University of Szeged

Faculty of Pharmacy

Institute of Pharmaceutical Chemistry

Synthesis and application of steviol-based regioisomeric 1,3-aminoalcohols and aminotriols

Ph.D. Thesis

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„The reward of the young scientist is the profound thrill of being the first person in the history of the world to see or understand something. Nothing compares to that experience.”

Dr. Cecilia Payne

(British-American astrophysicist, 1900–1979)

Table of contents

List of publications and lectures

Papers related to the thesis

I. **Dorottya Bai**, Zsuzsanna Schelz, Dóra Erdős, Anna K. Kiss, Viktória Nagy, István Zupkó, György T. Balogh, Zsolt Szakonyi
Stereoselective Synthesis and Antiproliferative Activities of Tetrafunctional Diterpene Steviol Derivatives
Int. J. Mol. Sci, **2023**, *24*, 1121, IF: 4.9
DOI: 10.3390/ijms24021121

II. **Dorottya Bai**, Zsuzsanna Schelz, Mária Fanni Boncz, István Zupkó, Zsolt Szakonyi
Stereoselective Synthesis and Antiproliferative Activity of Steviol-Based Diterpene 1,3-Aminoalcohol Regioisomers
Molecules, **2023**, *28*, 7962, IF: 4.2
DOI: 10.3390/molecules28247962

Scientific lectures

1. **Dorottya Bai**
Bi- és tetrafunkciós diterpén szteviol származékok sztereoszelektív szintézise
Szegedi Ifjú Kémikusok Támogatásáért Alapítvány előadóülése
Szeged, Hungary, 25 May, 2021, virtual conference, oral presentation

2. **Dorottya Bai**
Bi- és tetrafunkciós diterpén szteviol származékok sztereoszelektív szintézise
MTA Szteroid- és Terpenoidkémiai Munkabizottság előadóülése
Szeged, Hungary, 6 December, 2021, virtual conference, oral presentation

3. **Dorottya Bai**, István Zupkó, Zsolt Szakonyi
Bi- és tetrafunkciós diterpén szteviol származékok sztereoszelektív szintézise
XXV. Tavaszi Szél Konferencia
Pécs, Hungary, 6–8 May, 2022, poster

4. Zsolt Szakonyi, Dániel Ozsvár, **Dorottya Bai**, Viktória Nagy, István Zupkó
Stereoselective Synthesis and Antiproliferative Activity of Steviol and Isosteviol-Based Bi- and Trifunctionalized Diterpenoids
Southern Brazilian Journal of Chemistry 2021 Virtual Conference
Szeged, Hungary, 7 March, 2022, vitrual conference, oral presentation

5. **Dorottya Bai**, Zsuzsanna Schelz, István Zupkó, György T. Balogh, Zsolt Szakonyi
Antiproliferatív hatású diterpén 1,3-aminoalkoholok és aminotriolok sztereoszelektív szintézise
Gyógyszerkémiai és Gyógyszertechnológiai Szimpózium '22
Herceghalom, Hungary, 19–20 September, 2022, oral presentation

6. Zsolt Szakonyi, Zein Alabdeen Khdar, **Dorottya Bai**, Dániel Ozsvár
   Stereoselective synthesis of steviol-, isosteviol-, and allo-gibberellic acid-based bi- and trifunctionalized diterpenes
   The Polish Pharmaceutical Society Krakow Branch Meeting
   Krakow, Poland, 25 May, 2023, oral presentation

7. **Dorottya Bai**, Zsuzsanna Schelz, István Zupkó, Zsolt Szakonyi
   Antiproliferatív hatású ent-beyerán-vázas 1,3-aminoalkoholok sztereoszelektív szintézise
   MTA Heterociklusos és Elemorganikus Kémiai Munkabizottság előadóülése
   Balatonszemes, Hungary, 31 May – 2 June, 2023, oral presentation

8. **Dorottya Bai**, Zsuzsanna Schelz, István Zupkó, Zsolt Szakonyi
   Stereoselective synthesis and antiproliferative activity of bifunctional diterpene steviol derivatives
   22nd European Symposium on Organic Chemistry
   Ghent, Belgium, 9–13 July, 2023, poster

9. **Dorottya Bai**, **Zein Alabdeen Khdar**, Zsuzsanna Schelz, István Zupkó, Zsolt Szakonyi
   Diterpene-based aminoalcohols: synthesis and antiproliferative activity
   EUGLOH Annual Summit 2024
   Szeged, Hungary, 12 June, 2024, poster

List of abbreviations

ADME	absorption, distribution, metabolism, and excretion
ATP-ase	adenosine triphosphatase
BACE1	beta-site APP (Amyloid precursor protein) cleaving enzyme 1
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DMAP	4-dimethylaminopyridine
DMAPP	dimethylallyl pyrophosphate
DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulfonyl oxide
EtOH	ethyl alcohol (ethanol)
GGPP	2*E*,6*E*,10*E*-geranylgeranyl-pyrophosphate
HMBC	Heteronuclear Multiple Bond Correlation
HRMS	High Resolution Mass Spectroscopy
m-CPBA	*meta*-chloroperoxybenzoic acid
MeCN	acetonitrile
MeOH	methyl alcohol (methanol)
MsCl	methanesulfonyl chloride (mesyl chloride)
NBS	*N*-bromosuccinimide
NCS	*N*-chlorossuccinimide
NMR	Nuclear Magnetic Resonance Spectroscopy
PDC	pyridinium dichromate
PIDA	phenyliodine(III) diacetate
ppm	part per million
TBAB	tetrabutylammonium bromide
t-BuOH	*tert*-butyl alcohol
t-BuOOH	*tert*-butyl hydroperoxide
TCC	triclocarban
TEA	trimethylamine
TEMPO	(2,2,6,6-tetramethylpiperidin-1-yl)oxyl
THF	tetrahydrofuran
TMSI	trimethylsilyl iodide
p-TsCl	*para*-toluenesufonyl chloride (tosyl chloride)
p-TsOH	*para*-toluenesulfonic acid
TLC	thin-layer chromatography
$VO(acac)_2$	vanadyl acetoacetate

1. Introduction and aims

In the last couple of decades, the development of anticancer agents has been a significant focus of research for scientists worldwide. The challenges of evolving multidrug resistance and still high mortality rates demand new approaches in drug design [1–3]. Latest studies pay substantial attention to the synthesis of diterpene-based compounds due to their promising bioactivity [4–12].

Terpenoids are the largest, structurally diverse class of chiral products built of isoprene units found in most plants. Therefore, these molecules are easily available directly from their natural sources or by large-scale preparations. In organic syntheses they are often chosen as starting materials for asymmetric transformations as well as chiral catalysts in enantioselective reactions [13–18]. Several investigations have confirmed the multifaceted pharmacological properties exhibited by these compounds. These include antibacterial, antihyperglycemic, anti-inflammatory, cardiovascular protective and, most importantly, antitumor activities [4,19–26]. Additionally, some heterocyclic derivatives display antifungal, BACE1-inhibiting and antiproliferative action on a panel of human cancer cell lines [15,27–30]. Terpenes mainly contribute to the depolarisation of the cancer cell membrane, and to the activation of apoptosis in the membrane of mitochondria via caspases or the inactivation of the PI3K/Akt/NF-κB pathway, along with the inhibition of angiogenesis [31].

As a result of their coordination capacity and high affinity towards chiral building blocks of cells (e.g. amino acids) through polar functional groups, aminoalcohol derivatives have recently earned great interest. Bioactive aminoalcohols of natural origin, such as pactamycin, an antibiotic with antiproliferative properties [32], the immunosuppressant antibiotic and antifungal myriocin [33,34], or the actomyosin ATP-ase activator penaresdin A and B [35] for the treatment of Alzheimer's disease, are widely studied. The newest generation of terpenoid-type aminoalcohols are derived from commercially available monoterpenes like (–)-isopulegol and α- or β-pinene [36–39]. One of the few chiral sources of diterpenes that are accessible in large scale is stevioside, a triglycoside isolated on an industrial scale from the perennial herbal shrub *Stevia rebaudiana* [40]. It is frequently used for the synthesis of cytotoxic diterpenoid derivatives, since it can be easily transformed into aglycons, steviol and isosteviol, which show similar biological effects [4].

The Institute of Pharmaceutical Chemistry has a long history of research related to mono- and diterpenes, focusing on the synthesis and pharmacological evaluation of steviol- and isosteviol-based aminoalcohol and aminodiol derivatives in the past years. Numerous compounds with *ent*-kaurane or beyerane skeleton were found to express high inhibition of cancer cell growth on human cell lines, especially those that bear an *N*-benzyl moiety [9,10].

Our aim in my PhD work was to synthesise a versatile, novel library of 1,3-aminoalcohols, and by inserting a third hydroxy group, to form aminotriols starting from steviol through Wagner–Meerwein rearrangement and *spiro*-epoxide formation, respectively. In addition, considering their complex biological benefits, we intended to expand our study with heterocyclic derivatives via click reaction. Furthermore, we planned to investigate the antiproliferative activity of the new compounds *in vitro* on human gynaecological cancer cell lines (HeLa, SiHa, A2780, MCF-7, MDA-MB-231) through collaboration, to compare the results and to determine the structure–activity relationship.

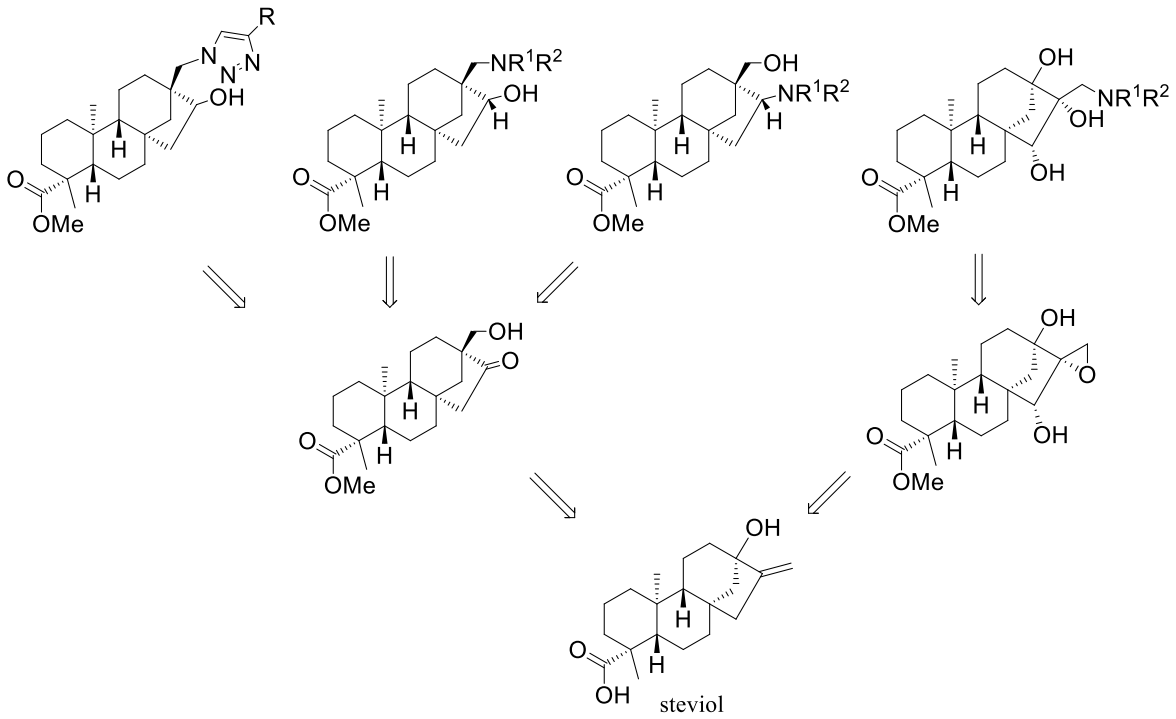


Figure 1: Retrosynthetic pathways for the synthesis of 1,3-aminoalcohols, aminotriols and triazoles

2. Literature Survey

2.1. Introduction of diterpenoids

Terpenoids form the basis of many essential oils, pigments and steroids. These diverse hydrocarbon compounds are classified based on the number and structural organisation of carbons formed by the linear arrangement of isoprene units (2-methylbuta-1,3-diene (C_5H_8)), the "building block" of terpenoids. It is often followed by cyclisation and rearrangements of the carbon skeleton with an empirical feature known as the isoprene rule [31]. Diterpenoids consist of four units of the isoprene structure and regarding the degree of cyclisation, they can be further assorted into subclasses of acyclic, mono-, bi- and tricyclic (e.g. taxanes, like Paclitaxel and docetaxel, which are widely used agents in chemotherapy) and tetracyclic (beyerenes, kaurenes) compounds. Many terrestrial and marine organisms can serve as natural sources for diterpenes, where they usually reside in polyoxygenated form bearing keto and hydroxy groups, the latter regularly esterified by small acid molecules [41,42].

There are three known pathways for the synthesis of primary isoprenyl fragments, the basis of all isoprenoid type compounds. These are the mevalonate, mevalonate-independent, and amino acid routes [43,44]. Since the mevalonate path is prevalent in nature, it is the one presented henceforth. *R*-(+)-Mevalonic acid, the universal biogenic precursor for most of the isoprenoids, is formed by the interaction of acetoacetyl-coenzyme A and acetyl-CoA (**Figure 2**). Phosphorylation and elimination reactions provide 3-isopentenyl pyrophosphate (IPP, also called "active isoprene") besides CO_2 and water and then enzymatic isomerisation results in dimethylallyl pyrophosphate (DMAPP). These two molecules are the primary building blocks for the biosynthesis of nearly every known isoprenoid as of present.

The biosynthesis starts with the reaction of IPP and DMAPP through nucleophilic substitution of the pyrophosphate group in DMAPP by the terminal olefin carbon of IPP. Further transformations take place with the involvement of geranylgeranyl diphosphate synthase, resulting in geranyl pyrophosphate (GPP), a precursor of all monoterpenoids. Next, farnesyl pyrophosphate (FPP) is synthesised in the reaction of GPP and IPP, from which all triterpenoids and steroids originate. Finally, farnesyl pyrophosphate reacts with another IPP molecule, providing geranylgeranyl pyrophosphate (GGPP), the precursor to diterpenoids and carotenoids [41].

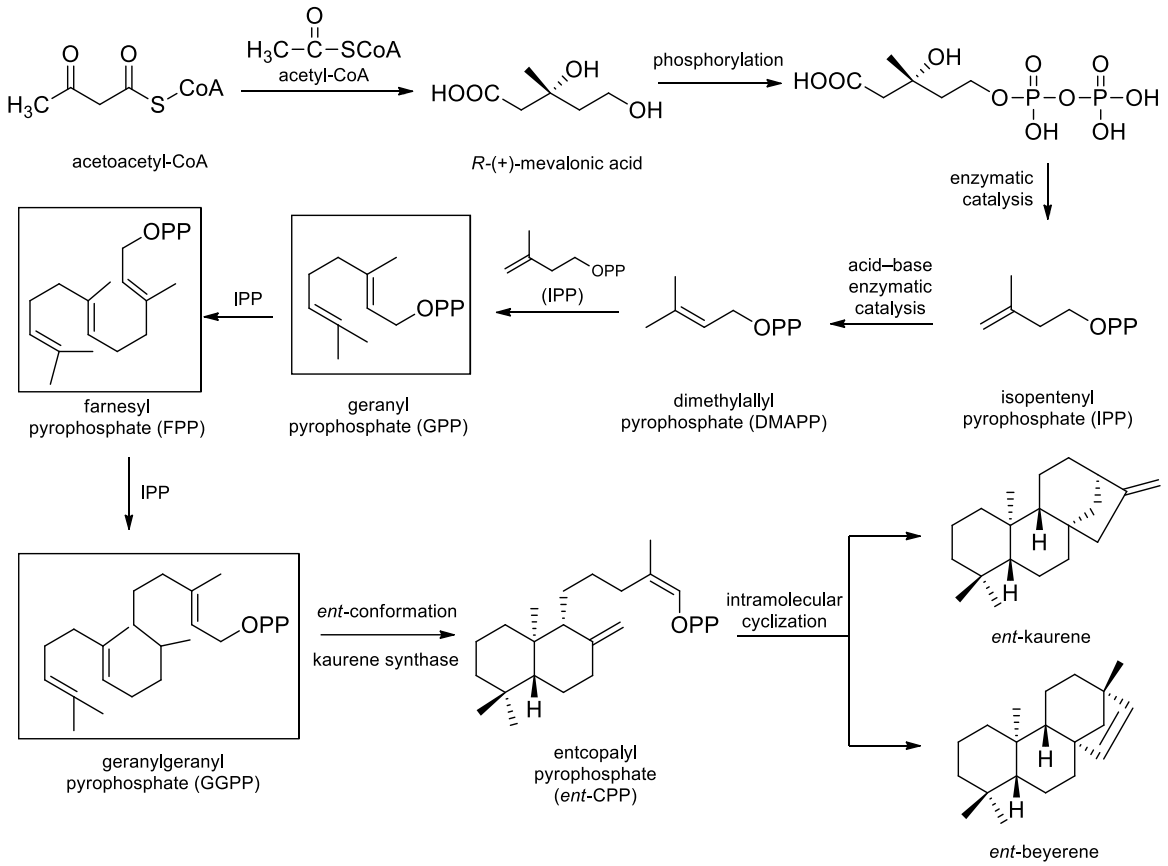


Figure 2: Biosynthesis of diterpenoids through the mevalonic pathway

We should consider the GGPP biotransformation pathway leading to tetracyclic diterpenoids, when the cyclisation begins with the protonation of the C_{14}–C_{15} double bond, then nucleophilic attacks of C_{10} on C_{15} and C_7 on C_{11} follow. The resulting conformations induce the synthesis of the diterpene *ent*-kaurene, which has the "enantio" (*ent*) conformation (**Figure 2**). The synthesis entails the concerted cyclisation sequence of geranylgeranyl pyrophosphate (GGPP) by the enzyme kaurene synthase, affording *ent*-copalyl pyrophosphate (*ent*-CPP). The latter undergoes intramolecular cyclisation and there are two possibilities for the stabilisation of the generated carbocation that could form either *ent*-kaurene or *ent*-beyerene [41]. These structures are the building blocks of several natural, diverse bioactive diterpenes, such as the plant growth hormone gibberellic acid [45], anti-inflammatory oridonin [46], or the antitumorous sweetener stevioside [4], as well as synthetic anticancer agents prepared in enantioselective syntheses [47].

