 14.2. MS (ESI) *m/z* 500 $(M + H)^+$. Anal. Calcd for $C_{31}H_{49}NO_4$: C, 74.51; H, 9.88; N, 2.80. Found: C, 74.49; H, 9.84; N, 2.77.

Heterocyclic hydroxamates. Synthesis of 14a as exemplificative. a) Formylation: to a solution of **5c** (300 mg, 0.65 mmol, 1 mol. equiv.) in toluene (70 mL), NaOEt (221 mg, 3.25 mmol, 5 mol. equiv.) and ethyl formate (241 mg, 3.25 mmol, 5 mol. equiv.) were sequentially added. The reaction mixture was stirred at 50 °C overnight, quenched with 2*N* H_2SO_4 and extracted with EtOAc. The organic phases were washed with brine, dried over Na_2SO_4 and evaporated under vacuum to afford

2-formyl-3-oxobetulinic acid [43] as a colorless oil, that was used without further purification for the next step. b) 1,2-Diazole formation: (reaction with hydroxylammonium chloride as exemplificative): to a stirred solution of crude 2-formyl-3-oxo-betulinic acid (310 mg, 0.64 mmol, 1 mol. equiv.) in ethanol/H_2O 9:1 (6 mL) hydroxylammonium chloride (400 mg, 5.76 mmol, 9 mol. equiv.) was added. The reaction mixture was heated at 80 °C for 5 h, diluted with H_2O and extracted with EtOAc. The organic phases were washed with brine, dried over Na_2SO_4 and evaporated under vacuum, affording the crude product as a brown oil, used without further purification. c) Hydroxyamidation: the reaction was carried out according to Protocol A, affording compound **14a** (41% from **5c**) as a white powder.

Lup-2-eno[2,3-*d*]-isoxazol-*N*-hydroxy-28-amide (14a): white powder. IR (KBr) cm-1: 2953, 2867, 1714, 1632, 1508, 1455, 1367, 956, 887. ^{1}H NMR (300 MHz, CD_3OD): δ = 10.37 (s, 1H, NH), 8.42 (s, 1H, OH), 8.26 (s, 1H), 4.67 (s, 1H), 4.55 (s, 1H), 3.00 (t, *J* = 9.3 Hz, 1H), 2.61 (t, *J* = 12.0 Hz, 1H), 1.64 (s, 3H), 1.40 (s, 3H), 1.22 (s, 3H), 1.11 (s, 3H), 0.93 (s, 3H), 0.74 (s, 3H); ^{13}C NMR (75 MHz, CD_3OD) δ = 173.5, 172.5, 151.3, 151.0, 109.9, 109.4, 54.0, 53.3, 50.6, 49.0, 48.9, 46.7, 42.4, 38.9, 37.3, 35.6, 34.8, 33.4, 32.6, 30.9, 29.0, 25.7, 21.7, 19.5, 18.7, 16.4, 16.2, 14.8. MS (ESI) *m/z* 495 $(M + H)^+$. Anal. Calcd for $C_{31}H_{46}N_2O_3$: C, 75.26; H, 9.37; N, 5.66. Found: C, 74.99; H, 9.30; N, 5.60.

1'H-Lup-20(29)-eno[3,2-c]-pyrazol-*N*-hydroxy-28-amide (14b): yellowish powder (overall 48% from **5a**). IR (KBr) cm-1: 2986, 2798, 1700, 1655, 1508, 1390, 1287, 1035, 851, 739. ^{1}H NMR (300 MHz, $(CD_3)_2CO$): δ = 10.35 (s, 1H), 7.15 (s, 1H), 4.68 (s, 1H), 4.56 (s, 1H), 3.03 (t, *J* = 9.1 Hz, 1H), 2.61 (m, 1H), 1.64 (s, 3H), 1.20 (s, 3H), 1.09 (s, 3H), 0.94 (s, 3H), 0.93 (s, 3H), 0.72 (s, 3H); ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ = 172.4, 151.0, 149.1, 132.8, 111.9, 109.0, 59.7, 50.0, 53.7, 50.5, 49.2, 46.8, 42.2, 40.7, 38.6, 37.9, 37.7, 36.6, 33.5, 33.4, 32.3, 30.8, 30.6, 25.7, 23.3, 21.4, 19.1, 18.7,

15.6, 14.2, 13.7. MS (ESI) *m/z* 494 $(M + H)^+$. Anal. Calcd for $C_{31}H_{47}N_3O_2$: C, 75.41; H, 9.60; N, 8.51. Found: C, 75.35; H, 9.55; N, 8.48.

2-(28-oleanoylamido)-3-(2-hydroxyphenyl) acrylic acid (15b): in a sealed tube, a solution of oleanoyl-glycineamide **15a** [39] (200 mg, 0.39 mmol, 1 mol. equiv.) in acetic anhydride (92 μL, 0.97 mmol, 2.5 mol. equiv.), was treated with salicylic aldehyde (72 mg, 0.58 mmol, 1.5 mol. equiv.) and NaOAc (24 mg, 0.29 mmol, 0.75 mol. equiv.). The reaction was heated at 140 °C for 1.5 h, then diluted with MeOH, EtOAc and brine. The organic phases were dried over Na_2SO_4 and evaporated under vacuum. To crude azalactone (154 mg, 0.26 mmol, 1 mol. equiv.) was dissolved in THF/MeOH 1:1 (4 mL) and 4*N* NaOH (5 mL, 20 mmol, 51 mol. equiv.). The mixture was heated at 40°C overnight, quenched with 2*N* H_2SO_4 and extracted with EtOAc. The organic phases were washed with brine, dried over Na_2SO_4, and evaporated under vacuum. The crude product was purified over silica gel (PE/EtOAc 9:1) affording 82 mg (35% from **15a**) **15b** as a yellowish solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.76 (s, 1H), 8.70 (s, 1H), 7.35 (m, 3H), 5.63 (brt, 1H), 3.18 (dd, *J* = 7.9, 1.5 Hz, 1H), 2.73 (dd, *J* = 11.4, 0.9 Hz, 1H), 1.18 (s, 3H), 0.95 (s, 3H), 0.93 (s, 6H), 0.85 (s, 3H), 0.73 (s, 3H), 0.63 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 178.3, 158.9, 149.9, 142.8, 129.4, 127.8, 125.1, 124.8, 124.2, 122.9, 120.1, 116.3, 79.0, 55.1, 47.9, 47.6, 46.5, 42.3, 41.9, 39.4, 38.8, 38.6, 36.9, 34.2, 33.0, 32.6, 32.4, 30.8, 28.1, 27.5, 27.2, 25.9, 24.2, 23.6, 18.3, 16.4, 15.6, 15.4. MS (ESI) *m/z* 618 $(M + H)^+$. Anal. Calcd for $C_{39}H_{55}NO_5$: C, 75.81; H, 8.97; N, 2.27. Found: C, 75.85; H, 9.01; N, 2.32.

***N*-Imidazolyl semiaminals 18 a and 18b:** to a stirred and cooled (-78 °C) solution of methyl imidazole (75 mg, 0.91 mmol, 2 mol.equiv.) in dry THF *n*-BuLi (0.8 M, 1.13 mL, 0.908 mmol, 2 mol.equiv.) was added dropwise. After 15 minutes, a solution of **17** (200 mg, 0.454 mmol, 1 mol. equiv.) in dry THF (3 mL) was slowly added and the reaction was stirred overnight at room temperature. The reaction was quenched

with H_2O, washed with EtOAc and the combined organic phases were dried over Na_2SO_4 and evaporated under vacuum. The crude product was purified over silica gel (PE/EtOAc 9:1 as eluent), affording **18a** (35 mg, 15%) and **18b** (58= mg, 25%).

18a: IR (KBr) cm-1: 3112, 2945, 2872, 1463, 1365, 1274, 1000, 936, 734. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.94 (s, 1H), 6.78 (s, 1H), 5.36 (t, *J* = 3.3 Hz, 1H), 4.85 (s, 1H), 3.71 (s, 3H), 3.20 (m, 1H), 2.73 (dd, J_1 = 3.9 Hz, *J* = 9.1 Hz, 1H), 1.19 (s, 3H), 1.03 (s, 3H), 0.98 (s, 3H), 0.93 (s, 6H), 0.88 (s, 3H), 0.77 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ= 147.3, 144.2, 126.8, 123.1, 121.6, 79.0, 68.8, 55.2, 47.6, 46.9, 41.7, 41.6, 41.0, 40.0, 38.8, 38.6, 37.0, 34.2, 33.9, 33.2, 32.5, 30.8, 28.1, 27.2, 26.3, 25.6, 25.4, 23.7, 23.4, 22.7, 18.3, 17.4, 15.6, 15.6. MS (ESI) *m/z* 523 $(M + H)^+$. Anal. Calcd for $C_{34}H_{54}N_2O_2$: C, 78.11; H, 10.41; N, 5.36. Found: C, 78.15; H, 10.45; N, 5.40.

18b: IR (KBr) cm-1: 3100, 2967, 2855, 1459, 1405, 1137, 1079, 1035, 920, 765. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.03 (s, 1H), 6.79 (s, 1H), 5.18 (t, *J* = 3.3 Hz, 1H), 4.71 (s, 1H), 3.66 (s, 3H), 3.21 (m, 1H), 2.12 (dt, *J*= 14.4 and 5.1 Hz, 1H), 1.20 (s, 3H), 1.01 (s, 3H), 0.99 (s, 3H), 0.92 (s, 3H), 0.80 (s, 3H), 0.79 (s, 3H), 0.50 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ = 149.2, 145.5, 127.0, 124.0, 121.0, 78.9, 68.4, 55.1, 47.7, 47.5, 42.0, 41.7, 39.9, 38.8, 38.5, 37.0, 34.2, 34.0, 32.9, 32.3, 30.7, 28.1, 27.2, 26.6, 25.7, 25.1, 23.7, 23.4, 23.0, 18.4, 17.1, 15.6, 15.4. MS (ESI) *m/z* 523 $(M + H)^+$. Anal. Calcd for $C_{34}H_{54}N_2O_2$: C, 78.11; H, 10.41; N, 5.36. Found: C, 78.14; H, 10.48; N, 5.43.

Synthesis of biotinylated derivative of 5b

6-((tert-butoxycarbonyl)amino)hexanoic acid was prepared according to literature. [44]

Synthesis of compound 24a (modified protocol of Yamaguchi esterification): to a stirred solution of 6-((tert-butoxycarbonyl)amino)hexanoic acid (1,2 eq) in dry THF (20 mL/gr), benzoyl chloride (1,2 eq), triethylamine (3 eq) and DMAP (cat) were

sequentially added. The reaction mixture was stirred for 10 minutes at room temperature and betulinic acid (1 eq) was finally added. After 24 h the reaction was quenched with H_2SO_4 2N. The solution was extracted twice with EtOAc. The combined organic phases were washed with $NaHCO_3$ sat. sol. (X2), dried over Na_2SO_4 and evaporated under reduced pressure. The crude was purified over silica gel, using Pe/EtOAc 9:1 as eluent. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.73 (s, 1H), 4.60 (s, 1H), 4.49 – 4.43 (m, 1H), 3.10- 3.80 (m, *2*H), 2.28 (dd, *J* = 14.3, 7.7 Hz, 2H), 1.68 (s, 3H), 1.43 (s, 3H), 0.96 (s, 3H), 0.92 (s, 3H), 0.82 (s, 3H) (only readily peaks are reported).

Deprotection of 24a (24b): to a stirred solution of **24a** (1 eq) in DCM dry (10 mL/g) at 0°C, boron trifluoride diethyl etherate (2 eq) was added dropwise. The reaction was warmed to room temperature and allowed to stir for 5 h. The reaction was quenched with a saturated solution of $NaHCO_3$ and a precipitated was formed. The organic layer was removed by extraction and the aqueous phase was filtered to obtain **24b** as white solid (57%). The product was used for the next step without further purification.

Synthesis of 24c: to a stirred solution of biotin in DCM (10 mL/g), EDC (2,5 eq) was added follow by HOBt (2,5 eq). The reaction was allowed to stir for 4 h. Then a solution of **24b** in DCM and TEA (3 eq) was added. After 24 h the reaction was extracted with DCM and brine (x2). The organic phase was dried over Na_2SO_4, evaporated under vacuum and then purified by GCC using EtOAc/MeOH 9:1 as eluent (21%). ^{1}H NMR (301 MHz, $CDCl_3$) δ 8.06 (d, *J* = 8.7 Hz, 1H), 7.44 (ddd, *J* = 22.4, 15.2, 8.0 Hz, 3H), 6.22 (brd s, 1H), 4.71 (s, 1H), 4.62 (s, 1H), 4.51 – 4.40 (m, 3H), 4.31 (brd s, 1H), 3.72 (dd, *J* = 6.6, 5.6 Hz, 3H), 3.25-3.10 (m, 4H), 2.30- 2.10 (m, 4H), 1.69 (s, 3H), 1.41 (s, 3H), 1.03 (s, 3H), 0.97 (s, 3H), 0.82 (s, 3H) (only readily peaks are reported).

Synthesis of product 19: to a solution of compound **24c** in pyridine (10 mL/g), NH_2OH*HCl was added. The reaction was heated at 80°C overnight and then quenched with a solution of H_2SO_4 2N. The organic phase was washed with H_2SO_4 2N (x3), dried over Na_2SO_4 and evaporated to obtain a crude product that was purified over silica gel, with EtOAc/MeOH 9:1 as eluent to afford a yellow solid (29%). ^{1}H NMR (300 MHz, $(CD_3)_2CO$) δ 5.97 (s, 1H), 4.72 (s, 1H), 4.58 (s, 1H), 4.54 – 4.40 (m, 2H), 4.35 (s, 1H), 3.28-3.15 (m, 4H), 2.28 (d, *J* = 7.6 Hz, 3H), 2.14 (d, *J* = 7.7 Hz, 3H), 2.04 (s, 3H), 1.95 (s, 3H), 1.28 (s, 3H), 1.02 (s, 3H), 0.95 (s, 3H), 0.88 (s, 3H), 0.84 (s, 3H)) (only readily peaks are reported).

Esterification of 5b with HOBt (25a): to a solution of betulinic acid in dry DCM, EDC (2 eq) was added followed by HOBt (2 eq). The reaction was allowed to stir at room temperature overnight. The solution was washed twice with brine and the organic phase was dried over Na_2SO_4 and evaporated under vacuum to obtain compound **25a**, that was used for the next reaction without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.07 (d, *J* = 8.4 Hz, 1H), 7.63 – 7.34 (m, 3H), 4.68 (d, *J* = 27.5 Hz, 2H), 3.18 (dd, *J* = 10.9, 5.3 Hz, 1H), 2.94 (td, *J* = 11.3, 5.4 Hz, 1H), 2.74 – 2.60 (m, 1H), 2.40 (dd, *J* = 12.9, 8.0 Hz, 1H), 2.29 – 2.01 (m, 2H), 1.70 (s, 3H), 1.24 (s, 3H), 1.04 (s, 3H), 0.97 (s, 3H), 0.80 (s, 3H), 0.75 (s, 3H) (only readily peaks are reported).

Synthesis of 5-(allyloxy)-3,3-dimethyl-5-oxopentanoic acid: to a suspension of 3,3-dimethylglutaric anhydride (3g, 26,3 mmol) in allylic alcohol (15 mL), DMAP (catalytic) was added and the reaction was allowed to stir at room temperature overnight. The reaction was extracted with EtOAc and brine (x3). The organic phase was dried over Na_2SO_4 and evaporated under reduced pressure to afford a yellow oil (61%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.13- 1.18 (s, 6H), 2.48 (s, 2H), 2.49 (s, 2H), 4.50 (d, *J*= 5.8 Hz, 2H), 5.25 (dd, *J*= 10.4, 1.3 Hz, 1H), 5.32 (dd, *J*= 17.3, 1.3 Hz, 1H), 5.9 (m, 1H).

Synthesis of compound 25b (modified protocol of Yamaguchi esterification): to a solution of 5-(allyloxy)-3,3-dimethyl-5-oxopentanoic acid (1,5 eq) in dry THF (20 mL/gr), benzoyl chloride (1,5 eq), triethylamine (2 eq) and DMAP (cat) were sequentially added. The reaction mixture was stirred for 10 minutes and compound **25a** (1 eq) was finally added. After 24 h the reaction was quenched with H_2SO_4 2N. The solution was extracted twice with EtOAc. The combined organic phases were washed with $NaHCO_3$ sat. sol. (X2), dried over Na_2SO_4 and evaporated under reduced pressure. The product was used for the next synthetic step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.06 (d, *J* = 8.4 Hz, 1H), 7.59 – 7.31 (m, 3H), 5.90 (ddd, *J* = 16.1, 10.9, 5.7 Hz, 1H), 5.25 (ddd, *J* = 13.8, 11.6, 1.4 Hz, 2H), 4.75 – 4.41 (m, 2H), 2.93 (td, *J* = 10.9, 5.0 Hz, 1H), 2.67 – 2.53 (m, 1H), 2.43 – 2.30 (m, 5H), 1.69 (s, 3H), 1.24 (s, 3H), 1.03 (s, 3H), 0.97 (s, 3H), 0.82 (s, 3H) (only readily peaks are reported).

Procedure for the deprotection of compound 25b (25c): to a solution of compound **25b** in THF, $Pd(OAc)_2$ (0,05 eq), morpholine (2 eq) and PPh_3 (1,5 eq) were added. After 24h at 40°C, the reaction was quenched with H_2SO_4 2N and extracted with EtOAc. The organic phase was dried using Na_2SO_4 and evaporated under reduced pressure. The product was purified over silica gel (Pe/ EtOAc 7:3) to afford **25c** as yellow solid (60%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.07 (d, *J* = 8.3 Hz, 1H), 7.72 – 7.32 (m, 3H), 4.67 (d, *J* = 27.5 Hz, 2H), 4.54 – 4.41 (m, 1H), 2.93 (dd, *J* = 11.1, 6.3 Hz, 1H), 2.62 (d, *J* = 9.4 Hz, 1H), 2.39 (dd, *J* = 15.3, 7.6 Hz, 5H), 1.69 (s, 3H), 1.24 (s, 3H), 1.03 (s, 3H), 0.97 (s, 3H), 0.82 (s, 3H) (only readily peaks are reported).

Compound 25d: to a solution of compound **25c** in pyridine, NH_2OH*HCl (6 eq) was added and stirred overnight at 80°C. The reaction was quenched with 2*N* H_2SO_4 and extracted with EtOAc. The combined organic phases were washed with brine, dried over Na_2SO_4, and evaporated under vacuum. The crude reaction product was

further purified over silica gel (Pe/ EtOAc 6:4) to afford a yellow solid (50%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.67 (d, *J* = 37.1 Hz, 2H), 4.48 (dd, *J* = 10.3, 5.2 Hz, 1H), 3.00 (dd, *J* = 12.9, 7.4 Hz, 1H), 2.42 (dd, *J* = 15.3, 7.6 Hz, 5H), 2.06 – 1.91 (m, 4H), 1.68 (s, 3H), 0.96 (s, 3H), 0.91 (s, 3H), 0.84 (s, 3H), 0.82 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 183.1, 179.2, 172.8, 150.4, 109.8, 81.2, 56.5, 55.4, 50.4, 49.3, 47.0, 42.4, 40.7, 38.46, 38.40, 37.9, 37.1, 34.2, 33.7, 33.2, 32.2, 30.6, 29.7, 28.1, 25.4, 23.8, 20.9, 20.1, 19.4, 18.2, 16.6, 16.2, 16.1, 14.7.

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Chapter 3

STRIGOTERPENOIDS, A CLASS OF CROSS-KINGDOM STRESS RESPONSE MODULATORS

3.1 INTRODUCTION

Oats, peas, beans and barley grow,
Oats, peas, beans and barley grow,
Can you, or I, or anyone know
How oats, peas, beans and barley grow?
- Old English Nursery Rhyme

Plant hormones are a structurally unrelated group of secondary metabolites able to influence at low concentration plant physiological processes such as growth, differentiation, development and response to abiotic and biotic stress. Among them, plant growth regulators such as auxin, gibberellin, cytokinin, abscisic acid, ethylene, together with defence hormones including salicylic acid, jasmonic acid and methyl malonate, have received attention regarding the mechanism governing the key processes of plant development and physiology.[1]

Frits Went, 1903-1990

Kenneth Thimann, 1904-1997

***Figure 1**: Frits Went and Kenneth Thimann, botanist pioneers in plant physiology known for describing how hormones control plants.*

Strigolactones (SLs) are a class of natural and synthetic compounds that in the past decade have been exciting the scientific community not only for their intriguing biological properties but also for their potential applications in agriculture.[2]

They are C15 apocarotenoid dilactones involved in shoot and root architecture and in plant responses to environmental stress, especially water and nutrient deprivation.

Figure 2: *plants of the witchweed Striga hermonthica parasitizing maize plant.*

Strigolactones were isolated from root exudates as stimulant of the germination of seeds of the parasitic plant *Striga*, the "witchweed".

Witchweeds were so-named by farmers in Africa because they appeared without warning from nowhere and attacked their crops. The Latin name for these witchweeds derives from Striga, a mythical witch apparently with origins in ancient Rome but known in several parts of Europe. The witch Striga was thought to be filled with hatred against others, especially children, feeding on their life essence, or consuming them without remorse.[3]

The first member of this family was Strigol, isolated in 1966. To date, at least 25 naturally occurring SLs have been characterized from root exudates and different synthetic analogues have been synthesized.[4]

The basic framework of natural SLs is a tricyclic lactone skeleton (ABC ring), linked to a butenolide D ring by an enol ether bridge. According to the stereochemistry of BCD-ring system they can be classified in two groups: the first one with the configuration of (+)-Strigol and the second one with the configuration of (-)-Orobanchol (*Figure 3*).

