Ph.D. Thesis

Studies on industrial *Cyanotis arachnoidea* extracts and semi-synthetic ecdysteroid derivatives

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List of publications related to the thesis

I. *17-Oxime ethers of oxidized ecdysteroid derivatives modulate oxidative stress in human brain endothelial cells and dose-dependently might protect or damage the blood-brain barrier.* Máté Vágvölgyi, Dávid Laczkó, Ana Raquel Santa-Maria, Judit P Vigh, Fruzsina R Walter, Róbert Berkecz, Mária A Deli, Gábor Tóth, Attila Hunyadi **PLoS One** 2024; 19 (2): e0290526.
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II. *LC-MS/MS fingerprinting analysis of Cyanotis arachnoidea extracts: process-related artifacts in anabolic food supplements.* Dávid Laczkó, En-Liang Chu, Ching-Chia Chang, Fang-Rong Chang, Gábor Girst, Tamás Gáti, Gábor Tóth, Árpád Könczöl, Attila Hunyadi **ACS Omega** 2025; 10 (18): 18605-18614.
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III. *Investigation of calonysterone and 20-hydroxyecdysone effects in high-fat, high-sugar diet-induced obesity rat model.* Alaa AM Osman, Dávid Laczkó, Máté Vágvölgyi, Adrien Seres-Bokor, Anita Sztojkov-Ivanov, Kata Kira Kemény, Attila Hunyadi, Eszter Ducza **Heliyon** 2025; 11 (3): e42435.
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List of abbreviations

^{13}C	NMR Carbon nuclear magnetic resonance
^{1}H NMR	Proton nuclear magnetic resonance
20E	20-hydroxyecdysone
20EOX	20-hydroxyecdysone autoxidated sample
anh.	Anhydrous
aq.	Aqueous
BBB	blood-brain barrier
CALF	Authentic leaf extract
CARO	Authentic root extract
CAPR1	Commercial extract of *Cyanotis arachnoidea* No. 1
CAPR2	Commercial extract of *Cyanotis arachnoidea* No. 2
CPC	Centrifugal partition chromatography
DMSO	Dimethyl sulfoxide
ESI	Electrospray ionization
EtOAc	Ethyl acetate
H_2O	Water
H_2O_2	Hydrogen peroxide
HPLC	High-performance liquid chromatography
HR-MS	High-resolution mass spectrometry
IC_{50}	Fifty percent inhibitory concentration
K_D	Partition coefficient
labCPC	Laboratory scale CPC instrument (250 mL) by Gilson
LC-MS	Liquid chromatography–mass spectrometry
MDR	Multi-drug resistance
MEK	Methyl ethyl ketone
MeOH	Methanol
MIBK	Methyl isobutyl ketone
MnO_2	Manganese dioxide
NaOH	Sodium hydroxide
PDA	Photodiode array
PIDA	(Diacetoxyiodo)benzene
PIFA	[Bis(trifluoroacetoxy)iodo]benzene
PMA	Phosphomolybdic acid

Py	Pyridine
QQQ	Triple quadrupole
rCPC	Semipilot scale CPC instrument (2100 mL) by RotaChrom Technologies
ROESY	Rotating-frame overhauser effect spectroscopy
ROS	Reactive oxygen species
RP-HPLC	Reverse-phase high pressure liquid chromatography
RT	Room temperature
TLC	Thin layer chromatography
UV	Ultraviolet

I. INTRODUCTION

Steroids are widespread in nature, but their significance and precise physiological effects are best understood in the animal kingdom. Ecdysteroids are a special group of natural steroids that were discovered in 1954. In the animal kingdom, these substances are typically found in invertebrates (e.g. arthropods, corals, worms). In arthropods, they play a hormonal role in the regulation of molting and development. Their common name is derived from the characteristic process of molting in insects, known as *ecdysis* [1]. Such compounds also occur in plants where they are structurally much more diverse, but in this case we have only a superficial understanding of their role; they most likely serve as a pesticide defense.

Accordingly, ecdysteroids cannot be classified strictly as animal or plant steroids, as some derivatives occur in both groups, albeit in different ratios. Among the many natural ecdysteroids discovered over the years, the most abundant, both in plants and insects, is 20-hydroxyecdysone (20E; compound **39**), i.e., the insect molting hormone [2]. The diversity of ecdysteroids in the living world has attracted the interest of pharmacologists from the very beginning. In connection with insect metamorphosis, they induce a high burst of protein synthesis. Interestingly, ecdysteroids also increase protein synthesis in vertebrates, which manifests in an anabolic effect even though they do not bind to nuclear sexual hormone receptors [3]. In addition, several other bioactivities have subsequently been discovered, including blood glucose and cholesterol lowering, immunomodulatory, and anti-inflammatory effects. Their antioxidant activity has only been known since the 1990s [2, 4, 5].

Due to their versatile and promising pharmacological properties, ecdysteroids have had many experimental applications in medicine, agriculture and cosmetics in recent years. Because of their significant anabolic effects, athletes have also been interested in ecdysteroid-containing products marketed as dietary supplements, often of dubious quality. Based on its anabolic and adaptogenic properties potentially exploited by athletes, 20E has been included in the monitoring program of the World Anti-Doping Agency (WADA) since 2020. Typically, this represents the first step toward inclusion on the doping list [6].

I.1. Natural occurrence and commercial availability of ecdysteroids

I.1.1 Structure

Ecdysteroids represent a special group of steroids in terms of structure. They usually have a skeleton of 27–29 carbon atoms (**Fig. 1**), but compounds with a skeleton of less carbons are also found in nature, formed by the cleavage of the side-chain at the C-17 (e.g., rubrosterone) or C-20 position (e.g., poststerone). The side-chain can also undergo various forms of ring closure. A common structural feature of ecdysteroids is the chromophoric enone moiety in their B-ring (double bond between the 7,8 carbon atoms and a carbonyl group on C-6) [7].

In contrast to sterols, these compounds are highly hydroxylated, with a minimum of 2 and a maximum of 8 hydroxyl groups, which results in a typically water-soluble, hydrophilic character. The arrangement of the steroid skeleton is characteristic: with a few exceptions, A/B is usually *cis*, C/D is usually *trans* [7].

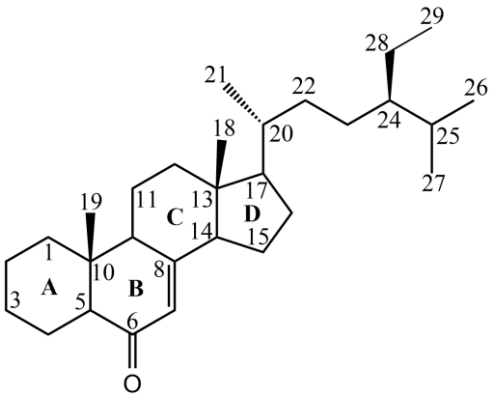


Figure 1 General formula of ecdysteroids with an intact side-chain.

I.1.2 Pharmacological aspects

Ecdysteroids have several bioactivities that appear to manifest in general positive effects on mammals, including humans. Both *in vitro* and *in vivo* experiments demonstrate that ecdysteroids can alter the function of mammalian cells, even in a matter of seconds, by binding to membrane receptors or ion channels. They can also activate signaling pathways that increase the expression of specific genes in the nucleus of cells, resulting in effects that can take hours to days to develop [8].

In addition to their numerous effects, they have been shown to exhibit low toxicity. 20E was administered orally to mice and, after measuring the LD_{50} value, it was found that the toxic dose was approximately 9g / body weight kg. In rabbits, 0.1 g / body weight kg did not induce a toxic reaction and treatment of rats at 2 g / body weight kg /day also did not result in toxic

symptoms. Although the LD_{50} in mice cannot be extrapolated to humans, the low toxicity observed in animal studies has contributed to the growing interest in 20E as a dietary supplement in recent decades [7].

Okui and Otaka are credited with discovering the effect of ecdysteroids on mouse livers, specifically their ability to increase protein synthesis and stimulate liver function. Some ecdysteroids caused a greater increase in protein synthesis than the reference 4-chlorotestosterone [9]. Later, several publications were published comparing the effects of ecdysteroids to those of androgenic hormones. In their experiments, Syrov and co-workers found that the anabolic effect of ecdysteroids depends on the physiological state induced by androgenic steroids. They observed that in castrated mature individuals, the effect of ecdysteroids was negligible compared to that of methandrostenolone. In this experiment, several compounds were compared, including, turkesterone and 20E. In the measurements, animals were given 5 mg / body weight kg of ecdysteroid orally for 10 days while the growth rate of the animals' heart, liver, kidney and muscle was measured [10].

Our research group has made significant progress in exploring the anti-tumor effects of ecdysteroids. Our research group's previous research has shown that typically less polar ecdysteroids, in combination with a well-chosen chemotherapeutic agent, can significantly reduce the drug resistance of both drug-susceptible and multi-drug resistant (MDR) tumor cells. Later, we found that this effect does not correlate with the weak ABCB1 efflux transporter (Pgp) inhibitory effect of some of these agents, and the latter (side) effect can be further reduced by the oxidative cleavage of the side-chain [11]. Recently, our research group has prepared a series of oximes, oxime ethers and a lactam derivative by the semi-synthetic transformation of 20E 2,3;20,22-diacetonide. The lactam derivative showed stronger chemosensitizing activity than our previous antitumor lead, 20E 2,3;20,22-diacetonide, without detectable inhibition of Pgp function [12].

Uchiyama et al. observed that 20E, when administered orally to rats, can significantly reduce the hyperglycemic effect of various agents elevating blood glucose levels (e.g., glucagon) while leaving normal blood glucose (euglycemia) unaltered. Experiments have also been carried out to elucidate the mechanism of action of ecdysteroids in lowering blood glucose. Yoshida's results show that 20E inhibits gluconeogenesis in the liver of mice and reduces the activity of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (PEPCK) enzymes [13, 14].

Furthermore, ecdysteroids are also able to reduce cholesterol levels by inhibiting its biosynthesis in the liver, enhancing cholesterol breakdown and stimulating its conversion to bile acids, which processes result in an anti-atherosclerotic effect [15].

For several decades, this group of compounds has also been known to exert beneficial immunomodulatory effects. In an early study by Trenin and Volodin, modulatory effects of 20E were demonstrated on lymphocytes and neutrophil granulocytes *in vivo* [15]. 20E is also thought to have adaptogenic effects, enabling organs to adapt to various external physicochemical stimuli (so-called stressors) and thus avoid organ damage [16].

It is also known that ecdysteroids inhibit lipid oxidation in vitamin D deficient states. There is a significant increase in oxidized free fatty acids in animals with hypovitaminosis D compared to healthy animals. 20E has been shown to exhibit antioxidant properties at levels as low as 0.02 mg / body weight kg, contributing to the inhibition of lipid oxidation [2, 17].