2.2. *Ent*-kaurene glycosides and their biological activity

Glycosides are built of an aglycone bonded to one or more carbohydrate moieties, which may vary from monosaccharides to polysaccharides. The aglycone can be a simple structure, such as short aliphatic alcohols or fatty acids, as well as more complex molecules, e.g. terpenoids, carotenoids, steroids, flavonoids, or alkaloids etc. Glycosides can be found in almost all living organisms, particularly in plants, where they are responsible for functions related to accumulation, storage and transport of hydrophobic substances. They have greater water solubility and lower reactivity than the free aglycones, which help protecting the organism from the toxicity of the skeletal molecule. The *ent*-kaurene-type glycosides carry an aglycone with a kaurenic structure called steviol. They have been studied in great depth due to their toxicity, therapeutic potential and economic value. The main examples of kaurenic glycosides are atractyloside **1**, carboxyatractyloside **2**, wedeloside **3** and stevioside **4** (**Figure 3**) [48].

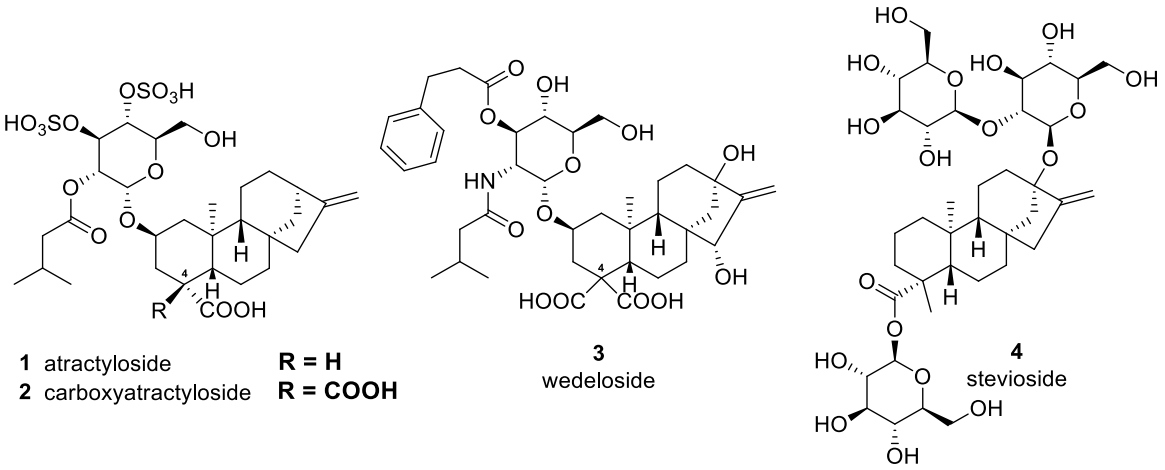


Figure 3: Bioactive *ent*-kaurene glycosides

Atractyloside **1** was initially isolated from the rhizomes of *Atractylis gummifera* L. and later from *Wedelia glauca* Ort. of the *Asteraceae* family in Argentina, Brazil and Uruguay. The enhanced bioactivity of the compound is due to the inhibition of mitochondrial oxidative phosphorylation, causing hypoglycemia, respiratory depression, nephrotoxicity, hypoxemia and cell injury in animals, as well as renal and hepatic failure in humans, which may be followed by necrosis [48].

Carboxyatractyloside **2** is also derived from *Atractylis gummifera* L., exerting toxicity 10 times higher than atractyloside in the *in vivo* assays, which is presumably the result of a second carboxylic function at C_4. The compound showed great inhibition of nucleotide

translation through the mitochondrial membrane and anticancer action against melanoma cells (100 μM) and Erlich tumour (3 μM), in addition to the established insecticidal and herbicidal activities [48].

Wedeloside **3** is an aminoglycoside isolated from *Wedelia asperrima* Benth., the sunflower daisy native to Australia. The acylaminedeoxyhexose unit linked to the diterpene makes this glycoside especially unique. The compound is considered to be the main toxic component of the plant and its bioactivity is comparable to that of carboxyatractyloside, as it causes the inhibition of mitochondrial ADP/ATP transport [48].

Stevioside **4** is a complex diterpenoid triglycoside molecule comprised of the *ent*-kaurene aglycone steviol and three molecules of glucose. The glycoside is extracted from the plant *Stevia rebaudiana* (Bertoni), a perennial herbal shrub of the *Asteraceae* family found in Paraguay and Brazil in South America. Diterpene glycosides make up from 4% to 20%, while stevioside itself accounts for 3–9% of the components in the dry leaf depending on the cultivar and growing conditions [48–51]. The minor steviol glycosides, such as rebaudioside A, differ from stevioside in the structure and the number of sugar moieties, while the aglycon part is the same. The more saccharid units there are, the sweeter the glycoside is. The sweet components of Stevia discovered as of today are shown in **Table 1**.

Table 1. Composition of glycosides obtained from *Stevia rebaudiana* [52–56]

Glycoside	R^1 (C_{13})	R^2 (C_{19})	m_{tot} (%)[a]
Stevioside	Glc(β1-2)Glc(β1)-	Glc(β1)-	9.1
Steviolbioside	Glc(β1-2)Glc(β1)-	H	0.1
Rebaudioside A	Glc(β1-2)[Glc(β1-3)]Glc(β1)-	Glc(β1)-	3.8
Rebaudioside B	Glc(β1-2)[Glc(β1-3)]Glc(β1)-	H	ND
Rebaudioside C	Rha(α1-2)[Glc(β1-3)]Glc(β1)-	Glc(β1)-	0.6
Rebaudioside D	Glc(β1-2)[Glc(β1-3)]Glc(β1)-	Glc(β1-2)Glc(β1)-	0.2
Rebaudioside E	Glc(β1-2)- Glc(β1)-	Glc(β1-2)Glc(β1)-	0.2
Rebaudioside F	Xyl(β1-2)[Glc(β1-3)]Glc(β1)-	Glc(β1)-	0.2
Rebaudioside M	Glc(β1-2)[Glc(β1-3)]Glc(β1)-	Glc(β1-2)[Glc(β1-3)]Glc(β1)-	ND
Rebaudioside VIIIa	$(Glc(\beta1\text{-}2))_2$-Glc(β1-3)Glc(β1)-	Glc(β1-2)[Glc(β1-3)]Rha(α1-3)Glc(β1)	ND
Rebaudioside VIIIb	Glc(β1-2)[Glc(β1-3)] Rha(α1-3)Glc(β1)	Glc(β1-2)[Glc(β1-3)]Rha(α1-3)Glc(β1)	ND
Dulcoside A	Rha(α1-2) Glc(β1)-	Glc(β1)-	0.3

[a] % in dry matter

Glc: D-glucose; Rha: L-rhamnose; Xyl: D-xylose; ND: not determined

Stevia leaves, stevioside and highly refined extracts are considered to be low-calorie commercial sweeteners, being 250 to 300 times sweeter than sucrose without showing cariogenic activity. Thus, it is frequently used in dental products, such as toothpaste or mouth wash apart from food and beverages [4,48,51,52]. Besides its application in the food sector, the glycoside exhibits therapeutic properties, e.g. antihypertensive [57,58], antihyperglycemic [59,60], anti-inflammatory [61–63] and antitumour [64–66] activities. Since cancer remains a global health issue, taking lives with the highest rate of all diseases, the antiproliferative activity of stevioside and its derivatives has been widely studied in recent years. Breast cancer is one of the most common type of cancer, affecting millions of women up to this day; therefore, research in this field is of great importance. The first indication of stevioside's mammary anticancer action was found by Toyoda et al. in the 1990's when investigating the carcinogenic effects of the glycoside. The occurrence of spontaneous adenomas in the breasts of female F344 rats was found to decrease upon the treatment [67].

Following the initial results, studies concerning the bioactivity of steviol glycosides were extended. Paul et al. in 2012 observed *in vitro* cytotoxic effect of stevioside on human breast cancer cell line MCF-7 in time- and dose-dependent manner at 2.5–30 μM concentrations. The scientists conducted cell cycle studies that showed the increase of the cell population in the G0/G1 phase. Since the cells were not in a replication or in a resting phase, it was concluded that the mechanism ended in cellular death. The hypothesis was proved by Annexin V staining, which revealed ROS-mediated (reactive oxygen species) apoptosis at 10 μM of stevioside. After 72 hours the apoptotic cells made up around 70% of the complete cell population [65].

In a different study, Khare et al. tested the antiproliferative effects of stevioside on the MDA-MB-231 and the $HER2^+$ SKBR-3 breast cancer cells at 5–100 μM concentrations for 48 hours. Cell viability decreased by 60% in both cell lines, while their chemosensitivity to chemotherapy drug 5-Fluorouracil (5-FU) amplified. It was also revealed by further investigation that the combined treatment with the two compounds generated increase in the Bax/Bcl-2 protein ratio. As the Bax protein is responsible for inducing cell death and Bcl-2 for inhibiting the activity of Bax, the increase of their ratio leads to Bax being overexpressed, triggering apoptosis [68].

Besides mammary cell lines, gastrointestinal and other types of tumours were also assessed. Ren et al. reported antiproliferative effect of stevioside on human colon cancer HT-29 cells in a dose-dependent manner at 48 and 72 hours, with cell cycle arrest at the

G2/M phase. Additionally, Caspase-9 and Caspase-3 activities as well as ROS production increased. Monitoring the mitochondrial membrane potential, the downturn of numbers was taken as evidence that the apoptotic effect is activated through the mitochondrial-mediated pathway [66]. Chen et al. found similar results regarding cancerous bladder cells. Stevioside selectively induced mitochondrial stress and apoptosis by ROS accumulation and activating the Bax protein without affecting normal cells. The glycoside was also found to be preventing tumour cell growth *in vivo* in xenograft models [69]. Proliferation of PC-3 prostatic adenocarcinoma cells was also significantly suppressed by stevioside as found by Raj et al. The expression of Caspase-3 increased while the Bcl-2 gene, which is supposed to inhibit Bax, decreased giving way to the apoptosis [70]. Stevia glycosides were also assayed on Epstein–Barr virus early antigen (EBV-EA) activation, where stevioside exhibited strong inhibition without noteworthy toxicity on Raji cells [64]. The Epstein–Barr virus is a double-stranded DNA virus from the family of γ-herpesviruses, and the first identified oncogenic virus, which causes infectious mononucleosis in humans, directly associated with many malignant diseases. The results are exceptional as none of the attempted vaccine formulations proved to be successful so far [71].

2.3. Transformations of stevioside

The chemical transformations of stevioside, as opposed to its aglycone, are quite limited in the literature. The main reason for this is probably the hydrophilic character of the glycoside, which restricts the selection of organic solvents typically used for syntheses. The majority of the articles document transglycosylation reactions, which is the transfer of one or more glucose units to stevioside. The unmodified glycoside has a bitter aftertaste; therefore, the aim of these procedures is to improve the general sweet taste of steviol glycosides. The first modification of this kind was reported by Kaneda et al. in 1977, when the group accomplished the transformation of stevioside to Rebaudioside A. [72]. Recently, the enzymatic reactions by cyclodextrin glucanotransferase (CGTase) gained more attention [73,74]. An example of a mono-glycosylated derivative by Yu et al. is presented on **Figure 4** [75].

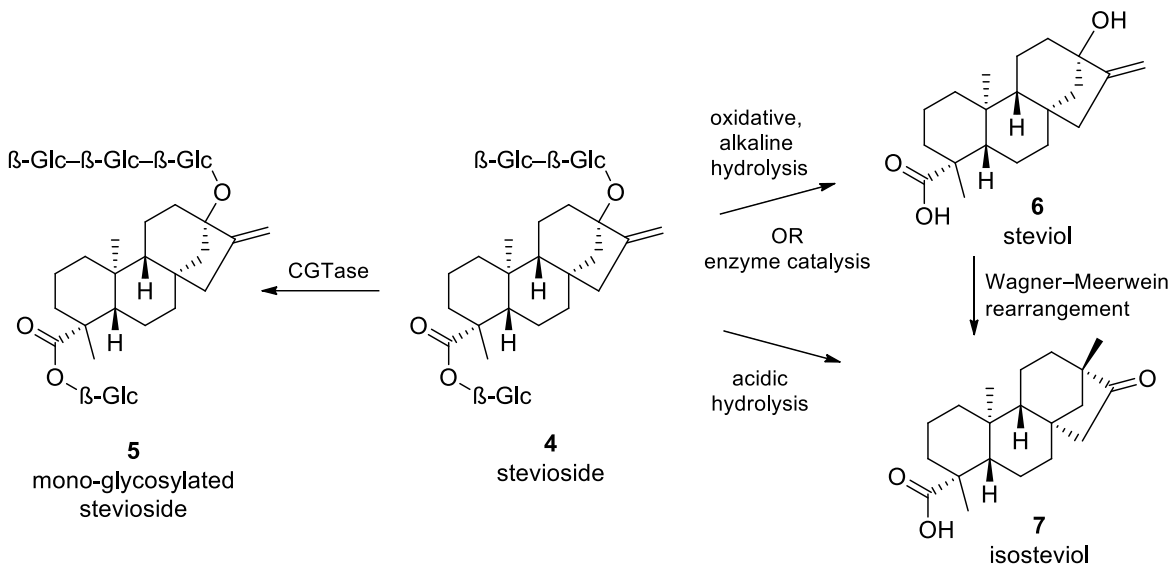


Figure 4: Enzymatic and chemical transformations of stevioside

The other possibility for modification of stevioside is removing the glycose units completely either by enzymatic or chemical methods. The first enzymatic synthesis of steviol **6** (**Figure 4**) from glycosides, described by Bridel and Lavieille dates back to 1931, later optimised by Mosettig et al. in 1955. They reported a yield of 54% using enzymes derived from the snail *Helix Pomatia* [76]. Pezzuto et al. (1985) [77] and more recently Takasaki et al. (2009) experimented with pectinases, that produced steviol in 68–78% yield [64]. As an alternative way, the enzymatic reaction was performed with hesperidinase, providing a steviol yield of 66% [78,79]. Wan et al. used β-glucosidase for the synthesis but reached the same yield even after optimalisation [80]. The most efficient method to synthesise steviol up to this date uses a mixture of enzymes (pectinase, CYTOLASE PCL5®, and *Helix Pomatia* enzymes) resulting in a yield up to 98%. The patent was filed by DSM, a Dutch multinational corporation engaged in the fields of health, nutrition, and materials. Despite the delicate nature of the procedure, it was carried out on a multigram scale (>50 g) [74].

The conventional chemical procedure for the synthesis of steviol entails oxidation by sodium periodate in water then adding a large amount of a strong base (e.g. KOH) at reflux temperature. The synthesis developed by Ogawa et al. in 1980 gives a 56% yield of steviol [81]. This method has been used continuously over the years and despite efforts in improvement, higher yield could not be produced [82,83]. Shi et al. described a yield of 70%; however, they failed to mention details of corrections or experimental data to prove the results [84]. The procedure is rather challenging for organic chemists, especially

because the workup includes an acidification step by acetic acid. The acidic medium can induce an undesired Wagner–Meerwein rearrangement of steviol, that results in an *ent*-beyerane structure, providing isosteviol **7** [74,81]. Therefore, removing the glucose units of the steviol glycosides by acid is better to avoid during the synthesis of steviol. The traditional way of preparing isosteviol is relatively simple, as the glycoside only needs to be stirred at reflux temperature in the presence of hydrochloric acid, reaching a yield as high as 80% [79,83,85,86].

Appraising the versatile chemopreventive and antiproliferative aspects of stevioside, it can be considered a good lead compound in drug design for several reasons:

- it is commercially available for large-scale development
- the hydrolysis of stevioside provides the *ent*-kaurene diterpenoid steviol and the *ent*-beyerane-type isosteviol. These compounds have also been widely examined and they showed a similar range of pharmacological effects as the glycoside [87–90]
- the molecular weight of both steviol and isosteviol are 318.2, which is optimal for further structural modifications according to Lipinski's rule of five (the molecular weight must be lower than 500), since the molecular weight usually increases in the long process of a lead compound becoming a drug candidate [91]
- the diverse structures of steviol and isosteviol provide several reaction sites (carboxyl, hydroxy and carbonyl groups as well as double bond) to access a versatile library of new compounds through synthetic pathways
- toxicity tests have confirmed the non-toxic nature of the compounds especially at low doses [67,92–94].

2.4. Synthesis of diterpene derivatives with anticancer properties

Terpene-based synthetic research efforts have mainly centred around stereoselective transformations that result in O-, N- and occasionally S-functionalised compounds such as, but not limited to, aminoalcohols, in the hope of gaining antiproliferative activity. The significance of aminoalcohols in biochemical processes is unquestionable. Besides the crucial responsibility for cell growth in the cell membrane (e.g. sphingosine), aminoalcohols are important bioactive synthons in synthetic antihypertensive, respiratory, analgesic etc. pharmaceutical agents [95–99], as well as chiral catalysts and auxiliaries for asymmetric syntheses of new pharmacons [10,100–103]. Combining the benefits of

diterpenes and aminoalcohols could unlock the path to a new generation of anticancer drugs.

In the last decades, our research group has been focused on the synthesis of terpenoid-derived aminoalcohols and the examination of their biological activities through collaborations. Some of the synthesised monoterpene-based aminoalcohol and aminotriol derivatives were found to express potent antimicrobial effects, too. The compounds with isopulegol structure **8** and **9** showed high selectivity against *B. subtilis*, while modified pinane **10** caused strong inhibition of HeLa cell growth besides effectively targeting both *B. subtilis* and *P. aeruginosa* bacteria (**Figure 5**) [27,104,105]. The selected *allo*-gibberic acid-based derivatives **11** and **12** performed with noteworthy selectivity and outstanding activity on HeLa and MCF-7, respectively [11,12]. The discovered properties paved the way for further modifications.

