University of Szeged

Doctoral School of Pharmaceutical Sciences

Pharmaceutical Chemistry and Drug Research Programme

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Institute of Pharmaceutical Chemistry

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

Summary of Ph.D. Thesis

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Szeged, Hungary

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1. Introduction and aims

In the last couple of decades, the development of anticancer agents has been a major focus of research for scientists all over the world. The challenges of evolving multidrug resistance and still high mortality rates demand new approaches in drug design. The latest studies pay significant attention to the synthesis of diterpene-based compounds due to their promising bioactivity.

Terpenoids are the largest, structurally diverse class of chiral products built of isoprene units that can be found in most plants, therefore these molecules are easily available directly from their natural sources or by large-scale preparation. In organic syntheses they are often chosen as starting materials for asymmetric transformations as well as chiral catalysts in enantioselective reactions. Several investigations have confirmed the multifaceted pharmacological properties the compounds exhibit, such as antibacterial, antihyperglycemic, anti-inflammatory cardiovascular protective and most importantly, antitumor activities. Additionally, some heterocyclic derivatives display remarkable antifungal, BACE1-inhibiting and antiproliferative action on a panel of human cancer cell lines. Terpenes mainly contribute to the depolarization 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.

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, aminodiol or aminotriol derivatives have earned great interest recently. Bioactive aminoalcohols of natural origin such as pactamycin, an antibiotic with antiproliferative properties, the immunosuppressant antibiotic and antifungal myriocin, or the actomyosin ATP-ase activator penaresdin A and B 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. One of the few chiral sources of diterpenes that are accessible in large scale is stevioside, a triglycoside isolated at an industrial scale from the perennial herbal shrub *Stevia rebaudiana*. It is frequently used for the synthesis of cytotoxic diterpenoid derivatives since it can be easily transformed to its aglycons, steviol and isosteviol, which show similar biological effects.

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.

Our aim in my PhD work was to synthesise a versatile, novel library of 1,3-aminoalcohols, and by inserting a third hydroxyl group, 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, compare the results and determine the structure-activity relationship.

2. Methods

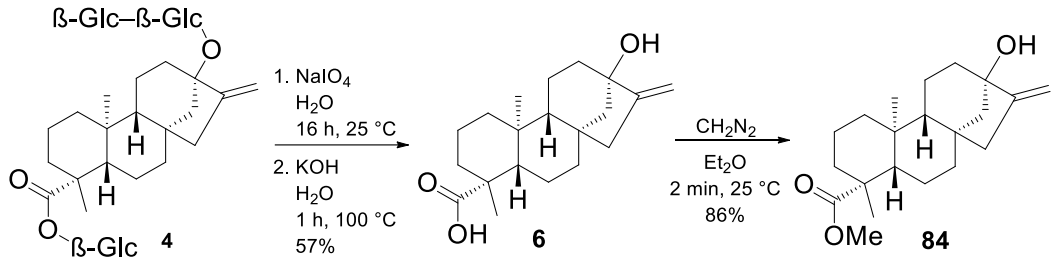
The reactions were performed on a mmol scale, the compounds were purified by normal phase column chromatography on silica gel or by crystallization. All new compounds were charaterized by their melting point, optical rotation, elemental analysis, 1D- and 2D-NMR. The antiproliferative properties were determined by a standard MTT assay. The absorbance was measured at 545 nm by using a microplate reader (SPECTROStar Nano, BMG Labtech, Offenburg, Germany). Calculations were performed using the GraphPad Prism 9 software (GraphPad Software Inc., San Diego, CA, USA). Filtrates and calibration solutions (5–500 µM) of the kinetic solubility study and starting donor and acceptor solutions of the PAMPA-GI study were investigated by HPLC-DAD-MS. LC-MS analysis was performed using a Waters 2795 HPLC with a Waters 2487 DAD detector coupled with a Micromass Quattro Ultima TMPt quadrupole mass spectrometer equipped with an ESI source.