The biogenetic pathway of SLs starts in plastids with the conversion of all-*trans*-β-carotene into carlactone by the action of an isomerase (D27) and two carotenoids cleavage dioxygenases (CCD7 and CCD8). Carlactone is then transferred into the cytoplasm and oxidized to carlalactonic acid that is finally converted into 5-deoxystrigol or orobanchol, the key precursors of natural occurring SLs (*Figure 3*). [5]

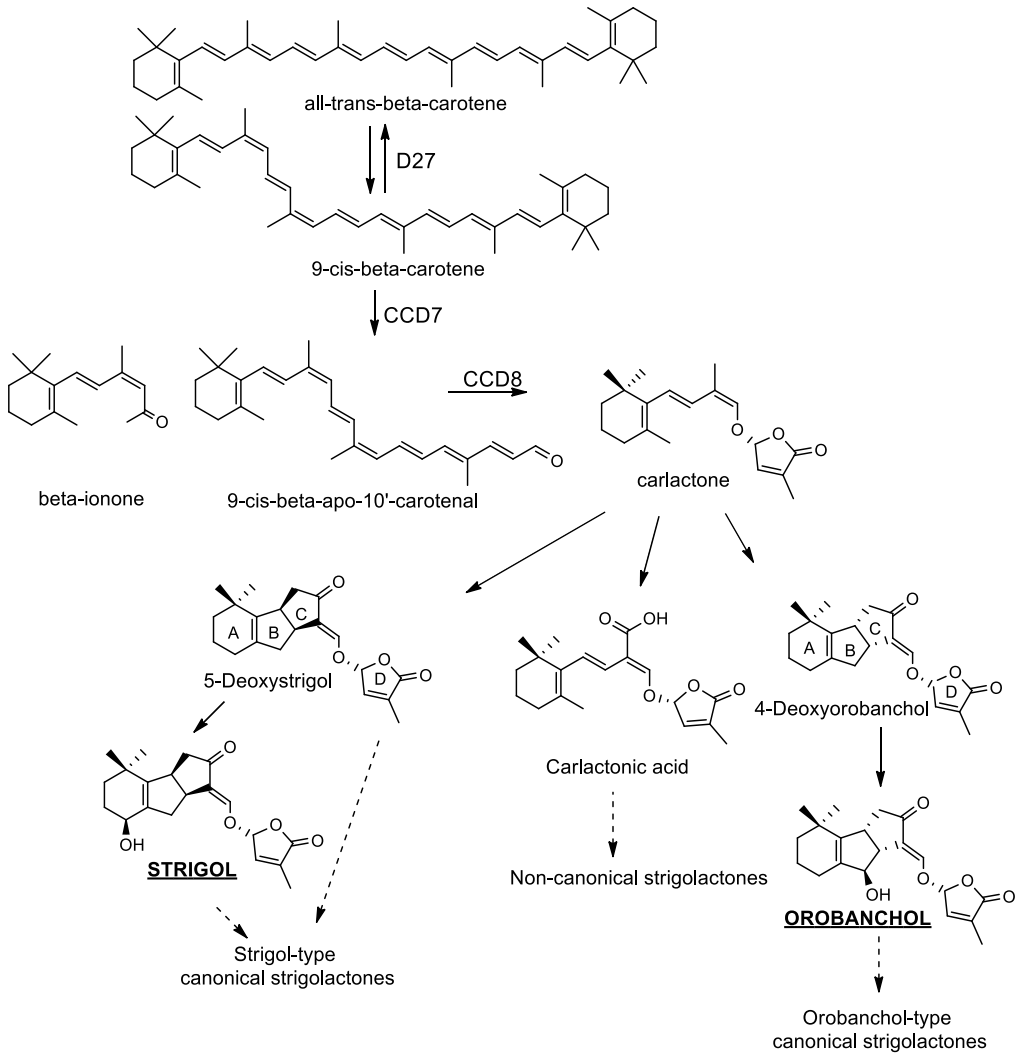


Figure 3: *SLs biosynthesis.*[6]

There is convincing evidence that the reactive CD ring system is responsible for the plant hormone activity of SLs, that is mediated by covalent binding to a reactive serine residue of the catalytic triad (serine-histidine-aspartate) in their macromolecular targets D14 and D14-like/KAI2. It is still debated if the hydrolysis of the butenolide moiety by the catalytic triad and mediated by water, is needed for the signalling (pathway a, *Figure 4*). Indeed, the potentially interaction between the C=O of the lactone and the serine oxygen atom could be sufficient to mediate a binding of SL to the receptor and an allosteric effect leading to the signalling (pathway b, *Figure 4*).[5]

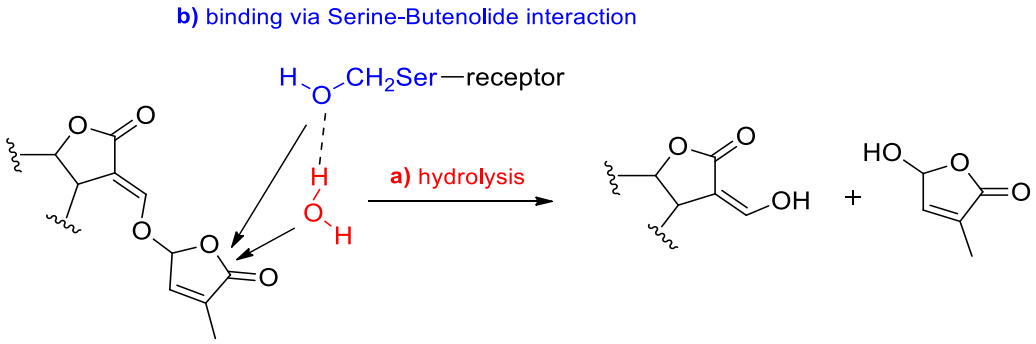


***Figure 4**: putative SL signalling pathways.*[5]

An alternative pathway would require the addition of water mediated by the serine or of a nucleophilic group present at the receptor site, that reacts in a Michael fashion with enone moiety in the CD-part, followed by a retro-Michael with a concomitant elimination of the D-ring unit (*Figure 5*).[7]

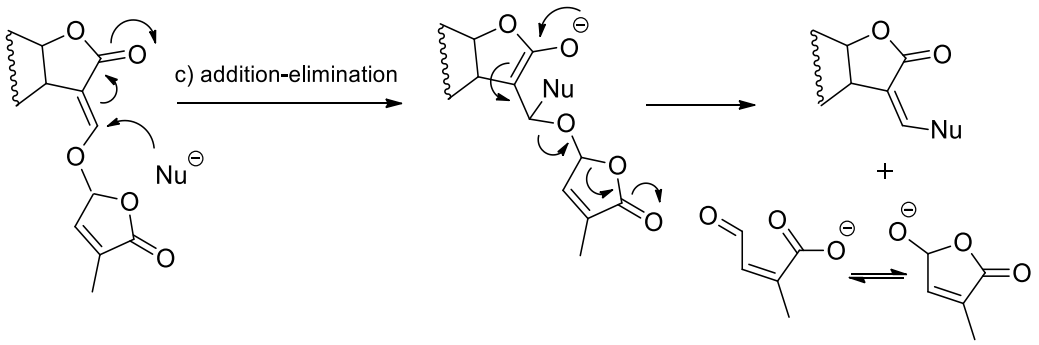


Figure 5: *pathway addition-elimination.*[7]

It is important to underline the relationship between the plant and the organisms in contact with it through strigolactones, and the actions of these molecules on the architecture of the plant itself:

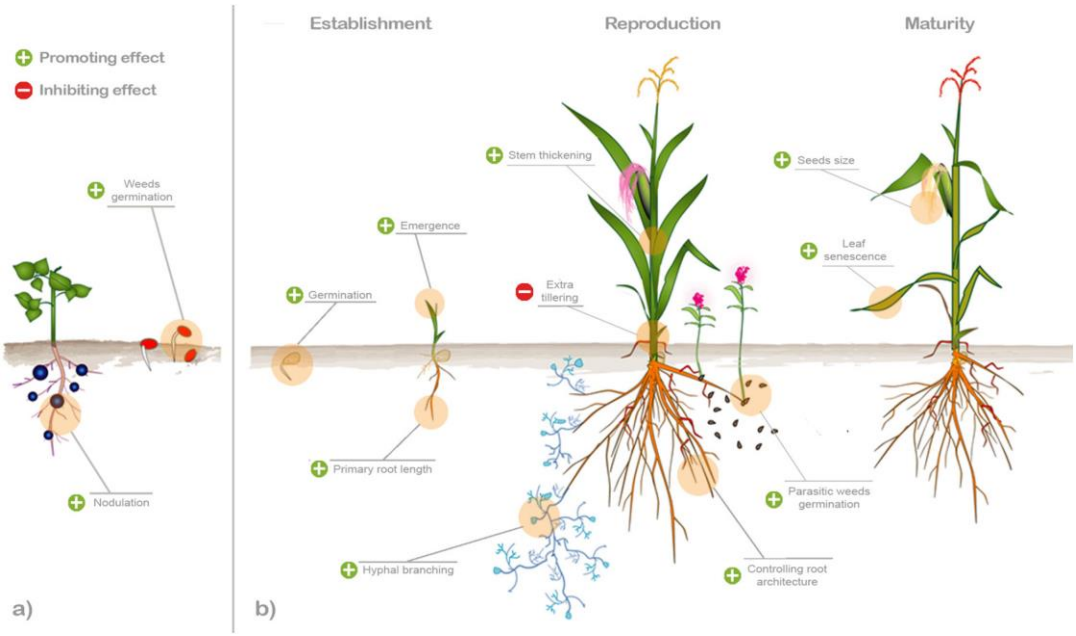


Figure 6: *roles of strigolactones in the rhizosphere (a) and in plant development (b).*[5]

- They are potent germination stimulants of parasitic weed seeds, that anchors itself on the host plant roots through haustorial penetration to

extract nutrients required for its survival and development, leading to plant death and economic losses.[5]

- They are produced by plants to stimulate the symbiosis with arbuscular mycorrhizal fungi (AM fungi), that undergo symbiotic association with plants' roots, helping the plant by improving the uptake of inorganic phosphate and minerals, and hence can sustain plant growth.[3],[2]
- The number of nodules formed on the roots and the shape of roots itself of some vegetables are influenced by SLs, leading to a better nitrogen fixation.[5]
- SLs act like signalling molecules not only in the rhizosphere but also in the aerial part of the plant and could mediate plant architecture changes (such as repression of lateral bud outgrowth).[5]
- They induct leaf senescence, allowing the relocation of nutrients like phosphorous, nitrogen and metals from photosynthetically less active parts of the plant to developing tissues, flowers, seeds and leaves.[5]
- SLs affect root architecture and root development depending on nutrients availability: under normal phosphate conditions, stimulate the growth of the primary root and of root hairs but they inhibit lateral roots development. In contrast, under low phosphate levels, these phytohormones increase the development of lateral roots to favour the adaptation of the plant architecture to abiotic stress conditions (drought, low soil nutrient content conditions). [5]

Natural SLs can be obtained in minute amounts from plant root exudates or by long multistep chemical syntheses. Simplified synthetic SL analogues and mimics were developed to investigate the structure-activity relationship in plants[8] and to overcome their intrinsic instability in soil due the hydrolysis of the enolether bridge,

with consequent inactivation.[7] Among these, the most important simplified analogue is GR24, that retains the germination activity of the parent compounds.

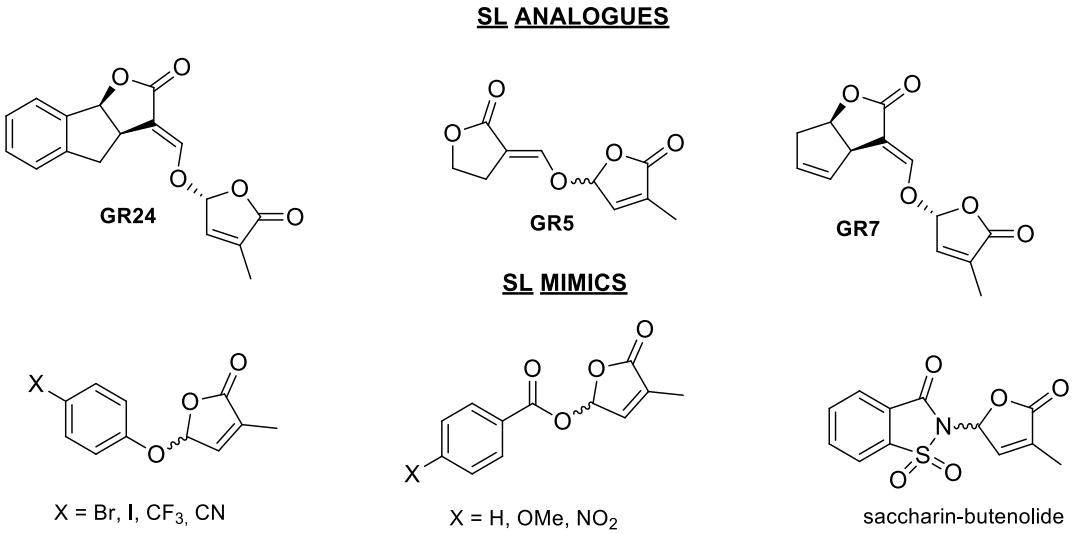


Figure 7: *some examples of SL analogues and SL mimics.*

SLs mimics are a second group of SLs that don't have the typical structural motif of natural SLs, but they are characterized by a simplified structure in which ABC system is replaced by an aryloxy-, arylthia- or aroyloxy-substituent linked to butenolide ring via an ether bridge.[8]

Therefore, an alternative mechanism was proposed for the mimic involving a Michael addition, followed by a proton shift and the subsequent elimination of the aryloxy group as in case of 4-bromodebranone (*Figure 8*).

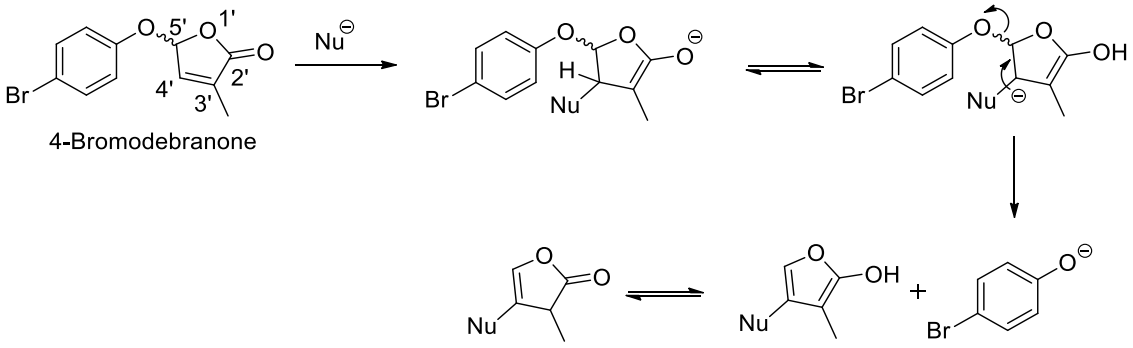


***Figure 8**: proposed mechanism for SL mimics mode of action.*[7]

3.2 RATIONALE OF THE PROJECT

Among plant hormones (*Figure 9*), plant growth regulators such as auxin, gibberellin (GA), cytokinin (CTK), abscisic acid (ABA), indole 3-acetic acid (IAA) and ethylene, together with defence hormones like salicylic acid (SA), jasmonic acid (JA) and methyl jasmonate (MJ), have received attention for their interesting biological profile both in plants and mammalians. [1]

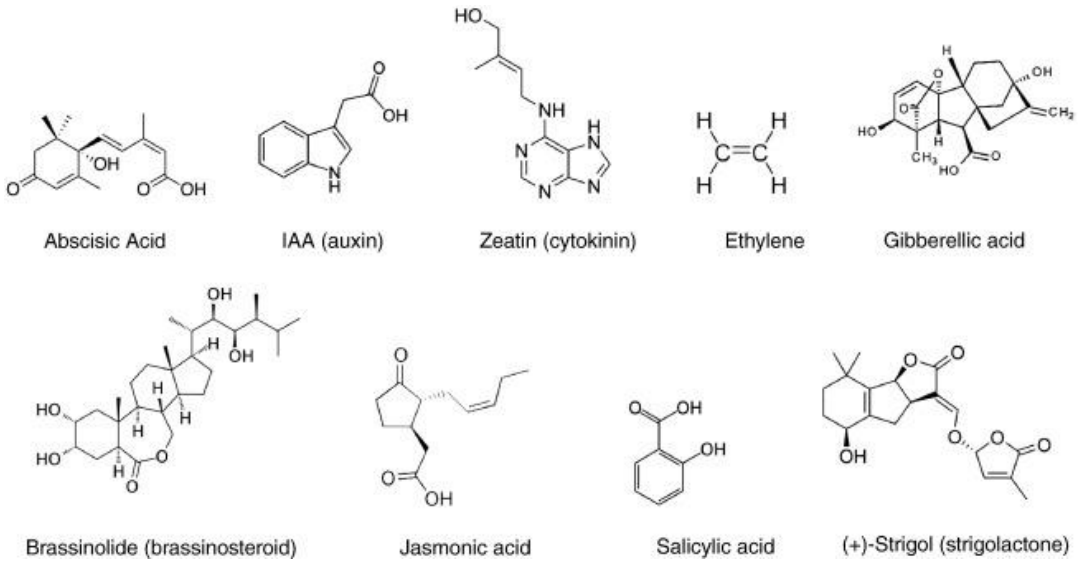


Figure 9: *major classes of phytohormones.*

In a recent study, D-ring was attached to gibberellin and kaurenoic acid as scaffold creating hybrid compounds which are as active as GR24 as seed germination stimulant of parasitic weeds. [9]

Moreover, plant hormones were used as a scaffold for drug discovery, as shown by aspirin and, more recently, by the cyclin-dependent kinase inhibitors olomoucine and roscovitine, whose structure was inspired by cytokinins, a class of N^6-substituted adenine derivatives.[10]

Synthetic and natural SLs have been extensively investigated as germination stimulants of parasitic weed seeds as well as biopesticides for crop protection, but only limited knowledge exists on their involvement in animal cell functions and their potential cross-kingdom activity, despite promising results in the realm of anticancer drug discovery.[11][12][13]

Within the possible mammalian targets of SLs, Nrf2 (nuclear factor (erythroid-derived 2)-like 2) seemed of particular relevance because of its sensitivity to nucleophilic trapping and its role in the regulation of many cytoprotective enzymes involved in the adaptive oxidative stress response.[14]

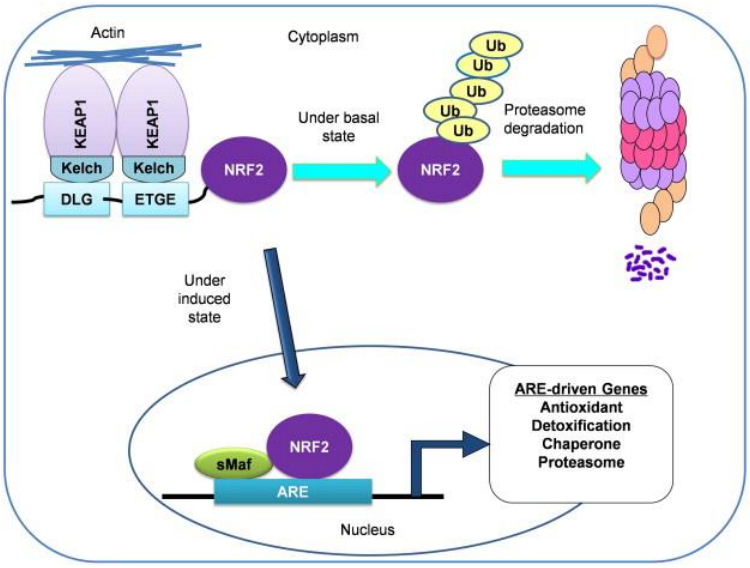
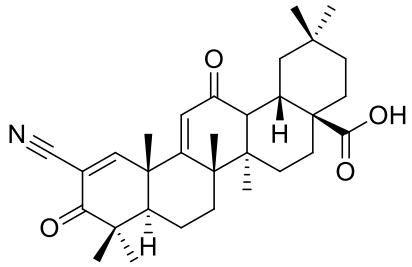


Figure 10: *Nrf2 pathway.*

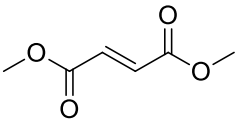
Nrf2 is a transcription factor that under normal conditions is kept in the cytoplasm and degraded through ubiquitination.

However, under oxidative stress conditions, it travels into the nucleus where it binds to DNA for the transcription of cytoprotective proteins involved in adaptive oxidative stress response.[14]

Nrf2 can be activated by electrophilic and non-electrophilic small molecules and it is the target of novel drugs exemplified by dimethyl fumarate and bardoxolone.



bardoxolone



dimethyl fumarate

Bardoxolone is an antioxidant inflammation modulator that also inhibits the activation of the proinflammatory transcription factor NF-kB.[15]

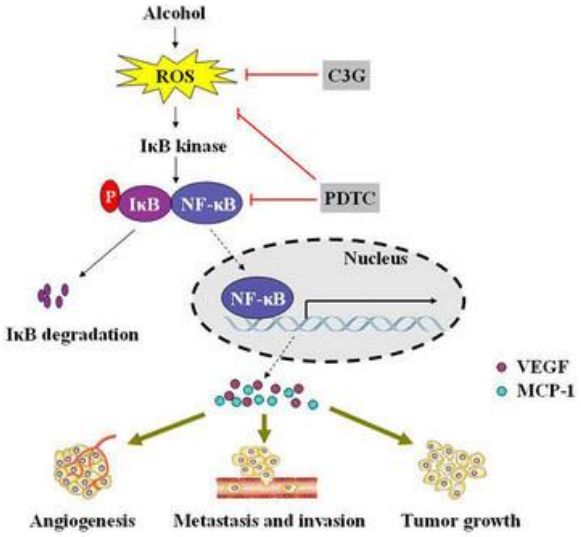


Figure 11: *NF-kB pathway.*[16]

NF-κB is another transcription factor present in the cytoplasm as a heterotrimer consisting of p50, p65 and an inhibitory subunit IκBα. NF-κB is activated by free radicals, inflammatory stimuli, cytokines, TNF-α, carcinogens, tumour promoters,

endotoxins, γ-radiation, ultraviolet light, and x-rays. On activation, the IκBα protein, an inhibitor of NF-κB, undergoes phosphorylation (by the kinase IKK), ubiquitination and degradation. p50 and p65 are then released to be translocated to the nucleus, binding to specific DNA sequences present in the promoters of various genes and initiating the transcription of more than 400 genes.[17]

In our essay, (+)-strigol (**1**) turned out to be a potent activator of Nrf2 pathway (EC_{50} = 1.2 ± 0.9 μM) providing a rationale for further studies in the area.