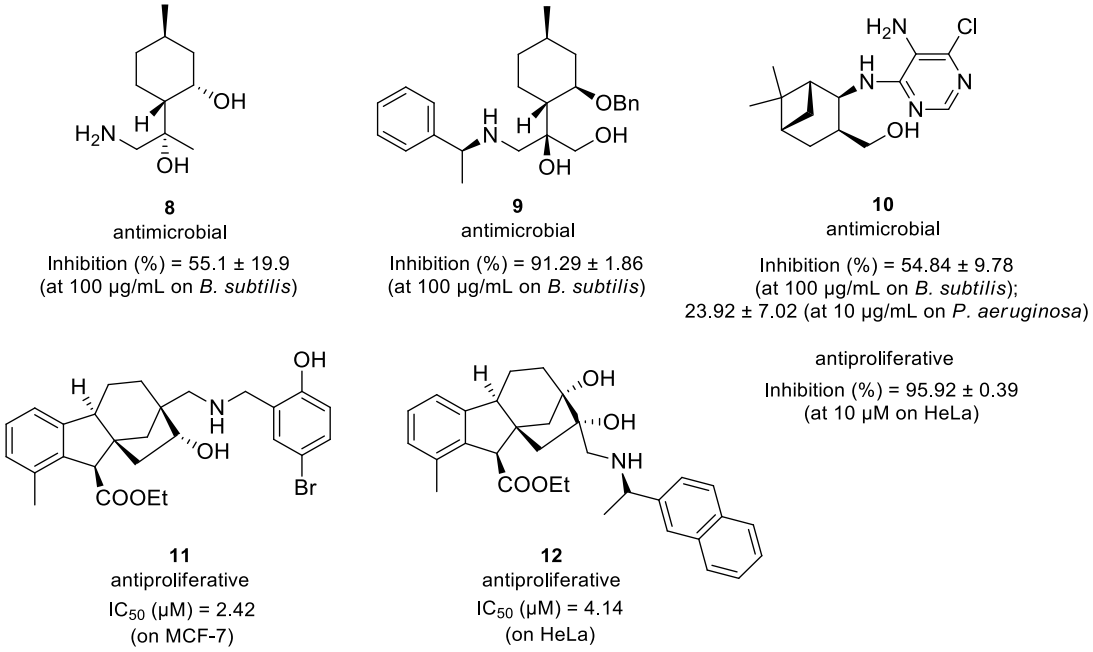


Figure 5: Mono-and diterpene-based aminoalcohols and aminodiols with antimicrobial and antiproliferative activity

2.4.1. Synthesis of *ent*-beyerane-type derivatives

Since the synthesis of isosteviol is rather rapid, easy and it provides high yield, the reactions of the diterpene and its derivatives have been studied more extensively. Zhang et al. synthesised a series of prolinamide, thiourea, aminoalcohol and *N*-acetyl derivatives from isosteviol and they evaluated their antitumour activities against human cancer cell lines *in vitro*. The primary 1,3-aminoalcohol was found to be exceptionally effective

against esophageal carcinoma (EC9706) with an IC_{50} value of 4.01 μM. To prepare the target compound, first esterification was carried out with CH_3CH_2Br and KOH in DMSO, which provided isosteviol ethyl ester in high yield (**Figure 6**). The corresponding diol was synthesised in a one-pot aldol-Cannizzaro reaction in the presence of formaldehyde and sodium ethylate. TEMPO-catalysed oxidation of the diol with *N*-bromosuccinimide resulted in aldehyde **15**, supposedly in 90% yield. The following oximation reaction of **15** with hydroxylamine hydrochloride gave compound **16**, which underwent Raney Ni-catalysed hydrogenation in THF to deliver primary 1,3-aminoalcohol **17** [106].

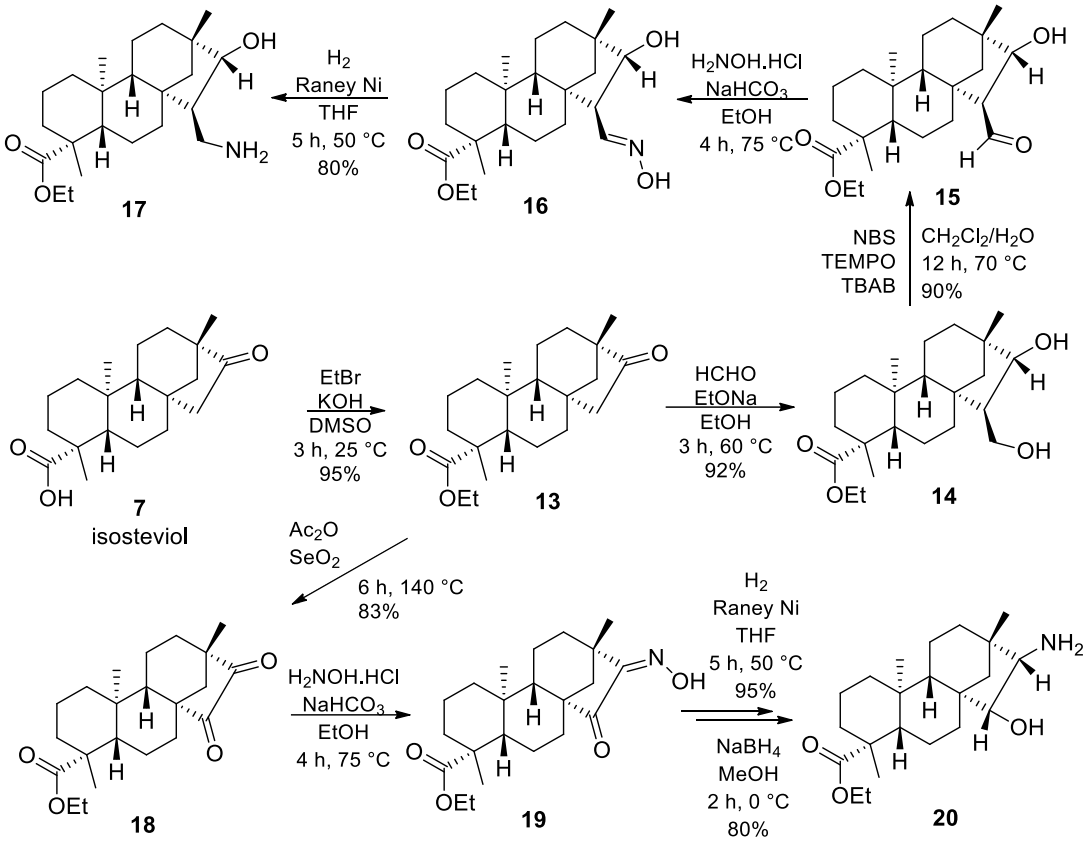


Figure 6: Synthesis of isosteviol-based primary 1,2- and 1,3-aminoalcohols

While the acylated products did not show noteworthy antiproliferative effects, the research group also prepared the 1,2-aminoalcohol affording moderate, slightly lower activity than the 1,3-isomer (IC_{50} = 19 μM) (**Figure 6**). The isosteviol ethyl ester was transformed into the diketone derivative upon treatment with acetic anhydride and selenium dioxide. Reaction with hydroxylamine hydrochloride afforded oxime **19**, then hydrogenation by Raney Ni and reduction with sodium borohydride led to primary 1,2-aminoalcohol **20** [106].

Liu et al. conducted a very similar research to synthesise some regioisomeric counterparts of the previous derivatives and the compounds were tested on different cell lines (HCT-116, HGC-27, JEKO-1). The most potent one was a thiourea derivative with *p*-NO_2 function on the benzene ring with an IC_{50} value of 1.45 μM against colon carcinoma (**Figure 7**). To prepare the compound, the diol synthesised according to the method by Zhang was oxidised to keto-alcohol by TCC and the carbonyl group was transformed into oxime by hydroxylamine hydrochloride. Reduction of the oxime with $NaBH_4$ and MoO_3 as catalyst gave primary aminoalcohol **23**. The appropriate derivative **24** was synthesised by 4-nitrophenyl isothiocyanate in dichloromethane at room temperature in one hour with 88% yield [107].

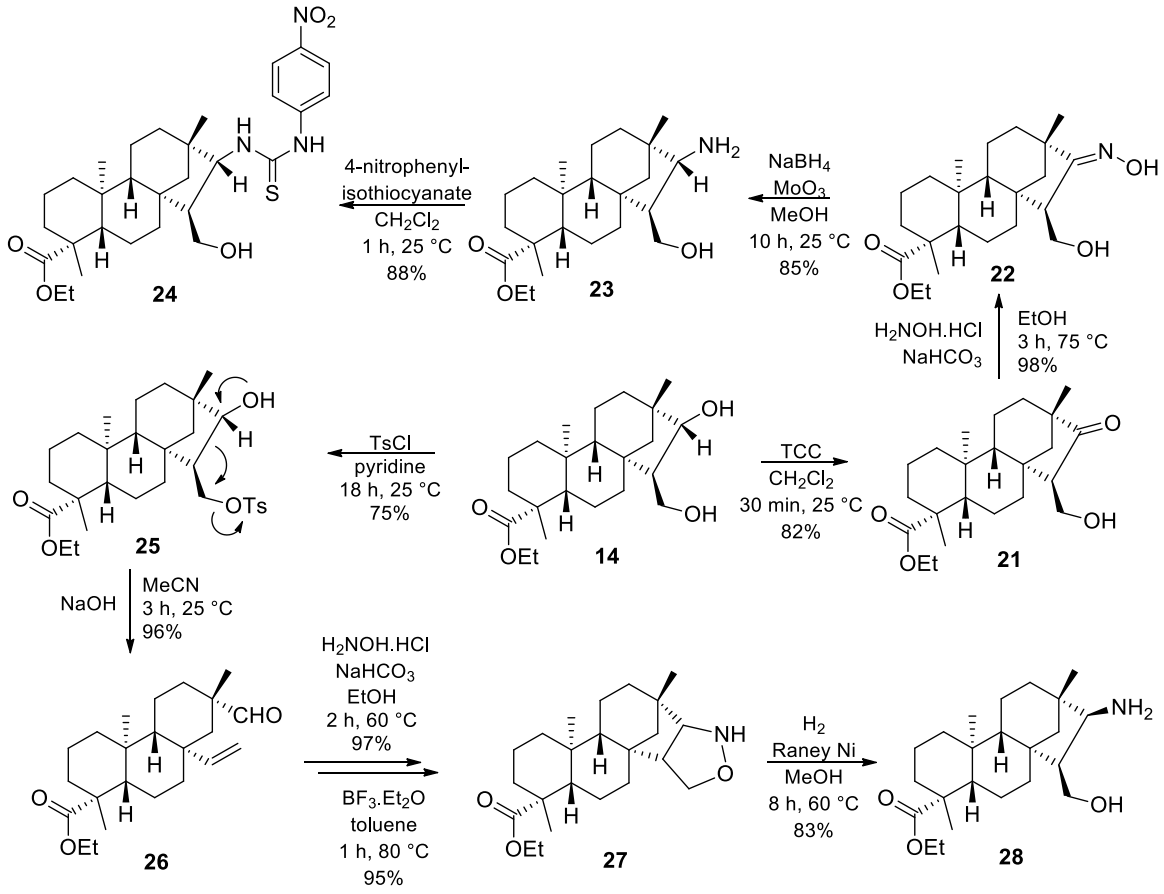


Figure 7: Preparation of regioisomeric thiourea derivative **24** and compound **28**

In similar nature, the bioactivity of the epimer of primary aminoalcohol **23** (**28**) was measured to be 4,9 μM of IC_{50} on HCT-116. The isomer was prepared through a five-step synthesis starting from diol **14** (**Figure 7**). Tosylation followed by Grob fragmentation resulted in the opening of ring D, leaving alkene and aldehyde functions at positions C_8 and C_{13} [108]. The ring was closed by Lewis acid-generated cyclisation of the oxime, and

at last, the isoxazolidine was converted to final product **28** by heterogeneous catalytic hydrogenation [107].

A series of amino- and keto-alcohols were prepared from isosteviol by Lohoelter et al. through epoxide intermediates [109]. Although the novel compounds have yet to be tested for cytotoxicity, the synthetic work is worth mentioning. The original aim of the group was to expand the D ring of the beyerane skeleton. In the first step an oxirane ring was introduced by the Corey–Chaykovsky reaction in a stereospecific manner (**Figure 8**). The acidic conditions applied next generated an undesired Wagner–Meerwein rearrangement and instead of the expected cyclohexanone, exclusive formation of aldehyde **31** was observed. As an alternative solution, the epoxide was treated with sodium azide to give β-azidoalcohol **33**, and then hydrogenation with palladium catalysis led to β-aminoalcohol **34**. This compound underwent the Tiffeneau–Demjanov rearrangement in the presence of acetic acid and sodium nitrite, providing cyclohexanone **35**. Bromination followed by treatment with sodium hydroxide in DMF resulted in a variety of products depending on the concentration of the applied alkaline solution. The structures were identified by single crystal X-ray analysis.

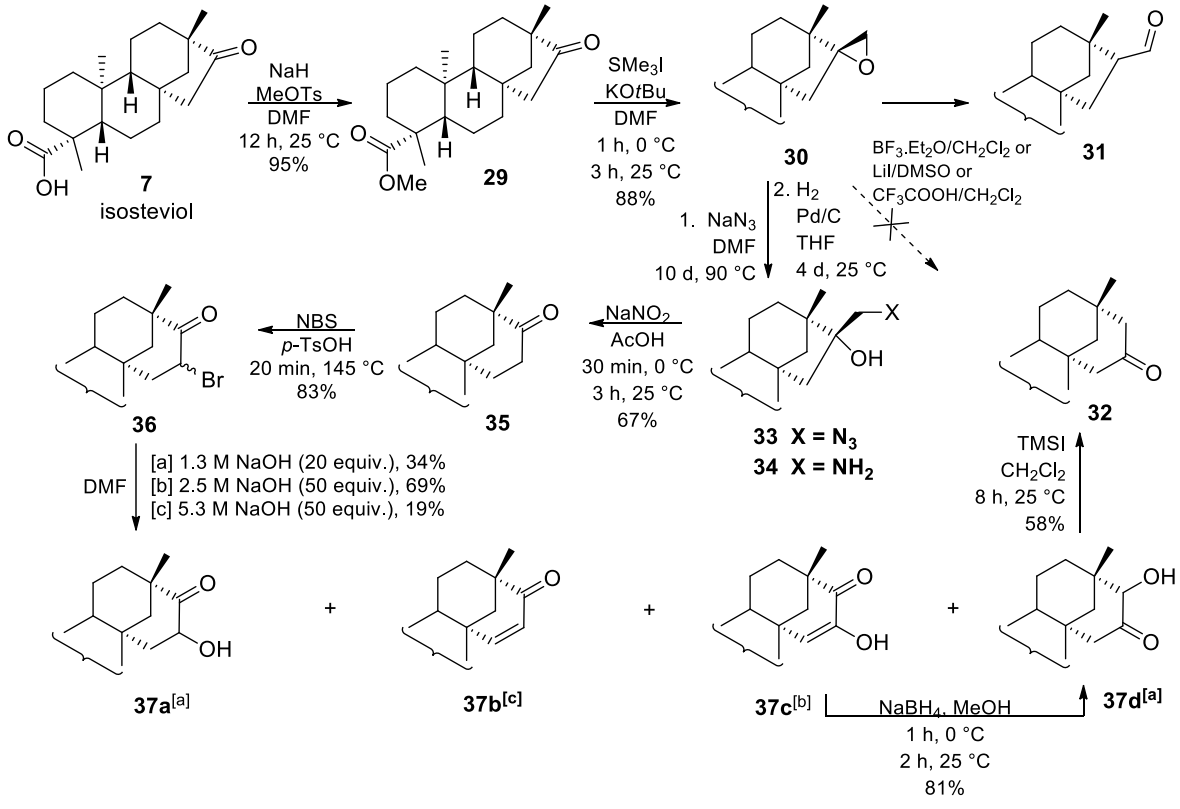


Figure 8: Synthesis of amino- and keto-alcohols derived from isosteviol

1,2-Diketone **38** was derived from isosteviol via Riley oxidation and according to the concentration of trimethylsulfonium iodide, the Corey–Chaykovsky procedure gave mono- and bis-epoxides **39** and **40** (**Figure 9**). Compound **39** was transformed into the corresponding azido- and aminoalcohol by the method mentioned previously. In the last step, the Tiffeneau–Demjanov rearrangement led to keto-enol **43** [109].

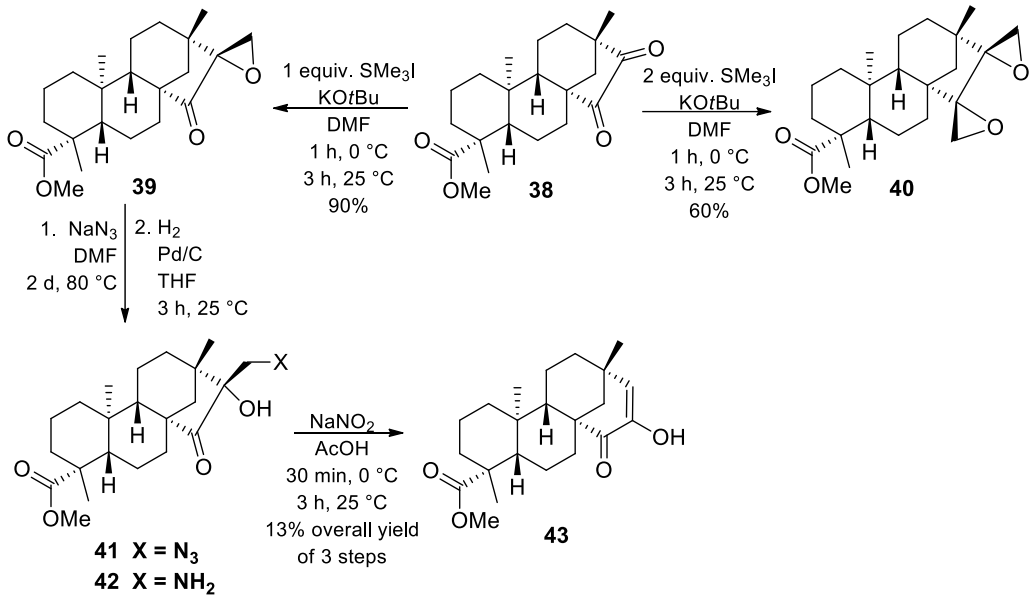


Figure 9: Expansion of ring D through 16-epoxy-15-oxo-derivative **39**

Wu et al. developed further modifications, going as far as synthesizing heterocyclic compounds such as condensed lactams and indoles to investigate their improved α-glucosidase inhibitor abilities. Studies showed the efficiency of α-glucosidase inhibitors in lowering the risk of colorectal cancer and cerebrovascular diseases in patients with diabetes mellitus [110]. The best-performing compound prepared by the Wu group was the ethyl ester of the indole derivative, which was obtained in the reaction of isosteviol ethyl ester and phenylhydrazine in acidic medium (**Figure 10**) [111]. On the other hand, Beckmann rearrangement of the oxime did not only provide the lactam but fragmentation also took place into olefinic nitrile products. The results were examined under different acidic conditions, but the nitrile compounds appeared simultaneously with the lactam each time. However, the reaction turned out to be selective for nitrile formation when applying H_2SO_4 in acetone at 40 °C, resulting in a 3:1 isomeric ratio. The compounds, due to the similar physical properties and polarities, could not be separated by chromatography techniques.