3. Results and Discussion

3.1. Stereoselective synthesis of bifunctional steviol derivatives

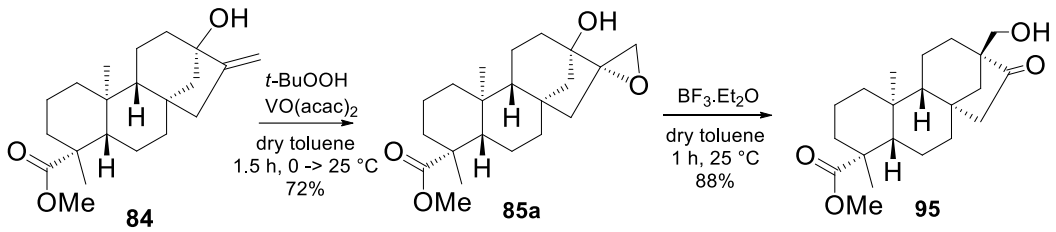
3.1.1. Synthesis of key intermediate β-keto-alcohol

For the starting point of our research, we chose commercially available natural glycoside stevioside **4** which was transformed into its aglycone, steviol **6** in a two-step synthesis. The oxidative, alkaline hydrolysis was carried out in the presence of $NaIO_4$ and KOH as described in the literature (**Scheme 1**). Esterification of steviol was accomplished with diazomethane prepared *in situ*, in diethyl ether resulting in methyl ester **84** in a few minutes (**Scheme 1**).



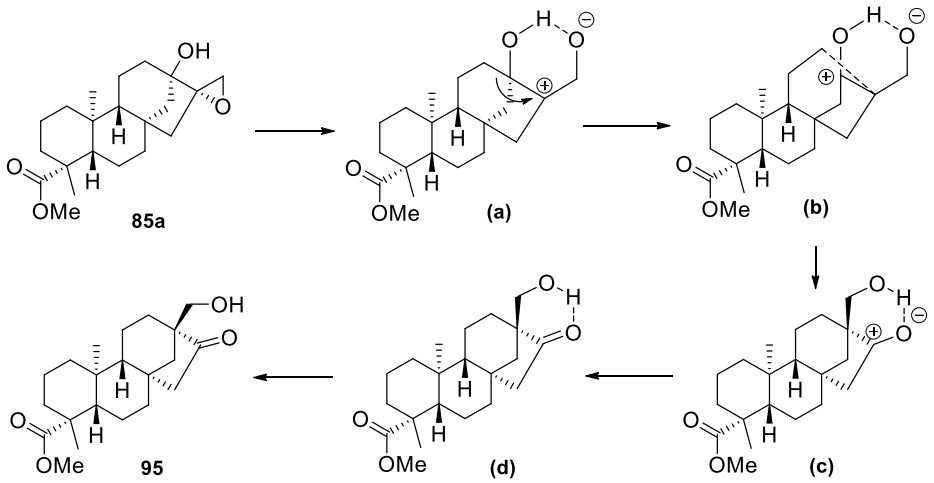
Scheme 1: Preparation of steviol methyl ester

In the next step, epoxidation of the methyl ester was conducted by a method applying *t*-BuOOH as the oxidising agent and vanadyl acetylacetonate ($VO(acac)_2$) as the catalyst. The reaction gave *cis*-epoxyalcohol **85a** in a stereospecific manner. The stereochemistry of the compound was described in literature previously (**Scheme 2**). The epoxide was then treated with $BF_3.Et_2O$ causing a rearrangement to take place at room temperature in merely an hour. The reaction resulted in a single derivative (**95**) and inspection by 2D-NMR spectroscopy confirmed the change of stereochemistry in the structure, where the kaurane skeleton converted to isosteviol-type *ent*-beyerane (**Scheme 2**).



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

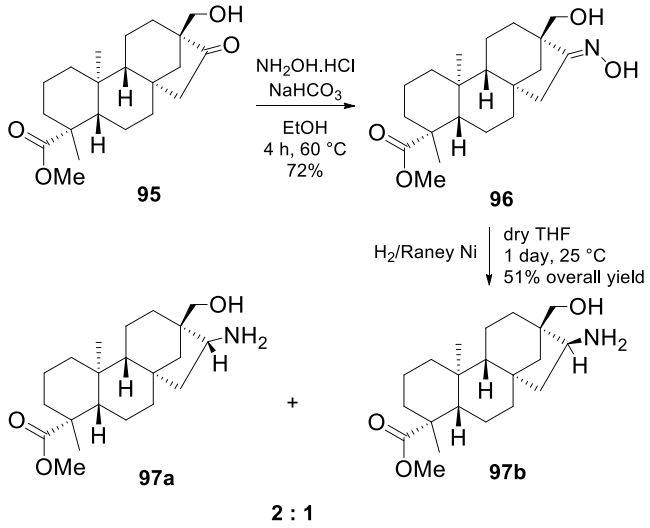
Based on the findings of Schreiber et al. on 8*S*,15-epoxy-gibberellic acid, we propose the following process for the reaction mechanism. The oxirane ring opens by the coordination of the Lewis acid to the oxygen of the epoxide and a six-membered ring is built through interaction with the neighbouring hydroxyl 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**).