Strigol and SLs in general have a very limited availability, and we therefore attempted to identify a surrogate of apocarotenoid A-C ring system of the natural hormone within more easily available isoprenoids and, to this purpose, analogues where ring D is implanted in various isoprenoids scaffolds were designed (*Figure12*).

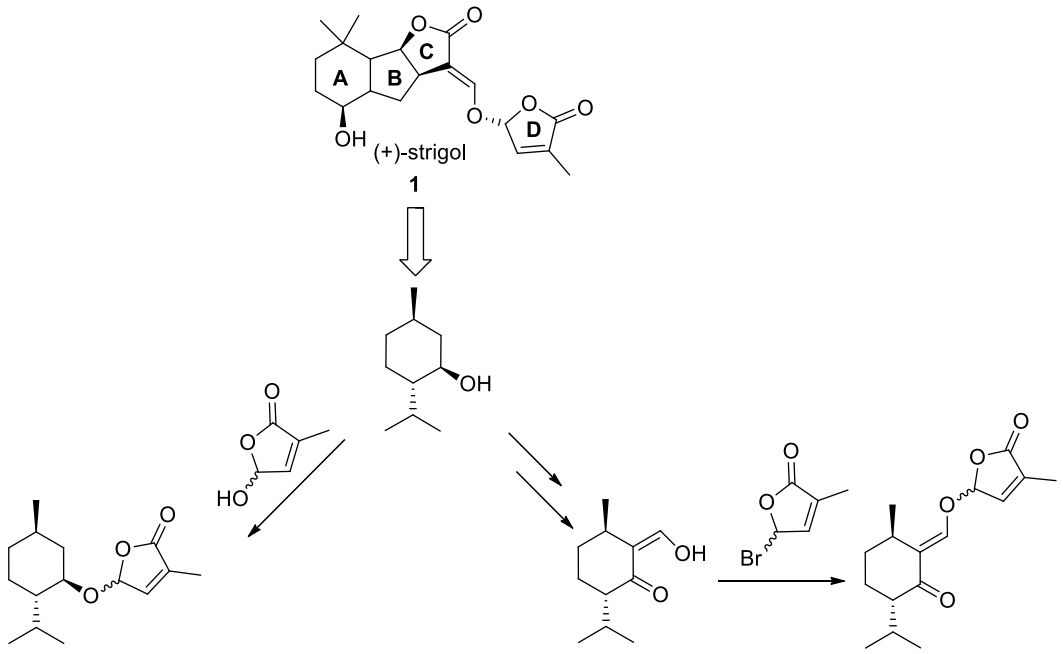


Figure 12: *general synthesis of strigoterpenes* (*synthesis of menthol derivatives as example).*

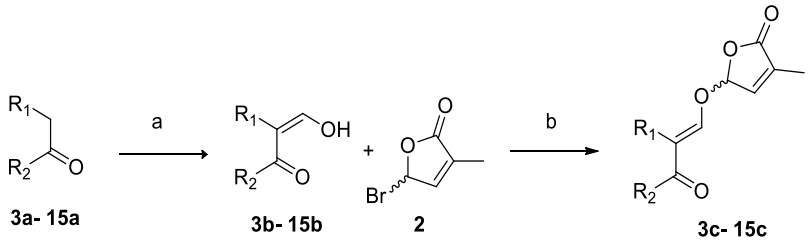
Lastly, to evaluate a possible synergistic effect, we have proceeded with the synthesis of new chimera derivatives where butenolide ring was condensed with easily available plant hormones such as indoleacetic acid and jasmonic acid.

3.3 RESULTS AND DISCUSSION

3.3.1 Chemistry

Two series of analogues were prepared, differing for the way ring D and the isoprenoid core are linked (oxymethine- or oxygen tether) (*Scheme 1* and *Scheme 2*).

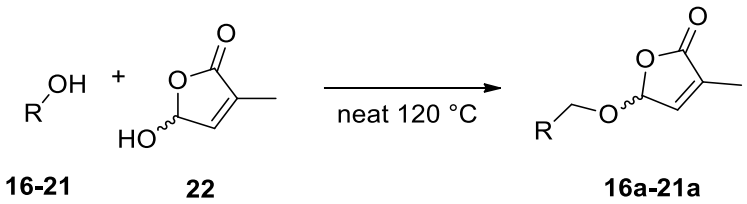
The first group of derivatives with an oxymethine tethered were built by Claisen formylation of an isoprenoid ketone and then coupling with the bromofuranone **2** obtained starting from diethyl-2-methyl malonate following a general procedure reported in literature[18] (*Scheme 1*).



Scheme 1: *synthesis of homoterpeno-strigoids.* (a) Ethyl formate, NaOEt, toluene 40°C; (b) NaH 60%, THF dry.

This synthetic approach has been applied to various isoprenoids obtaining in all cases an almost equimolecular and inseparable mixture of isomers at the furanone C-5 carbon and the diastereomeric mixture was assayed as such. The reaction was, however, stereoselective regarding the configuration of the oxymethine linker, with the predictable exclusive formation of the *E*-isomer, as evident from the downfield shift of the oxymethine (δ ca 7.40), diagnostic of a *syn*-relationship with the carbonyl. Thus, the nucleophilic displacement reaction occurred with formal inversion of configuration of the enol double bond, that was in the intramolecularly hydrogen-bonded *Z*-configuration in the starting enol (*Table 1*).

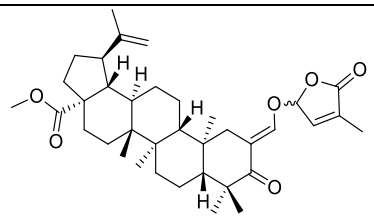
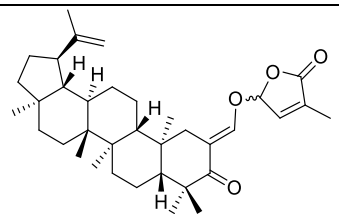
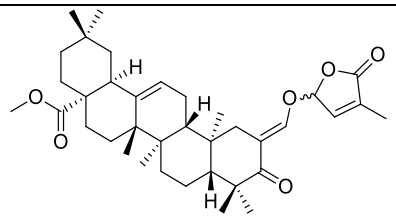
The second series of terpenostrigoids was obtained by condensing the butenolide lactol with a series of isoprenoid alcohols according to Feringa protocol[19] (heating at 120° C in the absence of solvent) (*Scheme 2*). Also, in this case, the reaction gave a mixture of diastereomeric furanones (**16a-21a**) that were assayed as such (*Table 1*).

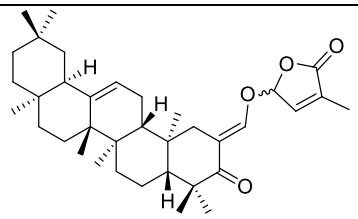
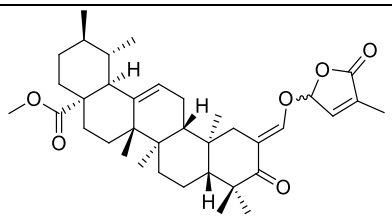
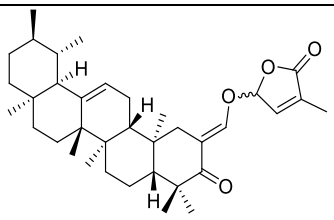
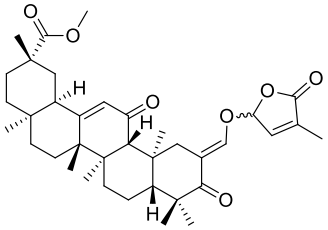
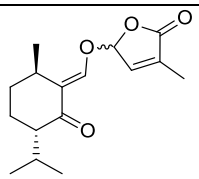
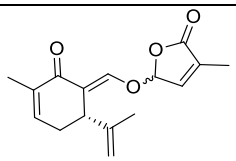


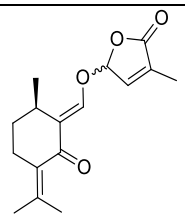
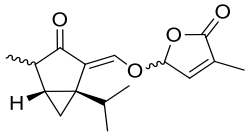
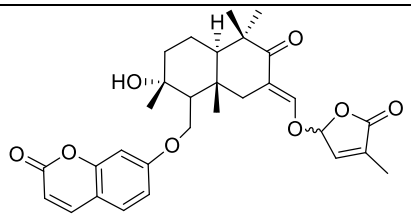
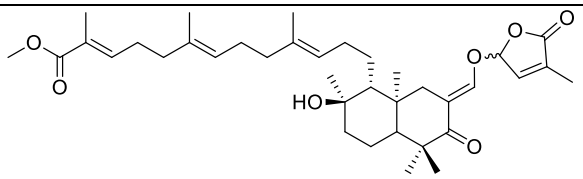
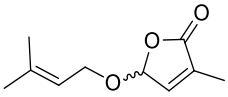
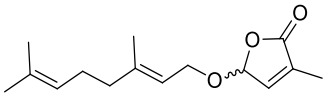
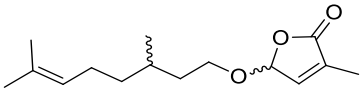
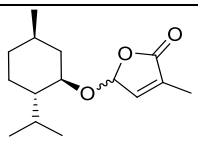
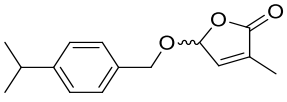
16-21 **22** **16a-21a**

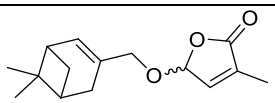
Scheme 2: *synthesis of terpenostrigoids.*

Table 1: *activity of homoterpeno-strigoids and terpenostrigoids on Nrf2 and TNFα-induced NF-κB activation. Strigol (1) EC_{50}: [a]NRF2 (EC_{50}) 1.2 ±0.9; [b] NF-κB (IC_{50}) >50*

	Compound	*Yield (%)*	*NRF2 (EC_{50})[a]*	*NF-κB (IC_{50})[b]*
3c		*70*	*>50*	*>50*
4c		*51*	*>50*	*>50*
5c		*65*	*>50*	*>50*

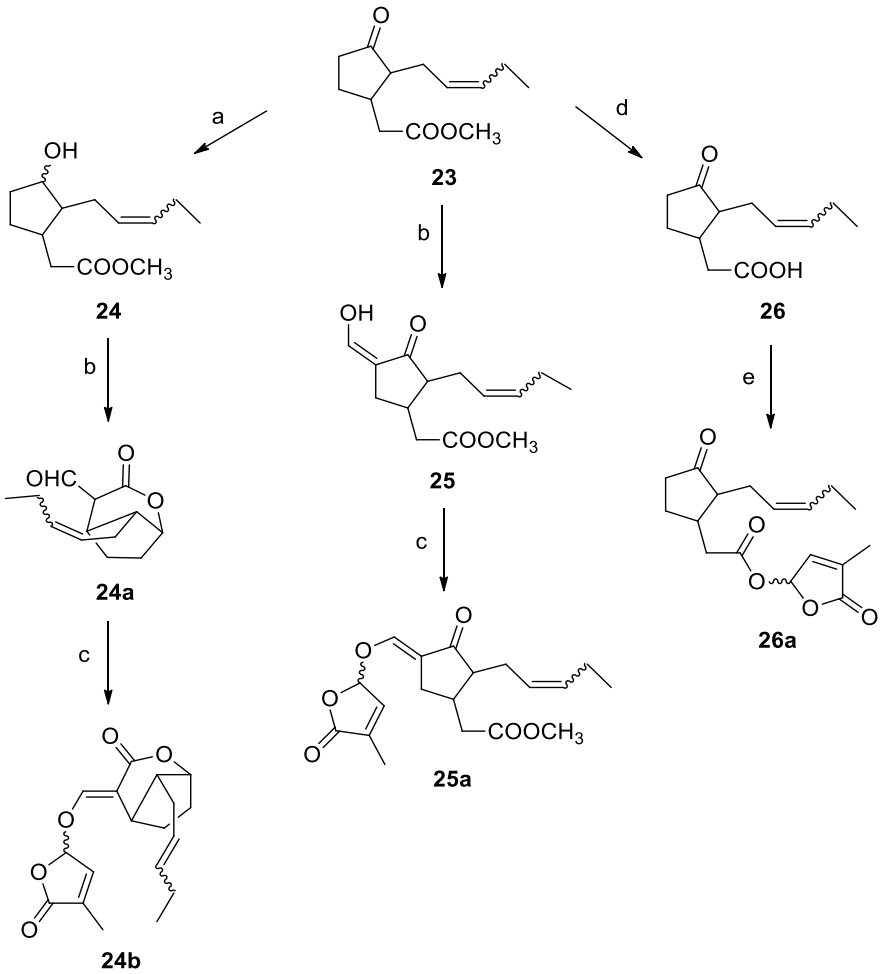
6c		*73*	*>50*	*>50*
7c		*44*	*>50*	*>50*
8c		*28*	*>50*	*>50*
9c		*56*	*>50*	*>50*
10c		*68*	*17.8 ± 1.7*	*>50*
11c		*64*	*1.6 ± 0.3*	*>50*

12c		*66*	*5 ± 0.2*	*>50*
13c		*37*	*1.9 ± 0.6*	*>50*
14c		*48*	*1.2 ± 0.03*	*7.9 ± 1.1*
15c		*26*	*2.5 ± 0.2*	*N.D.*
16a		*20*	*29.5 ± 6.4*	*>50*
17a		*31*	*16.2 ± 2.3*	*>50*
18a		*24*	*22.8 ± 3.4*	*>50*
19a		*28*	*26.3 ± 4*	*>50*
20a		*44*	*16.3 ± 2.7*	*>50*

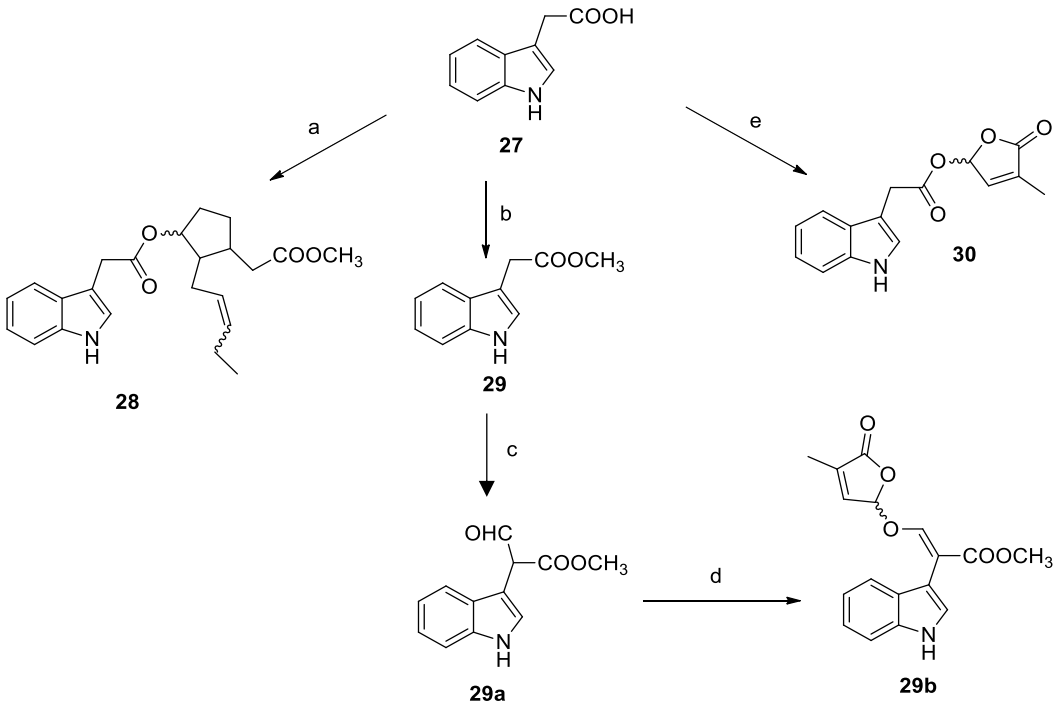
21a		*14*	*15.5 ± 1.9*	*>50*

To conclude our proof of principle library we synthesized six chimera compounds deriving from the condensation of known bioactive phytohormones such as indoleacetic acid and jasmonic acid with bromofuranone **2** (*Scheme 3* and *Scheme 4*). Compounds **23** and **27** were firstly transformed into corresponding α-hydroxymethylene intermediates **24a, 25** and **29a,** that were finally coupled with **2** to give the desired **24b, 25a** and **29b**, while in parallel, **23** and **27** were directly esterified with **22** under modified Yamaguchi conditions yielding compounds **26a** and **30**.

By using the same reaction conditions, we finally synthesized compound **28**, an hybrid between the two phytohormones.



Scheme 3: *synthesis of chimeric compounds between methyl jasmonate (**23**) and D ring.* (a) $NaBH_4$, MeOH; (b) NaH 60%, methyl formate, THF dry; (c) **2**, NaH 60%, THF dry 0°C to RT; (d) LiOH, THF/H2O; (e) **22**, Benzoyl chloride, TEA, DMAP, THF dry



Scheme 4: *synthesis of chimeric compounds between indolacetic acid (**27**) and D ring.* (a) **24**, Benzoyl chloride, TEA, DMAP, THF dry; (b) K_2CO_3, DMS, DMF; (c) NaH 60%, methyl formate, THF dry; (d) **2**, NaH 60%, THF dry 0°C to RT; (e) **22**, Benzoyl chloride, TEA, DMAP, THF dry.

Table 2: *activity of chimeric derivatives on Nrf2 and TNFα-induced NF-κB activation.*

Strigol (1) EC_{50}: [a]NRF2 (EC_{50}) 1.2 ±0.9; [b] NF-κB (IC_{50}) >50

Compound	*Yield (%)*	*NRF2 (EC_{50})*	*NF-κB (IC_{50})*
24b	*48*	*>50*	*>50*
25a	*51*	*1,2*	*39,81*
26a	*25*	*5,62*	*>50*
28	*53*	*>50*	*>50*
29b	*32*	*7.2 ±1.7*	*>50*
30	*31*	*7,76*	*>50*

3.3.2 Biological Evaluation

The strigoids obtained from tetracyclic triterpene ketones (**3c-9c**) were totally devoid of activity, suggesting that the isoprenoid scaffold was too large to access the site hosting the reactive thiol group of Nrf2. In accordance with this assumption, bicyclic and monocyclic isoprenoid ketones afforded active strigoids, some of which showed potency similar to strigol, with EC_{50} in the one-digit μM range.

Within *p*-menthane derivatives, the cross-conjugated dienones **11c** and **12c**, obtained from, respectively, carvone and pulegone, were significantly more potent than the enone **10c**, derived from menthone (EC_{50} = 1.6 ±0.3 μM and 5 ±0.2 μM vs 17.8 ±1,7 μM, respectively), suggesting that electronic factors are important for activity. Also, **13c** derived from α-thujone was significantly active (EC_{50} = 1.9 ± 0.6 μM) and one-digit mM activity was also retained in the bicyclic strigoids **14c** (EC_{50} =1.2 ± 0.03 μM and **15c** (EC_{50} = 2.5 ± 0.2 μM) derived, respectively, from the sesquiterpene coumarin ether samarcandone and the triterpenoid mirrhanone.

Overall, the oxygen-tethered terpenostrigoids were one order of magnitude less potent than the oxymethine-linked homoterpeno-strigoids.

Lastly, chimeric compounds were active with a similar potency of strigol, and in particular compound **25a** was able to inhibit both Nrf2 and NF-kB.

Compound **14c** was chosen as lead for further studies for its interesting dual profile of activity. We have first clarified the Nrf2 activation mechanism, that can be electrophilic (direct thiol trapping) or oxidative (oxidation of the cysteine sulfur atom) and mediated by reactive oxygen species (ROS). To this purpose, we have investigated the relationship between the induction of Nrf2 activity and the increase of cellular ROS.