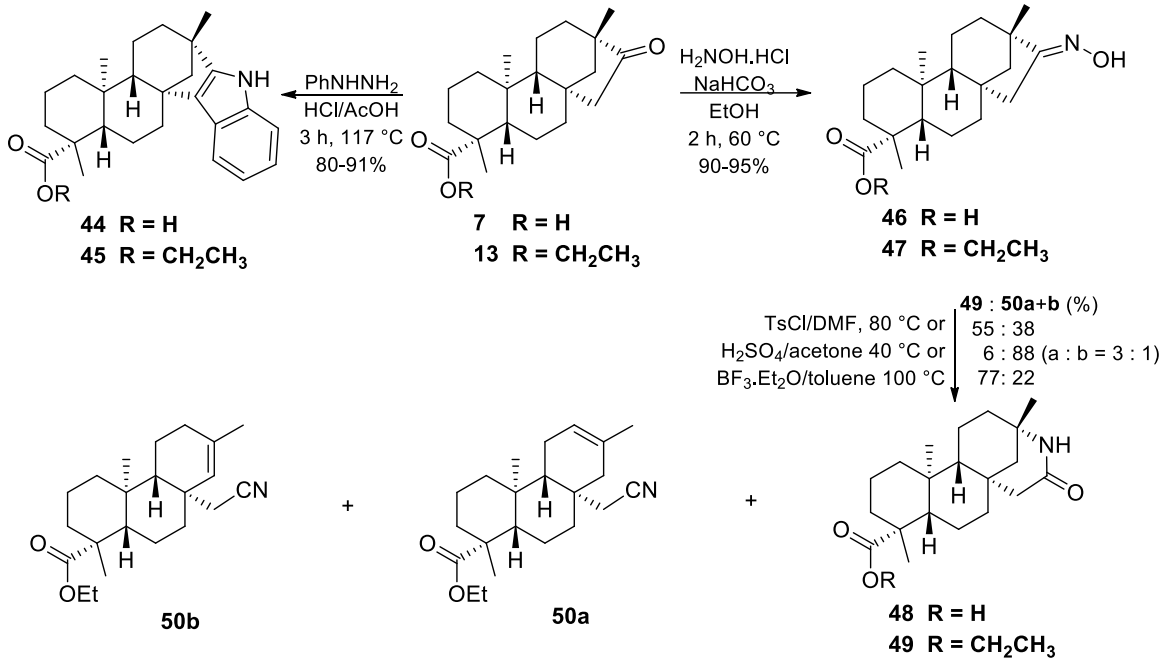


Figure 10: Isosteviol-based bioactive heterocyclic derivatives

As seen in previous examples, our research group shows great interest in the synthesis of aminoalcohols with potential antiproliferative activity, derived from natural terpene molecules. In the field of isosteviol- and steviol-based transformations our group carries out pioneer research on aminoalcohol derivatives as it is quite rare in literature. The first project covered the structural modifications of isosteviol to gain cytotoxic 1,3-aminoalcohols. The diol was prepared in a stereoselective aldol-Cannizzaro reaction from the beyerane molecule shown above, then diazomethane in diethyl ether was added to produce the methyl ester in high yield in a few of minutes (**Figure 11**). The aldehyde was synthesised in a regioselective, NCS-mediated oxidation in the presence of TBAB and TEMPO. In the following steps oximation with hydroxylamine hydrochloride and Raney Ni-catalysed hydrogenation provided the primary 1,3-aminoalcohol. A selection of primary amines and aldehydes were used to prepare derivatives **56a**–**f** either from aldehyde **53** or aminoalcohol **55** through Schiff bases [9].

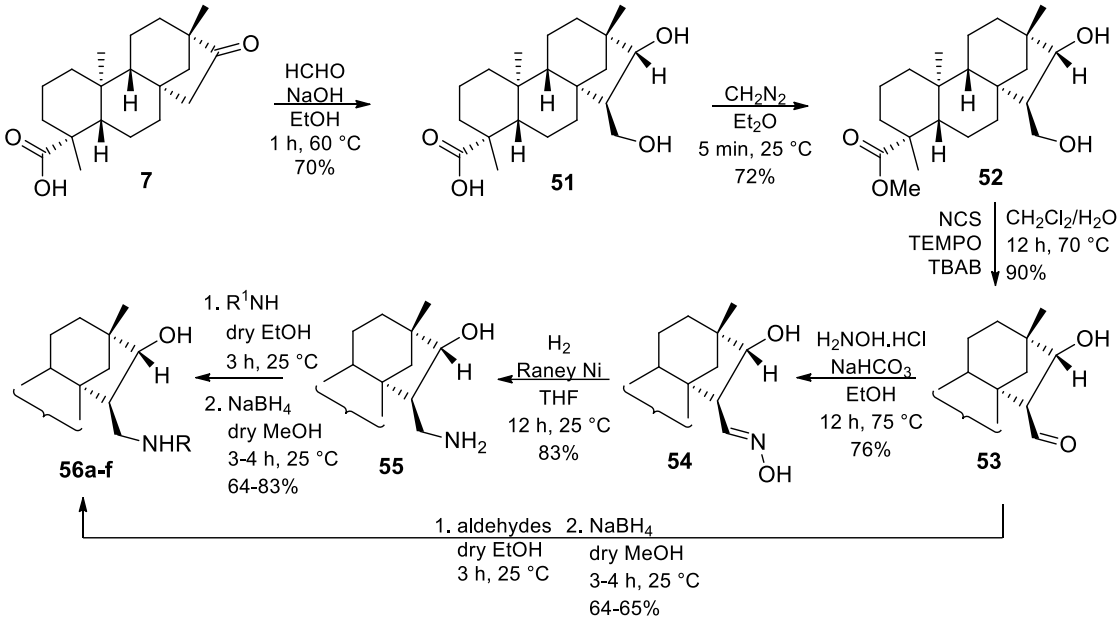
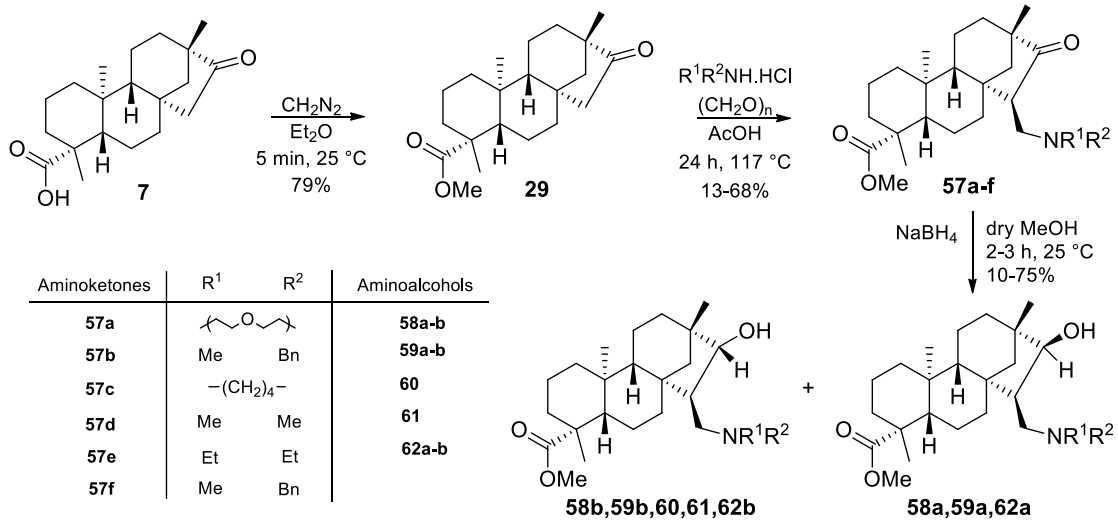


Figure 11: Stereoselective synthesis of isosteviol-based 1,3-aminoalcohols

The beyerene skeleton was modified with more complex substituents through Mannich condensation performed in glacial acetic acid with a series of secondary amine HCl salts and paraformaldehyde (**Figure 12**). A single diastereomer of the aminoketone could be detected after the reaction, while reduction by $NaBH_4$ resulted in isomeric mixtures in most cases. The pyrrolidine- and dimethylamino-functionalised derivatives produced **60** and **61**. The compound bearing the *N*-methyl-*N*-benzyl moiety gave a 5:1 ratio of diastereomers with the α-OH-isomer being the most abundant. This was probably due to the low steric hindrance, which enabled complex formation with the solvent, generating the site of the attack. The bulky substituents, in turn, are not capable of forming the corresponding complex [9].



Aminoketones	R^1	R^2	Aminoalcohols
57a			**58a-b**
57b	Me	Bn	**59a-b**
57c	$-(CH_2)_4-$		**60**
57d	Me	Me	**61**
57e	Et	Et	**62a-b**
57f	Me	Bn	

Figure 12: Synthesis route to 1,3-aminoalcohols through Mannich condensation

Additionally, the condensation that generated *N*-methyl-*N*-benzylaminoketone **57f** was originally conducted with dibenzylamine hydrochloride, but instead of creating the *N*,*N*-dibenzyl-substituted derivative, isomerisation took place in the iminium salt, because the special steric hindrance blocked its reaction with the enolate of the ketone (**Figure 13**). Thus, benzaldehyde was eliminated after water addition. This step clearly shows the limitation of the use of secondary amines [9].

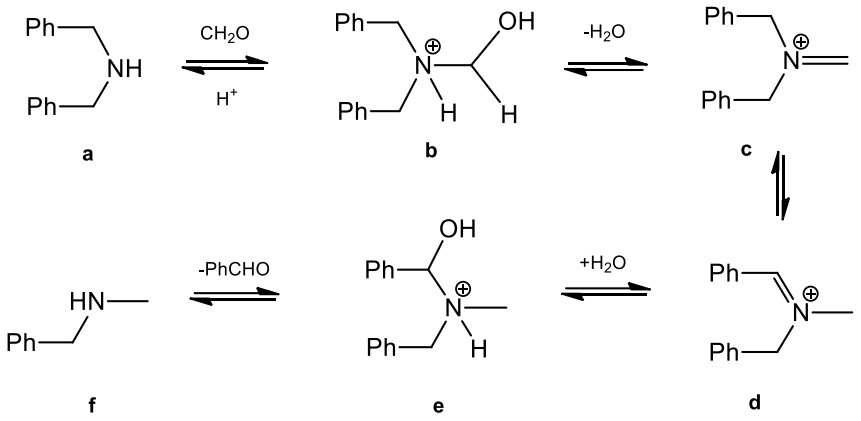


Figure 13: Proposed mechanism of Mannich condensation with dibenzylamine

Relying on previous outstanding experimental results of 1,3-oxazines and thiazines [15,112], their diterpenoid counterparts were prepared from primary 1,3-aminoalcohol **55**. Coupling compound **55** with phenyl isothiocyanate in dichloromethane at room temperature gave thiourea **63** (**Figure 14**). To create the heterocyclic function, the latter was treated with methyl iodide, followed by elimination of methyl mercaptol in the presence of KOH. Interestingly, this last step proved to be unsuccessful. The cyclisation was attempted in an alternative way, by adding EtOH that contained 18% hydrochloric acid, which resulted in thioethyl ether **64** as the sole product in moderate yield. The explanation in this case might be the steric hindrance of the diterpene skeleton blocking the attack of sulphur on C_{16}, while EtCl is generated *in situ* in the reaction of HCl and EtOH. EtCl interacts with thiourea **63** in a similar way as MeI, providing thioether **65** [9].

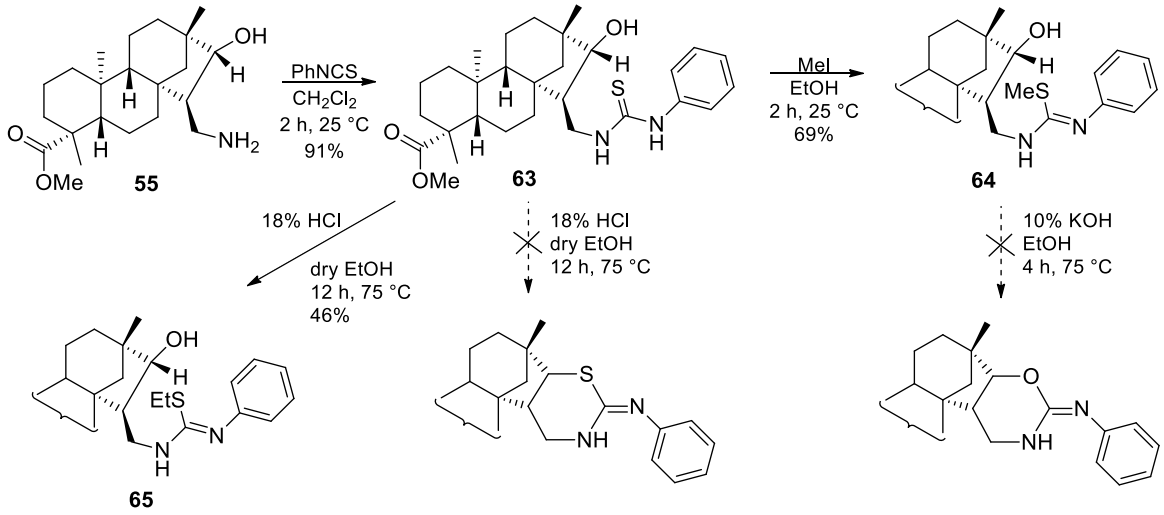


Figure 14: Preparation of thioether derivatives

The new compounds were assessed *in vitro* on human cancer cell lines HeLa, SiHa (cervical), MDA-MB-231, MCF-7 (breast) and A2780 (ovarian) and their antiproliferative activity was compared to that of Cisplatin. The intermediates (**51**–**55**) and aminoketones **57a**–**f** do not exert relevant inhibition of cell growth, indicating that the amino function is essential for expressing the cytotoxic effect. Thioethers **64** and **65** exhibited no action, whereas thiourea **63** showed moderate activity at 10–23 μM of IC_{50} values. The compound with the best performance was the *p*-fluorobenzyl-substituted 1,3-aminoalcohol, but no significant selectivity could be observed for any of the other derivatives. Based on the results they arrived at the conclusion that the aminoalcohol and the *N*-benzyl substituents play a crucial role in the inhibition, while the aliphatic function is not essential in the process [9].

2.4.2. Synthesis of *ent*-kaurane-type derivatives

Steviol-based aminoalcohols are nearly non-existent in literature and the lack of transformations could be assigned to the difficult synthesis and high commercial price of the aglycone. Li et al. carried out research concerning both isosteviol and steviol derivatives, building a library of diterpenoids with *exo*-methylene cyclopentanone in ring D. First, protection of the 13-hydroxy function of steviol was carried out by applying acetic anhydride and then the double bond was oxidised with O_3, followed by hydroxymethylation of the 16-ketone (**Figure 15**). Since selective oxidation of the secondary alcohol function of 13,15,16-triol with sodium hypochlorite and acetic anhydride was unsuccessful, the primary alcohol was protected with TsCl and the

carboxylic acid was treated with benzyl bromide before oxidation of the 16-hydroxy group with PDC. TsOH was removed in the presence of pyridine and DMAP, which provided hydroxyketone **70**. After debenzylation with 10% Pd/C carboxylic acid **71** was isolated. The derivatives were assayed on human cancer cell lines MDA-MB-231, HepG2 (hepatocellular carcinoma) and MGC-803 (gastric carcinoma) for their *in vitro* cytotoxicity with adriamycin and oridonin being control drugs. Benzyl ester **72** showed the highest inhibition at 0.95–2.41 μM of IC_{50} values [113].

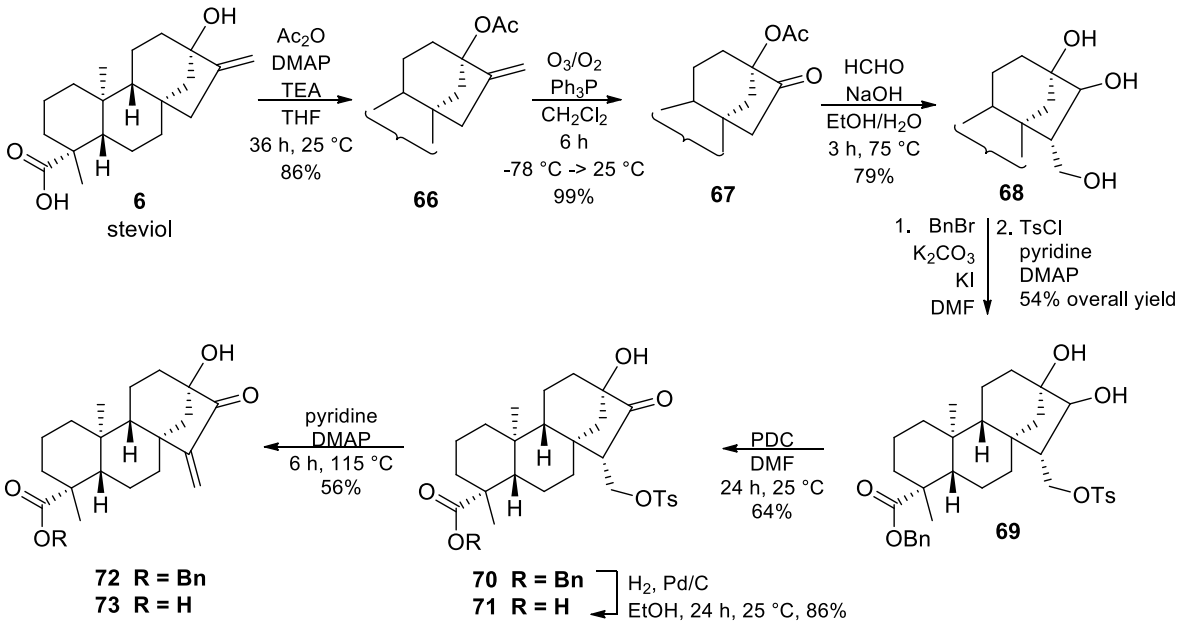


Figure 15: Synthesis of cytotoxic *exo*-methylene cyclopentanone derivatives

One year later Zeng et al. carried out reactions to expand the D ring, similar to the research of Lohoelter, but this time the aim was to create α-methylenelactones. This compound is technically a cyclic carboxylic ester with proven antitumour activity due to its alkylating properties, when interacting with biological nucleophiles (e.g. thiol-containing amino acids and enzymes) [114]. Some of the newly prepared derivatives were also tested for anticancer effects against six cancer cell lines (PC-3, HCT-116, MDA-MB-231, K562, HepG2 and MGC-803) *in vitro* by the MTT method. The most potent derivative (**83**) exhibited IC_{50} values of 0.09 and 0.22 μM on HepG2 and MDA cells. The compound was closely followed by its methyl ester derivative in antiproliferative activity [115].