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

3.1.2. Synthesis of 1,3-aminoalcohols

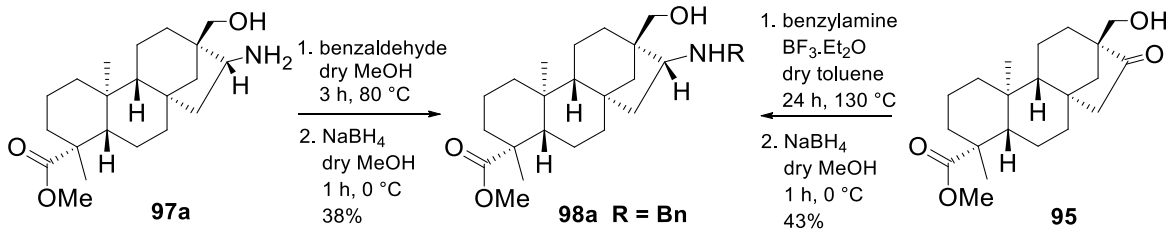
Our key molecule **95** was first transformed into oxime (**96**) with the help of hydroxylamine hydrochloride in the presence of $NaHCO_3$. The product then underwent hydrogenation catalysed by Raney Ni in THF at room temperature resulting in a mixture of primary aminoalcohols **97a** and **97b**. The diastereomers were obtained in a 2:1 ratio as identified by NMR spectroscopy, and they could be successfully separated by preparative column chromatography in CH_2Cl_2/MeOH = 1:1 mixture (**Scheme 4**).



Scheme 4: Stereoselective synthesis of primary aminoalcohols

To create a series of *N*-substituted aminoalcohols, first the reaction of major primary aminoalcohol **97a** with benzaldehyde was carried out. The resulting Schiff base was reduced by $NaBH_4$ at 0 °C, providing the *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. 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 the stereoselectivity. Reduction of the product was accomplished with $NaBH_4$ once again, without isolating the intermediate, resulting in **98a**. This method proved to be ideal for the preparation of different *N*-substituted aminoalcohols, because it gave us the compounds in less steps and we could avoid extensive work with the delicate primary aminoalcohols. The novel 1,3-aminoalcohols **98b**–**j** were synthesized by the above mentioned procedure in moderate to good yields (**Table 1**, **Scheme 5**).



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

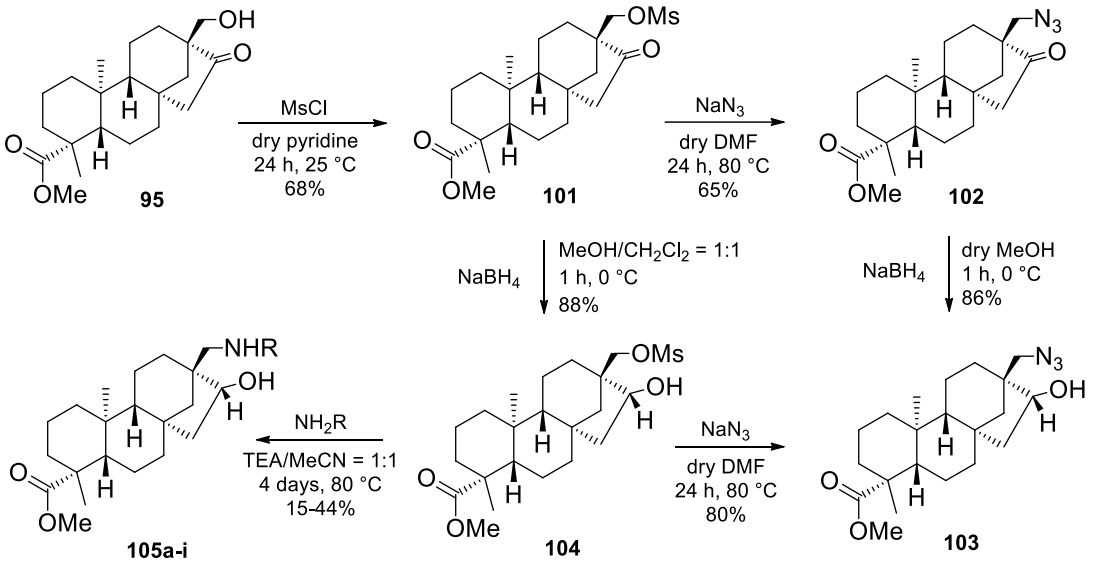
Table 1. Library of steviol-based 1,3-aminoalcohols