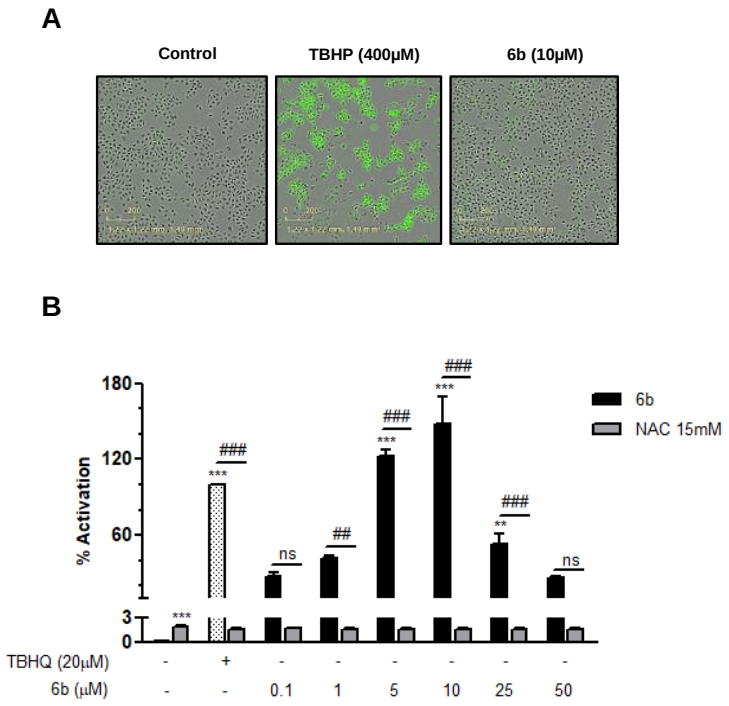


Figure 13: 14c *activates Nrf2 without inducing ROS. (A) ROS production in HaCaT cells. Images were obtained after 3 h of treatment. (B) Nrf2 transcription activity was analyzed in HaCaT-ARE-Luc. Cells were treated with* ***14c*** *in absence or presence of NAC (15mM) at the doses indicated during 6 h.*

Figure 13A shows that, in contrast to tert-butyl hydroperoxide (TBHP), **14c** was unable to affect the intracellular levels of ROS. Interestingly, pre-treatment with N-acetyl cysteine (NAC) inhibited the activity of **14c** on Nrf2 activation (*Fig. 13B*). NAC is a scavenger of oxygen free radicals and a precursor of L-cysteine. Since **14c** was not able to induce ROS, NAC might react with its coumarin moiety, that has Michael-acceptor properties, to generate an inactive adduct.[20]

Next, we have investigated the effect of **14c** on the canonical pathway of NF-κB activation by analyzing the steady-state levels of phosphorylated IκBα and p65 (a subunit of the more common form of NF-κB heterodimers). Both IκBα and p65 proteins are phosphorylated by the IκB kinase β (IKKβ), which is activated by TNFα

through the so-called canonical pathway. The drimane strigoid **14c** clearly inhibits the phosphorylation of both IκBα and p65 induced by TNFα in NIH-3T3-KBF-Luc cells. Phosphorylation of IκBα is required for its degradation and we found that **14c** could also prevent TNFα-induced IκBα degradation (*Figure 14A*).

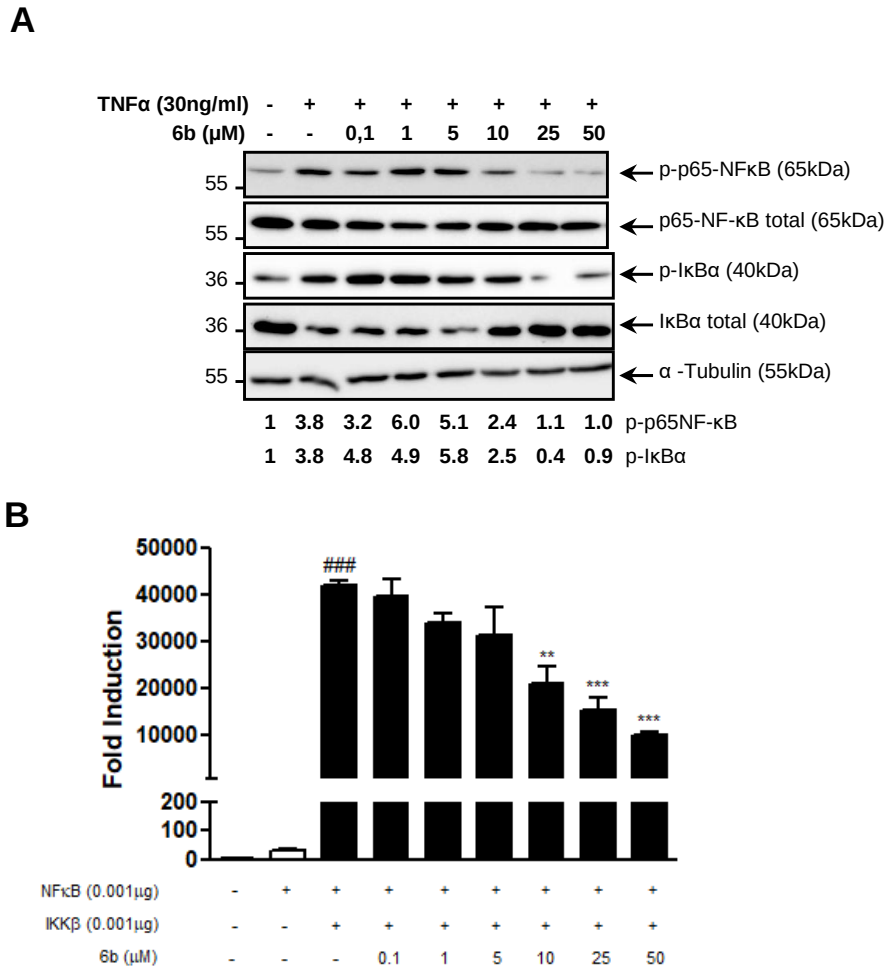


Figure 14: *effects of **13c** on NF-κB activation. (A) Levels of NF-κB proteins expression and phosphorylation by immunoblot. (B) IKKβ-induced NF-κB activation is inhibited by **14c.***

Furthermore, **14c** could also inhibit specifically the NF-kB activation induced by overexpression of IKKβ (Figure 2B). Taken together, these observations suggest that **14c** could directly interact with the Cys-179 of this kinase, inhibiting its activity.

3.3.3 Conclusions

We have identified a sesquiterpene-coumarin strigoid (**14c**) that not only replicated the activity of natural strigol (**1**) on the activation of Nrf2, but also targeted the NF-kB pro-inflammatory pathway. Within terpeno-strigoids, the activation of Nrf2 was sensitive to the size of the isoprenoid moiety, tolerating mono- and bicyclic systems but not more complex polycyclic constructs, while the inhibition of NF-kB was specific of **14c**.

The cross-talk between inflammation and the oxidative response plays an important role in cancer, and compounds capable to modulate both pathways are interesting leads to prevent and treat malignancies, qualifying **14c** for further studies.[21]

3.4 EXPERIMENTAL SECTION

General Methods and Materials.

Commercially available reagents and solvents were purchased from Aldrich or Alfa-Aesar and were used without further purification. *N,N'*-Dimethylformamide (DMF) was dried over a neutral alumina pad and stored on 4 Å activated molecular sieves. Dichloromethane was dried by distillation from P_2O_5 and stored on 4 Å activated molecular sieves. Pyridine was dried over neutral alumina pad and stored on activated 4 Å molecular sieves under nitrogen. When needed, the reactions were performed in flame- or oven-dried glassware under a positive pressure of dry nitrogen. For spectroscopic characterization, a JEOL ECP 300 MHz spectrometer was used for ^{1}H and ^{13}C spectra. Chemical shifts are reported in parts per million (ppm) using the residual solvent peak as reference ($CHCl_3$ at δ 7.27). A Thermo Finningan LCQ-deca XP-plus equipped with an ESI source and an ion trap detector was employed for mass spectrometry. Flash column chromatography was performed on silica gel (Merck Kieselgel 60, 230−400 mesh ASTM). Thin-layer chromatography (TLC) was carried out on 5 × 20 cm plates with a layer thickness of 0.25 mm (Merck silica gel 60 F254). When necessary, $KMnO_4$ was used for visualization.

Synthesis of bromobutenolide from methyl malonic acid (2): **2** was prepared according to literature[18] to give a yellow oil (58%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.20 (1 H, s, H3'), 6.83 (1 H, s, H2'), 2.01 (3 H, s, H7'); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.7, 147.4, 130.8, 74.8, 10.5.

General procedure of oxidation of terpenic alcohol with Jones reagent (3a- 15a): a solution of alcohol and acetone/EtOAc 1:1 was placed in a round-bottom flask and cooled to 0°C (ice-water bath). To the magnetically stirred solution Jones reagent

was added dropwise. Jones solution was added until an orange tint persisted in the reaction mixture. Isopropyl alcohol was then added dropwise to destroy excess of Jones reagent. The reaction mixture was washed with water, sodium bicarbonate, and brine, dried over anhydrous sodium sulfate, concentrated and the corresponding ketones were purified over silica gel.

General procedure of the synthesis of the oxymethine deritives (3b-15b): to a stirred solution of alcohol (**3a-15a**) (1 eq) in dry toluene (30 mL/g), freshly prepared NaOEt (10 eq) was added. The reaction was stirred at room temperature for 10 minutes and then ethyl formate was added (11 eq) and the reaction was warmed at 50 °C overnight. The reaction mixture was diluted with H_2SO_4 2N, extracted with EtOAc and the organic phases were dried over Na_2SO_4 and evaporated under reduced pressure. The crude was purified over silica gel.

Compounds **3b,**[22] **4b**[23]**, 5b**[24] **7b**[25]**, 9b**[26]**, 10b**[23]**, 12b**[27]**, 11b**[27]**,** have previously been reported.

Compound 6b: white solid (53%), Pe/Et_2O 99:1. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.57 (s, 1H), 5.24 (s, 1H), 2.29 (d, *J* = 14.3 Hz, 1H), 1.17 (d, *J* = 14.0 Hz, 3H), 1.15 (s, 3H), 1.12 (s, 3H), 1.02 (s, 3H), 0.93 (s, 3H), 0.87 (s, 6H), 0.84 (s, 3H) (only readily peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 190.9, 188.2, 145.2, 121.6, 105.5, 52.1, 47.4, 46.9, 45.7, 41.9, 40.2, 39.7, 39.5, 37.2, 36.3, 34.8, 33.4, 32.6, 31.9, 31.1, 28.5, 28.5, 27.0, 26.2, 25.8, 23.7, 23.6, 21.0, 19.6, 16.7, 14.7.

Compound 8b: pale pink solid (45%), Pe/Et_2O 99:1. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.57 (s, 1H), 5.18 (s, 1H), 2.30 (t, *J* = 12.8 Hz, 1H), 1.19 (s, 3H), 1.12 (s, 3H), 1.08 (s, 3H), 1.06 (s, 3H), 0.94 (s, 3H), 0.91 (s, 3H), 0.81 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 190.9, 188.2, 139.7, 135.0, 124.3, 105.9, 59.3, 52.2, 42.3, 41.6, 40.2, 39.8, 39.7, 39.6, 36.2, 33.9, 32.2, 31.3, 28.8, 28.6, 28.0, 27.0, 23.5, 23.2, 21.4, 21.0, 19.6, 17.5, 16.8, 14.9.

Compound 15b: pale yellow oil (39%), Pe/EtOAc 9:1. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.60 (s, 1H), 6.71 (t, *J* = 8.1 Hz, 1H), 5.22 – 5.04 (m, 2H), 3.69 (s, 3H), 1.79 (s, 3H), 1.60 (s, 3H), 1.58 (s, 3H), 1.17 (s, 6H), 1.06 (s, 3H), 0.76 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 190.5, 188.4, 142.4, 142.0, 135.2, 133.8, 124.9, 125.0, 105.7, 73.6, 60.4, 59.1, 51.9, 51.7, 43.4, 40.6, 40.3, 38.6, 38.3, 38.2, 31.1, 28.4, 27.3, 26.6, 25.6, 23.9, 23.3, 21.4, 20.6, 16.2, 16.0, 14.1, 12.4.

Compound 13b (mixture of stereoisomers)**:** brown oil (60%), Pe/EtOAc 95:5. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.23 (s, 1H), 7.16 (s, 1H), 2.79 (dt, *J* = 13.9, 7.0 Hz, 1H), 2.47 – 2.28 (m, 2H), 1.82 – 1.60 (m, 3H), 0.31 (t, *J* = 4.0 Hz, 1H), 0.22 (t, *J* = 6.0 Hz, 1H); ^{1}C NMR (75 MHz, $CDCl_3$) δ 213.0, 212.8, 159.4, 158.0, 118.5, 117.9, 45.3, 44.0, 41.6, 32.5, 30.9, 30.5, 24.9, 24.0, 23.6, 23.3, 22.8, 21.6, 21.0, 20.8, 20.6, 19.3, 19.2, 18.1, 17.1, 14.6, 13.3.

Compound 14b: yellow solid (81%), Pe/EtOAc 6:4. ^{1}H NMR (300 MHz, $(CD_3)_2CO$) δ 9.30 (s, 1H), 8.60 (s, 1H), 7.93 (d, *J* = 16.0 Hz, 1H), 7.52 (d, *J* = 9.2 Hz, 1H), 6.70- 6.4 (m, 3H), 4.76 – 4.05 (m, 2H), 3.70 (s, 2H), 3.16 (brd s, 1H), 2.57 (d, *J* = 14.9 Hz, 1H), 2.19 (d, *J* = 15.0 Hz, 1H), 1.21 (s, 3H), 1.20 (s, 3H), 1.10 (s, 3H), 0.98 (s, 3H); ^{1}C NMR (75 MHz, $(CD_3)_2CO$) δ 190.7, 188.1, 167.6, 162.0, 158.1, 140.1, 130.2, 115.1, 114.6, 107.3, 102.3, 71.0, 65.5, 59.5, 57.8, 51.8, 50.6, 43.4, 38.8, 37.3, 29.3, 23.5, 20.1, 20.0, 14.2.

General procedure of the synthesis of strigolactone derivatives (3c-15c):

A stirred solution of formylated compound (**3b-14b**) (1 eq) in dry THF was cooled at 0 ° C with an ice bath and NaH (60% in mineral oil) (1,5 eq) was added. After 10 minutes a solution of bromobutenolide **2** (1,5 eq) in dry THF was added dropwise and the reaction was warmed to room temperature and stirred overnight. The reaction mixture was diluted with H_2SO_4 2N, extracted with EtOAc, dried over

Na_2SO_4 and evaporated under reduced pressure. The crude was purified over silica gel furnishing a mixture of two non-separable diastereoisomers.

Compound 3c (mixture of stereoisomers): off-white solid (77%), Pe/EtOAc 8:2. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35 (brd, 1H), 6.88 (brd, 1H), 6.08 (d, *J* = 4.6 Hz, 1H), 4.71 (s, 1H), 4.56 (s, 1H), 3.65 (s, 3H), 3.00 (m, 1H), 2.67 (t, *J* = 16.5 Hz, 1H), 1.98 (s, 3H), 1.60 (s, 3H), 1.30 (s, 3H), 1.05 (s, 9H), 0.93 (s, 1H), 0.92 (s, 3H) (only readable peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 207.0, 206.7, 176.6, 170.7, 170.6, 151.8, 151.5, 150.5, 141.6, 135.3, 135.1, 116.9, 116 .8, 109.6, 100.8, 100.6, 56.5, 52.8, 52.6, 51.3, 49.3, 49.2, 48.5, 48.4, 46.9, 45.0, 44.9, 42 .4, 40.48, 40.44, 39.5, 39.3, 38.4, 36.9, 35.6, 35.5, 33.2, 33.1, 32.0, 30.5, 29.6, 29.2, 29.0,25.6, 22.2, 22.1, 20.32, 20.30, 19.39, 19.35, 15.8, 15.7, 15.5, 15.4, 14.6, 10.6; MS (ESI) *m/z* 615 $(M + Na)^+$. Anal. Calcd for $C_{37}H_{52}O_6$: C, 74.97; H, 8.84. Found: C, 75.08; H, 8.92.

Compound 4c (mixture of stereoisomers): off-white solid (51%), Pe/EtOAc 8:2. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36 (brd, 1H), 6.88 (brd, 1H), 6.09 (d, *J* = 4.4 Hz, 1H), 4.68 (s,1H), 4.54 (s, 1H), 2.68 (t, *J* = 16.2 Hz, 1H), 2.39 (m, 1H), 1.99 (s, 3H), 1.67 (s, 3H), 1.43 (s, 3H), 1.06 (s, 9H), 0.95 (s, 1H), 0.79 (s, 6H) (only readable peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$)δ 208.0, 151.6, 151.4, 151.0, 141.5, 135.4, 135.3, 117.1, 117.0, 109.4, 100.8, 10 0.6, 52.9, 52.6, 48.7, 48.3, 48.0, 45.1, 45.0, 43.0, 42.9, 40.69, 40.67, 40.0, 39.5, 39.3, 3 8.2, 35.7, 45.5, 33.2, 33.1, 29.8, 29.2, 29.1, 27.4, 25.3, 22.3, 22.1, 21.7, 20.3, 19.3, 18.1, 15.9, 15.8, 15.5,8, 15.56, 14.5, 10.7; MS (ESI) *m/z* 571 $(M + Na)^+$. Anal. Calcd for $C_{36}H_{52}O_4$: C, 78.79; H, 9.55. Found: C, 78.83; H, 9.59.

Compound 5c (mixture of stereoisomers): white solid (65%), Pe/EtOAc 8:2. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36 (s, 1H), 6.89 (d, *J* = 7.9 Hz, 1H), 6.10 (brd, 1H), 5.29 (brt, 1H), 3.59 (s, 3H), 2.85 (d, *J* = 13.1 Hz, 1H), 2.62 (t, *J* = 12.8 Hz, 1H), 1.97 (s, 3H), 1.12 (s, 3H), 1.06 (s, 6H), 0.90 (s, 1H), 0.87 (s, 3H), 0.84 (s, 3H), 0.83 (s, 3H), 0.75 (s, 3H)

(only readily peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 206.8, 206.5, 178.2, 170.6, 151.6, 151.4, 143.7, 141.6, 135.4, 135.2, 122.2, 116.7, 116.6, 100.7, 100.6, 53.0, 52.8, 51.6, 46.8, 45.9, 45.4, 45.3, 45.0, 44.9, 41.9, 41.5, 39.1, 38.9, 35.5, 35.4, 33.9, 33.1, 32.3, 31.9, 30.7, 29.4, 29.2, 27.6, 25.7, 23.6, 23.5, 23.1, 22.5, 22.4, 20.9, 16.5, 15.3, 15.2, 10.7; MS (ESI) *m/z* 615 $(M + Na)^+$. Anal. Calcd for $C_{37}H_{52}O_6$: C, 74.97; H, 8.84. Found: C, 74.92; H, 8.79.

Compound 6c (mixture of stereoisomers): white solid (73%), Pe/EtOAc 8:2. ^1H NMR (300 MHz, $CDCl_3$) δ 7.37 (s, 1H), 6.89 (dd, J_1 = 7.0 Hz J_2 = 1.5 Hz, 1H), 6.10 (brd, 1H), 5.20 (brt, 1H), 2.65 (t, *J* = 12.5 Hz, 1H), 1.98 (s, 3H), 1.09 (s, 3H), 1.08 (s, 6H), 1.00 (s, 3H), 0.82 (s, 6H), 0.79 (s, 3H) (only readable peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 206.7, 206.5, 170.6, 170.5, 151.5, 151.4, 145.1, 141.5, 135.4, 135.2, 121.7, 121.6, 116.8, 116.7, 100.7, 100.6, 53.0, 52.8, 47.4, 46.8, 45.3, 45.2, 45.0, 44.9, 42.0, 41.9, 39.67, 39.63, 39.3, 39.2, 37.1, 35.4, 35.3, 34.7, 33.4, 32.6, 31.8, 31.1, 31.0, 29.4, 29.3, 28.5, 27.0, 26.0, 25.8, 25.7, 23.78, 23.73, 23.66, 22.5, 22.4, 20.3, 19.7, 16.5, 15.5, 15.4, 14.2, 10.7; MS (ESI) *m/z* 571 $(M + Na)^+$. Anal. Calcd for $C_{36}H_{52}O_4$: C, 78.79; H, 9.55. Found: C, 78.84; H, 9.61.

Compound 7c (mixture of stereoisomers): off-white solid (44%), Pe/EtOAc 9:1. ^1H NMR (300 MHz, $CDCl_3$) δ 7.38 (brs, 1H), 6.90 (d, *J* = 6.7 Hz, 1H), 6.10 (d, *J* = 4.8 Hz, 1H), 5.29 (brt, 1H), 3.59 (s, 3H), 2.66 (t, *J* = 14.4 Hz, 1H), 2.23 (d, *J* = 11.3 Hz, 1H), 1.99 (s, 3H), 1.06 (s, 12H), 0.93 (s, 3H), 0.84 (s, 3H), 0.76 (s, 3H) (only readable peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 206.8, 206.5, 178.09, 178.06, 170.3, 151.8, 151.6, 141.5, 138.27, 138.20, 135.4, 135.3, 125.5, 122.2, 116.68, 116.64, 100.8, 100.7, 53.1, 53.0, 52.8, 51.5, 48.2, 45.2, 45.1, 45.0, 44.9, 42.2, 39.3, 39.1, 38.9, 36.6, 35.4, 35.3, 32.28, 32.22, 30.7, 29.4, 29.3, 28.0, 24.2, 23.5, 23.4, 22.5, 22.4, 21.2, 20.2, 17.0, 16.7, 15.5, 15.4, 10.7; MS (ESI) *m/z* 615 $(M + Na)^+$. Anal. Calcd for $C_{37}H_{52}O_6$: C, 74.97; H, 8.84. Found: C, 74.89; H, 8.80.

Compound 8c (mixture of stereoisomers): off-white solid (28%), Pe/EtOAc 8:2. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.38 (brs, 1H), 6.90 (d, *J* = 6.7 Hz, 1H), 6.10 (d, *J* = 4.8 Hz, 1H), 5.29 (brt, 1H), 3.59 (s, 3H), 2.66 (t, *J* = 14.4 Hz, 1H), 2.23 (d, *J* = 11.3 Hz, 1H), 1.99 (s, 3H), 1.06 (s, 12H), 0.93 (s, 3H), 0.84 (s, 3H), 0.76 (s, 3H) (only readable peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 206.8, 206.5, 178.09, 178.06, 170.3, 151.8, 151.6, 141.5, 138.27, 138.20, 135.4, 135.3, 125.5, 122.2, 116.68, 116.64, 100.8, 100.7, 53.1, 53.0, 52.8, 51.5, 48.2, 45.2, 45.1, 45.0, 44.9, 42.2, 39.3, 39.1, 38.9, 36.6, 35.4, 35.3, 32.28, 32.22, 30.7, 29.4, 29.3, 28.0, 24.2, 23.5, 23.4, 22.5, 22.4, 21.2, 20.2, 17.0, 16.7, 15.5, 15.4, 10.7; MS (ESI) *m/z* 615 $(M + Na)^+$. Anal. Calcd for $C_{37}H_{52}O_6$: C, 74.97; H, 8.84. Found: C, 74.89; H, 8.80.