Oxidative stress plays a significant role in the pathological processes of ischemic brain injury [18]. Several antioxidant compounds have been shown to reduce brain ischemic injury. Some years ago, the antioxidant effect of 20E was described. Jun Hu and co-workers used an *in vitro* oxidative damage model and an *in vivo* middle cerebral artery occlusion model for their experiments. In their model, they observed neuronal damage, intracellular reactive radical formation, mitochondrial membrane potential abnormalities, a decrease in the cells' own antioxidant potential, and elevations in Ca^{2+} and malondialdehyde levels following H_2O_2 treatment. 20E could attenuate the above-mentioned processes and inhibit the ASK1-MKK4/7-JNK stress signaling pathway [19]. The antioxidant activity of 20E was comparable to that of well-known lipid peroxidation inhibitors, such as diethyl-paraphenylenediamine.

The neuroprotective effect of 20E has also been demonstrated *in vivo*, which highlighted the compound's ability to reduce the extent of infarction and neurological damage, restore antioxidant function, and inhibit the increase in malondialdehyde levels. [19].

Recent research has made great advances in understanding the mechanism of action of ecdysteroids, suggesting an interaction mechanism between the MAS receptor and a membrane-bound palmitoylated estrogen receptor. The pleiotropic pharmacological effects observed in mammals are fully consistent with the activation of the MAS receptor by a safe steroid molecule. All in all, the proposed mechanism may indeed explain the close similarity between the effects of 20E and angiotensin(1−7) [20]. These results may have paved the way for potential therapeutic developments in the future, and several related clinical studies have been initiated to exploit 20E as a drug.

Severe pneumonia caused by COVID-19 potentially can be linked to SARS-CoV-2 binding to ACE2. The objective of a study was to determine if 20E-induced Mas-receptor activation could stabilize the Renin-Angiotensin System (RAS) and reduce the incidence of respiratory failure and death in persons with severe COVID-19 who were admitted to the hospital. To prove this, a double-blind, randomized, and placebo-controlled phase 2/3 trial was carried out. Compared to the placebo group, the 20E-treated group experienced a more than 40% decreased incidence of respiratory failure or early death by day 28 [21].

Sarcopenia is a progressive muscular disease that can impair movement capabilities. There are currently no medicinal therapies available; instead, treatment consists of diet and exercise. The purpose of SARA-INT was to find out if 20E, an activator of the MAS receptor, is safe and enhances the physical performance and muscle function of elderly sarcopenic patients. Close to the minimal clinically meaningful difference (MCID) in sarcopenia (0.1 m/s), 350 mg daily intake of 20E demonstrated substantial trends after 6 to 9 months of treatment that were consistent with a clinically relevant effect on the 400-m walking test gait speed [22].

I.1.3 Commercially available *Cyanotis arachnoidea* extracts

Until now, thousands of plant species have been screened for their ecdysteroid content. They have been found in some marine algae, fungi, mosses and mainly in gymnosperms and angiosperms. They may be present in extremely high concentrations in some plants. One of the prerequisites for industrial formulation and human use, or other applications, is the availability of a cheap, abundant pure ecdysteroid active substance or a standardized plant concentrate. These can only be obtained from ecdysteroid-rich and stable plants of good quality. Today, such raw materials are mainly available in, for example, East Asia (China, Japan) [23]. A significant demand for ecdysteroid containing dietary supplements, mostly due to their non-hormonal anabolic effect, can be observed by a quick search on the Internet [3]. It emerges that 20E is by far the most studied ecdysteroid, and that far less is known about the safety and (supposedly, but not necessarily similar) bioactivities of less common ecdysteroids. Tons of concentrated 20E or 20E-containing extracts are available from many companies, often with a minimum order limit of hundreds of kilograms at prices as low as around 1 USD/kg [23, 24].

These extracts are most typically prepared from the roots of *Cyanotis arachnoidea* (Commelinaceae), a plant native in China, which contain up to 3–4% ecdysteroids, compared to the much lower amounts (0.005–0.08%) found in dietary sources such as spinach [25]. In recent years, the use of ecdysteroids and plant extracts containing them has become common practice in sports and bodybuilding worldwide. The inexpensive and sometimes irregular

supply of plant-based products from around the world has raised the possibility that the production of products containing such compounds or plant extracts has also emerged in our country, primarily in the form of dietary supplements, and in some cases even without official notification. In Europe, *Cyanotis* extracts fall under the legal category of novel foods and cannot be marketed for human consumption without full authorization. In connection with this, our research group has previously identified an unusual case of dietary supplement adulteration, in which *Cyanotis* extracts were marketed under spinach extract label, hence bypassing current European regulations [23].

I.1.4 Chemical Fingerprint analysis of ecdysteroid containing samples

Complex plant extract characterization and quantification are increasingly relying on high-resolution analytical approaches such as chemical fingerprint analysis. This is particularly true for botanical supplements and medicinal plants meant for human consumption. Multilateral objectives of fingerprint analysis of botanical materials may include quality control [26], standardization [27], inspection of authenticity [28], taxonomical evaluation [29], metabolomic analysis [30], and interpretation of the processing methods (e.g., by identifying extraction-related artifacts) [31]. The most effective methods to date include targeted and untargeted LC-MS and NMR techniques combined with chemometrics (e.g., principal component analysis) [28].

The problem of accurately separating ecdysteroid mixtures analytically has been extensively studied, since this problem exists because of the complexity and structural diversity of the samples. The best resolution of more complex ecdysteroid-containing samples can be achieved using reversed-phase HPLC-MS techniques [23].

Mixtures including up to 20 phytoecdysteroids can be analyzed with high confidence using classical RP-HPLC-MS/MS techniques [32]. However, the ecdysteroid content of samples can be very complex and with many isobaric compounds that frequently demonstrate similar fragmentation patterns dominated by sequential water losses. Fingerprint analysis may remedy the issue for samples containing up to 50 components [33]. Several coupling techniques have been explored in the past to enable a more accurate analysis [34, 35, 36].

I.2. Occurrence and isolation of ecdysteroids

The German scientists Butenandt and Karlson discovered ecdysteroids in 1954 when they were able to isolate α-ecdysone (or ecdysone as it is now widely known), from silkworm pupae [37]. The following discovery of four novel analogs, ponasterone A, B, C and D,

indicated the existence of the so-called phytoecdysteroids and resulted from the search for bioactive chemicals of *Podocarpus nakaii* hay extract [38]. Subsequently, a large number of different ecdysteroids have been identified through extensive screening: currently, 590 natural ecdysteroids are known according to the regularly updated online ecdysteroid database [39]. With ever-expanding studies, many new minor ecdysteroids are being discovered that require complex chromatographic solutions, often using a combination of several different methods. In addition to the previously mentioned sources, ecdysteroids have been isolated from algae [40], fungi [41], non-crustacean marine organisms [42], nematodes [43], cestodes [44], trematodes [45], among others. Within the framework of this PhD-thesis, the focus will be on phytoecdysteroids, and only they will be discussed in detail.

Although *Cyanotis arachnoidea* is of particular interest to industry due to its high ecdysteroid content, phytoecdysteroids are present in many different plant species. It was postulated that theoretically all plants may have the necessary genetic capacity to produce ecdysteroids, but complex regulatory processes determine their presence, quantity, and distribution [8]. This indicates the extensive possibilities of isolating ecdysteroids with diverse structures, illustrating this with a few plant examples below.

Species of the Polypodiaceae family often contain various ecdysteroids. *Microsorum membranifolium*, a plant used in Polynesian traditional medicine, contains 20E and several 20E derivatives, and specific (*E/Z)*-isomeric phytoecdysteroid conjugates were also identified [46]. *Lepidogrammitis drymoglossoides* also belongs to the family Polypodiaceae, a plant used in Chinese medicine, contains ecdysteroid glycosides such as ponasteroside B in addition to the well-known 20E [47]. Probably the best known ecdysteroid-containing plant of this family is *Polypodium vulgare*, used in North America as an expectorant and laxative. Numerous different ecdysteroids, including several previously unknown ones, have been identified during the analysis of the root's extracts [48].

Several *Serratula* species of the Asteraceae family are known for their ecdysteroid content. *Serratula cichoracea* contains antimicrobial ajugasterone C and other ajugasterone derivatives such as 22-epi-ajugasterone C [49], while the butanolic extract of *Serratula chinensis* has been shown to contain novel carbohydrate-containing ecdysteroids. The latter is also used in traditional Chinese medicine for the treatment of pharyngitis [50]. However, *Serratula coronata* contains higher levels of ecdysteroids than the above-mentioned species, with 17 minor ecdysteroids identified in the root extract [51], including ponasterone A, apioside and 3-epi-shidasterone [52].

Achyranthes bidentata and *Chenopodium quinoa* are both plants of the Amaranthaceae family. The root of *Achyranthes bidentata* is mainly used in China, which contains 20E, inokosterone, serfurosterone, and other furanoecdysteroids [53, 54]. *Chenopodium quinoa* is a healthy food crop native to South America, containing several known (20E, polypodine B, makisterone) and new ecdysteroids (26,27-dehydroinocosterone, 5b-hydroxy-24(28)-dehydromakisterone A). The majority of 20E in quinoa remains intact after heat treatment of the plant, so presumably it has significant pharmacological effects in those who consume it regularly [55].

The plants detailed above represent only a small fraction of the thousands of plant species from which ecdysteroid derivatives have been identified [39]. Based on recent publications, it is still evident that there is a continuous research interest in identifying new, bioactive ecdysteroid derivatives. The continuously increasing number of scientific publications describing analytical and biological studies on ecdysteroids allow the rapid extension of the chemical space of these compounds. Furthermore, the excellent commercial availability of the most abundant ecdysteroid, 20E, provides a basis for semi-synthetic chemical transformations involving these compounds [56]. Within the framework of this thesis, our main focus was set on calonysterone and its derivatives in this regard.

I.2.1 Centrifugal partition chromatography in the isolation of ecdysteroids

The literature on the isolation of ecdysteroids dates to the 1960s, and various chromatographic methods can be used to isolate these compounds from their mixtures. Among these, various liquid-liquid chromatographic techniques such as droplet counter-current chromatography (DCCC), high-speed countercurrent chromatograph (HSCCC) and centrifugal partition chromatography (CPC) deserve particular attention. In DCCC, HSCCC and CPC both the stationary and the mobile phase are the layers of an immiscible two-phase solvent system, therefore, no solid stationary phase is present. In the implementation of CPC, a centrifugal force field is used to immobilize one phase (stationary phase) typically in a rotor-mounted cell cascade. Due to its loading capacity, flexibility and cost-effectiveness, CPC is widely used for fractionation and purification of natural metabolites from laboratory scale to industrial scale [57]. Depending on the stationary and mobile phases and the direction of the flow, we distinguish between ascending and descending modes (which correlate to the normal and reversed phase separations). One of the important elements of this study is the scale-up of the isolation of calonysterone, thus providing the opportunity for wide-ranging investigations. This scale-up involves 3 steps: a laboratory scale CPC (250 mL rotor volume), a semi pilot scale

CPC (2100 mL rotor volume) and an *in silico* process flow simulation of the industrial scale CPC (23 L rotor volume). However, within the framework of this thesis, we do not go into detail about the possibilities of a full scale-up and flow simulation.