I present the synthesis of compound **83** on **Figure 16**. Steviol was treated with excess chloro(methoxy)methane (MOM-Cl) to protect the 13- hydroxy function before reduction by $LiAlH_4$. Next, the acylation of the carboxylic acid and the introduction of a hydroxy

group in position C_{16} gave compound **77**. Oxidation with PDC and protection of the α-methylene function with *p*-thiocresol after removing the MOM ether with 10% HCl, afforded β-thioketone **80**, which was further oxidised with excessive *m*-CPBA according to the Baeyer–Villiger procedure. The sulfonyl unit was removed from the lactone by applying DBU in THF. Finally, treatment with 10% $KHCO_3$ in CH_3OH at reflux temperature transformed compound **82** to α-methylenelactone **83** [115].

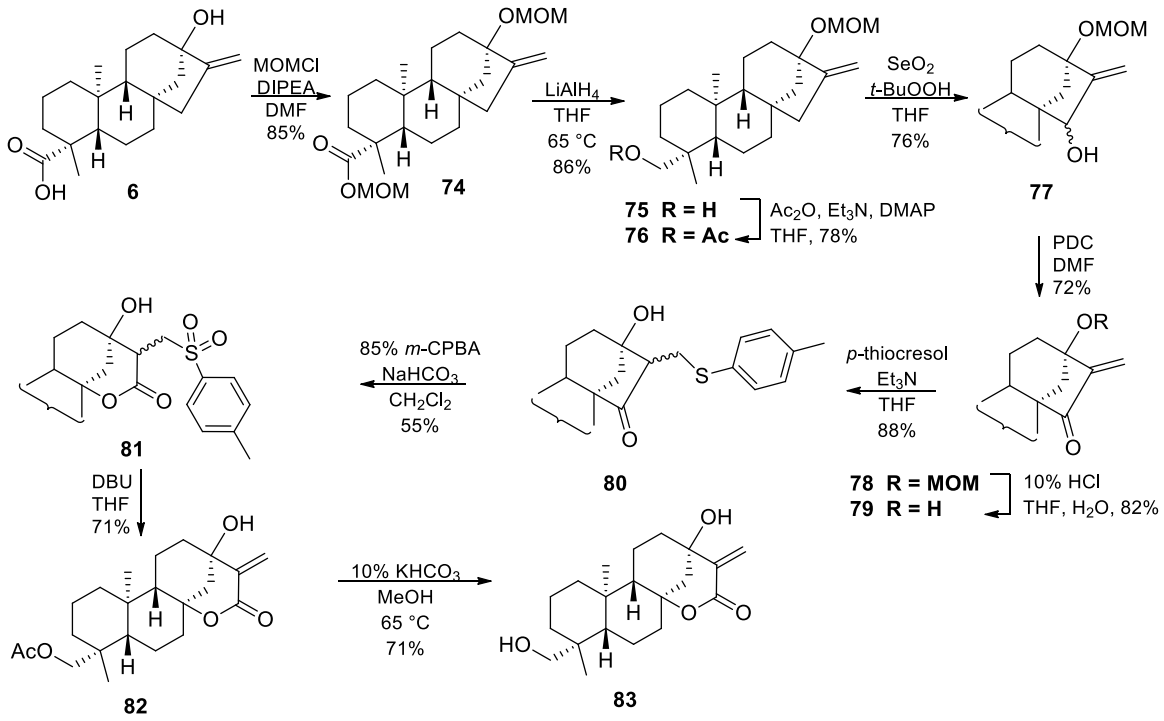


Figure 16: Synthesis strategy for the preparation of α-methylenelactone

As *N*-functionalised monoterpenes and isosteviol derivatives demonstrated their value as potential anticancer agents, our research group worked on broadening our knowledge on the steviol-derived analogues. Ozsvár et al. managed to synthesise a key intermediate *cis*-epoxide (**85a**) from steviol methyl ester in a reaction with *t*-BuOOH using vanadyl acetoacetate ($VO(acac)_2$) as catalyst in a stereospecific manner (**Figure 17**). Alternatively, when dimethyldioxirane prepared *in situ* was and added to the reaction mixture, the diastereomers were obtained in 2:1 ratio with **85b** as the minor product. The isomers were successfully separated by column chromatography and then the *cis*-epoxyalcohol was treated with sodium azide to afford azidoalcohol **86**. The azide function was reacted with several alkynes to produce substituted triazoles **87a**–**d** in click reaction [10].

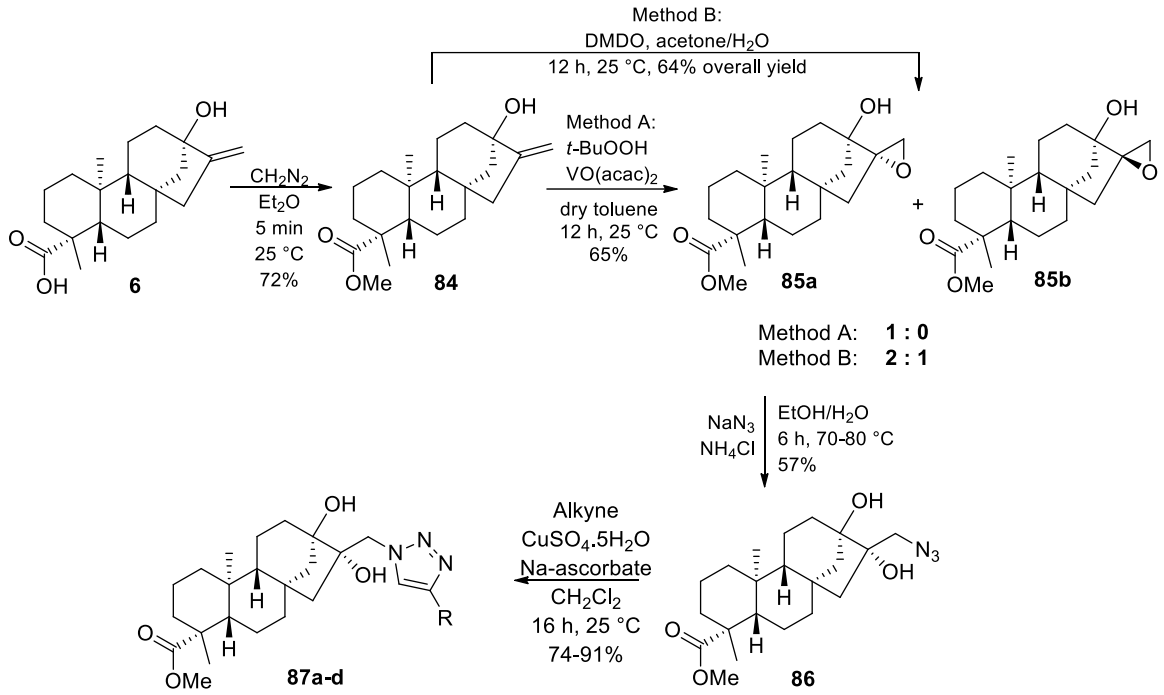


Figure 17: Click reaction of azidodiol derived from isosteviol-based epoxide

The *cis*-epoxide was transformed further by opening the oxirane ring with a selection of primary and secondary amines at room temperature in nucleophilic addition (**Figure 18**). The reaction proceeded in a stereoselective fashion. Extending the library of heterocyclic derivatives, compound **88g** with an *N*-propargyl function was coupled with (2-azidoethyl)benzene in the presence of $CuSO_4.H_2O$ and sodium ascorbate and the reaction gave click product **91** in moderate yield. The *N*-benzyl-substituted derivative was chosen for hydrogenation with Pd/C catalyst to prepare primary aminodiol **89** followed by ring closure with formaldehyde. The latter step was completed in an hour and showed high regioselectivity for *spiro*-oxazolidine **90** [10].

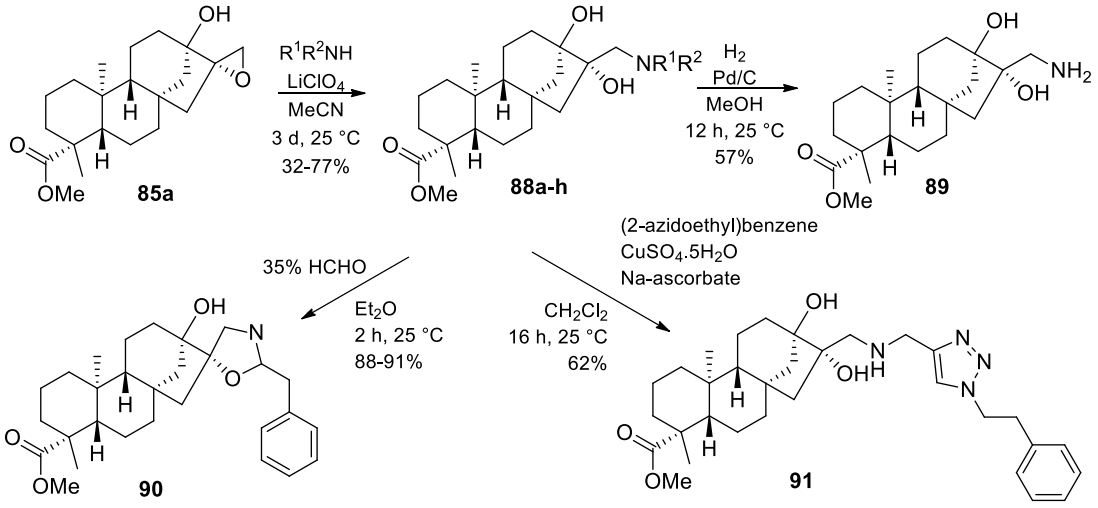


Figure 18: Stereoselective synthesis of steviol-derived aminodiols

The diastereomeric counterpart of **88a** (**92**) was synthesised from the *trans*-epoxyalcohol, and the ring closure resulted in two regioisomers in a 3:1 ratio with the oxazolidine being the major product (**Figure 19**). Unfortunately, these isomers could not be separated by chromatography. The primary aminodiol was prepared according to the previous method by hydrogenolysis with Pd/C catalyst [10].

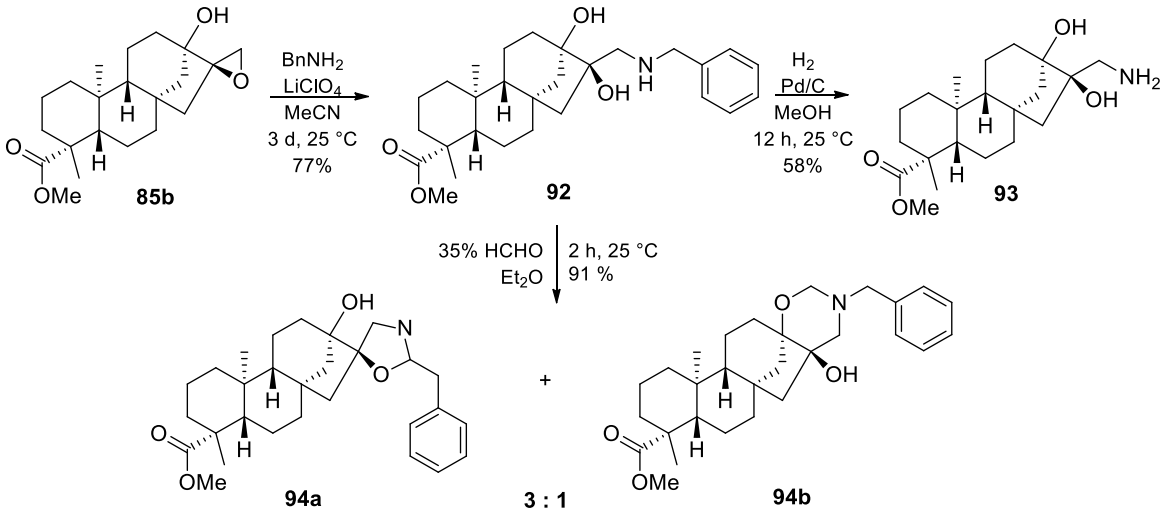


Figure 19: Ring closure of diastereomeric aminodiol **92**

The *in vitro* antiproliferative assay of gynaecological cell lines presented the same pattern as for the isosteviol-based aminoalcohols. The primary and aliphatic aminodiols and azidoalcohol intermediates expressed low activity, indicating that the presence of aromatic unit is necessary. The triazoles, similar to the *N*-methyl-*N*-benzylamino derivative, exhibited weaker effects than expected, which confirmed the importance of the secondary amino group. The α-methylbenzyl and bis-(trifuoromethyl)methylbenzyl substituents caused one of the strongest inhibition with IC_{50} values of 4–8 μM. Oxazolidine **90** was found to be the most effective with the cytotoxicity on ovarian cell line A2780 being comparable to that of Cisplatin [10].

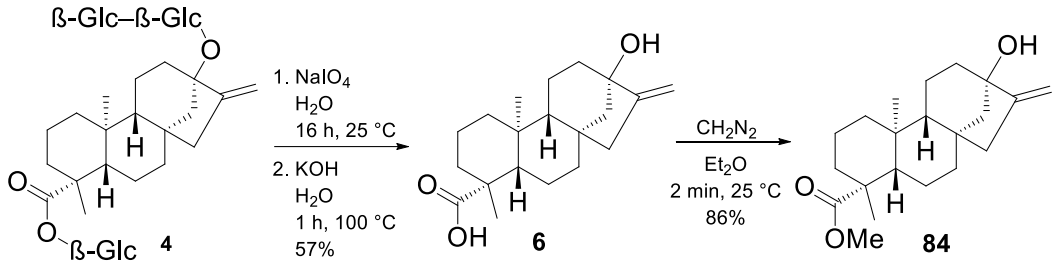
3. Results and Discussion

3.1. Stereoselective synthesis of bifunctional steviol derivatives

3.1.1. Synthesis of key intermediate β-keto-alcohol

For the starting point of our research, we chose commercially available natural glycoside stevioside **4** and in a two-step synthesis it was transformed into its aglycone, steviol **6**. The oxidative alkaline hydrolysis was carried out in the presence of $NaIO_4$ and KOH as described in the literature (**Scheme 1**) [83,84]. Due to the long reaction time and relatively low yield, optimisation of the reaction conditions was attempted before in our research group, but even by changing different parameters, better results than the ones documented by Ukiya et al. could not be achieved. Synthesis from the financially favourable mixture of stevia glycosides provided an even lower yield.

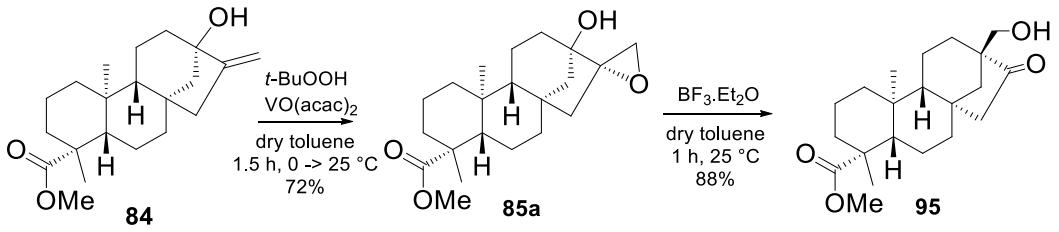
Esterification of steviol was accomplished with diazomethane in diethyl ether resulting in methyl ester **84** in a few-minute reaction (**Scheme 1**). Diazomethane was prepared *in situ* in the reaction of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald®) and KOH. The ethereal solution was added dropwise to steviol immediately. Fortunately, formation of the cyclopropane derivative as a possible side reaction was not observed.



Scheme 1: Preparation of steviol methyl ester

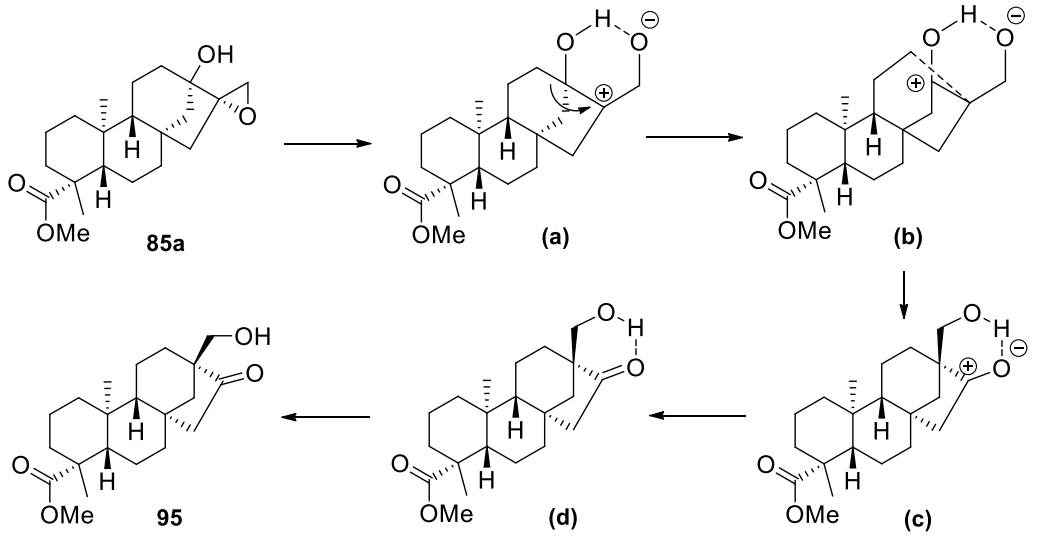
In the next step, epoxidation of the methyl ester was conducted by a method using *t*-BuOOH as the oxidising agent and $VO(acac)_2$ as the catalyst in anhydrous toluene. Based on the study of Van Speybroeck et al., the reaction is most likely to form alkylperoxo species in a concerted Sharpless mechanism, of which $V^{+IV}O(L)(OOtBu)$ and $V^{+V}O(L_1)(L_2)(OOtBu)$ are the most abundant. Throughout the process, the oxidation state of vanadium changes periodically in the catalytic cycle between the +IV and +V oxidation states, causing the mixture to change its colour from emerald green to maroon, when adding the peroxide, then to amber as the reaction progressed [10,116]. This visible change helped us define when the transformation completed. The reaction gave *cis*-epoxyalcohol

85a in a stereospecific manner. The stereochemistry of the compound was described in the literature previously (**Scheme 2**) [117,118]. The epoxide was then treated with $BF_3.Et_2O$ causing a rearrangement taking place at room temperature in merely an hour. The reaction resulted in a single derivative (**95**) and 2D-NMR spectroscopy confirmed the change of stereochemistry in the structure, where the kaurane skeleton converted to isosteviol-type *ent*-beyerane (**Scheme 2**) [119,120].