Compound 9c (mixture of stereoisomers): white solid (56%), Pe/EtOAc 8:2. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.38 (d, *J* =11.9 Hz, 1H), 6.90 (d, *J* = 20.7 Hz, 1H), 6.10 (d, *J* = 9.4 Hz, 1H), 5.69 (brt, 1H), 3.67 (s, 3H), 2.46 (s, 1H), 1.97 (s, 3H), 1.40 (s, 3H), 1.13 (s, 6H), 1.11 (s, 9H), 0.76 (s, 3H) (only readable peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 206.3, 206.1, 199.5, 199.4, 176.9, 176.4, 170.9, 170.7, 170.3, 170.2, 170.0, 152.0, 151.0, 141.9, 141.8, 135.4, 134.8, 128.4, 128.2, 116.6, 116.5, 100.9, 100.3, 60.3, 59.3, 59.2, 53.1, 53.0, 51.8, 48.4, 45.1, 45.05, 45.00, 44.0, 43.8, 43.4, 41.2, 39.6, 39.4, 37.7, 35.5, 35.3, 31.8, 31.6, 31.5, 31.1, 29.5, 29.3, 28.6, 28.3, 26.5, 26.4, 23.2, 23.1, 22.4, 22.3, 19.5, 18.07, 18.01, 15.4, 14.3, 10.7, 10.6; MS (ESI) *m/z* 629 $(M + Na)^+$. Anal. Calcd for $C_{37}H_{50}O_7$: C, 73.24; H, 8.31. Found: C, 73.19; H, 8.27.

Compound 10c (mixture of stereoisomers): pale-yellow oil (69%), Pe/EtOAc 95:5. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.24 (d, *J* = 5.9 Hz, 1H), 6.89 (s, 1H), 6.09 (brs, 1H), 3.03-2.85 (m, 1H), 2.47 (m, 1H), 1.96 (s, 3H), 1.72-1.66 (m, 3H), 1.00 (d, *J* = 7.5 Hz, 3H), 0.89 (d, *J* = 7.3 Hz, 3H), 0.77 (d, *J* = 6.9 Hz, 3H) (only readable peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 201.9, 170.7, 150.1, 149.9, 141.8, 135.1, 135.0, 125.5, 125.1, 125.0, 100.7, 54.5, 54.4, 54.3, 29.1, 28.9, 27.9, 27.8, 27.0, 26.8, 20.8, 20.7,

20.6, 20.2, 19.6, 18.3, 17.7, 17.6, 17.5, 10.6; MS (ESI) *m/z* 301 $(M + Na)^+$. Anal. Calcd for $C_{16}H_{22}O_4$: C, 69.04; H, 7.97. Found: C, 69.09; H, 8.05.

Compound 11c (mixture of stereoisomers): brown oil (64%), Pe/EtOAc 95:5. ^1H NMR (300 MHz, $CDCl_3$) δ 7.45 (s, 1H, first stereoisomer), 7.43 (s, 1H, second stereoisomer), 6.89 (s, 1H), 6.59 (brs, 1H), 6.13 (s, 1H), 4.71 (s, 1H), 4.58 (s, 1H), 3.69 (brd, 1H), 2.48 (m, 2H), 1.97 (s, 3H), 1.77 (s, 3H), 1.67 (s, 3H, first stereoisomer), 1.65 (s, 3H, second stereoisomer); ^{13}C NMR (75 MHz, $CDCl_3$) δ 187.8, 187.7, 170.7, 170.6, 149.9, 149.3, 145.3, 145.0, 142.2, 142.1, 141.9, 136.0, 135.9, 135.1, 134.9, 119.9, 111.7, 111.6, 100.9, 100.7, 60.3, 39.3, 39.2, 28.3, 21.4, 16.0, 10.6; MS (ESI) *m/z* 297 $(M + Na)^+$. Anal. Calcd for $C_{16}H_{18}O_4$: C, 70.06; H, 6.61. Found: C, 70.10; H, 6.71.

Compound 12c (mixture of stereoisomers): off-white solid (66%), Pe/EtOAc 8:2. ^1H NMR (300 MHz, $CDCl_3$) δ 7.28 (d, J = 4.3 Hz, 1H), 6.90 (s, 1H), 6.10 (brd, 1H), 2.95 (m, 1H), 2.52 (m, 1H), 2.45 (m, 1H), 2.10 (s, 3H), 1.96 (s, 3H), 1.79 (s, 3H), 1.74 (m, 1H), 1.56 (m, 1H), 1.03 (dd, J_1 = 7.0 Hz J_2 = 2.5 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 191.4, 170.8, 149.6, 149.5, 147.1, 141.9, 135.0, 130.2, 130.1, 126.4, 100.7, 28.6, 28.3, 27.9, 27.7, 24.5, 24.4, 23.8, 23.5, 23.4, 18.6, 18.4, 10.6; MS (ESI) *m/z* 299 $(M + Na)^+$. Anal. Calcd for $C_{16}H_{20}O_4$: C, 69.55; H, 7.30. Found: C, 69.48; H, 7.27.

Compound 13c (mixture of stereoisomers): brown oil (37%), Pe/EtOAc 9:1. ^1H NMR (300 MHz, $CDCl_3$) δ 7.28 (brs, 1H), 6.92 (brs, 1H), 6.09 (brs, 1H), 2.30 (m, 2H), 1.97 (s, 3H), 1.92 (m, 1H), (only readily peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 211.0, 209.0, 170.7, 148.3, 148.0, 147.8, 147.4, 142.2, 141.6, 141.5, 135.4, 135.2, 123.8, 123.6, 122.8, 122.7, 101.1, 100.8, 46.0, 44.8, 34.0, 32.7, 27.0, 26.9, 22.2, 22.1, 21.7, 21.5, 18.0, 17.9, 16.5, 16.4, 13.6, 13.5, 10.7; MS (ESI) *m/z* 299 $(M + Na)^+$. Anal. Calcd for $C_{16}H_{20}O_4$: C, 69.55; H, 7.30. Found: C, 69.57; H, 7.35.

Compound 14c (mixture of stereoisomers): amorphous solid (48%), Pe/EtOAc 6:4. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.81 (d, *J* = 16.2 Hz, 1H), 7.44 (m, 2H), 7.05 (s, 1H), 6.93 (m, 2H), 6.69 (m, 1H), 6.32 (m, 2H), 6.14 (s, 1H), 4.46 (brt, 1H), 4.22 (brt, 1H), 3.76 (s, 2H), 2.87 (d, *J* = 16.2 Hz, 1H), 2.01 (s, 3H), 1.30 (s, 6H), 1.19 (s, 3H), 0.87 (s, 3H) (only readable peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 205.7, 205.5, 198.2, 171.1, 170.6, 167.8, 167.4, 161.2, 161.1, 156.2, 156.1, 152.0, 151 .8, 151.4, 146.1, 145.9, 142.2, 141.7, 141.6, 138.7, 138.4, 135.6, 134.8, 134,3, 129.7, 129.6, 129.4, 117.9, 117.8, 117.5, 116.8, 115.7, 110.8, 110.4, 110.2, 109.4, 103.0, 100.6, 100.5, 98.2, 98.6, 72.3, 66.5, 66.3, 65.5, 60.4, 57.0, 56.6, 56.5, 55.0, 52.7, 52.6, 51.7, 45. 5, 45.2, 45.1, 42.9, 39.8, 39.7, 38.7, 38.6, 38.5, 29.7, 29.5, 29.4, 24.1, 23.9, 22.5, 22.4, 22.0, 15.5, 15.5, 14.4, 10.7; MS (ESI) *m/z* 545 $(M + Na)^+$. Anal. Calcd for $C_{30}H_{34}O_8$: C, 68.95; H, 6.56. Found: C, 69.01; H, 6.60.

Compound 15c (mixture of stereoisomers): yellow oil (26%), Pe/EtOAc 8:2. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.41 (s, 1H), 6.70 (t, *J* = 7.0 Hz, 1H), 5.13 (m, 2H), 3.69 (s, 3H), 1.79 (m, 3H), 1.59 (s, 3H), 1.57 (s, 3H), 1.16 (s, 6H), 1.06 (s, 3H), 0.76 (s, 3H) (only readable peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 206.5, 206.3, 170.7, 168.4, 152.1, 144.4, 142.5, 142.1, 141.9, 135.56, 135.50, 135.2, 134.0, 133.6, 125.0, 116.1, 100.7, 96.7, 73.7, 73.6, 60.5, 59.0, 58.9, 52.8, 52,6, 51.8, 45.2, 45.1, 43.1, 39.6, 38.2, 37.4, 31.3, 29.7, 29.4, 29.2, 27.3, 27.1, 25.7, 25.6, 23.1, 23.0, 22 .4, 22.3, 16.1, 16.0, 14.7, 14.6, 14.3, 12.4, 10.7, 10.6; MS (ESI) *m/z* 633 $(M + Na)^+$. Anal. Calcd for $C_{37}H_{54}O_7$: C, 72.75; H, 8.91. Found: C, 72.67; H, 8.87.

General procedure for the synthesis of strigolactone mimics (16a-21a): a mixture of hydroxybutenolide **22** (1.5 eq) and alcohol (**16-21**) (1 eq) were heated at 120°C for 3-7 days. The crude was directly purified over silica gel.

Compounds **19a** have previously been reported.[19]

Compound 16a (racemic): yellow oil (20%), Pe/EtOAc 95:5. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.74 (t, *J* = 1.5 Hz, 1H), 5.75 (t, *J* = 1.2 Hz, 1H), 5.26 (dt, J_1 = 7.6 J_2 =0.9 Hz, 1H), 4.18 (m, 2H), 1.83 (s, 3H), 1.68 (s, 3H), 1.61 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.0, 143.3, 139.7, 133.7, 119.1, 100.4, 66.1, 25.8, 18.0, 10.5; MS (ESI) *m/z* 205 (M + Na)$^{+}$. Anal. Calcd for $C_{10}H_{14}O_3$: C, 65.92; H, 7.74. Found: C, 65.98; H, 7.81.

Compound 17a (racemic): yellow oil (29%), Pe/Et_2O 99:1^{1}H NMR (300 MHz, $CDCl_3$) δ 6.79 (brd, 1H), 5.82 (d, *J* = 0.9 Hz, 1H), 5.26 (t, *J* = 7.3 Hz, 1H), 5.06 (brt, 1H), 4.29 (m, 2H), 2.06 (m, 4H), 1.92 (s, 3H), 1.69 (s, 3H), 1.65 (s, 3H), 1.58 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.1, 143.2, 133.9, 131.9, 123.8, 118.7, 100.2, 66.2, 39.6, 26.3, 25.7, 17.7, 16.5, 10.6; MS (ESI) *m/z* 273 (M + Na)$^{+}$. Anal. Calcd for $C_{15}H_{22}O_3$: C, 71.97; H, 8.86. Found: C, 72.01; H, 8.90.

Compound 18a (mixture of stereoisomers): colourless oil (24%), Pe/Et_2O 99:1 ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.79 (brd, 1H), 5.77 (s, 1H), 5.07 (t, *J* = 7.0 Hz, 1H), 3.88 (m, 1H), 3.67 (m, 1H), 1.95 (m, 2H), 1.93 (s, 3H), 1.69 (s, 3H), 1.58 (s, 3H), 1.35 (m, 5 H), 0.88 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.9, 143.0, 134.0, 131.3, 124.1, 101.7, 101.6, 68.7, 37.1, 36.4, 29.4, 25.7, 25.4, 19.4, 17.6, 10.6; MS (ESI) *m/z* 275 (M + Na)$^{+}$. Anal. Calcd for $C_{15}H_{24}O_3$: C, 71.39; H, 9.59. Found: C, 71.44; H, 9.63.

Compound 20a (racemic): yellow oil (44%), Pe/Et_2O 99:1. ^{1}H NMR (300 MHz, $CDCl_3$) 7.29 (d, *J* = 7.9 Hz, 2H), 7.22 (d, *J* = 7.9 Hz, 2H), 6.81 (s, 1H), 5.86 (s, 1H), 4.88 (d, *J* = 11.3 Hz, 1H), 4.67 (d, *J* = 11.0 Hz, 1H), 2.90 (m, 1H), 1.93 (s, 3H), 1.24 (d, *J* = 7.0 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.0, 149.3, 143.2, 134.0, 133.5, 128.6, 126.8, 100.3, 71.6, 33.9, 24.0, 10.6; MS (ESI) *m/z* 269 (M + Na)$^{+}$. Anal. Calcd for $C_{15}H_{18}O_3$: C, 73.15; H, 7.37. Found: C, 73.10; H, 7.40.

Compound 21a (mixture of stereoisomers): yellow oil (14%), Pe/Et_2O 99:1. ^1H NMR (300 MHz, $CDCl_3$) δ 6.79 (s, 1H), 5.81 (s, 1H), 5.60 (brd, 1H), 4.14 (m, 2H), 1.93 (s, 3H), 1.28 (s, 3H), 0.81 (s, 3H) (only readable peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.0, 143.7, 143.5, 134.1, 134.0, 122.8, 122.2, 100.5, 100.1, 72.8, 72.7, 43.6, 43.3, 40.76, 40.71, 38.1, 38.0, 31.6, 31.5, 31.43, 31.40, 26.2, 21.2, 21.0 10.7; MS (ESI) *m/z* 271 $(M + Na)^+$. Anal. Calcd for $C_{15}H_{20}O_3$: C, 72.55; H, 8.12. Found: C, 72.60; H, 8.16.

Synthesis of compound 24: methyl jasmonate (1 eq.) was dissolved in EtOH/DCM 10:1 and $NaBH_4$ (1 eq.) was added. The reaction mixture was stirred for 2 h, quenched with H_2SO_4 2N and extracted with EtOAc. The organic phase was dried over Na_2SO4, evaporated under reduced pressure and the crude product was used without further purification for the next reaction. Our data are in agreement with those reported in literature. [28]

Synthesis of compound 26: To a solution of methyl jasmonate (1 eq) in THF/H_2O, LiOH*H_2O was added (2 eq). The reaction mixture was stirred at room temperature for 1 h, quenched with H_2SO_4 2N and extracted with EtOAc. The organic phase was dried over Na_2SO_4 and evaporated under reduced pressure, and the crude product was used without further purification for the next reaction. Our data are in agreement with those reported in literature.[29]

General procedure of the synthesis of chimeric esters 26a, 28, 30 (modified protocol of Yamaguchi esterification): to a stirred solution of acid (**26** and **27**) (1,5 eq) in dry THF (20 mL/gr), benzoyl chloride (1,5 eq), triethylamine (2 eq) and DMAP (cat) were sequentially added. The reaction mixture was stirred for 10 minutes at room temperature and the alcohol (**22** and **24**) (1 eq) was finally added. After 24 h the reaction was quenched with H_2SO_4 2N and extracted twice with EtOAc. The

combined organic phases were washed with $NaHCO_3$ sat. sol. (X2), dried over Na_2SO_4 and evaporated under reduced pressure. The crude was purified over silica gel.

Compound 28: brown oil (53%), Pe/EtOAc 8:2. ^{1}H NMR δ 8.39 (br s, NH), 7.62 (d, *J* = 7.8 Hz, 1H), 7.34 – 7.01 (m, 4H), 5.46 – 5.20 (m, 2H), 4.93 – 4.85 (m, 1H), 3.76 (s, 2H), 3.74 (s, 2H), 3.69 (S, 3H), 3.66 (s, 3H), 2.52 (ddd, *J* = 20.0, 14.5, 8.0 Hz, 1H), 2.23 – 1.85 (m, H), 1.74 – 1.62 (m, H), 1.42 – 1.24 (m, 2H), 0.96 – 0.87 (m, H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.7, 172.0, 136.2, 133.6, 132.8, 127.3, 126.9, 125.8, 123.3, 122.0, 119.5, 118.9, 111.4, 108.2, 80.9, 77.8, 51.6, 50.8, 49.7, 39.8, 39.8, 39.3, 39.1, 31.8, 31.7, 31.2, 3.1, 30.1, 29.6, 29.3, 24.4, 20.6, 20.6, 14.3, 14.3.

Compound 26a (mixture of stereoisomers): yellow oil (25%), Pe/EtOAc 8:2. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.87 (m, 2H), 5.40 (m, 1H), 5.21 (m, 1H), 2.76 (m, 1H), 2.29 (m, 4H), 1.95 (s, 3H), 0.90 (t, *J* = 7.3 Hz, 3H) (only readily peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 218.4, 171.0, 170.4, 170.3, 141.9, 134.6, 134.5, 134.3, 134.2, 124.9, 124.8, 92.4, 92.3, 53.8, 38.7, 38.6, 37.8, 37.7, 37.6, 27.15, 27.11, 25.5, 20.6, 14.1, 10.6; MS (ESI) *m/z* 329 $(M + Na)^+$.

Compound 30 (racemic): brown oil (31%), Pe/EtOAc 8:2. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.35 (brs, 1H), 7.56 (d, *J* = 7.5 Hz, 1H), 7.30 (d, *J* = 7.8 Hz, 1H), 7.17 (m, 2H), 7.06 (brs, 1H), 6.81 (dd, J_1= 13.2 Hz J_2= 1.2 Hz, 2H), 3.83 (s, 2H), 1.92 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.5, 170.5, 142.4, 136.2, 134.3, 127.0, 123.7, 122.3, 119.8, 118.6, 111.6, 106.7, 92.8, 31.0, 10.6; MS (ESI) *m/z* 294 $(M + Na)^+$.

General procedure for the synthesis α-hydroxymethylene intermediates (24a, 25, 29a): to a cooled (0°C) and stirred solution of **24, 23, 29** (1 eq) in dry THF (10 mL/gr) NaH (60% in mineral oil) (3 eq) was added. After 10 minutes methyl formate (5 eq)

was added and the reaction was stirred at room temperature overnight. The mixture was quenched with H_2SO_4 2N, extracted with EtOAc, and the organic phase was dried over Na_2SO_4 and evaporated under reduced pressure. The crude was purified over silica gel.

Compounds **25**[30] and **29a**[31] have previously been reported and our data are in agreement with those reported in literature.

Compound 24a: colourless oil (43%), Pe/EtOAc 95:5. ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.62 (d, *J* = 12.5 Hz, 1H), 6.98 (d, *J* = 12.6 Hz, 1H), 5.57 – 5.16 (m, 2H), 4.57 (br s, 1H), 2.56 (br s, 1H), 2.29 – 1.52 (m, 9H), 0.94 (t, *J* = 7.6 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.8, 159.2, 134.1, 134.0, 125.4, 125.0, 108.5, 83.9, 47.7, 38.9, 30.7, 30.6, 26.5, 20.7, 14.3.

General procedure of the synthesis lactone derivatives (24b, 25a, 29b): to a cooled (0°C) solution of formylated compound (**24a, 25, 29a**) (1 eq) in dry THF, NaH (60% in mineral oil) (4 eq) was added. The reaction was allowed to react for 5 minutes and then bromobutenolide **2** (1,5 eq) was added and the reaction was stirred at room temperature overnight. The mixture was quenched with H_2SO_4 2N, extracted with EtOAc, and the organic phase was dried over Na_2SO_4 and evaporated under reduced pressure. The crude was purified over silica gel.

Compound 24b (mixture of stereoisomers): yellow amorphous solid (48%), Pe/EtOAc 8:2. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40 (d, *J* = 4.6 Hz, 1H), 6.93 (s, 1H), 6.14 (s, 1H), 5.53 – 5.20 (m, 2H), 4.56 (s, 1H), 3.06 (s, 1H), 1.99 (s, 3H), 1.72 – 1.57 (m, 1H), 0.93 (t, *J* = 7.5 Hz, 3H) (only readily peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.5, 166.3, 150.2, 141.2, 135.7, 134.0, 125.0, 117.8, 100.6, 47.3, 36.3, 36.2, 30.0, 29.9, 28.2, 28.1, 26.4, 20.7, 14.3, 10.7.

Compound 25a (mixture of stereoisomers): yellow oil (50%), Pe/EtOAc 8:2. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35 (s, 1H), 6.89 (brs, 1H), 6.11 (brs, 1H), 5.42 (m, 1H), 5.25 (m, 1H), 3.64 (s, 3H), 2.33 (m, 4H), 0.91 (t, *J* = 7.7 Hz, 3H) (only readily peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 206.7, 172.5, 172.4, 170.5, 147.9, 147.5, 14.5, 141.4, 135.4, 134.9, 134.5, 134.2, 140.0, 125.1, 124.9, 118.4, 100.7, 54.5, 53.9, 51.6, 39.0, 35.5, 29.7, 25.5, 20.6, 14.1, 10.6; MS (ESI) *m/z* 371 $(M + Na)^+$.

Compound 29b (mixture of stereoisomers): brown oil (32%), Pe/EtOAc 7:3. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.37 (brs, 1H), 7.78 (s, 1H), 7.23 (m, 4H), 6.75 (s, 1H), 6.12 (s, 1H), 3.78 (s, 3H), 3.76 (s, 2H), 1.89 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.9, 170.8, 168.0, 166.4, 152.0, 148.7, 141.9, 141.6, 136.2, 135.6, 135.2, 126.6, 126.3, 125.5, 123.9, 122.4, 122.0, 120.9, 120.6, 120.2, 119.7, 119.4, 111.6, 111.4, 109.7, 109.3, 106.4, 100.6, 100.5, 52.0, 51.9, 10.7, 10.6; MS (ESI) *m/z* 336 $(M + Na)^+$.

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Chapter 4

SYNTHESIS OF 1,2,3- TRIAZOLE ANALOGUES OF ANTI-HIV DRUG BEVIRIMAT

4.1 INTRODUCTION

The human immunodeficiency virus (HIV) is a lentivirus (a subgroup of retrovirus) that causes HIV infection that evolves in the so called acquired immunodeficiency syndrome (AIDS).