Several ecdysteroids have been previously isolated by the above-mentioned liquid-liquid chromatographic methods. Among the first techniques were the DCCC solutions [58, 59], but these were primarily chloroform, methanol and water containing solvent systems. 20E, 2-deoxy-20E and 20E-22-benzoate were isolated from *Silene tatarica* using chloroform – methanol – water (65:20:20, *v/v*) solvent system [60]; furthermore, ajugasterone C and 20E were isolated using chloroform – methanol – water (13:7:4, *v/v*) solvent system in ascending mode from *Vitex madiensis* [61]. However, these older technologies are ill-suited to today's industrial separation standards. Majority of these systems contain halogenated solvents, which are no longer recommended for use due to their toxicity and the difficulty of their disposal. Furthermore, the separation lengths in these systems can be remarkably high, even up to several hours, and sample quantities for injection are small. In addition, a few preliminary chromatographic purification steps are often necessary to prepare the starting material. HSCCC is less widely represented, but the isolation of 20E from *Serratula chinensis* roots in EtOAc – *n*-butanol – water (4:1:5, *v/v*) solvent system has been published previously [62].

Previously, CPC purification was applied by our group, using a chloroform – methanol – water (10:7:3, *v/v*) solvent system in descending mode to isolate photochemically modified ecdysteroid derivatives [63]. Another application is a multi-step purification procedure to separate oxidized ecdysteroid compounds in ascending mode using EtOAc – methanol – water (20:1:20, *v/v*) solvent system [64]. Later, dacryhainansterone and calonysterone were isolated with high purity from *Cyanotis arachnoidea* extract using *n*-hexane – ethyl acetate – methanol – water (1:5:1:5, *v/v*) in ascending mode [65], later *tert*-butyl alcohol – ethyl acetate – water (0.45:0.9:1, *v/v*) in ascending mode was utilized to isolate ecdysteroid derivatives obtained from the gamma-radiolysis of 20E [66].

I.2.2 Calonysterone

Calonysterone (compound **99** in this thesis) is a naturally occurring phytoecdysteroid with an unusual chemical structure, that has gained our attention for its potential pharmacological effects and applications. It has the chemical formula $C_{27}H_{44}O_7$ and a molecular weight of 480.64 g/mol (**Fig. 2**) [67]. It contains a polyunsaturated skeleton that is unusual among ecdysteroids, and a rare *o*-quinol B-ring [68]. Calonysterone has been isolated

from several plants, including *Cyanotis arachnoidea* [24]. Based on our own, partially published and patent pending results, this compound could be a valuable new lead. To facilitate further development, within this work we intend to develop a scale-up procedure in preparing this compound so that we can supply enough of it for in vivo studies.

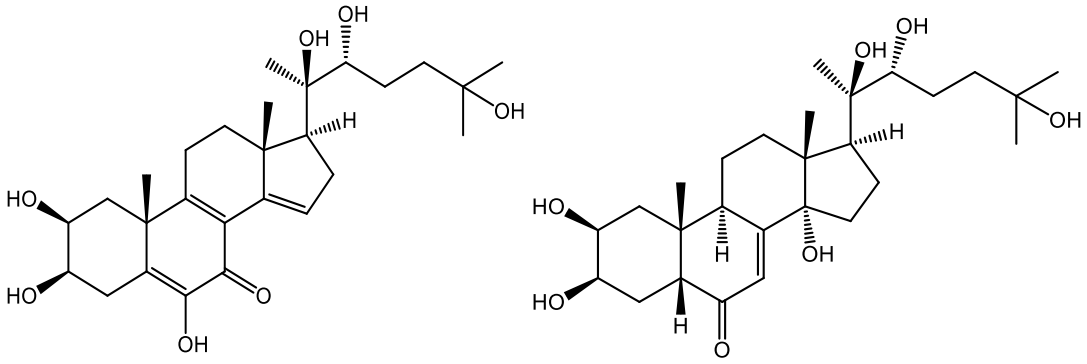


Figure 2 Chemical structure of calonysterone (**99**; left) and 20E (**39**; right).

I.3. Semi-synthetic ecdysteroids in research

I.3.1 Possibilities and intentions for chemical transformations

Due to their many beneficial bioactivities and low toxicity in mammals, the therapeutic potential of ecdysteroids is being intensively investigated [7]. The broadening of the chemical space of compounds is aided by the increasing number of semi-synthetic transformations that have been performed on various ecdysteroid derivatives over the years, supported by growing scientific literature [56]. Since this work deals with autoxidized and sidechain-shortened derivatives and oximes, we will discuss these in more detail.

The autoxidation of ecdysteroids in an alkaline environment was first reported over 20 years ago. This reaction has since become more deeply studied, as the autoxidation process often leads to the formation of various ecdysteroid derivatives with diverse structures (including, e.g., calonysterone). The specific derivatives formed depend on the reaction conditions and workup, opening the door to a wide range of further studies [69]. 20E and ponasterone A diacetonide, among others, have been subjected to autoxidation reactions in NaOH and potassium carbonate, which have shown that the hydroxylation processes occur after epimerization [70]. The autoxidation of poststerone in alkaline environment was also investigated by our research group [71]. As an alternative to the alkaline environment, ecdysteroids can be oxidized with an ozone/oxygen mixture, which in the case of 20E leads to a stereo- and chemoselective 2-dehydro-3-*epi*-20-hydroxyecdysone product [72]. Using oxidative agents like [bis(trifluoroacetoxy)iodo]benzene (PIFA) and (diacetoxyiodo)benzene

(PIDA) the oxidative cleavage of the C20-C22 bonds of ecdysteroids can be carried out [71, 73].

To modulate the bioactivity and possible metabolic fate of ecdysteroids, the modification of their polarity is an effective strategy. The synthesis of less-polar derivatives can be easily achieved, e.g., by forming ethers or esters. During the last more than one decade, our group prepared and studied over 120 apolar ecdysteroid derivatives [12, 74, 75].

Oximes have a variety of biological properties and are commonly employed in chemical synthesis. There has been a surge in interest in synthetic steroidal oximes recently. This field received a major boost with the isolation of natural oximes from *Cinachyrella* marine sponges [76]. Oximes are discussed in more detail in **Section I.3.2**.

Fluorinated analogues of natural compounds have always been of interest because of the chemical properties of the fluorine atom, which often allows the synthesis of derivatives with excellent metabolic stability [77]. Using DAST as a reagent, Csábi et al. prepared 25-fluoro and 14,25-difluoro ecdysteroid diacetonide derivatives with antitumor properties [78].

Modifications of the sterane skeleton can be used to produce derivatives with high structural versatility. To accomplish this, one possibility is to use laser irradiation to induce phototransformation [63]. Another advancement in ecdysteroid chemistry is the synthesis of conjugates of ecdysteroids with other clinically active compounds to achieve a possible therapeutic benefit. For example, the conjugation of ecdysteroid compounds with specific ascorbic acid derivatives [79].

I.3.2 The chemistry behind side-chain cleaved oxime derivatives

Oxime compounds belong to the group of imines and can be generally described by the formula RR'C=N–OH, where R is an organic side-chain and R' is either a second organic side-chain or a hydrogen (**Fig. 3**) [80]. Their synthetic production is a widely studied process. Aldoxime and ketoxime functions are most easily produced by the treatment of hydroxylamine analogues with aldehyde and ketone functional groups, respectively. By using this method, a variety of functional groups can be incorporated into the molecule during oxime formation by using a reagent with an appropriate structure [81, 82].

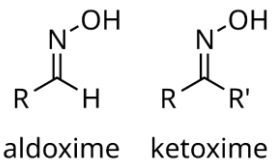


Figure 3 General formula of oximes.

The preparation of an oxime function is a popular medicinal chemical transformation as the incorporation of this moiety can significantly influence the pharmacological properties of a bioactive starting material [83]. Accordingly, the oxime function was observed to contribute to the anti-fungal, antioxidant, anti-inflammatory, antibacterial and anti-cancer effects of bioactive molecules [12, 84, 85, 86]. Extensive literature is available on the preparation of oxime derivatives of bioactive steroid compounds.

Savchenko and his co-workers prepared regioselective oxime derivatives of ecdysteroids, in which the keto group on the B-ring of 20E was left intact under the applied reaction conditions, while the keto group on the side-chain on the D-ring was involved in the oxime formation. Oxidative transformations of the side-chain at the C-17 position can form a keto group at the C-25 position of the side-chain, which requires the protection of the C-20 and C-22 hydroxyl groups by an acetonide protecting group. In absence of the protecting group, oxidative removal of the side-chain results in the formation of a poststerone (**71**), and hence the formation of the keto group at the C-20 position. For oxime formation, the hydroxylamine salts mentioned above were used [87].

In the case of the oxime products obtained, isomerism occurs through the C=N bond, allowing a distinction between the (*Z*)- and (*E*)-configurations. NMR measurements by Savchenko et al were complemented by Bogdán et al, whereby the previously limited non-overlapping ^{1}H signals were supplemented by in-depth high-resolution NMR studies that the resulting products are indeed of (*E*)-configuration. Therefore, the entire ^{1}H and ^{13}C NMR signal assignment of ecdysteroid C-20-oxime derivatives was obtained for the first time [88].

Kovganko et al. synthesized oxime ether derivatives from 20E using *O*-substituted hydroxylamine compounds. The oxime function is implemented on the C-6 keto group on the 20E B-ring; however, it is known that the 6-ketone in ecdysteroids is sterically hindered, thus the reaction was reported to be slow and required higher reagent amounts to process, than in the case of the more reactive side-chain cleaved C-20 ketones [89]. Our research group had successfully prepared fourteen 20E diacetonide oxime, oxime ether, and a lactam derivative, and studied the *in vitro* antitumor effects of the products. The oxime derivatives were formed on the C-6 keto group of the 20E B-ring while the C-2–C-3 and C-20–C-22 hydroxyl groups had acetonide protecting groups. The reaction was carried out using hydroxylamine hydrochloride and the various alkoxyamine hydrochlorides dissolved in pyridine at 70°C for 3 days. All the compounds exerted chemosensitizing activity towards doxorubicin on a mouse lymphoma cell line [12].

In line with their worldwide human consumption, the metabolism and pharmacokinetics of ecdysteroids is of high and constantly increasing interest. Most studies were performed with 20E. The rate of its metabolism is initially low until it reaches the large intestines, where gut bacteria may dehydroxylate ecdysteroids at the C-14 position, reduce the keto group at the C-6 position, and/or cleave the side-chain at the C-20–C-22 position. The modified compounds then enter the enterohepatic circulation similarly to bile acids, resulting in glucuronide conjugation in the liver and deconjugation in the colon. The enterohepatic cycle results in an increasingly complex pattern of applied ecdysteroid metabolites and helps to maintain low but biologically significant levels of ecdysteroids in plasma [90, 91]. Poststerone is one of the major degradation products of 20E [92], and its anabolic effect is comparable to its parent compound. The C-20–C-22-diol structural element is therefore a metabolically sensitive function, which gives way to side-chain cleaved derivatives that are also bioactive [93]. A semi-synthetic removal of the ecdysteroid side-chain, possibly followed by a subsequent modification of the resulting acetyl function, may be a promising strategy for designing new bioactive derivatives with an improved metabolic profile.