Scheme 2: Synthesis of key intermediate β-keto-alcohol through *spiro*-epoxide

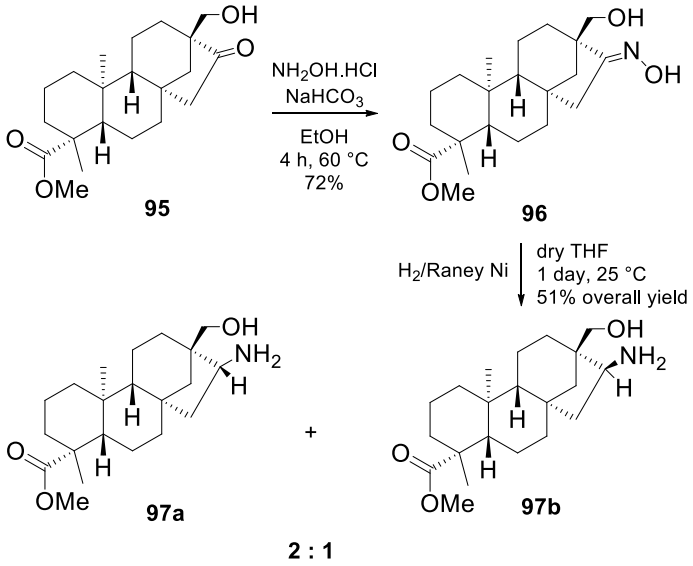
Schreiber et al. described the mechanism of a similar reaction for 8*S*,15-epoxy-gibberellic acid. To get a better understanding of changes during the interaction, we considered their findings and proposed the following process. The oxirane ring opens by the coordination of the Lewis acid to the epoxide oxygen and then building a six-membered ring through interaction with the neighbouring hydroxy group (**a**) (**Scheme 3**). The carbocation is stabilised through Wagner–Meerwein rearrangement, and the bond between C_{12} and C_{13} breaks, while a new bond is created between C_{12} and C_{16} (**b**). The semipolar bond of the carbonyl function (**d**) is created through displacement of the negative charge over the hydrogen bridge (**c**) [121].



Scheme 3: Proposed mechanism for Wagner–Meerwein rearrangement of epoxide **85a**

3.1.2. Synthesis of 1,3-aminoalcohols

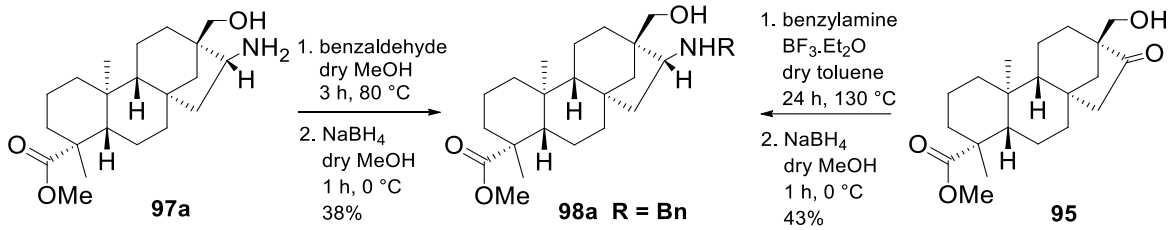
Our key molecule was first transformed into oxime with the help of hydroxylamine hydrochloride in the presence of $NaHCO_3$ in ethanol (**96**) [122]. The product then underwent hydrogenation catalysed by Raney Ni in THF at room temperature resulting in primary aminoalcohols **97a** and **97b** [9]. The diastereomers were obtained in a 2:1 ratio as identified by NMR spectroscopy and they could be successfully separated by preparative column chromatography with a 1:1 mixture of CH_2Cl_2/MeOH (**Scheme 4**).



Scheme 4: Stereoselective synthesis of primary aminoalcohols

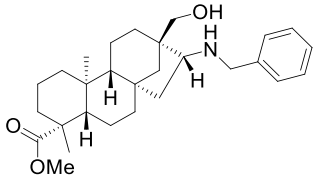
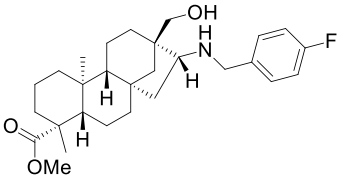
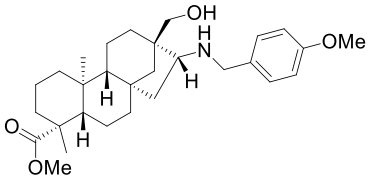
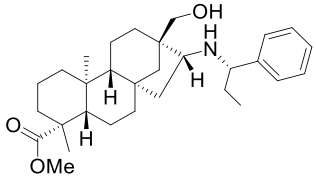
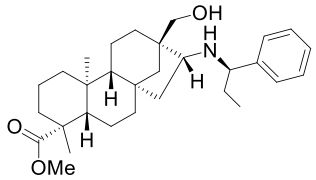
Nucleophilic addition of amines to carbonyl compounds followed by dehydration is a convenient way to prepare enamines. To create a series of *N*-substituted aminoalcohols, first we attempted to carry out the reaction with major primary aminoalcohol **97a** and benzaldehyde in ethanol. The resulting Schiff base was reduced by $NaBH_4$ at 0 °C, providing *N*-benzyl-substituted derivative **98a** (**Scheme 5**). The transformation was also accomplished from β-keto-alcohol **95** in the presence of benzylamine and $BF_3.Et_2O$ in anhydrous toluene as described by Hamada and Shioiri [123]. The Lewis acid catalyst efficiently induced the interaction with the nucleophilic amine by forming adducts with the carbonyl oxygen, which was responsible for both the decent reaction time and stereoselectivity. The system was assembled using the Dean–Stark apparatus to remove water from the mixture, but we found molecular sieve to be just as effective for small scale. Reduction of the product, once again, was accomplished with $NaBH_4$ without isolating the intermediate, resulting in derivative **98a** (**Scheme 5**). This method proved to

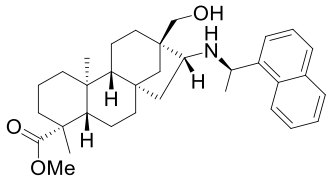
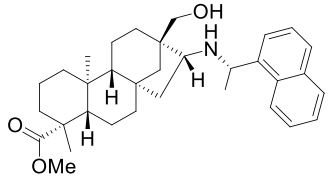
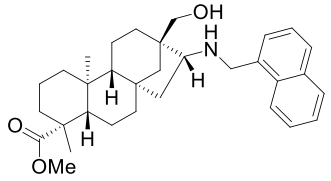
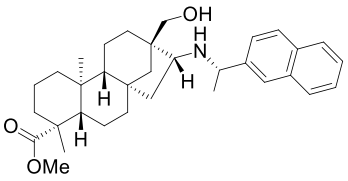
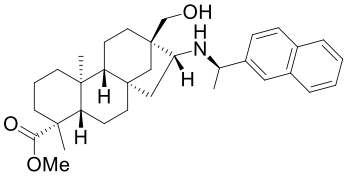
be ideal for the preparation of different aminoalcohols, because it gave us the compounds in fewer steps and we could avoid extensive work with the delicate primary aminoalcohols. The novel 1,3-aminoalcohols (**98b**–**j**) were synthesised by the present procedure in moderate to good yields (**Table 2**).



Scheme 5: Synthesis of *N*-benzyl substituted 1,3-aminoalcohol **98a**

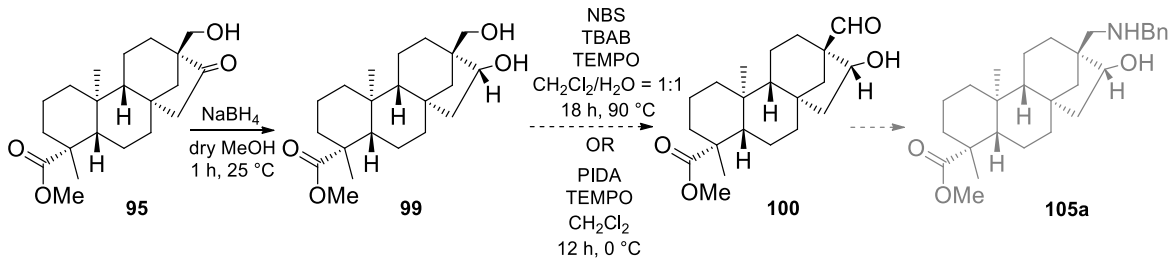
Table 2. Library of steviol-based 1,3-aminoalcohols, prepared according to the reaction process presented on **Scheme 5**

Entry	Compound	R	Product	Yield (%)
1	**98a**	benzyl		43
2	**98b**	4-fluorobenzyl		48
3	**98c**	4-methoxybenzyl		70
4	**98d**	(*R*)-α-ethylbenzyl		65
5	**98e**	(*S*)-α-ethylbenzyl		60

6	**98f**	(*R*)-1-(1-naphthyl)ethyl		41
7	**98g**	(*S*)-1-(1-naphthyl)ethyl		38
8	**98h**	1-naphthylmethyl		78
9	**98i**	(*R*)-1-(2-naphthyl)ethyl		60
10	**98j**	(*S*)-1-(2-naphthyl)ethyl		56

3.1.3. Synthesis of 1,3-aminoalcohol regioisomers

The potential of further transformations of β-keto-alcohol **95** led us to the synthesis of the regioisomeric analogues of the prepared aminoalcohols. Originally, we planned to achieve this goal by Schiff base synthesis through the aldehyde derivative of **95**. First, the β-keto-alcohol was converted to diol **99** by reduction with $NaBH_4$. Oxidation of the primary alcohol function was attempted with *N*-chlorosuccinimide (NCS), but despite of increasing the amount of the reagent, catalysts and reaction time, the transformation did not happen. Thus, we opted for using the more reactive *N*-bromosuccinimide (NBS), which gave the aldehyde (**100**) in an incomplete reaction with extremely low yield (**Scheme 6**) [9]. Slightly better results could be achieved when PIDA (phenyliodine(III) diacetate) was added instead, but in this case the secondary alcohol was affected as well, providing a ketone function at C_{16} [124]. Due to the poor conversion and the variable outcome of the Schiff reaction resulting in many side products, this synthesis route was dropped [125].



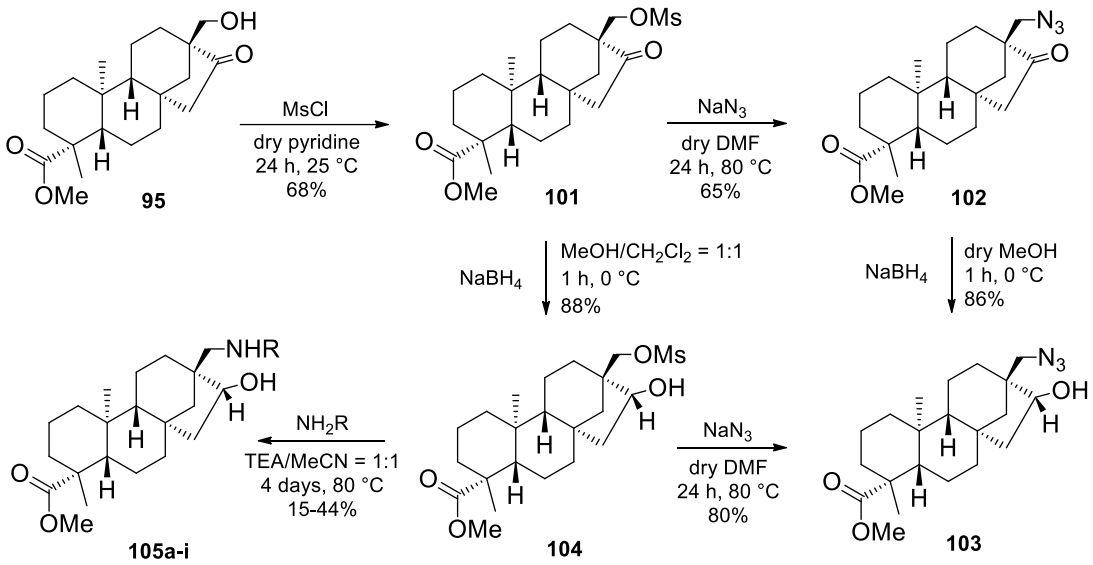
Scheme 6: Attempted synthesis of aminoalcohol regioisomer through aldehyde **100**

Looking for a more effective way to prepare the regioisomers, compound **95** was treated with methanesulfonyl chloride in anhydrous pyridine to change the hydroxy function to a better leaving Ms group (**Scheme 7**) [126]. In the following steps, two pathways were considered. Worrying about losing the *O*-mesyl function to reduction of the ketone with $NaBH_4$, the compound was converted to azide **102** with sodium azide in anhydrous DMF before the reduction was carried out [127]. Alternatively, to decrease the reactivity of the mesylate in the presence of hydride ions, a 1:1 ratio of MeOH and dichloromethane was used as reaction medium instead of pure methanol, resulting in hydroxy-mesylate derivative **104**. As the process proved to be slightly more effective with the steps switched, compound **104** served as starting material for the preparation of aminoalcohols **105a**–**i** (**Table 4**). To be able to compare the derivatives and draw conclusions, the nucleophilic substitution was accomplished with the same selection of *N*-substituted primary amines as before, in acetonitrile and triethylamine in a 1:1 ratio, which was determined experimentally to minimise the development of side products (based on observation of the TLC plates) and maximise the yield (**Table 3**, **Scheme 7**) [128].

Table 3. Optimisation of the mesylate-amine exchange[a]

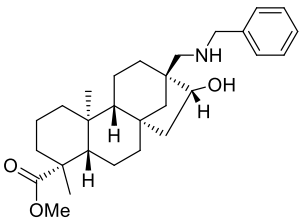
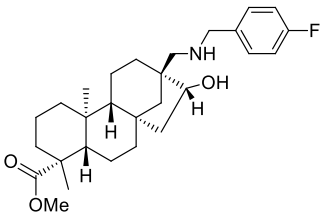
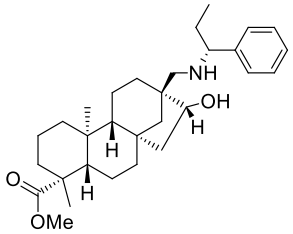
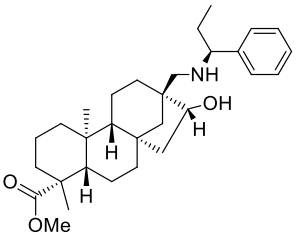
Entry	MeCN/TEA	**105a** (%)
1	20 : 1[b]	ND[c]
2	0 : 1	ND
3	1 : 2	15
4	1 : 1	22

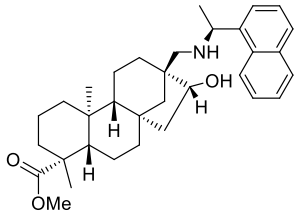
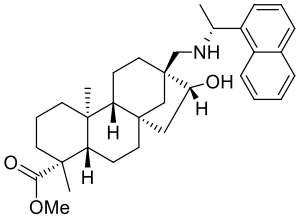
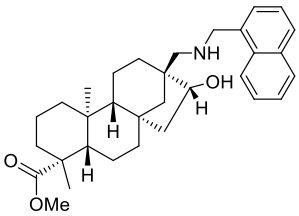
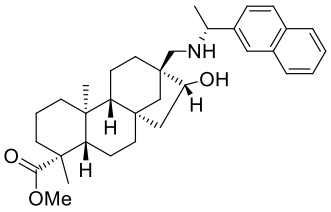
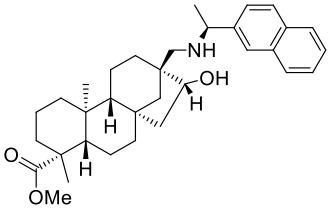
[a]The reactions were carried out at 80 °C for 4 days. [b]TEA was added in portions as catalyst while the reaction was monitored by TLC. [c]No product was detected that could be isolated.



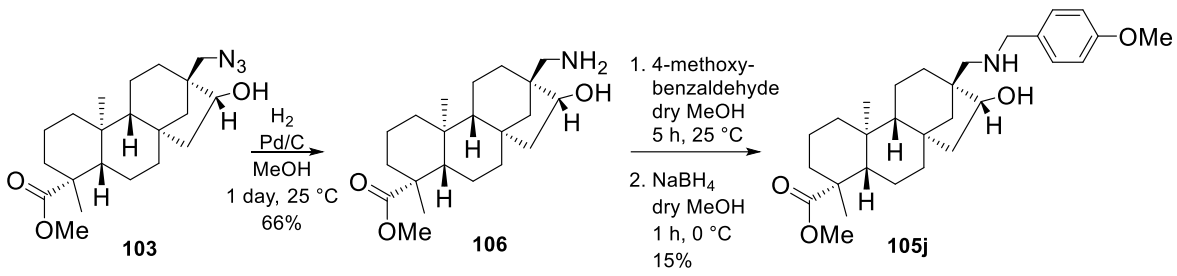
Scheme 7: Synthesis pathways for the preparation of 1,3-aminoalcohol regioisomers

Table 4. Library of 1,3-aminoalcohol regioisomers

Entry	Compound	R	Product	Yield (%)
1	**105a**	benzyl		22
2	**105b**	4-fluorobenzyl		41
3	**105c**	(*R*)-α-ethylbenzyl		40
4	**105d**	(*S*)-α-ethylbenzyl		36

5	**105e**	(*R*)-1-(1-naphthyl)ethyl		29
6	**105f**	(*S*)-1-(1-naphthyl)ethyl		21
7	**105g**	1-naphthylmethyl		44
8	**105h**	(*R*)-1-(2-naphthyl)ethyl		19
9	**105i**	(*S*)-1-(2-naphthyl)ethyl		15

When applying 4-methoxybenzylamine, despite testing multiple different conditions affecting the temperature, equivalency, and catalyst/solvent ratio, no product could be detected on TLC. We decided to synthesise the desired derivative through primary aminoalcohol **106** as an alternative pathway, which was prepared by palladium-catalysed hydrogenation of hydroxy-azide **103** in methanol. Next, 4-methoxybenzaldehyde was added to compound **106** to form the Schiff base followed by reduction without isolation by $NaBH_4$, providing aminoalcohol **105j** (**Scheme 8**) [9].