In most cases, HIV is a sexually transmitted infection and it occurs by contact or transfer of blood, pre-ejaculate, semen, and vaginal fluids. Non-sexual transmission can occur from an infected mother to her child during pregnancy or childbirth by exposure to her blood or vaginal fluid and through breast milk. Within these bodily fluids, HIV is present as both free virus particles and virus within infected immune cells.[1]

Two types of HIV have been characterized: HIV-1 and HIV-2. HIV-1 is the most virulent and infective one and it is mainly localized in Europe, America and Central Africa, while HIV-2 is found more widespread in West Africa and Asia and it has a clinically more moderate syndrome.[2]

HIV-1 infects cells that have on their membrane the CD4 receptor and the co-receptors CXCR4 and CCR5, belonging to the family of G protein-coupled receptors (GPCRs).

The cells affected by HIV are cells of the human immune system, such as T helper cell (specifically $CD4^+$ T cells), monocytes, $CD68^+$ macrophages (in lymph nodes, spleen, liver, brain, lungs, bone marrow), dendritic cells in lymph node germination centers and lymphoepithelial surfaces (e.g. in the vagina, in the rectum and in the tonsils).[3]

In general, HIV causes a systemic and generalized infection with a progressive elimination of T helper lymphocytes ($CD4^+$), our sentinels in the recognition of foreign pathogens such as viruses, bacteria, tumour cells and fungi, leading to the immunodeficiency syndrome.

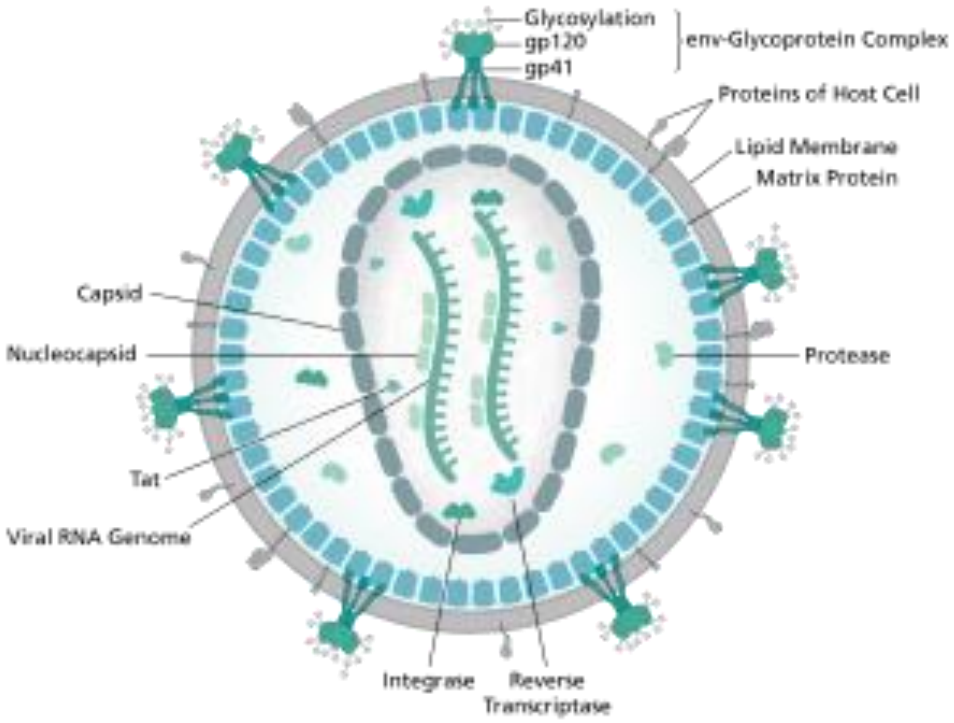


Figure 1: d*iagram of HIV virion.*

The virion of HIV has a spherical structure with a diameter of about 100-120 nm. It is an enveloped virus in which the envelope is a lipid bilayer surrounding the viral matrix, and the core consists of a conoid shaped capside, enclosing the viral genome. The envelop, using gp120 and gp41 proteins, is responsible for the hooking and the fusion of the virus with the cell, favouring the penetration. [4]

The genetic material of the virion and the three genes essential for the replication are contained in a central core called capsid, that consists entirely of a single protein, p24. A matrix composed of the viral protein p17 surrounds the capsid ensuring the integrity of the virion particle and the release of the new replicated viruses as it interacts with the cell membrane allowing the budding. [5]

Gag (group-specific-antigen) encodes for the virion core proteins (p24, p17, p9, p7), Pol (polymerase) encodes for the reverse transcriptase, the protease and the integrase, while Env (envelope) encodes for the proteins of the outer envelope.[2]

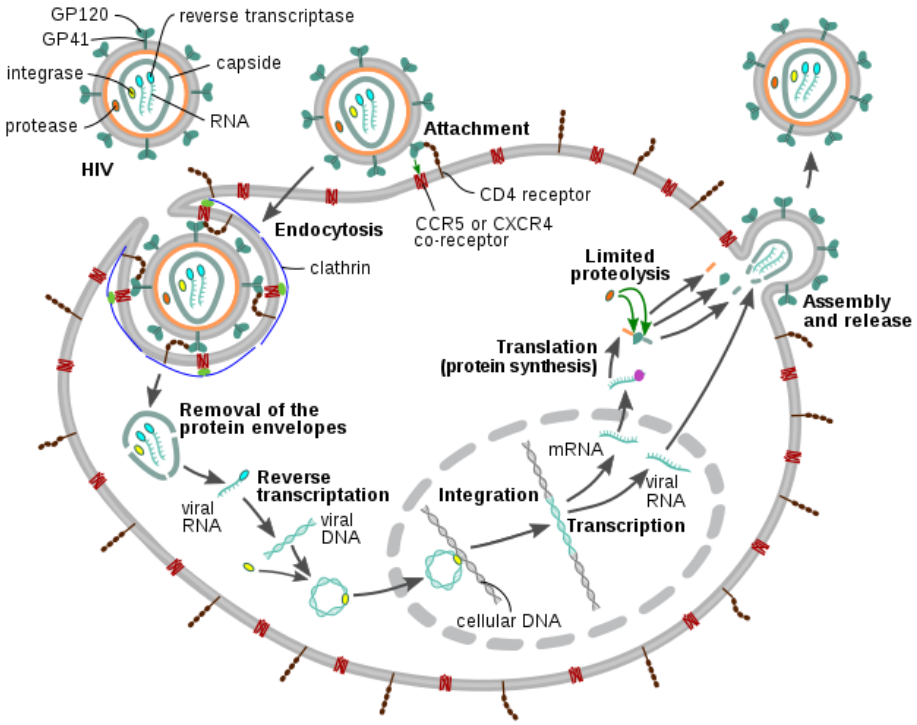


Figure 2: *the HIV replication cycle.*

The HIV virion enters macrophages and $CD4^+$ T cells by the adsorption of gp120 on its surface to CD4, CXCR4 and CCR5 on the target cell followed by fusion of the viral envelope with the target cell membrane.[6],[7]

After the hooking HIV RNA and various enzymes (including reverse transcriptase, integrase, ribonuclease, and protease) are injected into the cell and the viral RNA genome is transcribed into DNA, which is then integrated into the host chromosome followed by new structural HIV proteins synthesis.

Finally, the virus is released from the cell and a protease splits new synthesized proteins to create a new mature infectious virus.

AIDS continues to remain a major health crisis affecting more than 40 million people worldwide. Highly active antiretroviral therapy (HAART) combines antiretroviral therapies with different modes of action, improving the prognosis of HIV infected patients. However, patients are more affected by the long-term side effects of antiretroviral therapy, drug toxicity and drug-drug interactions.[8]

The main classes of anti-HIV drugs are:

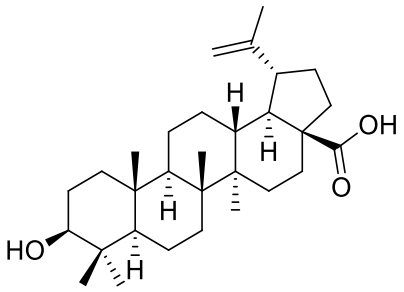
- Nucleoside/ Nucleotide Reverse Transcriptase Inhibitor (NRTI): both classes are pro-drugs acting as false precursors of nucleosides that are incorporated in the chain of nascent viral DNA by reverse transcriptase (zidovudina and tenofovir are examples).
- Inhibitors of the HIV Protease: these compounds act on the aspartyl-protease of HIV, an essential enzyme that cleaves the Gag-Pol polyprotein into the individual structural proteins and enzymes necessary for the formation of mature virions (ritonavir, saquinavir and lopinavir are examples).
- Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI): they bind to reverse transcriptase with consequent inhibition (nevirapine, efavirenz and etravirine).
- Fusion inhibitors: drugs that block the entry of the virus into the cell through binding of gp41 (enfuvirtide) or CCR5 (maraviroc).[9]

Although current therapies have led to a chronicity of the disease, increasing patients' life expectancy, the inaccuracy of the virus in the replication process has generated new mutated forms resistant to treatments.

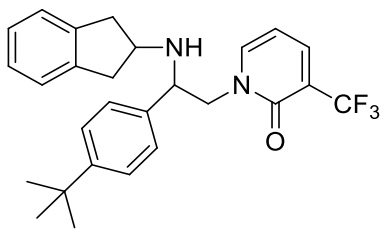
Primary infection with antiretroviral-resistant HIV-1 has become an important clinical problem rendering available treatments less effective. Therefore, there is a need for new antiretrovirals with novel mechanism of action, which will not be subjected to cross-resistance with existing agents.[8]

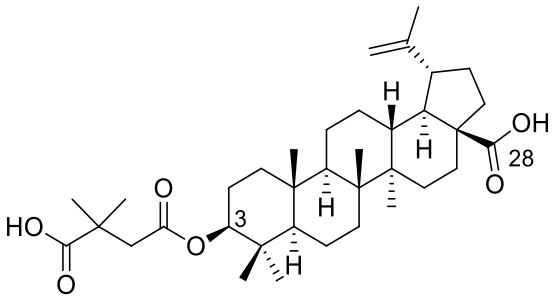
A new promising approach is to inhibit the maturation of virion by targeting Gag protein, an important player in virus life cycle responsible of the virion maturation. To date only two different groups of small molecules have been identified as HIV-1 maturation inhibitors:

1. betulinic acid (BA) derivatives



2. piridone based compound PF-46396





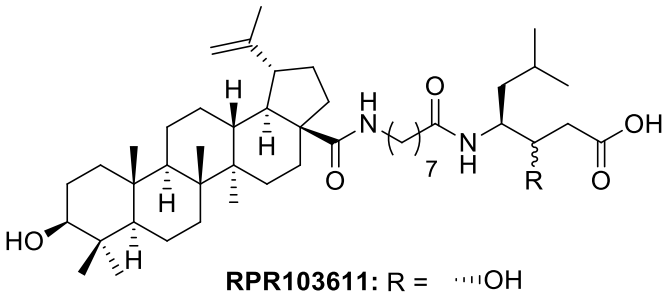
bevirimat

Bevirimat (BVM) is the first-in-class HIV-1 maturation inhibitor based on betulinic acid skeleton able to prevent the cleavage of capsid protein (CA) from its precursor SP1 leading to the accumulation of CA-SP1.

Bevirimat is HIV-1 specific (with IC_{50} of 10,3 nM), with no meaningful activity against HIV-2 or simian immunodeficiency viruses. Such findings support the high specificity of its mechanism of action for HIV-1.[8]

Clinical trials indicated that BVM caused a significant and clinically relevant reduction of the viral load, however a significant fraction (40-50%) of treated patients had resistant viruses that had significantly reduced sensitivity to BVM. This drug resistance was linked to naturally occurring HIV polymorphisms in the SP1 region of HIV-1 Gag and it poses serious limitations to the clinical potential and further development of BMV.[10]

To overcome the limitations connected to BVM, and to improve the potency of this class of promising compounds, the chemical space of betulinic acid was deeply explored through the installation of different groups at positions 3 and/or 28, underling how the viral targets could vary depending on the nature and position of the side chain.[11]



RPR103611: R = ····OH

IC9564: R = ◄OH

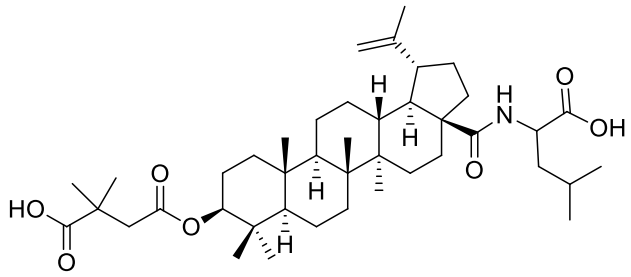
Despite BVM is the prototype of HIV-1 maturation inhibitors, the insertion of a β-amino acid moiety at position 28 of betulinic acid led to statine derivatives RPR103611 and IC0564, inhibiting the virus-cell fusion step.

Different mechanisms have been proposed for these compounds:

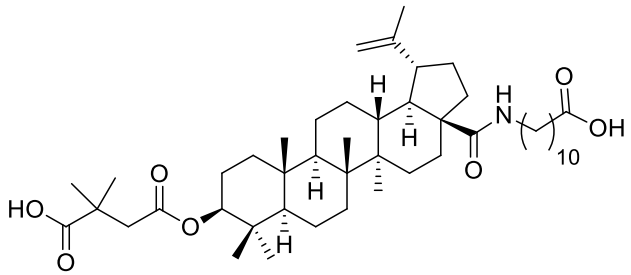
- They might insert into a groove formed at interface between gp41 and gp120 that is induced upon gp120 binding to CD4, resulting in reduced co-receptor binding.
- They could lock the gp120-gp41 complex into an inactive conformation by inhibiting signals.
- The direct interaction of BA derivatives with gp41 might lock the protein in its metastable conformation, inactivating the protein.

Despite a good level of activity these derivatives failed *in-vivo* studies due to the resistance induced by mutations in gp120 and gp41.

The modification of both positions 3 and 28 gave a new group of BA derivatives, exemplified by LH15 and LH55, having in position 3 the 3,3'-dimethylsuccinyl moiety typical of BVM, and in position 28 different acidic amides.[11]



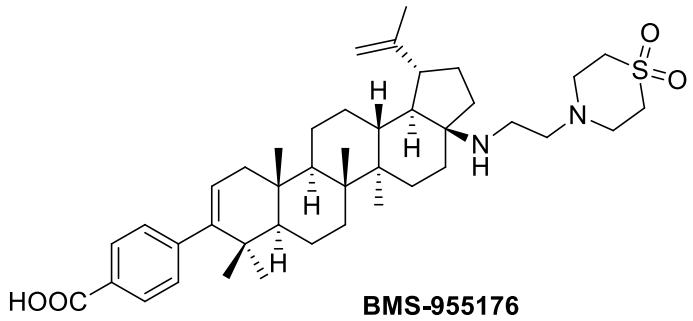
LH15



LH55

These molecules exhibit both anti-fusion and anti-maturation activities with an enhanced antiviral potency also against the resistant forms of the virus, displaying a potential to become useful in anti-HIV therapy.[11]

A second generation of maturation inhibitors has been developed by Bristol-Myers Squibb,[12] leading the discovery of the orally efficacious compound BMS-955176 that is currently in Phase IIb clinical trials.



BMS-955176

The *para*-substitution pattern in position 3 is critical for antiviral activity, while the 1,1- dioxidothiomorpholine heterocycle in position 17 is important for the oral exposure. The candidate compound has a low plasma protein binding, it can be administered once a day, it is safe and well tolerated and it is currently being evaluated in a phase II clinical trial as part of a treatment regimen with antiretroviral agents that have different mechanism of actions (EC_{50} 1,9 nM).[12]

4.2 RATIONALE OF THE PROJECT

The exploration of the chemical space of BA has led to the synthesis of numerous compounds that can be considered as mosaic tails in the definition of the SAR table (*Figure 3*) of BA based maturation inhibitors.[13], [14]

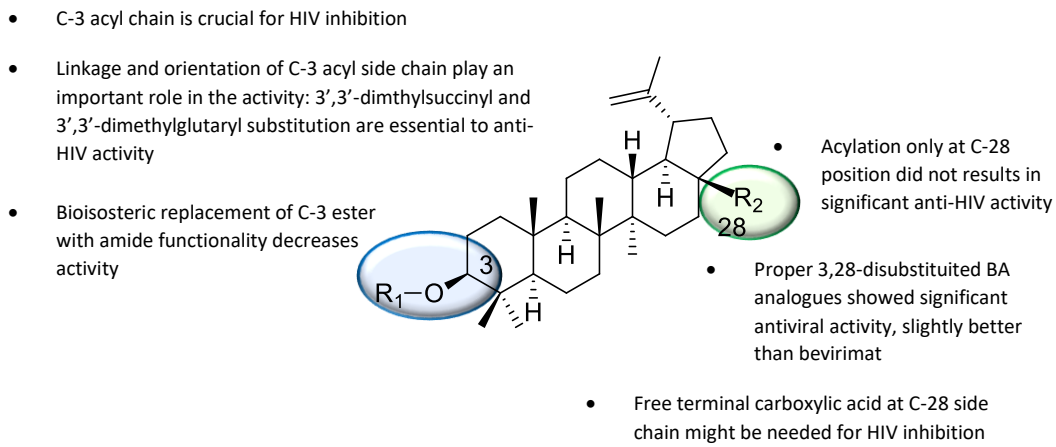


***Figure 3**: summary of SAR of BA analogues.*

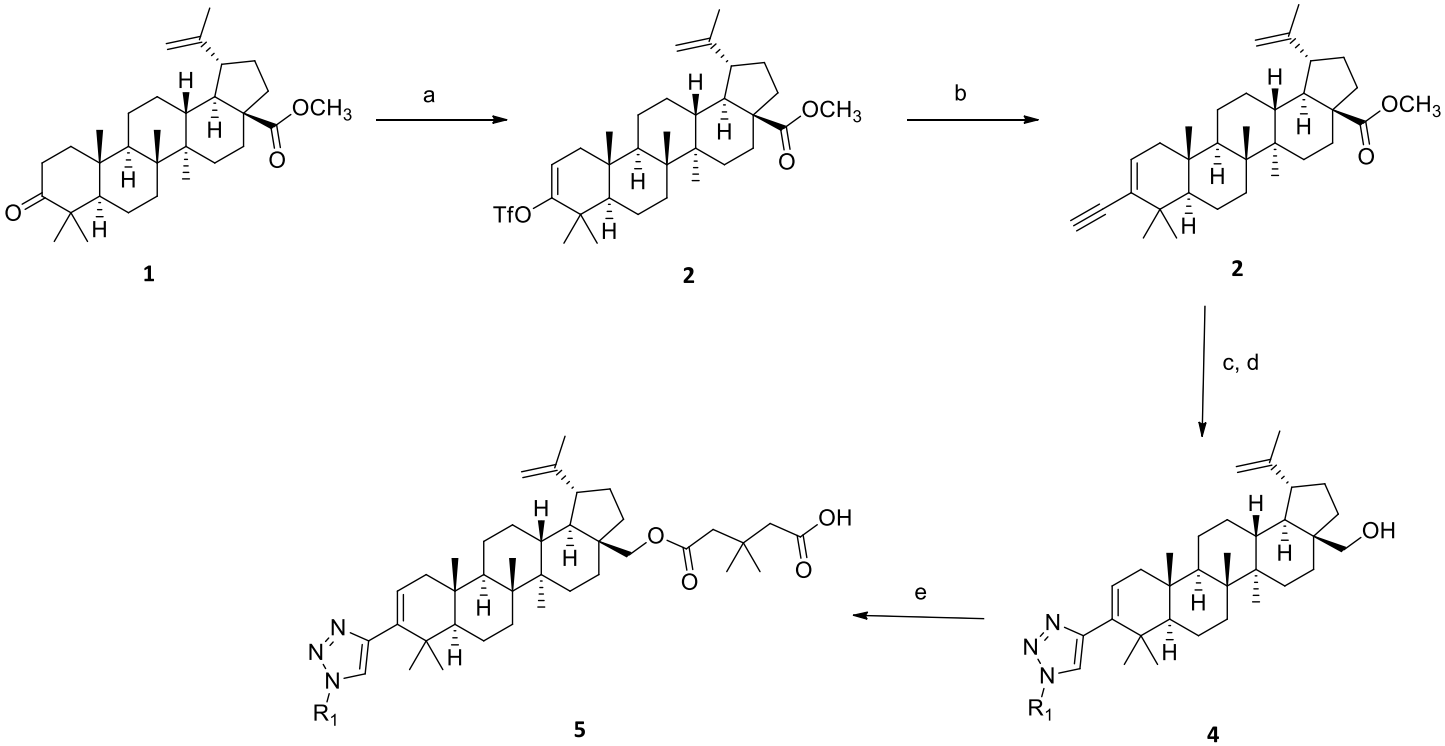
Considering all the information reported in the SAR table, we have decided to design new BA derivatives where the ester function at position 3 was replaced with 1,2,3- triazole ring as a bridge between the free carboxylic acid end and the terpenic core, while in position 28 we have decided to introduce a 3,3'-dimethylglutaryl group.

The introduction of a 1,2,3- triazole ring ensures greater metabolic stability: bevirimat and all the analogues having an ester function at C-3 are readily inactivated by phase 1 metabolism and it gives us the possibility to apply a "*click chemistry approach"* for testing various linear and aromatic azides having a free carboxylic acid function.

4.3 RESULTS AND DISCUSSION

4.3.1 Chemistry

The first approach was based on the Sonogashira cross-coupling reaction between vinyl triflate **2** and ethynyltrimethylsilane for the insertion of ethynyl moiety in position 3. Unfortunately, we were not able to obtain the desired compound **3**, although different reaction conditions and different catalysts have been tested (*Scheme 1*).