II. OBJECTIVES

II.1. Analytical evaluation of *Cyanotis arachnoidea* extracts

LC-MS fingerprinting is a powerful technique for the quality control of ecdysteroid-containing plant extracts and food supplements, and for the detection of possible artefacts and/or counterfeiting agents. Thus, we aimed to:

- develop a high-resolution LC-MS/MS technique that can identify up to 100 components and provide the detailed characterization of both naturally occurring and industrially processed plant extracts containing ecdysteroids, and
- conduct a comparative analysis using the method in-hand to look for possible artifacts with bioactivity relevance.

II.2. Scale-up preparation and isolation of calonysterone

The isolation of minor ecdysteroids from extracts in larger quantities is a complex and expensive process, which makes their synthetic production from commercially available 20E a reasonable alternative. This strategy might pave the way for their extensive pharmacological investigation that would otherwise not be possible. In this context, our aim was as to:

- develop a method suitable to produce large quantities of calonysterone, and
- develop a scalable and economical CPC purification method for the isolation of the product(s).

II.3. Preparation and investigation of semi-synthetic calonysterone derivatives

Based on the previously detailed neuro- and cerebrovascular protective activity of natural ecdysteroids against ROS, our aim was to:

- prepare a set of sidechain-cleaved oxime ether-containing derivatives of calonysterone and develop methods for their chromatographic purification, and
- in a research collaboration, to examine the compounds' pharmacological potential as blood-brain barrier protecting agents and establish possible structure-activity relationships.

III. MATERIALS AND METHODS

Table 1. Detailed description of instruments used.

Instrument:	**Purposes:**
Agilent 1260 Infinity II instrument coupled with an Agilent 6420 QQQ-ESI-MS (Agilent Technologies, Santa Clara, CA) with Kinetex F5, 150x4.6 mm; 2.6 µm column	Analytical evaluation of ecdysteroid containing extracts (**III.1.3.**)
250 mL capacity CPC column (CPC250) with a preparative LC system (PLC2250; Gilson, France)	Laboratory scale calonysterone isolation (**III.2.3.**)
RotaChrom rCPC (RotaChrom Technologies LLC., Hungary) instrument with a 2100 mL volume CPC column	Semipilot scale calonysterone isolation (**III.2.3.**)
Combiflash Rf+ device (Teledyne ISCO, Lincoln, NE, USA) equipped with diode array and evaporative light scattering detection (DAD-ELSD). We used it with commercially available prefilled "RediSep" columns	Sidechain-cleaved calonysterone purification (**III.3.1.**)
A dual-pump Jasco HPLC instrument (Jasco, Hachioji, Tokyo, Japan) equipped with an "MD-2010 Plus" PDA detector. Column: Phenomenex (Torrence, CA, USA) 250 x 4.6 mm 5 µm stationary phase (Biphenyl)	Product purity check and reaction tracking (**III.3.1** and **III.3.2.**)
Armen "Spot Prep II -250" preparative chromatographic system (Gilson Inc., Middleton, WI, USA) equipped with four individual solvent pumps and a dual-wavelength UV detector. Column: Phenomenex 250 x 21.2 mm 5 µm stationary phase (BiPhenyl)	Product preparative purification (**III.3.2.**)

III.1. Analytical evaluation of *Cyanotis arachnoidea* extracts

III.1.1 Solvents and standards

The organic solvents and additives used for the solvent system screening, CPC separations and for LC-MS were purchased from Molar Chemicals Ltd. (Halásztelek, Hungary). The ecdysteroid compounds used during the solvent system screening and for analytical standards were provided by the Institute of Pharmacognosy (University of Szeged, Hungary). These compounds possess a purity of ≥97% by HPLC and were isolated during former studies by our group [64][94].

III.1.2 Sample details

Two commercially available *Cyanotis arachnoidea* extracts were studied in this work. The first was purchased from Xi'an Olin Biological Technology Co., Ltd., (Xi'an, China) with a 50% claimed purity of 20E by means of UV absorbance (248±4 nm). Prior to this PhD work, this extract was extensively percolated with methanol at room temperature followed by an evaporation to dryness [65], and the residue was used to the current studies (CAPR1).

The other commercially available *C. arachnoidea* root extract (CAPR2) was purchased from a different company: Kingherbs Limited (China). This sample contained 10.85% percent of 20E by means of UV absorbance (248±4 nm). The sample was analyzed without any additional preparatory steps.

Authentic *C. arachnoidea* roots and leaves were collected and extracted with methanol manually in Taiwan within the framework of research cooperation. The samples did not undergo any preparatory steps other than methanol extraction. The *C. arachnoidea* root and leaf extracts will hereinafter be referred to as CARO and CALF, respectively.

The autoxidized 20E sample (20EOX) was prepared by the method detailed in section **III.2.1.**

III.1.3 Analytical method applied

The samples were analytically evaluated using RP-HPLC techniques. An Agilent 1260 Infinity II instrument coupled with an Agilent 6420 QQQ-ESI-MS were used for analytics. Using a flow rate of 0.9 mL/min, a Kinetex F5, 150x4.6 mm; 2.6 µm column had been used. The solvents used were respectively A: 95% 5 mM ammonium-formate, 0.1% formic acid + 5% ACN and B: 95% ACN + 5% 5 mM ammonium-formate, 0.1% formic acid. The gradient was as follows: 0–8 minutes from 13–20% B, 8–12 minutes isocratic 20% B, 12–24 minutes from 20–40% B, and 24–26 minutes from 40–90% B. The column temperature was set to 40°C and the UV detection was carried out on a wavelength of 210±4, 248±4 and 360±4 nm. The positive ionization mode of LC/ESI-MS was used for measuring from m/z 200–800, the heated capillary temperature was set to 300 °C, the electrospray voltage to 4 kV respectively. The mass spectrometer was used in data-dependent mode (DDA) composed of 2 scan events for the proper analysis of the reference compounds and data-independent acquisition mode (DIA) for the analysis of crude samples. After obtaining the full-scan mass spectrum, the selected abundant ion from the whole scan was dissociated via collision.

III.2. Preparation and isolation of calonysterone

III.2.1 Calonysterone synthesis

The following procedure was used to accomplish 20E autoxidation: after dissolving 3 g of 20E in 32 ml of methanol, 112 ml of water was added to the mixture. Separately, 24 ml of water was used to dissolve 2.4 g of sodium hydroxide (NaOH), then the two solutions were combined and stirred at room temperature for 6 hours. After the conversion, hydrochloric acid (HCl) was added to create an acidic environment, and the mixture was stirred overnight at room temperature. The pH was then neutralized using a NaOH solution and the obtained solution was evaporated under reduced pressure at 40 °C. The purification of calonysterone was performed by centrifugal partition chromatography. Fractions were analyzed by TLC and HPLC, calonysterone containing fractions were combined and evaporated under reduced pressure.

III.2.2 Solvent system selection

The first step in the selection of suitable solvent systems for liquid-liquid chromatography is the determination of partition coefficients (K_D values) for the main ecdysteroid component and impurities in two-phase solvent systems. The K_D values are determined by dividing the concentration of the component in the upper phase by the concentration of the component in the lower phase. The preparation is as follows: 4 mg of the crude extract was added into a 15 mL size test tube, then 4 mL of upper and 4 mL of lower phase was added from the pre-equilibrated biphasic solvent system. After mixing the contents of the test tube properly with a vortex mixer, the phases were allowed to separate properly for half an hour. Half mL aliquots of the upper and the lower phase were then filtered into HPLC vials, dried and redissolved in 1 mL of analytical grade methanol. Lastly, the peak areas were determined by HPLC, and the K_D values were obtained by the quotient of the two numbers.

III.2.3 CPC methods and instrumentation

All CPC separations were conducted at RotaChrom Technologies Plc (Headquarters: Kecskemét, Hungary) within the Cooperative Doctoral Program (KDP) that provided the framework to this PhD. Laboratory scale CPC runs were performed on a 250 mL capacity CPC rotor (labCPC) with a preparative LC system. The semi-pilot scale CPC runs were carried out with an instrument equipped with a 2100 mL volume CPC column (rCPC). After determining the volumes of the upper and lower phases necessary for the separation, the solvent systems were prepared in a separation funnel. The biphasic liquid systems were vigorously shaken, then

they were allowed to settle for 30 min at room temperature (22°C). The densities of the upper phase and lower phase were measured by a portable density meter (DensitoPro, Mettler Toledo, Columbus, Ohio, USA). In the case of the laboratory scale CPC, the rotor was filled with the stationary phase at a 50 mL/min flow rate and a rotation speed of 500 rpm, then the equilibration and the elution were carried out at a rotation speed of 2000 rpm and at 15 mL/min flow rate. 20 mL volume fractions were collected. UV detection was carried out at 248±4 nm. When rCPC was used, the rotor was filled with the stationary phase at a 200 mL/min flow rate with a rotation speed of 500 rpm, then during the equilibration, the rotation speed was increased up to 1000 rpm, and the mobile phase was continuously pumped with a 200 mL/min flow rate. The fractions were collected manually in every 30 sec (100 mL). The concentration of the sample was 1g/10mL dissolved in the solvent system's lower phase and 10 mL was injected when performing labCPC, and 80 mL was injected when applying rCPC.

III.3. Preparation and investigation of semi-synthetic calonysterone derivatives

III.3.1 Oxidative side-chain cleavage of calonysterone

After dissolving 2 g aliquot of calonysterone in 500 ml of methanol, one molar equivalent (1.34 g) of PIDA was added, and the reaction mixture was stirred for 60 minutes at room temperature. After neutralizing the solution with 10% aq. $NaHCO_3$, the solvent was evaporated using a rotary evaporator operating under reduced pressure. The residue was then redissolved in acetone and adsorbed on 10 g of silica gel to prepare the sample for dry loading flash chromatographic purification. The flash chromatographic purification of the product (**99a)** was carried out on a Combiflash Rf+ instrument. The gradient program applied used dichloromethane (A) and methanol (B) as eluents and the program was as follows: 0% to 1% of solvent B in 7 minutes, 1% to 2.5% from 7 to 13 minutes then 2.5% to 7% from 13 to 50 minutes, on a 40 g silica column (flow rate: 35 ml/min, run time: 60 min). The reaction was repeated multiple times to gather a larger quantity of **99a**, which was isolated with an average yield of 45.5%.

III.3.2 General procedure for the synthesis of side-chain cleaved calonysterone 20-oxime and oxime ether derivatives (99b–99g)

A 120 mg aliquot of **99a** (0.34 mmol) was first dissolved in 20 ml of ethanol, then depending on which functional group intended to be linked, 120 mg of either hydroxylamine hydrochloride or other alkoxyamine hydrochloride was added to the solution (**Table 2**) while it was being stirred. After stirring at 80 °C for 40 minutes, the solution was evaporated to

dryness under reduced pressure. Following the addition of 100 ml of water to the dry residue, the aqueous solution was extracted three times using 100 ml of ethyl acetate for each step, and the combined organic phases were then dried over anhydrous Na_2SO_4. The mixture was then filtered, and the solvent was evaporated under reduced pressure.

Table 2 Reagents used in oximation reactions.