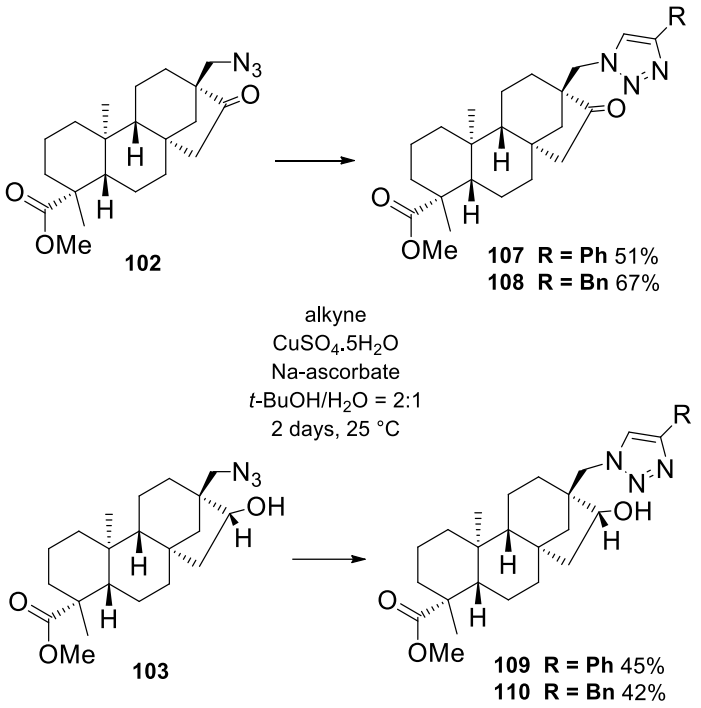


Scheme 8: Synthesis of 4-methoxybenzylamino derivative **105j** through primary aminoalcohol

3.1.4. Synthesis of 1,2,3-triazolo derivatives by click reaction

Triazole and its derivatives are one of the most important heterocyclic pharmacons due to their anticancer [129,130], antimicrobial [131,132], anti-inflammatory [133] and antidiabetic effects [134] amongst other therapeutic applications. The beneficial properties of the triazole unit for the elevated bioactivities may include hydrogen bonding capability under *in vivo* conditions, the ability to build various non-covalent interactions to improve solubility and decreasing the overall lipophilicity of the diterpene and binding to bimolecular targets [10,135,136]. Several triazole derivatives of natural origin, including the triterpene myrrhanone C were found to express selective toxicity against breast cancer MCF-7 of gynaecological cell lines [135,137–139]. Further study disclosed that the antitumor activity is attributed to the formation of reactive oxygen species (ROS) in the cell without significant nuclear DNA damage. For example, dehydroabietic acid analogues proved to be excellent against adriamycin-resistant MCF-7 cells at low concentrations in a dose-dependent manner. The effect of different substituents at the C_4 position of the 1,2,3-triazole function was also tested, and the structure–activity relationship (SAR) study confirmed the essential role of aromatic substituents exhibiting high cytotoxicity [135].

Considering their widely acclaimed biological activity, we experimented with the synthesis of the heteroaromatic derivatives as well. To prepare heterocyclic derivatives **107** and **108**, keto-azide **102** was coupled with aromatic alkynes in a *t*-BuOH/H_2O 2:1 medium for better solubility of catalyst $CuSO_4.5H_2O$ and sodium ascorbate, the latter generated *in situ* from ascorbic acid and NaOH in methanol (**Scheme 9**). When the reaction was carried out with the ascorbate pre-prepared, notable progress could not be observed. The synthesis was repeated with hydroxy-azide **103** as the starting material, forming compounds **109** and **110** in good yields [140,141].

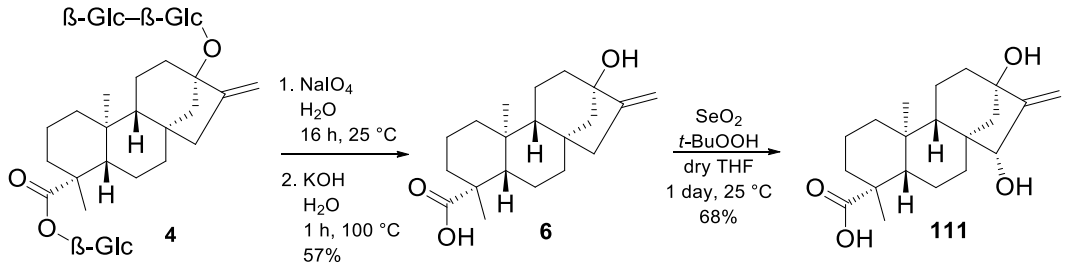


Scheme 9: Preparation of 1,2,3-triazolo derivatives via click reaction

3.2. Stereoselective synthesis of tetrafunctional steviol derivatives

3.2.1. Synthesis of key intermediate *spiro*-epoxide

We wanted to expand the collection of novel aminoalcohol-type diterpenes by adding another hydroxy group to the kaurane skeleton and to investigate its effect on biological activity. Starting from stevioside (**4**), steviol (**6**) was synthesised in two steps, according to literature methods applied previously. Allylic hydroxylation of compound **6** was accomplished by adding selenium(IV) dioxide and *tert*-butyl hydroperoxide in dry THF (**Scheme 10**) [113]. The reaction was found to be stereoselective for the 15-α-OH isomer (**111**) as described in the literature [82,117,142].

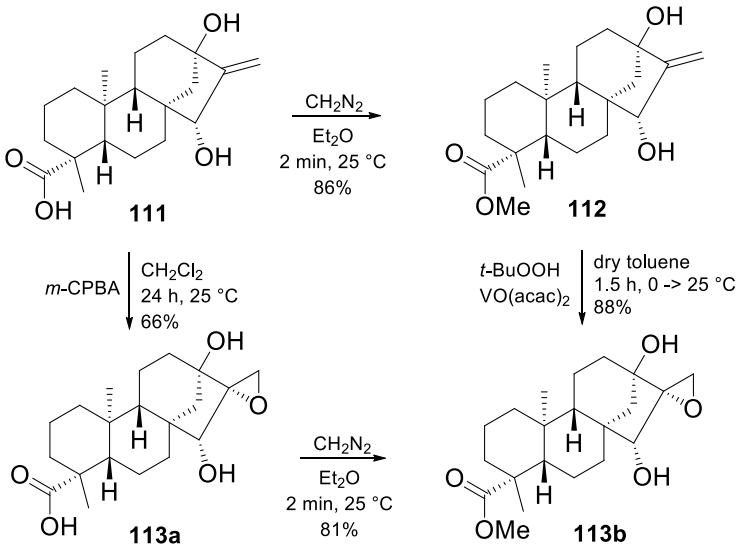


Scheme 10: Preparation of allylic alcohol **111** from steviol

The reaction was followed by esterification of **111** with the same method as before, using diazomethane, resulting in methyl ester **112** in excellent yield just in a few minutes

(**Scheme 11**). Fortunately, the cyclopropanation side reaction was not observed. Switching the last two steps was also considered, to match the synthesis route of the aminoalcohols, but it had been dismissed due to observation of lower yield of allylic hydroxylation from steviol methyl ester [83].

Next, *spiro*-epoxide **113b** was synthesised from methyl ester **112** in a stereospecific reaction with *t*-BuOOH as an oxidizing agent, catalysed by $VO(acac)_2$ [116]. To determine the stereochemistry of **113b**, the compound was prepared in an alternative pathway as well. As described in the literature, treatment of **111** with *meta*-chloroperoxybenzoic acid in dichloromethane gave derivative **113a** with known stereochemistry (**Scheme 11**) [117]. Ester synthesis was carried out again with diazomethane and the resulting product proved to possess the same structure as the one synthesised using *t*-BuOOH (**113b**) after studying the NMR spectra. Considering reaction time and the observed yields, we decided to continue working via the vanadium-catalysed method.

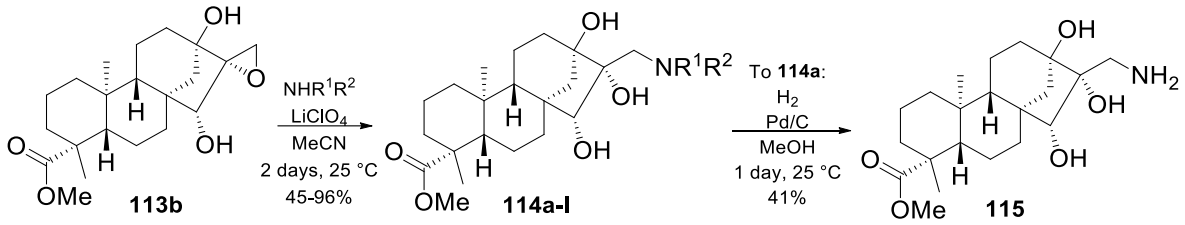


Scheme 11: Chemo- and stereoselective synthesis of key intermediate *spiro*-epoxide **113b**

3.2.2. Synthesis of 2-aminomethyl-1,2,3-triols

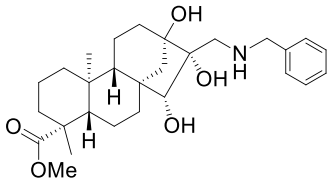
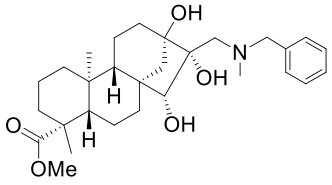
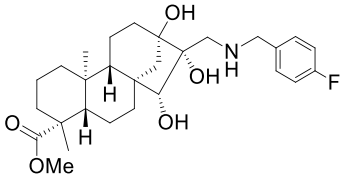
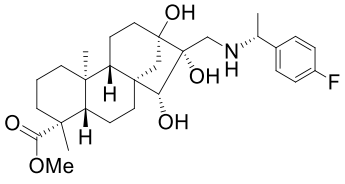
Nucleophilic addition of amines to epoxyalcohols is another favoured way for the synthesis of a versatile collection of aminoalcohols, and it had been previously reported in several papers of our research group [10,104,143,144]. Following the method as described, the oxirane ring of epoxide **113b** was opened with selected primary amines from the aminoalcohol series as well as additional primary and secondary amines in the presence of $LiClO_4$ applied as a Lewis acid catalyst (**Scheme 12**). The coordination of the lithium ion to the epoxide oxygen is presumed to be increasing the electrophilic character of the

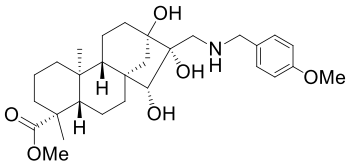
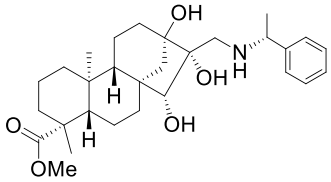
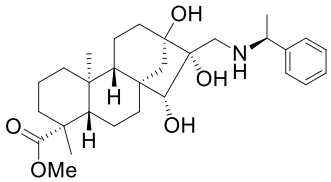
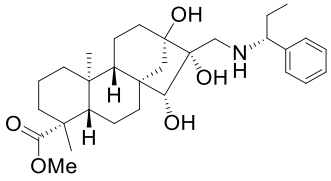
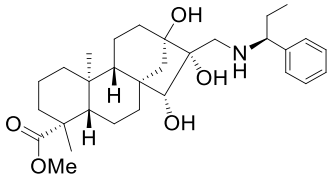
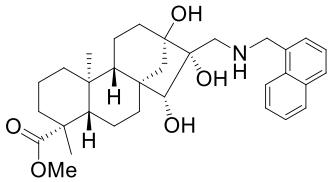
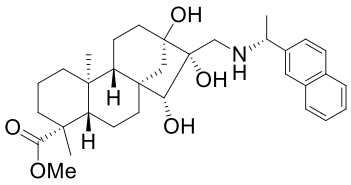
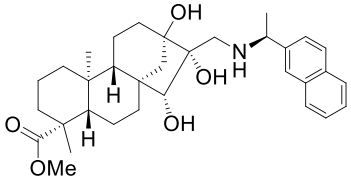
moiety against the nucleophilic attack of the amine. This way the compound is activated towards the ring opening, which is beneficial regarding the decent reaction time, yield and stereoselectivity [145,146]. The preparation of the novel aminotriols was accomplished with moderate to excellent yields as given in **Table 5**. Secondary *N*-benzylmethylamine was found to be less efficient for the synthesis and the resulting derivative (**114b**) showed lower bioactivity in our *in vitro* pharmacological study as well (see chapter 3.3). Furthermore, compound **114a** was subjected to debenzylation by hydrogenolysis over Pd/C to obtain primary aminotriol **115** in moderate yield (**Scheme 12**).



Scheme 12: Stereoselective synthesis of aminotriols

Table 5. Library of 2-aminomethyl-1,2,3-triols

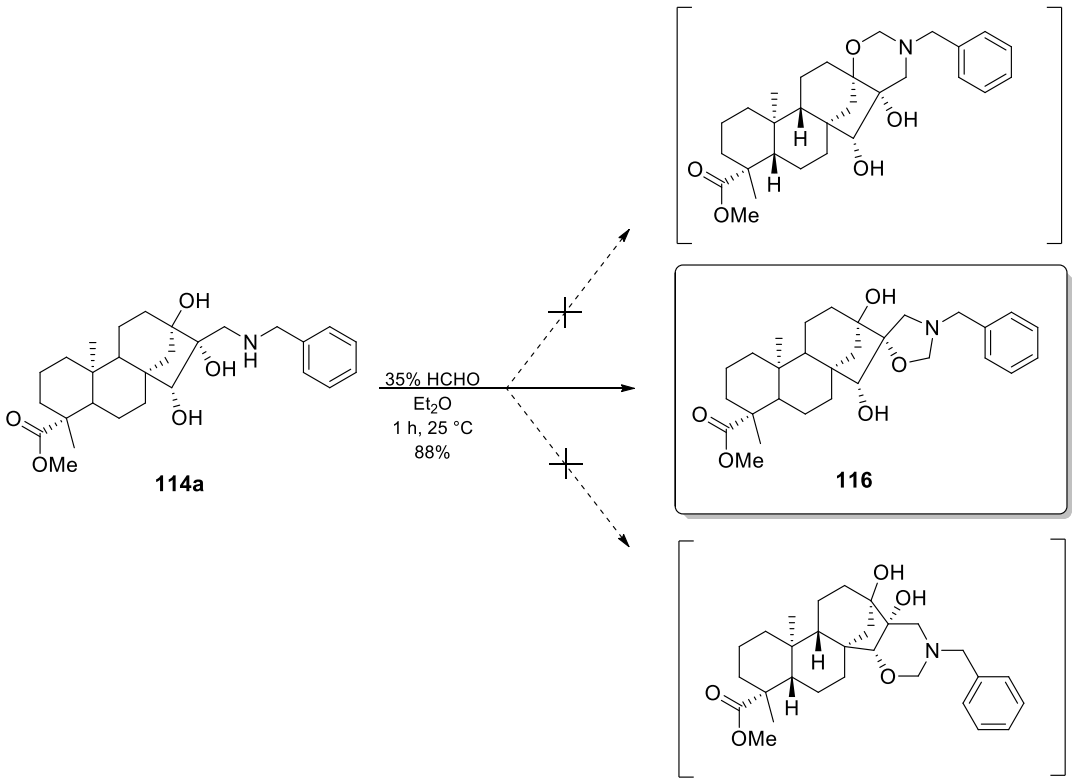
Entry	Compound	R^1	R^2	Product	Yield (%)
1	**114a**	H	benzyl		93
2	**114b**	Me	benzyl		45
3	**114c**	H	4-fluorobenzyl		95
4	**114d**	H	(*R*)-4-fluoro-α-methylbenzyl		50

5	**114e**	H	4-methoxybenzyl		96
6	**114f**	H	(*R*)-α-methylbenzyl		59
7	**114g**	H	(*S*)-α-methylbenzyl		60
8	**114h**	H	(*R*)-α-ethylbenzyl		80
9	**114i**	H	(*S*)-α-ethylbenzyl		65
10	**114j**	H	1-naphthylmethyl		60
11	**114k**	H	(*R*)-1-(2-naphthyl)ethyl		60
12	**114l**	H	(*S*)-1-(2-naphthyl)ethyl		55

3.2.3. Regioselective synthesis of heterocyclic oxazolidine derivative

The oxazolidine scaffold and its derivatives are best known for their antimicrobial properties [147], but they display a broad spectrum of activity such as anticonvulsant [148], anti-inflammatory [149] and anticancer effects [150]. Derekova et al. found cytotoxic action of oxazolidinone derivatives by using the apoptosis PARP molecular marker in HeLa (cervical cancer). The compounds were found to generate an accumulation of cells in the G1 phase of the cell cycle. Sarkar et al. showed that forming a complex with metals like palladium, copper or platinum presented a considerable cytotoxicity against breast adenocarcinoma (MCF-7) and low activity against mice embrionary fibroblast (NIH/3T3). However, the relatively new discovery of the antitumor activity of the oxazolidine derivatives calls for more profound studies to better understand their action mechanisms [151,152].

In a former study, a steviol-based oxazolidine derivative expressed remarkable cytotoxic activity on human gynaecological cancer cell lines [10]. Intrigued by our previous results, we decided to synthesise the heterocyclic counterpart of compound **114a** and to determine the regioselectivity of the ring closure. Treatment of aminotriol **114a** with aqueous formaldehyde at room temperature gave *spiro*-oxazolidine **116** in a highly regioselective reaction (**Scheme 13**).