Entry	Compound	R	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

Preparation of the regioisomeric analogue of the aminoalcohols discussed in chapter 3.1.2. started with the treatment of compound **95** with methanesulfonyl chloride in anhydrous pyridine to eanable the hydroxyl function exchange to a better leaving group (**Scheme 6**). In the following steps two pathways were considered: in fear of losing the *O*-mesyl function

during reduction of the ketone with $NaBH_4$, the mesyl group was converted to azide (**102**) with sodium azide before the reduction keto function was carried out. Alternatively, to decrease the reactivity of the mesylate in the presence of hydride ions, a 1:1 solvent ratio of MeOH and dichloromethane was used as reaction medium instead of pure methanol, resulting in hydroxyl-mesylate **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 2**). 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 and maximise the yield (**Scheme 6**).



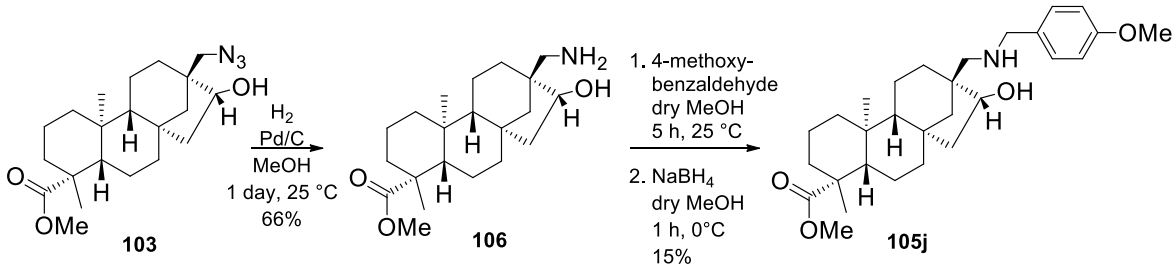
Scheme 6: Synthesis pathways for the preparation of 1,3-aminoalcohol regioisomers

Table 2. Library of 1,3-aminoalcohol regioisomers

Entry	Compound	R	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

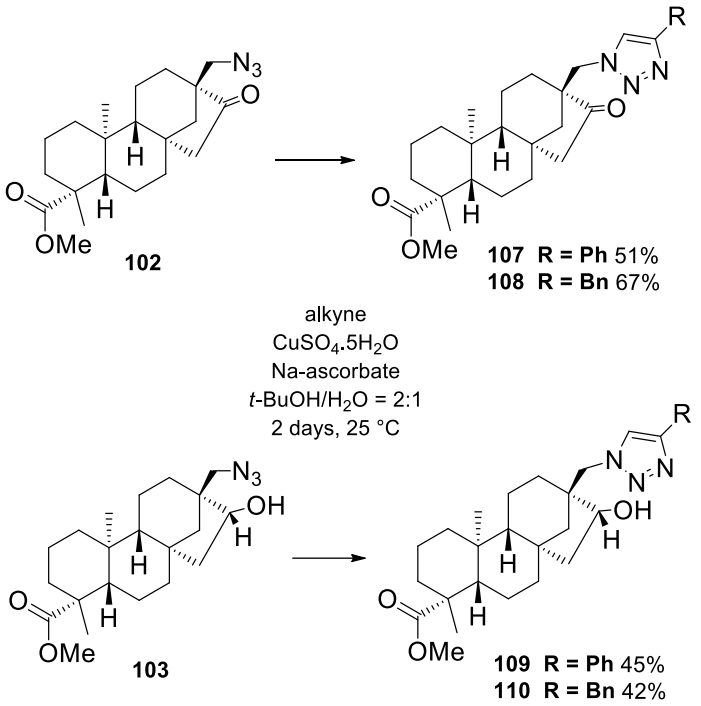
Applying 4-methoxybenzylamine, despite testing multiple different conditions, affecting the temperature, equivalency, and catalyst/solvent ratio, no product could be detected on TLC therefore we decided to synthesise the desired derivative through primary aminoalcohol **106** as an alternative pathway, which was prepared by palladium-catalysed hydrogenation of hydroxyl-azide **103** in methanol. Next, 4-methoxybenzaldehyde was added to compound **106** to form the Schiff base intermediate, followed by its reduction without isolation with $NaBH_4$, providing aminoalcohol **105j** (**Scheme 7**).