C20-NO*R*	**Starting material**	**Reagent**
99b (R=H)	120 mg **99a** (0.34 mmol)	120 mg hydroxylamine hydrochloride (1.73 mmol)
99c (R=Me)		120 mg methylamine hydrochloride (1.78 mmol)
99d (R=Et)		120 mg ethylamine hydrochloride (1.47 mmol)
99e (R=All)		120 mg allylamine hydrochloride (1.28 mmol)
99f (R=tBu)		120 mg *tert*-butylamine hydrochloride (1.10 mmol)
99g (R=Bn)		120 mg benzylamine hydrochloride (0.84 mmol)

Each reaction was monitored by TLC with silica gel plates on aluminum sheets. The eluents were a mixture of 93% dichloromethane and 7% ethanol – water (10:0.3, *v/v*). The plates used for reaction monitoring were examined under UV illumination at 254 nm. To obtain the appropriate ecdysteroid product, the products were purified using preparative RP-HPLC.

The compounds were purified by preparative HPLC using the liquid pump, detector and fraction collector of an Armen Spot Prep II instrument with the following experimental setup:

- Kinetex Biphenyl 100A 5 µm 250x21.2 mm column
- 15 ml/min eluent flow rate
- Detection at 254±4 nm wavelength
- 100 µl injection volume
- 120 min run time
- Isocratic eluent systems: water – acetonitrile 70:30 (*v/v*) for **99b**, **99c**; 66:34 (*v/v*) for **99d**, **99e**; 60:40 (*v/v*) for **99f** and 45:55 (*v/v*) for **99g**

The purity of the compounds was checked by a Jasco HPLC instrument as follows:

- Kinetex Biphenyl 100A 5 µm 250x4.6 mm column
- 1 ml/min eluent flow rate
- Detection at 254±4 nm wavelength
- 100 µl injection volume
- 30 min run time

- Isocratic eluent systems: water – acetonitrile 70:30 (*v/v*) for **99b**; 60:40 (*v/v*) for **99c, 99d**, **99e** and 50:50 (*v/v*) for **99f**, **99g**

III.3.3 Procedures for the structure elucidation of the obtained products

Detailed structure elucidation methodology is available in publication **No. I.** HR-MS analysis of the compounds was carried out on an Agilent 1100 LC-MS instrument coupled with Thermo Q-Exactive Plus orbitrap analyzer used in positive ionization mode. Solutions of 100 μg/ml concentration were prepared from samples with acetonitrile solvent containing 0.1% formic acid. ^{1}H NMR, ^{13}C DeptQ, edHSQC, HMBC, and one-dimensional selective ROESY spectra (τmix: 300ms) were recorded at 295 K on a Bruker Avance III HD 600 spectrometer equipped with a Prodigy cryo-probehead. DMSO-*d6* was used to dissolve samples and tetramethylsilane (TMS) was the internal standard. Chemical shifts (δ) and coupling constants (J) are given in ppm and in Hz, respectively.

Table 3 HR-MS of compounds **99b–99g**.

99b	HR-MS: $C_{21}H_{27}NO_5$,	$[M+H]^+$ Calcd.: 374.19730, found: 374.19696
99c	HR-MS: $C_{22}H_{29}NO_5$,	$[M+H]^+$ Calcd.: 388.21185, found: 388.21208
99d	HR-MS: $C_{23}H_{31}NO_5$,	$[M+H]^+$ Calcd.: 402.22750, found: 402.22795
99e	HR-MS: $C_{24}H_{31}NO_5$,	$[M+H]^+$ Calcd.: 414.22750, found: 414.22808
99f	HR-MS: $C_{25}H_{35}NO_5$,	$[M+H]^+$ Calcd.: 430.25880, found: 430.25890
99g	HR-MS: $C_{28}H_{33}NO_5$,	$[M+H]^+$ Calcd.: 464.24315, found: 464.24351

III.3.4 Brief summary of procedures for biological investigation

Biological measurements were carried out by the Biological Barriers Research Group led by Prof. Mária A. Deli (Institute of Biophysics, HUN-REN Biological Research Centre, Szeged, Hungary); for a detailed description of the methodology, please see publication **No. I.** Briefly, the hCMEC/D3 human brain microvascular endothelial cells were grown in dishes coated with rat tail collagen and maintained in an incubator at 37˚C with 5% CO_2. When the cultures reached almost 90% confluence, they were passaged to rat tail collagen-coated 96-well plates for impedance measurement assays. The impedance of brain endothelial cells was assessed using the real-time cell electronic sensing analysis (RTCA), and once the cells reached a stable growth, they were treated with compounds **99a–99g** at concentrations ranging from 0.01 to 10μM, then measure cell viability. Stock solutions were prepared by diluting the

compounds in DMSO to a final concentration of 10 mM and stored at -20˚C. Working solutions were freshly prepared by diluting the stock solutions in cell culture medium to obtain a concentration range of 0.01–10 μM. For barrier integrity assays, TEER measurements reflect the permeability of intercellular tight junctions for ions, and it was expressed relative to the surface area during measurements. TEER values of cell-free inserts were calculated and normalized to the control group. To measure the flux of paracellular permeability and the permeability, fluorescent marker FD4 and EBA dye were used respectively. hCMEC/D3 cells were for permeability assays, which were evaluated by a microplate reader. Total ROS generated after the treatments with compound **99g** and tBHP alone or in combination was measured by a fluorometric detection probe (DCFDA). During statistical evaluation the mean ± SD values were used to present the data, and at least four parallel samples were used, and changes were considered statistically significant when $p<0.05$.

IV. RESULTS AND DISCUSSION

IV.1. Analytical evaluation of *Cyanotis arachnoidea* extracts

IV.1.1 Analyzed ecdysteroid-containing samples

Within the framework of this thesis, five ecdysteroid-containing samples were analyzed. First, two different commercial extracts of *Cyanotis arachnoidea* (CAPR1 and CAPR2) were purchased from the People's Republic of China, from two independent companies. China is one of the largest distributors of *C. arachnoidea* extracts in the global market, partly because the plant is native to Asia. Second, a representative authentic sample of *C. arachnoidea* was needed for proper comparison, which was collected in Taiwan. Following collection, the roots and leaves of each plant were separated and extracted with methanol, referred to as CARO for root extract and CALF for leaf extract. Our preliminary experiments indicated that CAPR1 extract contained a much larger number of components than the authentic extracts. This gave us the hypothesis that artifacts might have been formed during industrial processing. The 20E base-catalyzed autoxidation product mixture (20EOX) was used as our fifth analyte to evaluate this. It was deemed reasonable to involve pure 20E and not one of the Cyanotis extracts in the oxidation experiment to search for the presence of metabolites of autoxidative origin, since 20E is by far the main phytoecdysteroid of Cyanotis extracts. These selected samples allow us to compare them from several aspects, revealing potential similarities and differences.

Reversed-phase HPLC in conjunction with tandem mass spectrometry was selected to analyze the ecdysteroid fingerprints, as it is one of the most widely applicable high-performance chromatographic method available for such purposes. The analytical evaluation was carried out using a Kinetex F5 column. This column with its superficially porous pentafluorophenyl propyl stationary phase represents an alternative to traditional C18 columns with similar selectivity and comparable retention. In the design of the separation parameters, we had to consider the large number of components in the samples and find a balance between long run times, which would provide noticeably broader peaks, and short run times, which would result in several co-eluting components and would make it more difficult to evaluate mass spectrometric data. Representative chromatograms of the five samples are shown in **Fig. 4**.

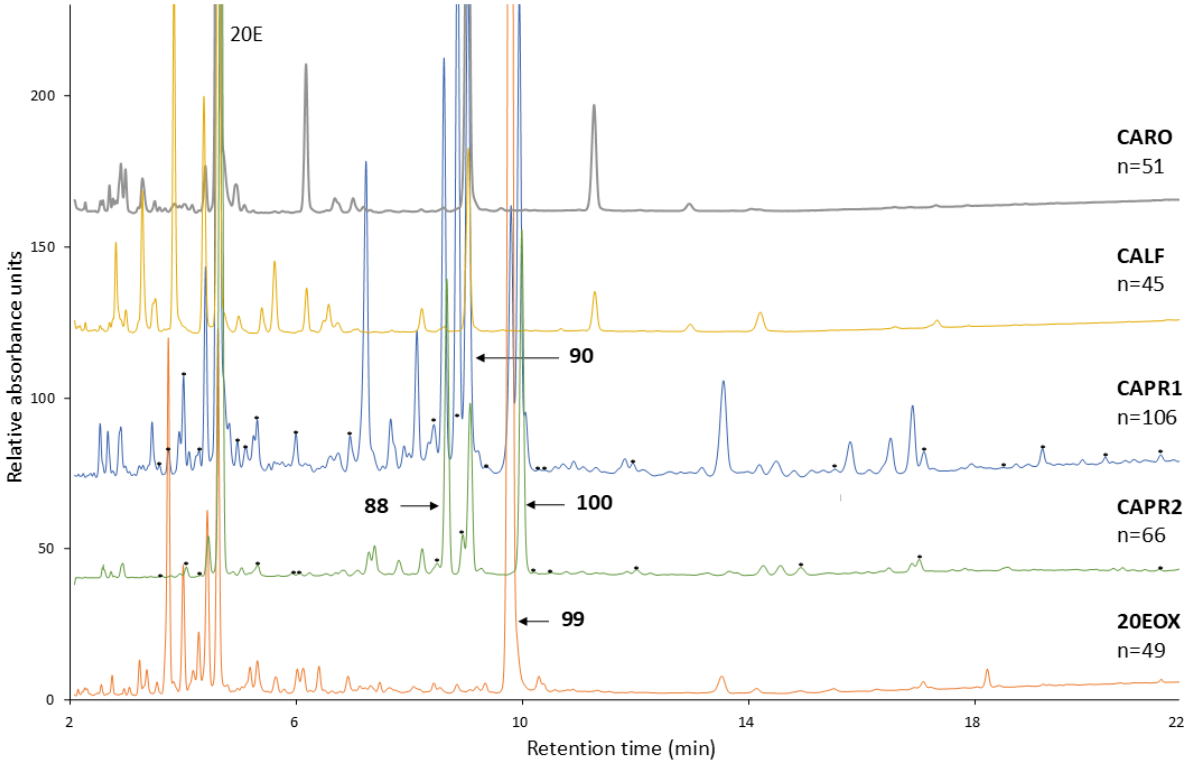


Figure 4 Analytical chromatograms of CARO, CALF, CAPR1, CAPR2 and 20EOX samples. UV detection was carried out at 248±4 nm. Peaks to be found only in commercial extracts and absent from authentic natural extracts are marked with asterisks (*) on the chromatograms of CAPR1 and CAPR2.

IV.1.2 LC-MS data of identified ecdysteroids

The analytical evaluation of the samples was greatly aided by the compounds isolated during the research group's previous work. Thus, several of the ecdysteroids found in the samples examined could have been identified using authentic reference materials that were obtained and thoroughly described in either our earlier research or the current thesis. Structures of these compounds and their LC-MS data are shown in **Fig. 5** and **Table 4**, respectively. We identified 25 substances unambiguously in the extracts. We considered those compounds to be artifacts that were identified in the autoxidized sample and at least one industrial extract but were not detectable in the authentic extracts.