Scheme 1: *first synthetic strategy.* (a) KHDMS, $PhNTF_2$, THF dry -78°C; (b) TEA, CuI, $Pd(TPP_2)Cl_2$, ethynyltrimethylsilane, toluene; (c) $LiAlH_4$, THF dry; (d) R_1-N_3; (e) 3,3-dimethyl glutaric anhydride. $PhNTF_2$= N-Phenyl-Bis (trifluoromethanesulfonimide), $Pd(TPP_2)Cl_2$= Bis (triphenylphosphine) palladium chloride

Since we were unable to implant the alkyne group in the terpenic scaffold, we have planned new analogues in which the triazole ring was linked at position 3 via an ether bond. (*Figure 4*).

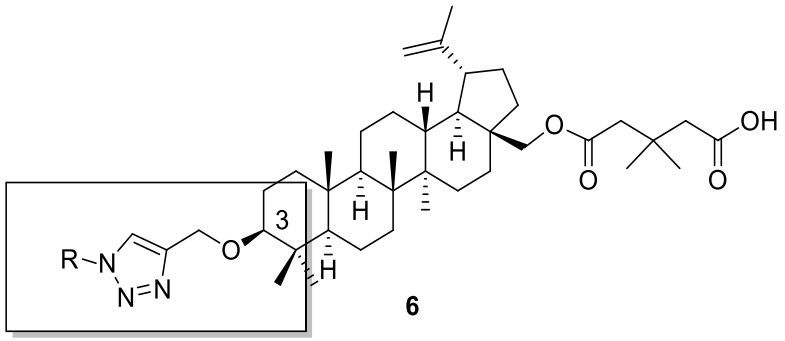
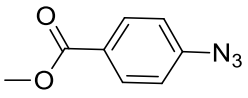
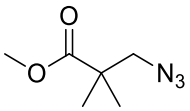
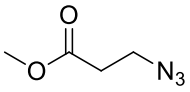
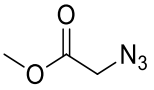
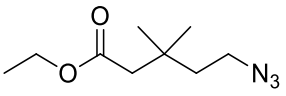


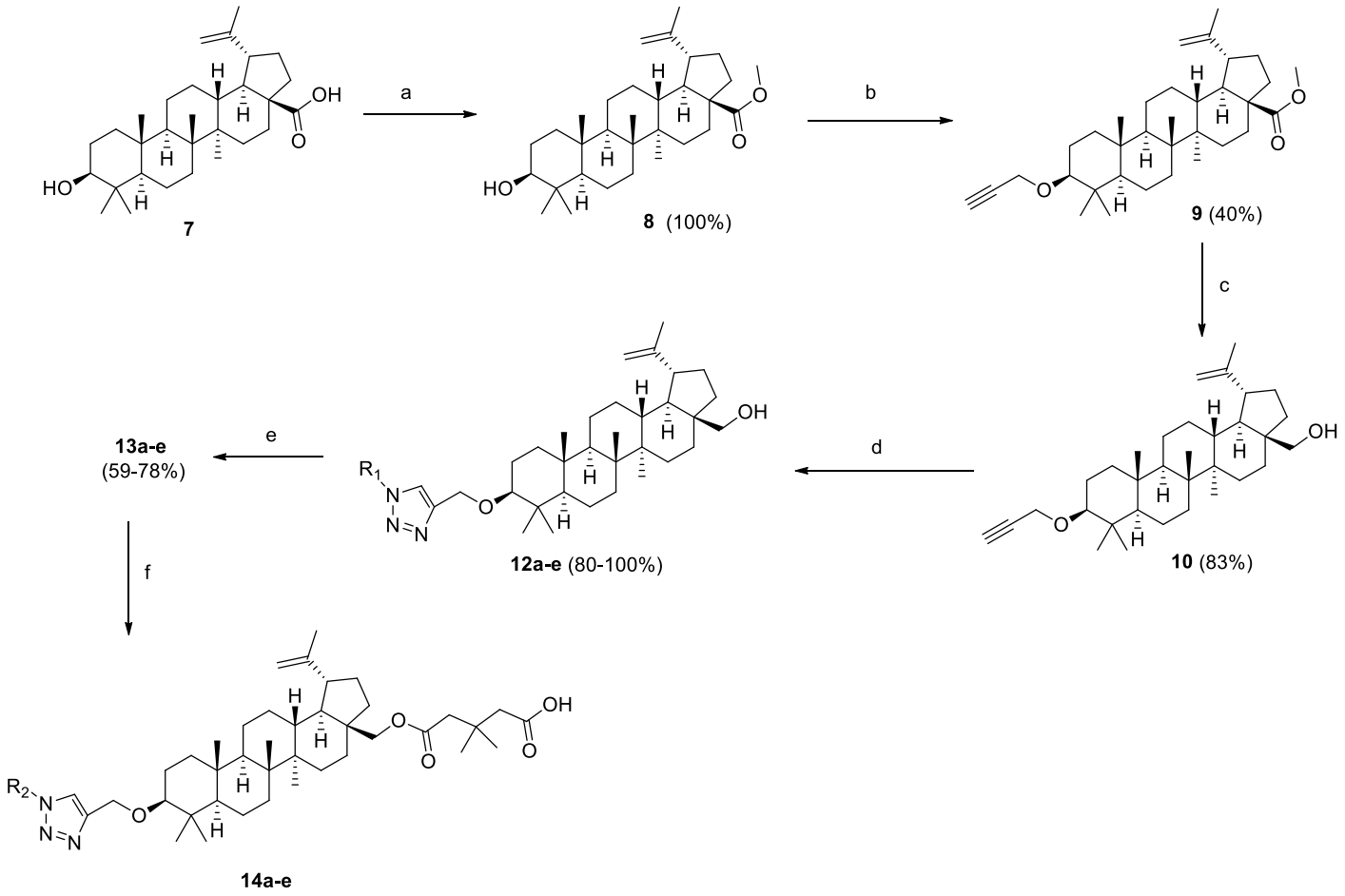
Figure 4

The methyl ester of betulinic acid **8** (*Scheme 2*) was treated with NaH 60% in dry THF in presence of propargyl bromide to give compound **9** in moderate yield (45%), that was finally reduced with $LiAlH_4$ to the corresponding alcohol **10** as key intermediate. Compound **10** was coupled with five different azides (**11a-e**, *Table 1*) under Medal-Sharpless conditions giving 1,2,3-triazole esters **12a-e**, that were hydrolysed (NaOH 10N, THF/H_2O, 85 °C) to the corresponding free carboxylic acids **13 a-e**.

Table 1: *aromatic and linear azides used for the synthesis of 1,2,3-triazole derivatives of BA.*

Compound	Azide
11a	
11b	
11c	
11d	
11e	

The final compounds (**14a-e)** were obtained by reacting compounds **13a-e** with 3,3-dimethyl glutaric anhydride in DCM in presence of DMAP (*Scheme 2*).



Scheme 2: *procedure for the synthesis of 1,2,3-triazole derivatives of BA.* (a) K_2CO_3, DMS, DMF; (b) NaH 60%, 18-crown-6, then propargyl bromide, THF dry 0°C to 95°C; (c) $LiAlH_4$, THF dry; (d) R_1-N_3 (**11 a- e**), sodium ascorbate, Cu_2SO_4, H_20:t-BuOH 1:3 40°C; (e) NaOH 10N, THF 85°C; (f) 3,3-dimethylglutaric anhydride DCM.

This synthetic process is not ideal to be scaled up, due to the low yield in the etherification reaction between propargyl bromide and betulinic methyl ester. We have investigated different conditions in order to improve the yield of the reaction, by varying solvents, temperature and nature of the base, without significant results (*Table 2)*.

Table 2*: reactions conducted to improve the yield of propargylation reaction.*

REAGENTS	**SOLVENT**	**RESULT**
1) NaH 60% 1,5 eq, 10', 0°C 2) Propargyl bromide 1,2 eq, 0°C-RT	THF dry	Starting material
1) NaH 60% 7 eq, 30', 0°C 2) Propargyl bromide 3 eq, NaI cat, 0°C-85°C	DMF dry	9% yield
1) NaH 60% 7 eq, 18-crown-16, 30', 0°C 2) Propargyl bromide 3 eq, 0°C-85°C	DMF dry	Starting material
1) LDA, 30', 0°C 2) betulinic methyl ester 1 eq, 0°C to RT, 5' 3) Propargyl bromide 3 eq, 0°C to 40°C	THF dry	Starting material
1) LDA, 0°C, 30' 2) 18-crown-16 cat, betulinic methyl ester 1 eq, 0°C to RT, 30' 3) Propargyl bromide, 0°C to 60°C	THF dry	Starting material
1) LDA, 0°C, 30' 2) 18-crown-16 cat, betulinic methyl ester 1 eq, 0°C to RT, 30' 3) Propargyl bromide, 0°C to 60°C	DMPU dry	Starting material

1) NaH 2 eq, 18-crown-16, 0°C to RT, 30' 2) Propargyl bromide 3 eq, 0°C to RT / 0°C to 60°C	DMPU dry	Starting material
1) LiHMDS 1.1 eq, 18-crown-16 cat, propargyl bromide 2 eq (one pot), 0°C to RT/ 0°C to 60°C	THF dry	Starting material
1) NaH 60% 1.1 eq, 0°C to RT 30' 2) Propargyl bromide 2 eq, 0°C to RT/ 0°C to 60°C	THF dry	Starting material
On betulinic acid 1) NaH 60% 1.1 eq, 0°C to RT 30' 2) Propargyl bromide 2 eq, 0°C to RT/ 0°C to 60°C	THF dry	Starting material
1) LiHMDS 1M 2 eq , 18-crown-16 cat, 30', 0°C 2) Propargyl bromide 2 eq, 0°C to 60°C	DMPU dry	See *Figure 5*
1) LiHMDS 2 eq , 18-crown-16 cat, 0°C 2) Propargyl bromide 2 eq, 0°C to 40°C	DMPU dry	Starting material

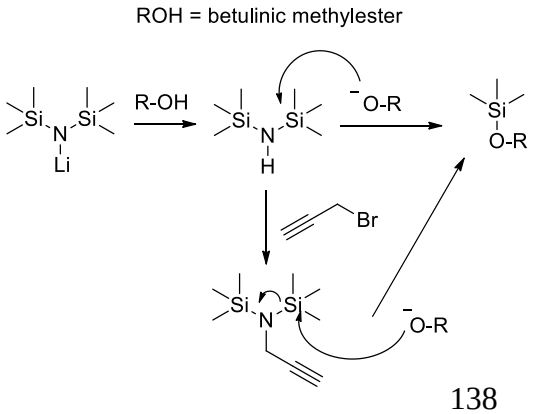


Figure 5: *mechanism of reaction with LiHMDS and propargyl bromide*

4.3.3 Conclusions

New 1,2,3- triazole analogues of anti-HIV drug bevirimat were synthetized in a 6 steps process. Both the triazole intermediate **12a-e** and the final compounds **13a-e** will be tested as possible anti-HIV agents by Vivacell, our biological partner in the TriForC consortium.

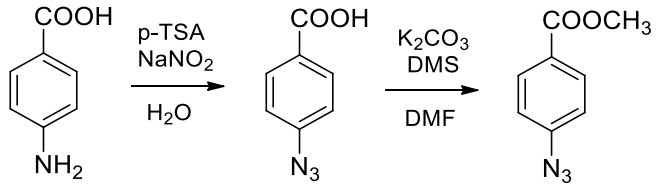
If the compounds will be active, we have planned in the future to expand the library of compounds using other azides and synthesizing analogues with different side chains.

4.4 EXPERIMENTAL SECTION

General Methods and Materials.

Commercially available reagents and solvents were purchased from Aldrich or Alfa-Aesar and were used without further purification. *N,N'*-Dimethylformamide (DMF) was dried over a neutral alumina pad and stored on 4 Å activated molecular sieves. Dichloromethane was dried by distillation from P_2O_5 and stored on 4 Å activated molecular sieves. Pyridine was dried over neutral alumina pad and stored on activated 4 Å molecular sieves under nitrogen. When needed, the reactions were performed in flame- or oven-dried glassware under a positive pressure of dry nitrogen. For spectroscopic characterization, a JEOL ECP 300 MHz spectrometer was used for ^{1}H and ^{13}C spectra. Chemical shifts are reported in parts per million (ppm) using the residual solvent peak as reference ($CHCl_3$ at δ 7.27). A Thermo Finningan LCQ-deca XP-plus equipped with an ESI source and an ion trap detector was employed for mass spectrometry. Flash column chromatography was performed on silica gel (Merck Kieselgel 60, 230−400 mesh ASTM). Thin-layer chromatography (TLC) was carried out on 5 × 20 cm plates with a layer thickness of 0.25 mm (Merck silica gel 60 F254). When necessary, $KMnO_4$ was used for visualization.

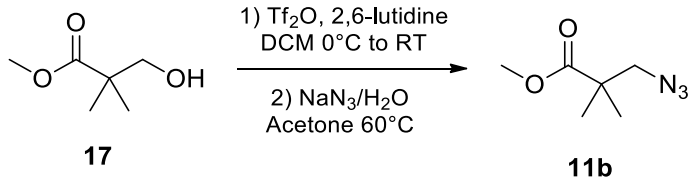
SYNTHESIS OF AZIDES 11a-e



15 **16** **11a**

Synthesis of compound 11a: to a stirred solution of *p*-TsOH·H_2O (9 eq) in H_2O (9 mL/1 mmol), *p*-aminobenzoic acid was added (1 eq). After stirring for 1 minute, an aqueous solution of $NaNO_2$ (9 eq) was added dropwise in 5 min. The resulting mixture was then stirred for 1 h and then NaN_3 (2 eq) was added. An immediate emission of N_2 was observed and the mixture was stirred for 4 h. Solid aryl azide was filtered off, washed with H_2O and dried. The aqueous phases were further extracted with EtOAc (x3), dried (Na_2SO_4) and evaporated at reduced pressure.

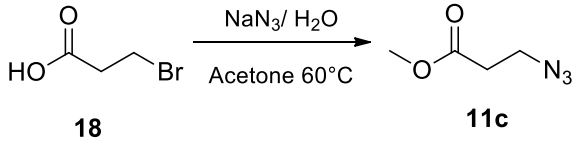
The crude carboxylic azide **16** (1 eq) was treated with sodium carbonate (5 eq) and dimethylsulfate (5 eq) in DMF (10 mL/ 1 g). The reaction was stirred at room temperature overnight, then quenched with H_2SO_4 2N and extracted with a 3:1 mixture of petroleum ether and diethyl ether. The organic layers were dried over Na_2SO_4, filtered and evaporated to afford the methyl ester **11a** as brown solid, without further purification (quantitative yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.98 (d, *J* = 8.9 Hz, 3H), 7.02 (d, *J* = 8.9 Hz, 3H), 3.87 (s, 3H)



17 **11b**

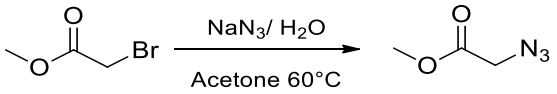
Synthesis of compound 11b: To a stirred solution of hydroxy propionate **17** (1 eq) in DCM (20 mL/10 mmol), Tf_2O (1,2 eq) and 2,6-lutidine (1,2 eq) were sequentially added at 0 °C. The resulting solution was stirred for 3 h, quenched with H_2SO_4 2N (x2) and extracted with DCM (100 mL/ 10 mmol). The organic phases were dried over Na_2SO_4 and evaporated at reduced pressure to give the crude triflate that was used without further purification. To the crude was dissolved in acetone (20 mL/ 10 mmol) and water (20 mL/ 10 mmol) and NaN_3 (2 eq) was added. The resulting solution was stirred at 60°C overnight. The mixture was extracted with EtOAc and the organic phase was dried over Na_2SO_4 and evaporated under reduce pressure giving crude **11b** as a brown oil.

^{1}H NMR (300 MHz, $CDCl_3$) δ 3.65 (s, 3H), 3.35 (s, 2H), 1.15 (s, 6H).



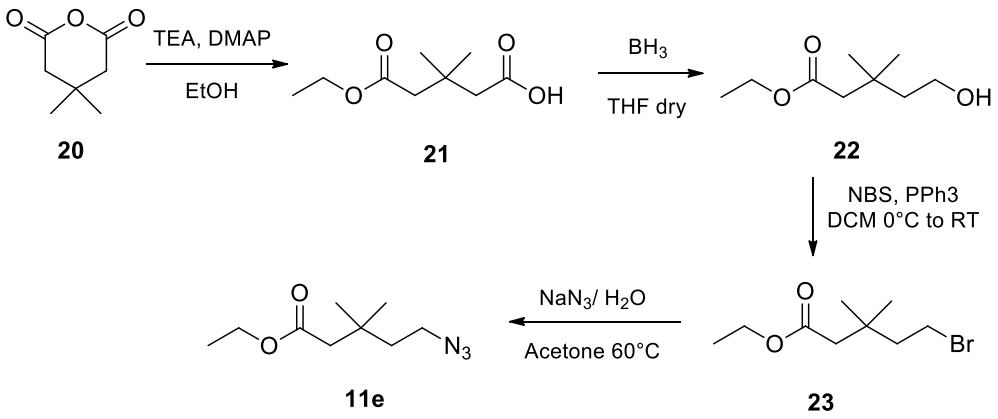
18 **11c**

Synthesis of compound 11c: to a stirred solution of methyl 3-bromopropanoate (1 eq) in acetone (50 mL/ 60 mmol) an aqueous solution of NaN_3 (2,5 eq) was added. The reaction was stirred at 60°C overnight, the solvent was evaporated and the crude mixture was diluted with water and extracted with DCM (x4). The organic phases were dried over Na_2SO_4 and evaporated under reduce pressure giving crude **11c** as a brown oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.64 (s, 3H), 3.50 (t, *J* = 6.5 Hz, 2H), 2.51 (t, *J* = 6.5 Hz, 2H).



19 **11d**

Synthesis of compound 11d: To a stirred solution of **19** (1 eq) in acetone (50 mL/ 60 mmol) an aqueous solution of NaN_3 (2,5 eq) was added. The reaction was stirred at 60°C overnight, the solvent was evaporated and the crude mixture was diluted with water and extracted with DCM (x4). The organic phases were dried over Na_2SO_4 and evaporated under reduce pressure giving crude **11d** as a yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.12 (q, *J* = 7.1 Hz, 2H), 3.75 (s, 2H), 1.16 (d, *J* = 7.1 Hz, 3H).



Synthesis of compound 11e: to a stirred solution of 3,3-dimethylglutaric anhydride **20** (1 eq) in EtOH, DMAP (0,1 eq) and TEA (1 eq) were sequentially added. The solution was stirred overnight at reflux, cooled to room temperature and the solvent evaporated under reduced pressure. The crude mixture was diluted with H_2SO_4 2N (x1) and extracted with EtOAc (x1). The organic phases were dried over Na_2SO_4 and evaporated under reduce pressure giving the crude monoester **21** (colourless oil), that was used in the next step without further purification.

To a cooled (-15°C) solution of the crude monoester **21** (1 eq) in dry THF (13 mL/ 13,1 mmol), BH_3-THF 1M was added dropwise (1 eq). The reaction was stirred at -15°C for 10 minutes at room temperature for 6 h, then cooled down at 0 ° C, diluted with Na_2CO_3 sat. sol. and extracted with EtOAc (x3). The organic phases were dried over Na_2SO_4, evaporated under reduce pressure giving the crude mono alcohol **22** (yellowish oil) without further purification.

To a stirred and cooled (0°C) solution of **22** in DCM (30 mL/ 1g), triphenylphosphine (1,1 eq) and NBS (1,2 eq) were sequentially added. After 24 h, DCM was removed under reduced pressure and a cold mixture of benzene/cyclohexane 1:1 was added to the round bottom flask and brought to

-18°C overnight. The precipitate was filtered over celite and washed with a cold mixture of benzene/cyclohexane 1:1 (x3). The organic phase was evaporated under reduced pressure affording crude compound **23** used without further purification.

To a stirred solution of **23** (1 eq) in acetone (50 mL/ 60 mmol) an aqueous solution of NaN_3 (2,5 eq) was added. The reaction was stirred at 60°C overnight, the solvent was evaporated and the crude mixture was diluted with water and extracted with DCM (x4). The organic phases were dried over Na_2SO_4 and evaporated under reduce pressure giving crude **11e** as yellow oil, ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.08 (q, *J* = 7.1 Hz, 2H), 3.25 (t, *J* = 7.1 Hz, 2H), 2.16 (s, 3H), 1.61 (t, *J* = 7.8 Hz, 2H), 1.19 (t, *J* = 6.8 Hz, 3H), 0.97 (s, 6H).

Procedure for the esterification of betulinic acid (8): to a stirred solution of betulinic acid **7** (1 eq) in DMF (10 mL), sodium carbonate (5 eq) and dimethylsulfate (5 eq) were added. The reaction was stirred at room temperature overnight, then quenched with H_2SO_4 2N and extracted with a 3:1 mixture of petroleum ether and diethyl ether. The organic layers were dried over Na_2SO_4, filtered and evaporated to afford the methyl ester without further purification (quantitative yield).