Scheme 7: Synthesis of 4-methoxybenzylamino derivative **105j** through primary aminoalcohol

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

To prepare heterocyclic derivatives **107** and **108**, keto-azide **102** was coupled with aromatic alkynes in *t*-BuOH/H_2O = 2:1 medium for better solubility of catalysts $CuSO_4.5H_2O$ and sodium ascorbate, the latter generated *in situ* from ascorbic acid and NaOH in methanol (**Scheme 8**). When the reaction was carried out with the ascorbate prepared well in advance, notable progress couldn't be observed. The synthesis was extended for hydroxyl-azide **103** as starting material, forming compounds **109** and **110** in good yields.

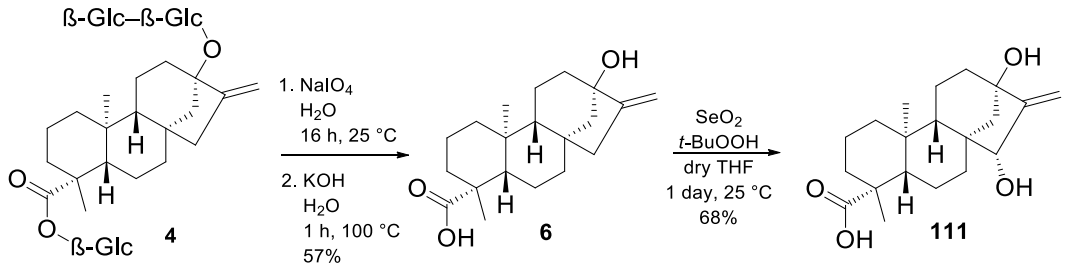


Scheme 8: Preparation of 1,2,3-triazolo derivatives via click reaction

3.2. Stereoselective synthesis of tetrafunctional steviol derivatives

3.2.1. Synthesis of key intermediate *spiro*-epoxide

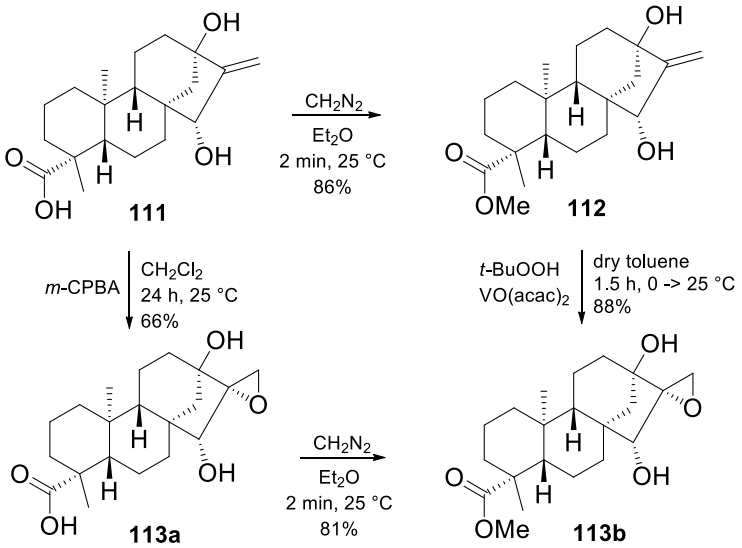
To expand the collection of novel aminoalcohol type diterpenes by adding further hydroxyl group to the kaurane skeleton, allylic hydroxylation of steviol **6** was accomplished by adding selenium(IV) dioxide and *tert*-butylhydroperoxide in dry THF (**Scheme 9**). The reaction was found to be stereoselective for the 15-α-OH isomer (**111**) as described in literature.