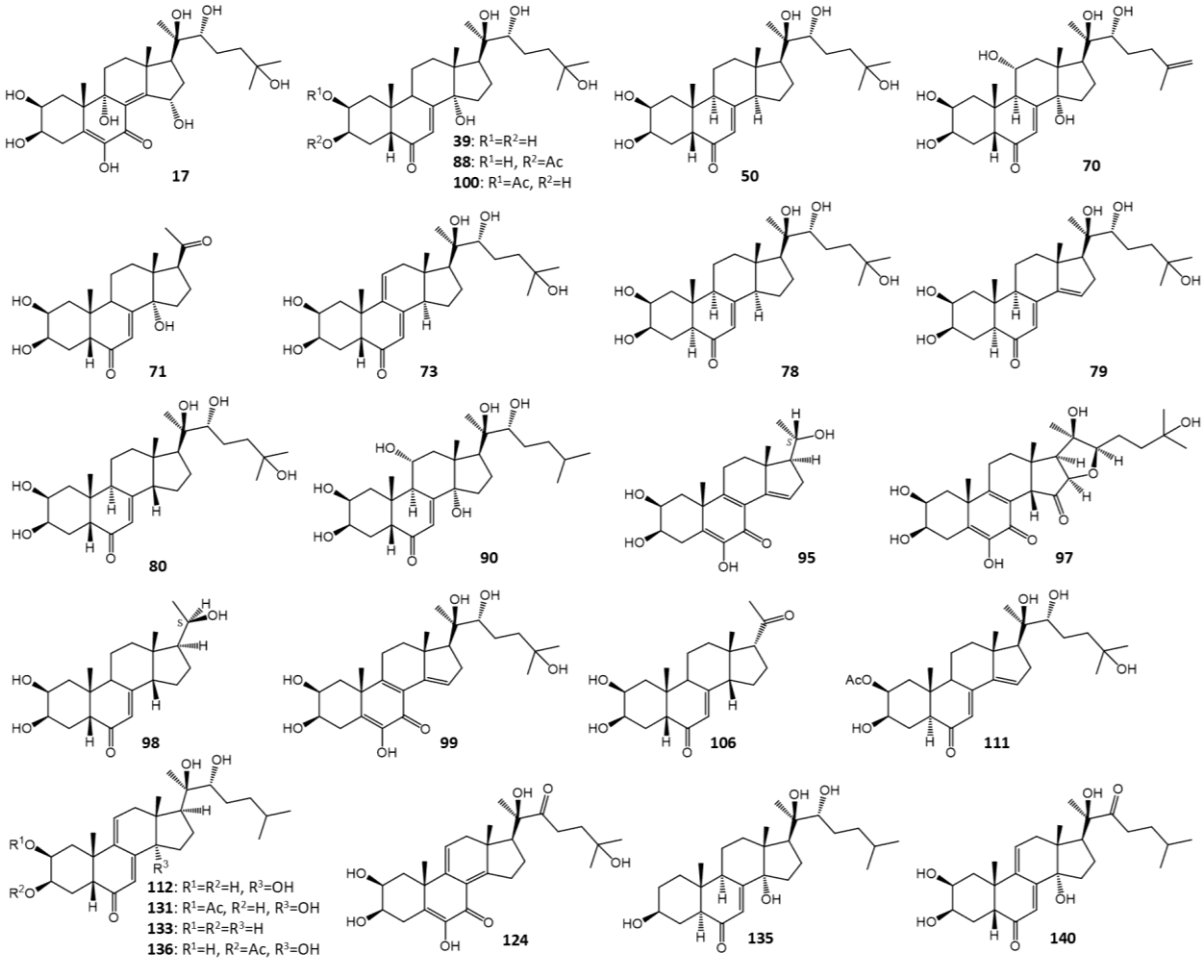


Figure 5 Chemical structures of ecdysteroids categorically identified in the extracts using fully characterized reference standards.

IV.1.3 Comparison of ecdysteroid containing samples

Based on the fingerprints, we compared the extracts in pairs and counted exactly how many common peaks they had at the LOD level. We then expressed as a percentage how many percent of the components in the samples compared were found in the other sample, where 100% means that all peaks in each extract were found in the other extract. Results of this analysis are summarized in **Table 5**.

Table 4. HPLC-MS characteristics of 25 ecdysteroids identified by fully characterized reference standards and their semiquantitative presence in the samples. Color codes for the suggested origin: *green*, genuine compound; *yellow*, oxidized artifact; *violet*, genuine compound present in quantities likely altered by oxidation. The genuine or artifact nature of compounds without a highlighting color could not be decided based on the available data. For a complete overview of all detected compounds, see publication **No II**. supplementary information (**Table S2**).

Comp. No.	Trivial name	Retention time (min)	$[M+H]^+$	Presence				
				CAPR1	CAPR2	CARO	CALF	20EOX
17	Oxycalonysterone C	3.28	493.3	+	-	-	-	++
39	20-Hydroxyecdysone	4.53	481.2	++++	++++	++++	++++	+++
70	Isovitexirone	6.93	479.1	+	+	++	+	-
71	Poststerone	7.10	363.1	+	+	+	-	-
72	14-Deoxy-20-hydroxyecdysone	7.15	465.3	+++	+++	-	-	-
73	14-Deoxy-25-hydroxydacryhainansterone	7.20	463.3	+	+	-	-	-
78	5α-14-Deoxy-20-hydroxyecdysone	7.82	465.2	+	-	-	-	-
79	5α-Stachysterone B	7.91	463.3	+	-	+	-	-
80	14-Epi-14-deoxy-20-hydroxyecdysone	8.05	463.2	++	++	-	-	-
88	20-Hydroxyecdysone 3-acetate	8.53	523.3	+++	+++	+	+	-
90	Ajugasterone C	8.95	481.3	+++	+++	+++	+++	-
91	14-Epi-14,15-dihydrocalonysterone	9.00	461.2	+	-	-	-	-
97	Oxycalonysterone A	9.61	491.2	+	-	-	-	+
98	14-Epi-14-deoxy-20(*S*)-dihydropoststerone	9.66	349.2	+	-	-	-	-
99	Calonysterone	9.72	477.2	+++	+	+	+	++++
100	20-Hydroxyecdysone 2-acetate	9.86	523.3	+++	+++	+	+	-
106	14β(H)17β(H)-14-deoxypoststerone	11.73	347.2	+		-	-	-
111	5α-Stachysterone B 2-acetate	13.09	505.3	+	+	-	-	-
112	Dacryhainansterone	13.46	463.3	+++	++	+	-	++
124	Oxycalonysterone B	17.17	475.2	+	-	-	-	-
131	Dacryhainansterone 3-acetate	19.82	505.2	+	-	-	-	-
133	14-Deoxydacryhainansterone	21.21	447.3	+	-	-	-	-
135	5α-2-Deoxyponasterone A	21.46	449.2	+	+	-	-	-
136	Dacryhainansterone-2-acetate	21.61	505.3	+	-	-	-	-
140	22-Oxodacryhainansterone	22.60	461.2	+	-	-	-	+

Table 5. Fingerprint comparison of the extracts. Intersection: the number of common compounds identified. Similarity 1st to 2nd, and 2nd to 1st are expressed as the percentage of detectable peaks in the 1st sample that are also present in the 2nd, and vice versa, respectively.

1st	2nd	Intersection	Similarity (%)	
			1st to 2nd	2nd to 1st
CAPR1	CAPR2	52	49	79
CAPR1	CARO	41	39	80
CAPR1	CALF	32	30	71
CAPR1	20EOX	33	31	67
CAPR2	CARO	30	45	58
CAPR2	CALF	21	32	46
CAPR2	20EOX	15	23	31
CARO	CALF	37	73	82
CARO	20EOX	2	4	4
CALF	20EOX	1	2	2

IV.1.3.1. CARO to CALF comparison

We aimed to compare authentic extracts of wild-collected *C. arachnoidea* roots and leaves (CARO and CALF) to industrially processed extracts. Approximately 50–60 components were detectable in both authentic extracts using the described analytical HPLC method. 20E was the main component in both CARO and CALF extracts, both of which also contained significant amounts of ajugasterone C in addition to minor compounds. Their qualitative composition is unsurprisingly very similar, however the root extract's ecdysteroid content is significantly higher than the leaf extract. In terms of industrial extract production, it varies whether the whole plant is processed or only the root of the plant, although commercially available *C. arachnoidea* extracts are usually declared to be root extracts.

IV.1.3.2. CAPR1 to CARO comparison

The first stage in the evaluation of the CAPR1 industrial extract was comparing it to the authentic root extract (CARO), as CAPR1 was obtained from *C. arachnoidea* roots according to the provider. This is also in line with the much higher market coverage of root extracts as compared to whole plant extracts. The CAPR1 extract showed distinct differences in the number of components and a shift in the main constituents toward relatively less polar derivatives. The CAPR1 extract contained 108 detectable components, compared to the CARO sample's 50–60 detectable components. Over 80% of the compounds detected in the CARO extract were also present in the CAPR1 extract, suggesting that these compounds most likely derived directly from the root of the plant. Yet, the CARO extract contained fewer than 40% of

all CAPR1 components that were detected. It should be noted that the CAPR1 and CARO plant samples were harvested at different times and from different geographical locations, which should be reflected in the qualitative and quantitative variations in their ecdysteroid compositions. However, it seems improbable that so many genuine substances would only be found in industrially processed plants and entirely absent from those that were collected in the wild. Rather, a much more plausible explanation for such a compositional difference should be artifact formation, during the industrial preparation process, e.g., extraction and partial purification.

IV.1.3.3. Comparison of CAPR1 to 20EOX

After the comparison of CAPR1 and 20EOX, it can be stated that nearly 70% of the alkaline autoxidation products of 20E were present in CAPR1. This implies that oxidative artifacts were formed during industrial processing. It is also worth emphasizing that i) alkaline autoxidation may take place for every ecdysteroid that has a 7-ene-6-one molecule in its B-ring, not only 20E is exposed to it, and ii) autoxidation is unlikely to occur at neutral pH but requires a strong alkaline medium. Since 20EOX originated from a single compound (i.e., 20E), it is a much less complex mixture than what could reasonably be expected from the autoxidation of a crude *C. arachnoidea* extract containing a wide range of further ecdysteroids. This may explain the large number of elements in the CAPR1 sample compared to the ca. 50 compounds in the 20EOX sample. Additionally, since we did not use any added base at any extraction step, no such artifacts were detectable in the authentic extracts CARO or CALF.

IV.1.3.4. Comparison of CAPR2 to the other extracts

The composition of CAPR2 extract turned out to be more like authentic CARO sample, in contrast to the case of CAPR1. However, CAPR2 also contained a significant proportion of compounds that are indicative of possible artifact formation. CAPR2 was particularly rich in acetates (see publication **No II**. supplementary information (**Table S1**)), which may be the sign of the use of acetic acid at some point in the processing, e.g., during a neutralization step.