Synthesis of compound 9: to a cooled solution of **8** in dry THF (10 mL/ 1g) NaH (60% in mineral oil) (7 eq) and a catalytic amount of 18-crown-6 were sequentially added. The solution was stirred at 0 ° C for 30 minutes and propargyl bromide 80% wt solution in benzene (3 eq) was added dropwise. The reaction was heated at 90° C overnight, cooled in an ice bath, quenched with H_2SO_4 2N and extracted with EtOAc. The organic phases were dried over Na_2SO_4, evaporated under reduce pressure and the crude product was purified over silica gel (Pe/EtOAc 98:2 as eluent) affording compound **9** (40%) as a yellow

solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.72 (s, 1H), 4.58 (s, 1H), 4.15 (qd, *J* = 15.9, 1.8 Hz, 2H), 3.64 (s, 3H), 2.98 (dd, *J* = 11.5, 4.5 Hz, 2H), 2.36 – 2.31 (m, 1H), 2.26 – 2.11 (m, 2H), 1.66 (s, 3H), 1.24 (s, 3H), 0.94 (s, 3H), 0.89 (s, 3H), 0.80 (s, 3H), 0.73 (s, 3H) (only readily peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.4, 150.3, 109.7, 85.7, 80.9, 73.4, 56.4, 56.2, 56.0, 51.2, 50.6, 49.5, 46.9, 42.3, 40.7, 38.5, 38.3, 37.1, 36.8, 34.3, 32.1, 30.6, 29.6, 27.9, 25.5, 22.4, 21.0, 19.4, 18.2, 16.2, 15.9, 14.7.

Synthesis of compound 10: to a cooled solution (0°c) of **9** in THF dry, $LiAlH_4$ (4 eq) was added. The reaction mixture was stirred at room temperature for 5 h, cooled in an ice bath, diluted with H_2SO_4 2N and extracted with EtOAc (x1). The organic phase was washed with $NaHCO_3$ sat. sol. dried over Na_2SO_4 and evaporated under reduce pressure. The crude product was purified over silica gel (Pe/EtOAc 9:1 as eluent) affording compound **10** (83%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.60 (d, *J* = 29.8 Hz, 2H), 4.25 – 4.03 (m, 3H), 3.76 (d, *J* = 10.7 Hz, 1H), 3.30 (d, *J* = 10.8 Hz, 1H), 2.98 (dd, *J* = 11.7, 4.1 Hz, 1H), 2.43 – 2.29 (m, 2H), 1.66 (s, 3H), 1.24 (s, 3H), 1.14 (s, 3H), 1.00 (s, 3H), 0.95 (s, 3H), 0.81 (s, 3H), 0.74 (s, 3H) (only readily peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 150.4, 109.7, 85.7, 80.8, 73.6, 60.5, 60.2, 56.4, 55.9, 50.4, 48.8, 47.8, 47.7, 42.7, 41.0, 40.9, 38.6, 37.2, 37.1, 34.2, 34.0, 29.8, 29.2, 28.0, 27.1, 25.2, 22.6, 20.9, 19.1, 18.3, 16.2, 16.1, 16.0, 14.8.

Click Chemistry General procedure. Synthesis of 12a-e: to a stirred solution of **10** in H_2O:t-butanol 1:3 and THF (1mL/ 400 mg) as cosolvent, azide (2 eq), a catalytic amount of sodium ascorbate and copper sulfate were sequentially added. The solution was stirred at 40 °C overnight, diluted with brine and extracted with EtOAc. The organic phases were dried over Na_2SO_4, evaporated under reduce pressure, followed by purification over silica gel.

Compound 12a: yellow solid (80%), Pe/EtOAc 8:2. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.19 (d, *J* = 8.7 Hz, 2H), 8.01 (s, 1H), 7.84 (d, *J* = 8.7 Hz, 2H), 4.86 (d, *J* = 12.4 Hz, 1H), 4.63 (dd, *J* = 21.5, 8.7 Hz, 3H), 3.94 (s, 3H), 3.78 (d, *J* = 10.8 Hz, 1H), 3.32 (d, *J* = 10.8 Hz, 1H), 2.99 (dd, *J* = 11.6, 4.2 Hz, 1H), 2.38 (dt, *J* = 16.1, 8.2 Hz, 1H), 1.67 (s, 3H), 1.00 (s, 4H), 0.96 (s, 3H), 0.94 (s, 3H), 0.82 (s, 3H), 0.78 (s, 3H)) (only readily peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.9, 150.5, 147.7, 140.2, 131.3, 130.1, 119.9, 109.6, 87.0, 63.1, 60.1, 55.7, 52.4, 50.3, 48.8, 47.8, 47.8, 42.7, 40.9, 38.9, 38.5, 37.3, 37.1, 34.2, 34.0, 29.8, 29.2, 28.1, 27.1, 25.2, 22.9, 20.9, 19.1, 18.3, 16.3, 16.1, 16.0, 14.7.

Compound 12b: yellow solid (85%), Pe/EtOAc 7:3. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.51 (s, 1H), 4.77 – 4.44 (m, 6H), 3.75 (d, *J* = 11.0 Hz, 1H), 3.68 (s, 3H), 3.29 (d, *J* = 10.8 Hz, 1H), 2.90 (dd, *J* = 11.5, 3.6 Hz, 1H), 2.34 (dd, *J* = 11.5, 6.1 Hz, 1H), 2.00 (s, 3H), 1.64 (s, 3H), 1.19 (s, 3H), 0.97 (s, 1H), 0.93 (s, 3H), 0.79 (d, *J* = 8.2 Hz, 3H), 0.71 (s, 3H) (only readily peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 179.1, 150.5, 146.3, 123.7, 109.7, 86.5, 62.9, 60.6, 57.3, 55.7, 50.4, 48.9, 47.8, 43.8, 42.8, 41.0, 38.8, 38.6, 37.4, 37.2, 34.3, 34.0, 29.8, 29.3, 28.0, 27.1, 25.3, 23.3, 23.2, 23.1, 20.9, 19.1, 18.3, 16.3, 16.1, 16.0, 14.8.

Compound 12c: pale brown solid (100%), Pe/EtOAc 7:3. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.60 (s, 1H), 4.76 – 4.47 (m, 6H), 3.77 (d, *J* = 10.7 Hz, 1H), 3.67 (s, 3H), 3.30 (d, *J* = 10.8 Hz, 1H), 3.01 – 2.87 (m, 3H), 2.43 – 2.31 (m, 1H), 1.65 (s, 3H), 0.98 (s, 3H), 0.94 (s, 3H), 0.85 (s, 3H), 0.79 (s, 3H), 0.73 (s, 3H) (only readily peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.9, 150.5, 123.2, 109.6, 86.4, 63.0, 60.3, 60.0, 55.6, 52.1, 50.3, 48.7, 47.8, 45.5, 42.6, 40.9, 38.7, 38.5, 37.2, 37.1, 34.4, 34.2, 34.0, 29.7, 29.2, 27.9, 27.0, 25.2, 22.8, 20.8, 19.1, 18.2, 16.2, 16.1, 16.0, 14.7.

Compound 12d: yellow solid (90%), Pe/EtOAc 7:3. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.64 (s, 1H), 5.12 (s, 2H), 4.81 – 4.49 (m, 6H), 4.22 (dd, *J* = 14.9, 7.6 Hz, 2H), 3.76 (d, *J* = 11.8 Hz, 1H), 3.29 (d, *J* = 11.5 Hz, 1H), 2.92 (dd, *J* = 12.1, 4.4 Hz, 1H), 2.48 – 2.28 (m, 1H), 1.64 (s, 3H), 1.26 (s, 3H), 0.97 (s, 3H), 0.93 (s, 3H), 0.86 (s, 3H), 0.78 (s, 3H), 0.72 (s, 3H) (only readily peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.3, 150.5, 147.0, 123.7, 109.7, 86.5, 63.1, 62.4, 60.4, 55.7, 50.9, 50.4, 48.8, 47.8, 42.7, 41.0, 38.8, 38.5, 37.3, 37.1, 34.2, 34.0, 29.8, 29.2, 28.1, 27.0, 25.2, 22.9, 20.9, 19.1, 18.3, 16.3, 16.1, 16.0, 14.7, 14.1.

Compound 12e: colourless solid (87%), Pe/EtOAc 7:3. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.52 (s, 1H), 4.85- 4.35 (m, 7H), 3.77 (d, *J* = 10.8 Hz, 1H), 3.30 (d, *J* = 10.7 Hz, 1H), 2.93 (d, *J* = 11.6 Hz, 1H), 2.41 – 2.29 (m, 1H), 1.66 (s, 3H), 1.24 (s, 3H), 1.06 (s, 3H), 0.99 (s, 3H), 0.94 (s, 3H), 0.87 (s, 3H), 0.80 (s, 3H), 0.74 (s, 3H) (only readily peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.6, 150.6, 146.4, 109.6, 100.0, 86.5, 63.2, 60.4, 60.3, 59.9, 55.7, 50.3, 48.8, 47.8, 46.6, 45.6, 42.7, 41.5, 40.9, 38.8, 38.5, 37.3, 37., 34.2, 34.1, 32.6, 29.8, 29.2, 28.0, 27.5, 27.1, 25.2, 22.9, 20.9, 19.1, 18.2, 16.3, 16.1, 16.0, 14.7, 14.3, 14.2.

General procedure of hydrolysis to compounds 13a-e: to a solution of **12a-e** in THF, 10 equivalents of 10N NaOH were added and the reaction was stirred at 85 ° C for 5 hours. The reaction was diluted with H_2SO_4 2N, extracted with EtOAc and washed with H_2SO_4 2N (X3). The organic phase was dried using Na_2SO_4 and evaporated under reduced pressure affording crude compounds **13a-e**, that were purified over silica gel.

Compound 13a: yellow solid (59%), EtOAc/ MeOH 8:2. ^{1}H NMR (300 MHz, DMSO-D6) δ 8.88 (s, 1H), 8.07 (bdd, 4H), 4.80 – 4.45 (m, 4H), 3.59- 4.40 (m, 2H), 3.02 (dd, *J* = 34.8, 8.7 Hz, 2H), 2.50- 2.39 (m, 1H), 1.63 (s, 3H), 1.23 (s, 3H), 0.95

(s, 3H), 0.92 (s, 3H), 0.85 (s, 3H), 0.79 (s, 3H), 0.70 (s, 3H)) (only readily peaks are reported); ^{13}C NMR (75 MHz, DMSO-D6) δ 183.0, 166.8, 151.0, 146.7, 140.1, 131.5, 130.0, 122.6, 120.2, 110.2, 85.8, 67.5, 62.2, 58.5, 55.6, 50.3, 48.7, 47.9, 47.8, 42.8, 38.9, 38.8, 37.3, 37.2, 34.3, 29.9, 29.6, 28.3, 27.2, 25.6, 25.4, 22.9, 20.9, 19.3, 18.4, 16.8, 16.5, 16.4, 16.2, 15.0.

Compound 13b: white solid (78%), EtOAc 100%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.50 (s, 1H), 4.81 – 4.50 (m, 6H), 3.79 (d, *J* = 11.2 Hz, 1H), 3.33 (d, *J* = 11.1 Hz, 1H), 2.96 (dd, *J* = 11.6, 3.7 Hz, 1H), 2.44 – 2.32 (m,1H), 1.68 (s, 3H), 1.26 (s, 3H), 1.00 (s, 3H), 0.96 (s, 3H), 0.83 (s, 3H), 0.81 (s, 3H), 0.73 (s, 3H) (only readily peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 179.1, 150.5, 146.3, 123.7, 109.7, 86.5, 62.9, 60.6, 57.3, 55.7, 50.4, 48.9, 47.8, 43.8, 42.8, 41.0, 38.8, 38.6, 37.4, 37.2, 34.3, 34.0, 29.8, 29.3, 28.0, 27.1, 25.3, 23.3, 23.2, 23.1, 20.9, 19.1, 18.3, 16.3, 16.1, 160, 14.8.

Compound 13c: white solid (66%), EtOAc 100%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.75- 4.50 (m, 6H), 3.78 (d, *J* = 11.9 Hz, 1H), 3.32 (d, *J* = 11.5 Hz, 1H), 3.01 – 2.86 (m, 3H), 2.35 (dd, *J* = 15.9, 10.2 Hz, 1H), 1.65 (s, 3H), 1.34 (s, 3H), 0.98 (s, 3H), 0.94 (s, 3H), 0.85 (s, 3H), 0.79 (s, 3H), 0.72 (s, 3H) (only readily peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.7, 150.5, 146.1, 123.5, 109.7, 86.8, 62.8, 60.4, 55.7, 53.5, 50.3, 48.8 47.8, 45.7, 42.7, 40.9, 38.8, 38.5, 37.3, 37.1, 34.6, 34.2, 34.0, 29.7, 29.2, 28.0, 27.0, 25.2, 22.9, 19.1, 18.3, 16.3, 16.2, 16.0, 14.8.

Compound 13d: pale yellow solid (68%), EtOAc 100%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.70 (s, 1H), 5.14 (s, 2H), 4.83 – 4.46 (m, 6H), 3.79 (d, *J* = 11.4 Hz, 1H), 3.32 (d, *J* = 10.7 Hz, 1H), 2.93 (d, *J* = 8.4 Hz, 1H), 2.34 (s, 1H), 1.66 (s, 3H), 1.24 (s, 3H), 0.98 (s, 3H), 0.94 (s, 3H), 0.87 (s, 3H), 0.80 (s, 3H), 0.73 (s, 3H) (only readily peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.4, 150.4, 146.2, 124.4, 109.8,

87.0, 62.9, 60.4, 55.7, 53.5, 50.3, 48.8, 48.0, 46.8, 42.7, 40.9, 38.8, 38.5, 37.3, 37.1, 34.2, 34.0, 29.7, 29.5, 28.1, 27.0, 25.2, 23.9, 22.9, 20.9, 19.1, 18.3, 16.3, 16.2, 16.1, 14.8.

Compound 13e: white solid (60%), Pe/EtOAc 6:4. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.53 (s, 1H), 4.75- 4.37 (m, 6H), 3.78 (d, *J* = 10.7 Hz, 1H), 3.32 (d, *J* = 11.0 Hz, 1H), 2.93 (dd, *J* = 11.6, 3.8 Hz, 1H), 1.66 (s, 3H), 1.09 (s, 3H), 0.99 (s, 3H), 0.95 (s, 3H), 0.87 (s, 3H), 0.80 (s, 3H), 0.74 (s, 3H) (only readily peaks are reported); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.1, 150.5, 146.3, 122.4, 109.7, 86.7, 63-0, 60.3, 55.7, 50.3, 48.8, 47.8, 47.7, 46.8, 45.5, 42.7, 41.5, 40.9, 38.8, 38.5, 37.3, 37.1, 34.2, 34.0, 32.5, 29.7, 29.2, 28.0, 27.5, 27.0, 25.2, 22.9, 20.96, 20.92, 19.1, 18.3, 16.3, 16.1, 16.0, 14.8.

General procedure for the synthesis of compounds 14a-e: to a stirred solution of compound **13a-e** in dry pyridine, 3,3- dimethylglutaric anhydride (2 eq) and DMAP (1 eq) were sequentially added and the reaction was stirred at 40 ° C overnight. The reaction mixture was quenched with H_2SO_4 2N and extracted with EtOAc. The combined organic phases were washed with brine, dried over Na_2SO_4, evaporated under reduced pressure, and the crude compound was purified over silica gel.

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5. FINAL CONCLUSIONS

PCTTAs occur very widely distributed in the vegetal kingdom, and for over 100 years they have attracted the attention and stimulated the curiosity of organic chemists, favoring a deep exploration of their chemical space.

Their multi-target biological profile has not gone unnoticed in the biomedical community, and despite they can be considered as "*old scaffolds"*, they are still providing bioactive compounds for the treatment of major diseases (bardoxolone methyl, bevirimat, BMS-955176).

In this PhD thesis, we have designed a new protecting-free and easily scalable semi-synthetic route to obtain α and β-amyrins and lupeol in high yield from easily accessible starting materials and we have identified the pentacyclic triterpenoid acid hydroxamates as a class of novel and selective modulators of HIF-1 signaling.

Moreover, we have synthesized triterpeno-strigoids that were devoid of activity on stress response modulators, but others mono and sesquiterpeno-strigoids shown interesting biological profiles comparable to that of natural strigolactones.

Finally, a small set of new 1,2,3-triazole analogues of bevirimat were synthesized, as possible anti-HIV candidates.

6. RINGRAZIAMENTI

Eccomi arrivata alla parte più difficile della tesi…i ringraziamenti!

Ho scelto di spendere poche parole e di non dilungarmi troppo e ho deciso di concludere con le foto di coloro che hanno percorso con me questi tre meravigliosi anni di dottorato!

Quindi grazie a…

Giovanni

&

Federica

Daiana,

un'amica "scoperta" solo da poco... che spero di continuare a "scoprire" anche in futuro

Ai miei genitori, che spero di rendere sempre orgogliosi.

Il vostro appoggio sarà fondamentale per le scelte che dovrò prendere in futuro…perché questo è solo l'inizio del mio cammino!

Concludo con coloro che mi hanno vista ridere, a volte piangere, crescere e cambiare. Grazie ad Albi e Diego per esserci stati sempre!

Vi voglio bene!

7. PUBLICATIONS

1. Pollastro F, Caprioglio D, Del Prete D, Rogati F, Minassi A, Tagliatela-Scafati O, Munoz E, Appendino G, Cannabichromene, ***Nat. Prod. Commun***., 2018, 13 *(9),* 1189- 1194
2. Rogati F, Cruz C, Prados M. E, Galera N, Jinénez C, Appendino G, Bellido M. L, Calzado M. A, Caprioglio D, Muñoz E, Minassi A, Triterpenoid Hydroxamates as HIF Prolyl Hydrolase Inhibitors, ***J. Nat. Prod.,*** 2018, 81 *(10),* 2235- 2243.
3. Rogati F, Millan E, Appendino G, Correa A, Caprioglio D, Minassi A, Munoz E, ***ACS Med. Chem. Lett.,*** 2019, DOI: 10.1021/acsmedchemlett.8b00604

8. POSTERS AND ORAL COMMUNICATIONS

1. Poster “Synthesis of triterpenic acid derivatives containing chelating groups” presented at XLII Attilio Corbella Summer School On Organic Synthesis, Gargnano (BS), 18-22 June 2017
2. Poster “Synthesis of triterpenic acid derivatives containing chelating groups” presented at International Summer School of Natural Product ISSNP, Napoli, 3-7 July 2017. Winner of the 3rd place poster award.
3. Oral presentation: “Strigoterpenes: a new class of compounds with cross-kingdom action?” At XLIII edition of *Attilio Corbella* International Summer School On Organic Synthesis (ISOS 2018), Gargnano (BS), 10-14 June 2018
4. Poster “Deoxygenation Of Ursolic, Oleanolic And Betulinic Acid To Their Corresponding C-28 Methyl Derivatives (α-amyrin, β-amyrin, Lupeol)” at XII Spanish-Italian Symposium on Organic Chemistry (SISOC-XII), Ferrara, 2-4 July 2018
5. Poster “Triterpenoid hydroxamates as HIF Prolyl Hydrolase inhibitors” at XXXVIII Convegno Nazionale della Divisione di Chimica Organica della Società Chimica Italiana (CDCO 2018). Milan, 9-13 September 2018
6. Oral presentation ‘’Strigoterpenes, a class of cross-kingdom stress response modulators’’ at 4th Sino-Italian Symposium on Bioactive Natural Products (SISBNP 2018). Turin, 4-5 October 2018
7. Oral presentation “Triterpenoid hydroxamates as HIF Prolyl Hydrolase inhibitors” at MERCK & ELSEVIER Young chemists symposium. Rimini, 19-21 November 2018

9. DIDACTIC SCTIVITIES

Schools and congresses:

1. XLI A. Corbella “International Summer School on Organic Synthesis”, Gargnano (BS), 12-17 June 2016
2. XLII A. Corbella “International Summer School on Organic Synthesis”, Gargnano (BS), 18-22 June 2017 (32 CFU)
3. Second edition “International Summer School of Natural Product ISSNP”, Napoli, 3-7 July 2017 (32 CFU)
4. “XLIII edition of *Attilio Corbella* International Summer School On Organic Synthesis” (ISOS 2018), Gargnano (BS), 10-14 June 2018
5. “XII Spanish-Italian Symposium on Organic Chemistry” (SISOC-XII), Ferrara 2-4 July 2018
6. “XXXVIII Convegno Nazionale della Divisione di Chimica Organica della Società Chimica Italiana” (CDCO 2018). Milan, 9-13 September 2018
7. “4th Sino-Italian Symposium on Bioactive Natural Products” (SISBNP 2018). Turin, 4-5 October 2018
8. “MERCK & ELSEVIER Young chemists symposium”, Rimini, 19-21 November 2018

Seminars and classes attended:

1. Corso di Piante medicinali, Prof. Federica Pollastro (Dipartimento di Scienze del Farmaco Novara), 2016
2. "Molecular properties in drug discovery", Prof. Giulia Caron (University of Torino), Novara, 11th February 2016.
3. "Asymmetric Hydroxylative Phenol Dearomatization Reactions using Chiral Iodanes", Prof. Stéphane Quideau (University of Bordeaux), Novara, 25th February 2016.

4. Corso base sui brevetti e sulla valorizzazione della ricerca scientifica, Scuola di Medicina, Novara, 29th January 2016.
5. Theoretical and practical course ''How to structure, write and assess a scientific project" with Prof. Cesare Patrone, Dipartimento di Scienze del Farmaco Novara.
6. Using Deuterium in Drug Discovery, Prof. Pirali, 21th June 2018.
7. Structural Biology and enzymology in drug discovery, Prof. Rizzi, 8th February 2018.
8. The click chemistry in medicinal chemistry: from drug discovery to bioconjugation and in vivo imaging, Prof. Tron, 11th July 2018.
9. Transannular cyclization reactions: a shortcut in the total synthesis of natural product, Prof. Minassi, 10th April 2018.

10. CURRICULUM VITAE

Federica Rogati was born in 1990 in Borgomanero (NO, Italy). After graduating from high school at "Contessa Tornielli Bellini" institute in Novara, she started her university studies in 2009 in Medicinal Chemistry and Technology at the University of Eastern Piedmont Amedeo Avogadro (department of Pharmacy), Novara.

She graduated with honors in March 2015 with a thesis in medicinal chemistry in the laboratories of Prof. Gian Cesare Tron.

After obtaining the qualification to practice as a pharmacist in June 2015, she obtained a six months fellowship in the laboratory of Prof. Giovanni Battista Appendino.

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