Scheme 13: Regioselective ring closure of aminotriol **114a** with formaldehyde

To uncover which of the possible heterocyclic structures formed in the reaction, both 1- and 2D-NMR data of **116** needed to be studied. The ^{1}H NMR spectrum in DMSO-d_6 clearly showed that 15-α-OH exists as a doublet and it does not participate in the ring closure. Cross-coupling could be observed in the HMBC spectrum between the hydrogens carried by the carbon atom of the heterocycle bonded to both the nitrogen and the oxygen, and C_{16}, but not with C_{13} and C_{15} (**Figure 20**).

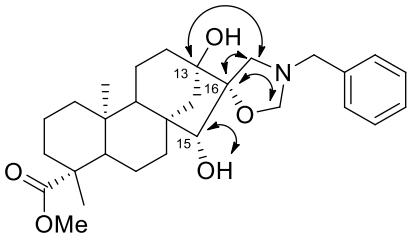


Figure 20: Determination of the structure of derivative **116** by HMBC

3.3. Biological investigation and drug-likeness studies

3.3.1. *In vitro* antiproliferative activity assay

The *in vitro* antiproliferative activities of the novel synthesised compounds against a panel of different human cancer cell lines of gynaecological origin, including cervical (HeLa and SiHa), breast (MCF-7 and MDA-MB-231), ovary (A2780) cancers and NIH/3T3 healthy fibroblasts were assayed by the MTT method in collaboration with the Department of Pharmacodynamics and Biopharmacy at the University of Szeged. Cisplatin, a clinically applied anticancer agent, was used as a reference compound.

Most molecules exerted relevant action against the non-malignant fibroblasts, indicating the limited selectivity of the tested substances. Analysis of the results led us to believe that the presence of the *N*-benzyl substituent at the amino function is required for the cytotoxic effect in the case of both aminoalcohols and aminotriols, similar to that noted previously on steviol-derived aminodiols [10]. The aromatic substituent on the diterpene core increased the bioactivity, since the compounds with primary amino function (**97a-b**, **115**) caused pronounced cell growth inhibition only at 30 μM, or none. However, the regioisomeric analogue **106** was more active, especially against MCF-7 cells. Compounds with an azido function at the diterpene skeleton (**102**, **103**), as well as intermediates **95**, **111** and **112** elicited no substantial action. Incorporating the originally basic nitrogen into a triazole ring (**107–110**) resulted in analogues with poor or modest activities and slight preference towards the MCF-7 cell line.

Concerning the aminoalcohol analogues with benzyl or naphthyl moieties, significant difference in antiproliferative activity was observed between the regioisomers. Molecules in which the aromatic building block was connected to the core through a methylene linker tended to elicit higher activities than analogues connected directly (e.g. **98a**, **98f**–**h** and **105a**, **105e**–**g**, respectively). The stereochemistry of the nitrogen has no notable impact on the antiproliferative effects (**98f** and **98g**, **105e** and **105f**, or **98i** and **98j**). Some of the presented compounds exhibited stronger growth-inhibitory actions than the reference agent cisplatin [153]. In the case of the most active agents, the assays were repeated by applying a range of concentrations (0.1–30 μM). Molecules **105b** and **105j** exerted outstanding activities against both cancer cells and fibroblasts. Compound **98j**, on the other hand, had similar effects on the malignant cells with limited action on fibroblasts, indicating considerable cancer selectivity. Since this latter agent seems to be superior to the clinically utilised cisplatin, it could be regarded as a potential hit compound and it may be subjected to further investigations. The structures and calculated IC_{50} values of the most potent derivatives are presented on **Figure 21** and in **Table 6**, respectively.

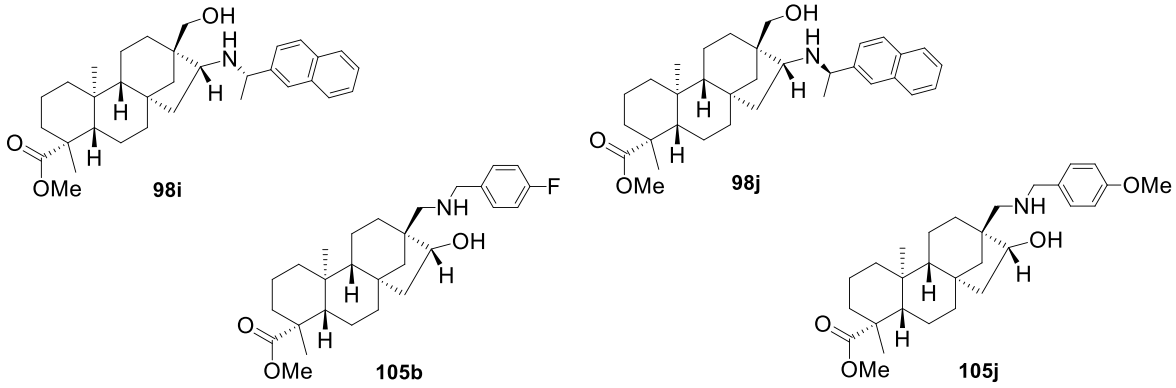


Figure 21: 1,3-Aminoalcohols with potent antiproliferative activity

Table 6. IC_{50} values of the aminoalcohols with outstanding antiproliferative activity

Compound	Calculated IC_{50} values (μM)					
	HeLa	SiHa	MCF-7	MDA-MB-231	A2780	NIH/3T3
98i	3.40	6.33	3.88	4.51	4.43	17.44
98j	3.51	5.37	2.47	5.62	4.06	8.70
105b	5.07	4.41	1.59	3.28	4.39	5.15
105j	3.09	5.77	1.04	2.30	3.78	3.71

Derivatives **113b**, **114a** and **114b** executed significant activity in higher concentrations only, with **113b** expressing potent inhibition on the HeLa line and **114b** having a slight

selectivity for MDA-MB-231 cells. On the MDA-MB-231 cell line, derivatives **114f** and **114g** displayed noteworthy selectivity, followed by slightly weaker action on MCF-7 and A2780. Compounds **114e** and **114i** (**Figure 22**) exhibited higher selectivity on the MCF-7 cell line at lower concentrations, with the latter also showing a moderate inhibition of HeLa cell growth. A strong and selective antiproliferative effect was found regarding compounds **114c** and **114h** on MCF-7, with decent activity on the MDA-MB-231 line. The heterocyclic derivative **116** also showed slight **s**electivity for MCF-7 cells in 30 μM, with moderate activity on the SiHa, MDA-MB-231 and A2780 lines. Additionally, **114c** expressed moderate inhibition on SiHa cell line, while **114h** exerted modest inhibition on HeLa and A2780.

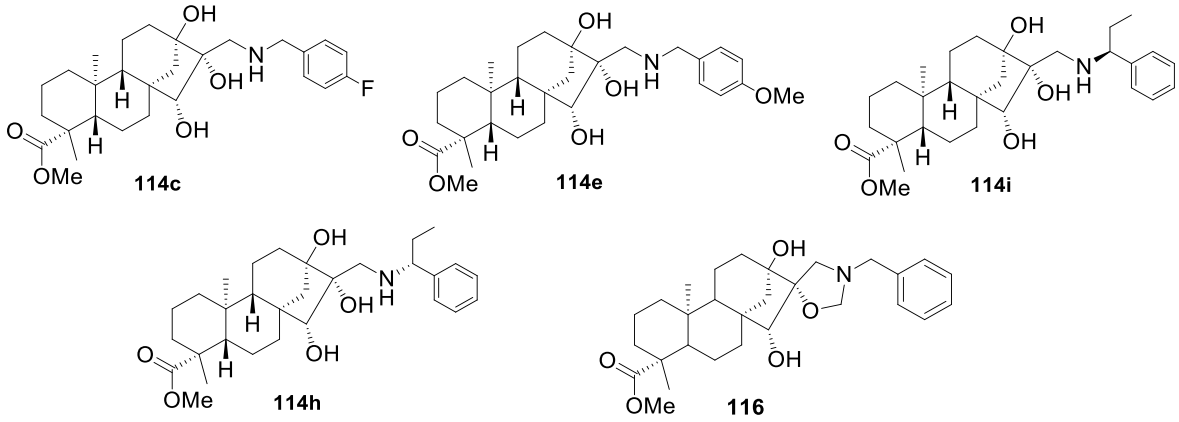


Figure 22: Steviol-based aminotriols with selective antiproliferative activity

Considering the antiproliferative activity profile of the studied aminotriol derivatives against NIH/3T3 healthy cell lines, the most selective aminotriols (**114c**, **114e**, **114h** and **114i**) performed best on the MCF-7 breast cancer cell line, which indicates group selectivity. In addition, a smaller but also selective antiproliferative effect was found on the other breast cancer cell line (MDA-MB-231) in the case of derivatives **114c**, **114h** and **116**. Compounds **114c**, **114e** and **114i** can be considered as primary *in vivo* preclinical candidates, while derivatives **114h** and **116** can be secondary candidates for further studies.

Based on recent data, naphthalene rings, while increasing the activity, lower the selectivity for a specific cell line. Substituents, such as methoxy and fluorine in *para*-position of the benzene ring, favour the MCF-7 cells regarding the inhibition of cell growth. The α-alkyl groups seem to be beneficial for the selectivity. Furthermore, the number of carbon atoms has influence on the affinity towards specific types of cells. However, when paired with the *p*-fluorine function, these properties are lost. Recognizable tendencies between the aminoalcohols and aminotriols showed stronger cytotoxic effects in

the case of 16-OH derivatives with lower sensitivity for a certain cell line than their regioisomers, while aminotriols expressed somewhat higher selectivity for cancer cells, in general, without significant damage to the fibroblasts.

3.3.2. *In silico* and *in vitro* drug-likeness study

We had the opportunity to collaborate with the Department of Chemical and Environmental Process Engineering at the University of Technology and Economics in Budapest to investigate the physicochemical classification and drug-likeness evaluation of the steviol-based aminotriols and a few intermediates. Physicochemical parameters such as Lipinski's rule of five (Ro5) [154], pK_a values and topological polar surface area (TPSA) were predicted by ACD/Labs Percepta software package for estimation of drug absorption. The Ro5 violation occurred in the case of the benzyl and naphthyl (**114h**, **j**, **l**) derivatives. The molecular weight of the latter and compounds **114d** and **114e**, as well as the lipophilicity (log*P*) of **114h** and **114i** exceeded the limitations as seen in **Table 7**. The TPSA value of all tested compounds is less than 140 Å^2, which corresponds to the oral bioavailability rule defined by Veber et al. [155]. Intermediates **112** and **113b** could not be ionised by MS. It is presumably due to the lack of mobile protons and, therefore, detection was not possible in further tests.

Table 7. Predicted physicochemical parameters of steviol-based aminotriols

Product	MW[a]	p$Ka_{,base}$ / p$Ka_{,acid}$	logP^a /log$D_{7.4}$	TPSA Å²	Lipinski Ro5 violation[a]
114a	472	9.5 / -	4.5 / 2.8	99.0	No
114b	486	8.6 / -	4.8 / 4.0	90.2	No
114c	490	9.5 / -	4.4 / 2.8	99.0	No
114d	504	9.6 / -	4.8 / 3.2	99.0	Moderate: Mw
114e	502	9.9 / -	4.3 / 2.3	108.3	Moderate: Mw
114f	486	9.6 / -	4.8 / 3.1	99.0	No
114g	486	9.6 / -	4.8 / 3.1	99.0	No
114h	500	9.6 / -	5.2 / 3.5	99.0	Moderate: log*P*
114i	500	9.6 / -	5.2 / 3.5	99.0	Moderate: log*P*
114j	522	9.2 / -	5.5 / 4.2	99.0	High: Mw, log*P*
114k	536	9.3 / -	5.7 / 4.3	99.0	High: Mw, log*P*
114l	536	9.3 / -	5.7 / 4.3	99.0	High: Mw, log*P*
115	382	10.2 / -	2.3 / -0.1	113.0	No
116	484	5.2 / -	4.1 / 4.1	79.2	No

[a]Lipinski's Ro5 violation for molecular weight (MW > 500) and lipophilicity (logP > 5)

Kinetic aqueous solubility and *in vitro* intestinal effective permeability were also determined by our colleagues, using intestinal-specific parallel artificial membrane

permeability assay (PAMPA-GI system) [156]. Concentrations of filtrates and calibration solutions (5–500 μM) of kinetic solubility study and starting donor and acceptor solutions of PAMPA-GI study were measured by HPLC-DAD-MS method. Considering the data, only compounds **114c**, **114i** and **114j**–**l** have moderate and poor aqueous solubility, respectively (**Table 8**). This result shows a good correlation with the high log*P* value of the naphthyl derivatives. These types of compounds, because of their lipophilicity, get stuck in the lipid membrane essentially damaging non-cancerous cells as well modifying the composition of the membrane and interacting with receptors, as proven by the fibroblast experiment. In addition, the inhibited membrane permeability can be explained by the increased membrane partition (MR>90%), while the hydrophilic character of **115** and **116** results in low penetration (MR<5%). In the case of the **114a**–**b** and **116** derivatives, the decrease in the basicity of the aminotriols was accompanied by a decrease in the effective permeability (P_e). For compounds **114a**–**b** this was also associated with a decrease in kinetic solubility. Regarding the physicochemical properties of the measured derivatives, **114a** and **114d**–**g** have at least moderate membrane penetration beside good solubility and they do not violate Lipinski's rule.

Table 8. Experimental physicochemical characterisation of steviol-based aminotriols. P_e: effective permeability, MR: membrane retention. Kinetic aqueous solubility, P_e and MR values of PAMPA-GI measurements represent mean ± S.E.M, n=3.

Product	Kinetic solubility[a]	PAMPA-GI	
	(μM)	P_e[b] (10^{-7} cm/s)	MR[c] (%)
114a	290.8±35.5	6.4±0.3	29.8±0.3
114b	114.3±16.0	2.3±0.8	67.3±8.6
114c	42.1±10.8	4.0±0.4	63.8±4.9
114d	145.4±14.1	4.8±0.5	22.4±2.0
114e	142.9±26.8	3.7±1.1	6.1±1.1
114f	181.6±3.2	6.4±0.7	28.8±5.8
114g	131.3±2.2	5.8±0.1	42.6±3.9
114h	145.0±28.2	1.4±0.4	46.8±3.3
114i	89.9±8.5	2.5±0.9	44.6±4.2
114j	18.9±2.4	ND[d]	94.2±2.0
114k	13.3±0.9	ND[d]	92.6±2.1
114l	11.9±1.8	ND[d]	92.6±1.1
115	535.4±38.4	ND[d]	1.5±0.3
116	321.1±88.5	ND[d]	2.7±0.4

[a]After 2 h, at 37 °C in PBS, pH 7.4; classification for kinetic solubility (μM): good (non-greyed ≥ 100) moderate (100 > light grey ≥ 40), poor (dark grey < 40), [b]Gradient pH system ($pH_{donor}6.5 \rightarrow pH_{acceptor}7.4$) after 4 h, at 37 °C; classification for PAMPA-GI permeability, P_e (10^{-6} cm/s): medium (non-greyed ≥ 5), low (5 > light grey ≥ 3), very-low (dark grey < 3), [c]Increased lipid membrane retention (MR% > 90), indicating a strong interaction between the active substance and the artificial lipid membrane, [d]ND: not determined (the compound cannot be detected in the acceptor side).

Summary

In my thesis work, stereoselective synthesis and *in vitro* biological study of novel diterpene-based aminoalcohols, aminotriols and their heterocyclic derivatives from the aglycone of natural glycoside stevioside were performed.

Starting from commercially available stevioside **4**, steviol **6** was obtained according to literature methods and then it was transformed into *spiro*-epoxide methyl ester **85a** in stereospecific reaction. Treating the epoxyalcohol with Lewis acid $BF_3.Et_2O$ resulted in the rearrangement of the kaurane skeleton, a process originally described by Wagner and Meerwein. β-Keto-alcohol **95** with a newfound *ent*-beyerane structure played centric role in the development of several biologically active derivatives.

The oximation and Raney Ni-catalysed hydrogenation of **95** afforded primary aminoalcohol diastereomers in a 2:1 ratio. Coupled with a series of primary amines, compound **95** gave a library of 1,3-aminoalcohols **98a**–**j** through Schiff bases that were reduced by $NaBH_4$. Regioisomeric aminoalcohols **105a**–**i** were synthesised through hydroxy-mesylate **104** via nucleophilic substitution of the same selection of amines as before. Azidoalcohol **103** derived from the mesylate was subjected to hydrogenation catalysed by Pd/C serving primary aminoalcohol **106**. *para*-Methoxybenzyl-substituted derivative **105j** was prepared exclusively from the primary aminoalcohol through Schiff base. Compound **103** and azidoketone **102** were used in click reaction to provide heteroaromatic 1,2,3-triazolo-amino- and keto-alcohols (**107**–**110**).

A new family of kaurane-based aminotriols were synthesised from steviol. Subsequently these underwent allylic hydroxylation and epoxidation by *t*-BuOOH, in the presence of selenium dioxide and vanadyl acetoacetate. The *spiro*-oxirane ring was opened with several primary and secondary amines to obtain aminotriols **114a**–**l** in highly stereoselective reactions. Debenzylation of *N*-benzyl-derivative **114a** afforded primary aminotriol **115** in moderate yield. Compound **114a** was also chosen as starting material to attempt formaldehyde-mediated ring closure, which proceeded in regioselective manner, granting *spiro*-oxazolidine **116**.

The antiproliferative activities of aminoalcohols and aminotriols, as well as the heterocyclic compounds were investigated in collaboration by *in vitro* MTT assays and the structure–activity relationships were determined by considering the substituent effects on the diterpenoid ring system. 1,3-Aminoalcohols **98i**, **98j**, **105b** and **105j** expressed potent

action on a panel of human gynaecological cancer cell lines with considerable selectivity for MCF-7. Among aminotriols, **114c**, **114e** and **114i** were found to be the most effective besides showing selectivity on the same breast cancer line. The analysis confirmed that the *N*-benzyl unit is essential for the inhibition of cell growth, and the *para*-substituted ring especially with α-alkyl substituents are beneficial for the selectivity. While the naphthyl functions highly elevated toxicity, any kind of selectivity was lost.

This effect was explained by the *in silico* and *in vitro* membrane permeability assay conducted in collaboration. The data collected showed poor aqueous solubility and high lipophilicity; therefore, the compounds get stuck in the lipid membrane. Based on the physicochemical properties, **114a** and **114d**–**g** presented at least moderate membrane penetration besides good solubility without violating Lipinski's rule. Considering the pharmacological results, **114e** is eligible for further studies in the preclinical stage.

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ANNEX