IV.1.4 Summary of sample comparisons

The qualitative and quantitative ecdysteroid profiles of industrial and authentic *C. arachnoidea* extracts differ significantly (**Fig. 6**). It is well recognized that a plant's secondary metabolite content can be significantly influenced by external elements like soil, climate, weather, water availability, etc., in addition to genetic and epigenetic factors. Therefore, due to the different origin of our samples, one cannot draw a perfectly sound judgment on the

genuine or artifact nature of the detected ecdysteroids. Nonetheless, our results suggest that several ecdysteroids found in industrial extracts are either autoxidized derivatives of 20E or associated with such known artifacts. This, along with the ecdysteroid fingerprint's visibly much greater complexity, indicates that extracts may contain artifacts formed during industrial processing. In contrast to other CHO compounds, ecdysteroids seem to be especially sensitive to extraction and storage, according to several comparable investigations on the formation of artificial products [95].

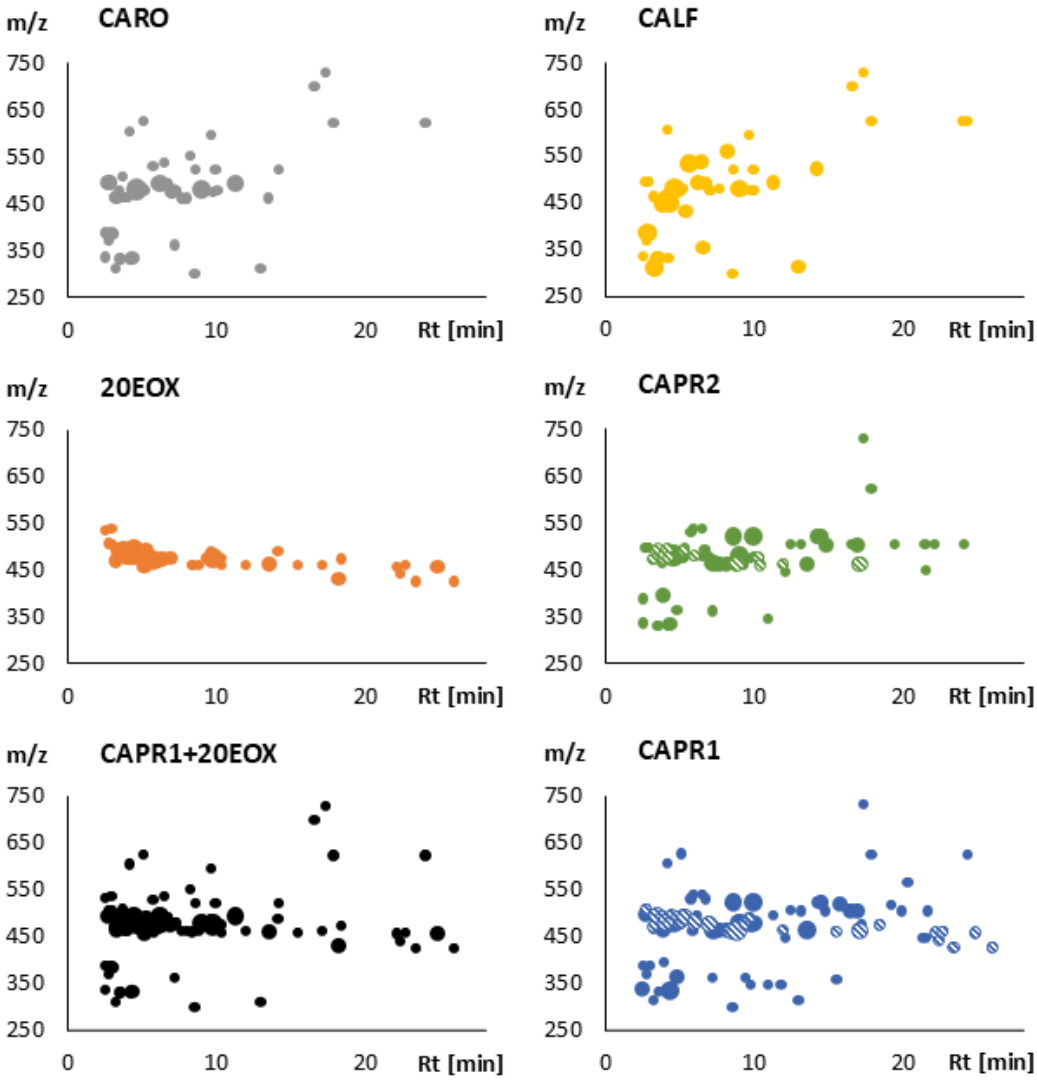


Figure 6. Overview of the LC-MS fingerprints of the samples analyzed: CARO, CALF, 20EOX, CAPR1 and CAPR2; CARO+20EOX: mathematical sum of CARO and 20EOX. The size of the bullets represents the relative peak area values of each peak. Striped bullets represent the presumable artifacts.

Very little is known about the pharmacology of minor phytoecdysteroids in mammals, even though the health advantages associated with 20E are widely established and some have even been clearly confirmed in clinical studies (e.g., against COVID-19 [21], or sarcopenia [22]. In our earlier research, we demonstrated that less polar ecdysteroids, including acetonides, have the opposite effect on cancer cell treatment resistance as 20E [11]. Compared to their parent molecule, autoxidized derivatives of 20E, such as compound **99**, have a significantly greater effect in activating protein kinase B (Akt) [64]. Other oxidized analogues of 20E likewise had stronger effects on two important pathways that control cell survival and death, AMPK and Akt [66, 96, 97]. These examples demonstrate that significant alteration in the ecdysteroid composition of a plant extract has a high potential to significantly change the overall bioactivity profile.

IV.2. Preparation and isolation of calonysterone

IV.2.1 Synthetic preparation of calonysterone

The basis for the implementation of calonysterone synthesis was based on previous work of the research team. In this work, the composition changes of the autoxidation of 20E in alkaline environment was monitored by capillary electrophoresis. A key intermediate for the synthesis of calonysterone was 14,15-dihydro-14α-hydroxycalonysterone (**HC**). As reported in an earlier publication of our group [64], this intermediate yields isocalonysterone through base-catalyzed elimination of the 14-OH, or calonysterone through acid catalysis. Our group has also studied the time dependence of 20E autoxidation (**Figure 7A**), and we knew that the amount of this precursor reaches its maximum, approximately 80%, at 5.5−6 h. To facilitate the formation of calonysterone at this point, the autoxidation process was stopped by acidification. Therefore, reaching 77% calonysterone content by means of HPLC at a wavelength of 248±4 nm (**Fig. 7B**).

The measurements show that a highly acidic environment (pH=1) is required for the water elimination reaction to proceed properly after autoxidation, however a higher pH value resulting in an inadequate rate of calonysterone transformation. This pH value needs to be maintained for about 16 hours, but longer exposure to acidic conditions will result in an increasing rate of degradation products, therefore it is exceptionally important to neutralize the acidic environment at the right time. During neutralization, it is advisable not to set the pH above 7 as calonysterone tends to convert to the desmotropic pair even in a slightly alkaline

environment, thus impairing recovery [64]. The subsequent isolation steps are not impaired if the pH is set to 6.

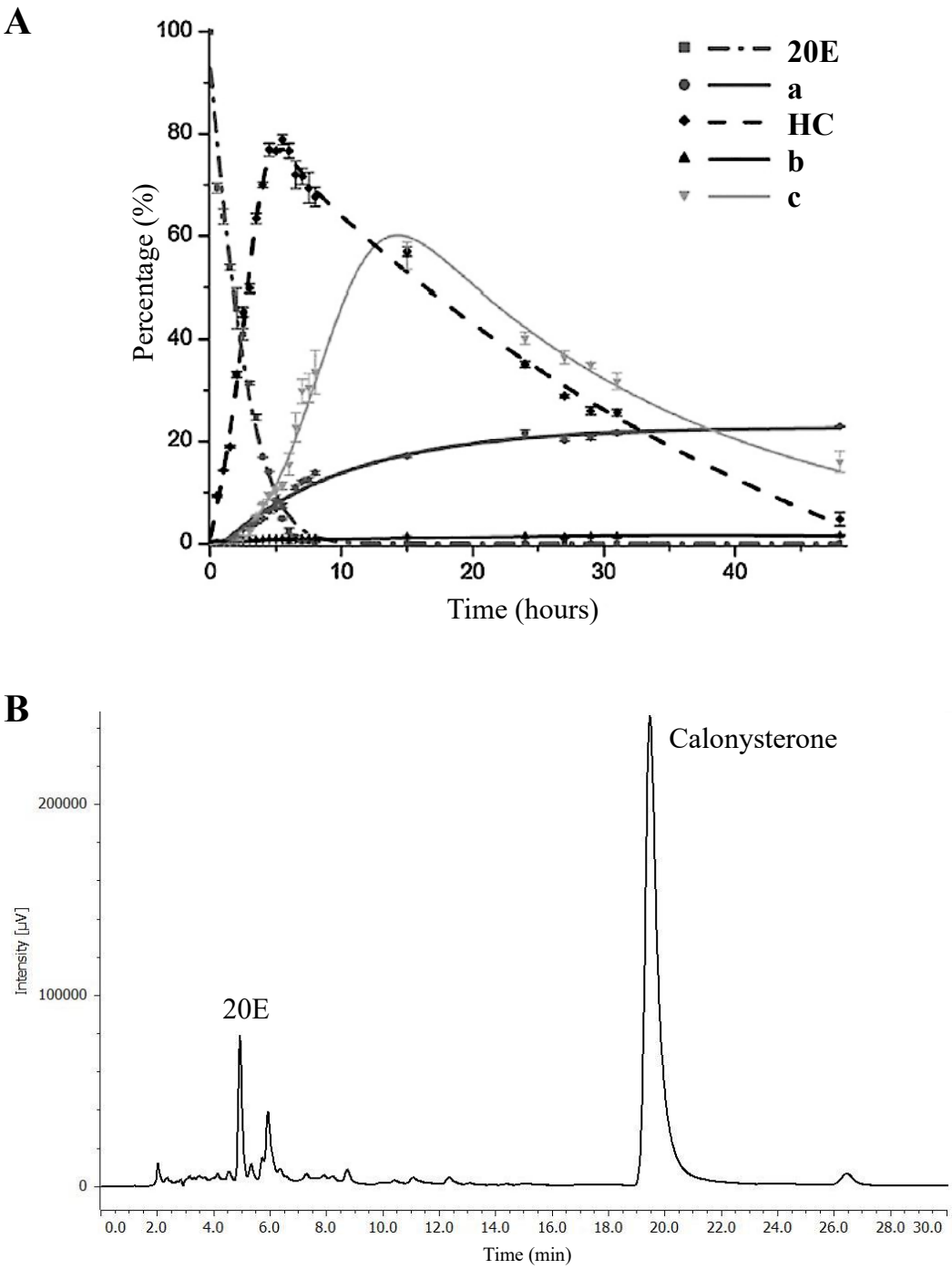


Figure 7 A: Composition changes of **20E**, **a**, **HC**, **b** and **c** in the reaction mixture during the autoxidation process of 20E [98]. **B:** HPLC analytical chromatogram of the reaction mixture. UV detection was carried out on a wavelength of 248±4 nm.