Scheme 9: Preparation of allylic alcohol **109** 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 under a few minutes (**Scheme 10**). Next, *spiro*-epoxide **113b** was synthesised from methyl ester **112** in stereospecific reaction with *t*-BuOOH as oxidizing agent and catalysed by vanadyl

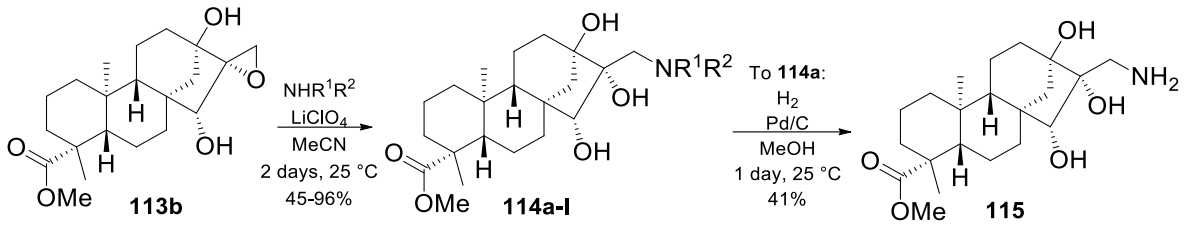
acetylacetonate ($VO(acac)_2$). To determine the stereochemistry of **113b**, the compound was prepared in an alternative (even less successful) pathway as well. As described in literature, treatment of **111** with *meta*-chloroperoxybenzoic acid in dichloromethane gave derivative **113a** with known stereochemistry (**Scheme 10**). Ester synthesis was carried out once again with diazomethane and the resulting product proved to be possessing 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 with the vanadium catalysed method.



Scheme 10: Chemo- and stereoselective synthesis of key intermediate spiro-epoxide **113b**

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

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 $LiClO_4$ as Lewis acid catalyst (**Scheme 11**). 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. The preparation of the novel aminotriols was accomplished with moderate to excellent yields given in **Table 3**. Secondary amine *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. Furthermore, compound **114a** was subjected to debenzylation by hydrogenolysis over Pd/C to obtain primary aminotriol **115** in moderate yield (**Scheme 11**).



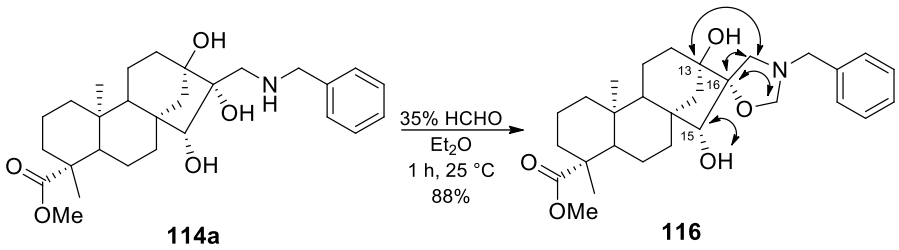
Scheme 11: Stereoselective synthesis of aminotriols

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

Entry	Compound	R^1	R^2	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

In a former study, a steviol-based oxazolidine derivative expressed remarkable cytotoxic activity on human gynaecological cancer cell lines. Intrigued by our previous results, we decided to synthesise the heterocyclic counterpart of compound **114a** and determine the regioselectivity of the ring closure. Treatment of aminotriol **114a** with aqueous formaldehyde at room temperature gave spiro-oxazolidine **116**, in highly regioselective reaction (**Scheme 12**). Determination of the structure was accomplished by the study of the 2D NMR data and the observation of cross-couplings in the HMBC spectrum.



Scheme 12: Regioselective ring closure of aminotriol **114a** with formaldehyde

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. 1,3-aminoalcohols **98i**, **98j**, **105b** and **105j** expressed potent action on several cancer cell lines with considerable selectivity for MCF-7. Calculated IC_{50} values of the most potent derivatives are presented in **Table 4**.

Table 4. 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

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, the *para*-substituted ring and α-alkyl groups are beneficial for the selectivity. While the naphthyl functions highly elevated the toxicity, any kind of selectivity was lost.

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

In collaboration with the Department of Chemical and Environmental Process Engineering at the University of Technology and Economics in Budapest we investigated the physicochemical classification and drug-likeness of the steviol-based aminotriols. Physicochemical parameters such as Lipinski's rule of five, pK_a values and topological polar surface area (TPSA) were predicted for estimation of drug absorption. Kinetic aqueous solubility and *in vitro* intestinal effective permeability were determined by intestinal-specific parallel artificial membrane permeability assay (PAMPA-GI system). The extreme inhibition of naphthyl compounds was explained by poor aqueous solubility and high lipophilicity, meaning the compounds get stuck in the lipid membrane and damage the fibroblasts as well. Based on the physicochemical properties, **114a** and **114d**–**g** presented at least moderate membrane penetration beside good solubility and don't violate Lipinski's rule. Considering the pharmacological results, **114e** is eligible for further studies in preclinical stage.

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
   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