During the optimization of the synthesis method, the right water-methanol solvent ratio was given exceptional priority. The solubility of ecdysteroids in water is somewhat limited, both for the starting material 20E and the product calonysterone, and methanol can significantly

increase the compounds' solubility without compromising the efficiency of the autoxidation process. Regarding the volume of methanol, the maximum ratio that could be added was 20 volume percentage, respectively. At lower methanol ratio, the dissolved material tended to precipitate out of solution, preventing the autoxidation from taking place.

It was also important to ensure that the mixing is sufficiently intensive, and that the solution contains enough oxygen during mixing for proper oxidation. We also explored the possibility of using H_2O_2 to ensure an adequate oxygen supply to the solution. Although calonysterone was also formed in the presence of H_2O_2 and MnO_2, the results have not shown an improvement neither in yield nor reaction time during synthesis over conventional mixing that would justify their use.

IV.2.2 Solvent system selection

The principal of a successful CPC separation method is to find the solvent system in which the distribution of the components to be separated is most ideal. In the evaluation of the partition, solubility, and selectivity data, special emphasis was made on sustainability of the solvent systems. There are general requirements that our two-phase solvent system must meet [99]: optimal distribution for the target components (K_D=0.5–2.0); high selectivity (α>1.5) between compounds; short settling time (<30 sec) and it is desirable the solvents to have low cost, low toxicity and high solubility. The choice of the solvent system involved a literature survey and studying a database of previous measurements of ecdysteroids. Concerning the latter, the partition behavior of various ecdysteroids was previously tested in more than 60 biphasic liquid-liquid chromatographic solvent systems, and this serves as a suitable basis for the preliminary selection of solvent systems. K_D measurements have shown that ketone/ester – alcohol – water systems have the best solubility and optimum distribution for ecdysteroid type components.

During method development and solvent system selection, we have considered key industrial factors. We have tried to use the greenest possible solvents and the use of more sustainable solvent alternatives, for example limonene instead of hexane. The information on green solvent was provided by the unified version of general solvent selection guides for medicinal chemists by GSK, Sanofi and Pfizer [100]. In addition, the FDA's ICH Q3C guideline was also considered, from which data on the level of solvent residues was obtained, which is also an important aspect in pharmaceutical industry. The length of the purification methods is also an important aspect, so we strove to keep the separation time under 30 min, which includes the loading of the rotor with the stationary phase and the equilibration. The

ascending mode was prioritized over the descending mode as it makes the processing of fractions easier and cheaper.

In this case, we have tried to set the K_D value of the compound of interest (calonysterone) to around 1, as the resolution of the separation is the highest at this point. Considering this, the following solvent systems were selected for further investigation:

- **labCPC No1.** MIBK – Aceton – H_2O (3:4:3, *v/v*)
- **labCPC No2.** EtOAc – MeOH – H_2O (4:2:4, *v/v*)
- **labCPC No3.** EtOAc – MeOH – H_2O (4:1,5:4, *v/v*)
- **labCPC No4.** MEK – EtOAc – H_2O (5:1:4, *v/v*)
- **labCPC No5.** MIBK – MeOH – H_2O (1:1:1, *v/v*)

IV.2.3 Centrifugal partition chromatographic isolation of calonysterone

Solvent systems nominated as promising during the solvent system screening are always tested on a laboratory scale CPC as a first step. The parameters for the separation are selected based on a generic set of settings, which can be changed when required. These settings include the rotor filling parameters, the rotation speed set to 2000 rpm, and the flow rate set to 15 mL/min. In the present measures there was no justification for changing these parameters. During the run, an ascending mode was used, and 20 mL fractions were collected from the upper organic solvent phase. The reason for using ascending mode was given by the partition coefficients of the sample components, primarily the compound of interest, calonysterone. In this case, the desired product was eluted faster in ascending mode, resulting in a narrower peak and shorter run time. ASC mode is also significant because evaporation of the upper phase is beneficial in both time and energy, as it contains solvents with lower boiling points. The crude reaction mixture contains a large amount of salt (NaCl) due to pH adjustment processes during the synthesis, which also requires the ascending mode for efficient removal, as the aqueous stationary phase retains the salt on the rotor, thus removing it from the sample efficiently and avoiding a preliminary, costly and time-consuming desalting step.

In the laboratory scale measurements, 1 gram of crude sample was dissolved in lower phase (set to 10 mL) and injected. Usually, the sample matrix solvent has minimal influence on the stability of the system, in this case only dissolution in the lower aqueous phase was an option due to the high salt content of the sample.

After the laboratory scale separations with the different selected solvent systems, we found that compared to the solvent systems containing MEK and MIBK (**labCPC No1.**, **No4.** and **No5.**), the systems containing EtOAc and MeOH gave the best peak shapes (**labCPC No2.**

and **No3.**). The two systems resulted in very similar separation and purity results, so the system containing more MeOH was selected for scale-up, because of minimum run time reduction and minimum solubility increase due to more MeOH.

On the chromatogram (**Fig. 8A**) and on the fractogram (**Fig. 8B**) it is visible that the compound of interest elutes in 25 minutes during the separation, allowing the separation to be completed at this point and the stationary phase to start to be removed from the rotor, which also means the filling of the rotor with fresh stationary phase for the next separation. After analytical measurements, the purity of the combined fractions is 98.0%. The calculated yield of the product in the pooled fractions is above 85%.

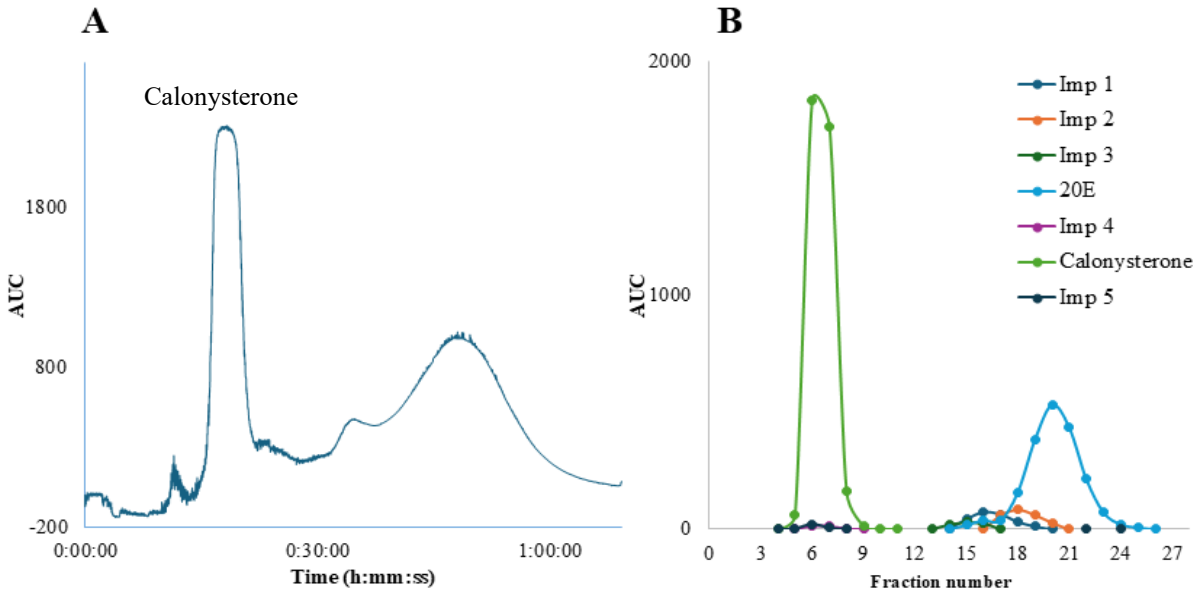


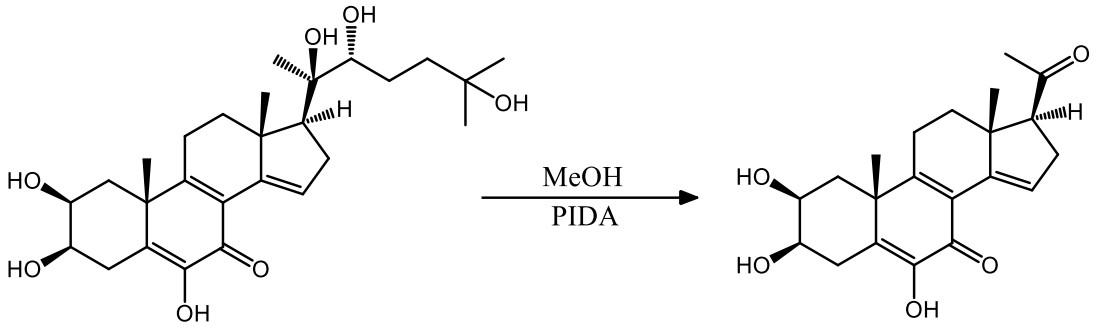
Figure 8 A: Chromatogram of **labCPC No2.** separation, EtOAc – MeOH – H2O (4:2:4, *v/v*). UV detection was carried out on a wavelength of 248±4 nm. **B:** Fractogram of **labCPC No2.** separation is compiled by the analytical measures of the fractions.

The EtOAc – MeOH – H_2O (4:2:4, *v/v*) solvent system was chosen for scale-up in ascending mode **(rCPC No1.**). This means an injected sample of 8 g on a 2100 ml rotor resulted in a short, 20 minutes long method. However, the chromatogram of the scaled-up separation provides wider peaks, it can be explained by the higher loading, the analytical evaluation proves that 95% purity can be achieved with a similar 85% calculated yield. The isolated yield of the entire process with scaled-up purification is 63.1%. This approach demonstrates the scalability of separation to ensure access to the material for a wide range of biological studies.

IV.3. Preparation and investigation of calonysterone semi-synthetic derivatives

IV.3.1 Oxidative cleavage of the side-chain

The regioselective cleavage of the calonysterone side-chain at the C-17 position between the 20,22-diol (**Scheme 1**) was performed with a hypervalent iodine compound, PIDA, which has been successfully used previously to remove the side-chain of 20E [71].



99 **99a**

Scheme 1 Side-chain cleavage reaction of calonysterone.

Our preliminary results have shown that PIDA achieves better yields than PIFA, which is a more powerful oxidant and would therefore produce more minor by-products [71]. The reaction is completely done in 1 hour based on TLC tracking. After the reaction was complete, the solution was neutralized with a 10% aqueous solution of $NaHCO_3$, which was necessary because PIDA produces acetic acid as a by-product. The solvent was then removed using a rotary vacuum evaporator and adsorb the remaining dry material onto silica gel with a low boiling solvent for chromatographic purification.

The adsorbed material was purified by normal phase flash chromatography (**Fig. 9**), which proved to be a more practical choice than reverse phase separation: polar silica gel allows a higher chromatographic load compared to its modified surface analogues, and it is typically worth working with lower boiling point, apolar organic eluent mixtures, which are easier to remove by distillation compared to aqueous mixtures. From a total starting material of 4.86 g (from multiple repetition), 1.79 g of product was successfully isolated after purification in the combined appropriate fractions. This corresponds to a yield of 45.5% for **99a**.

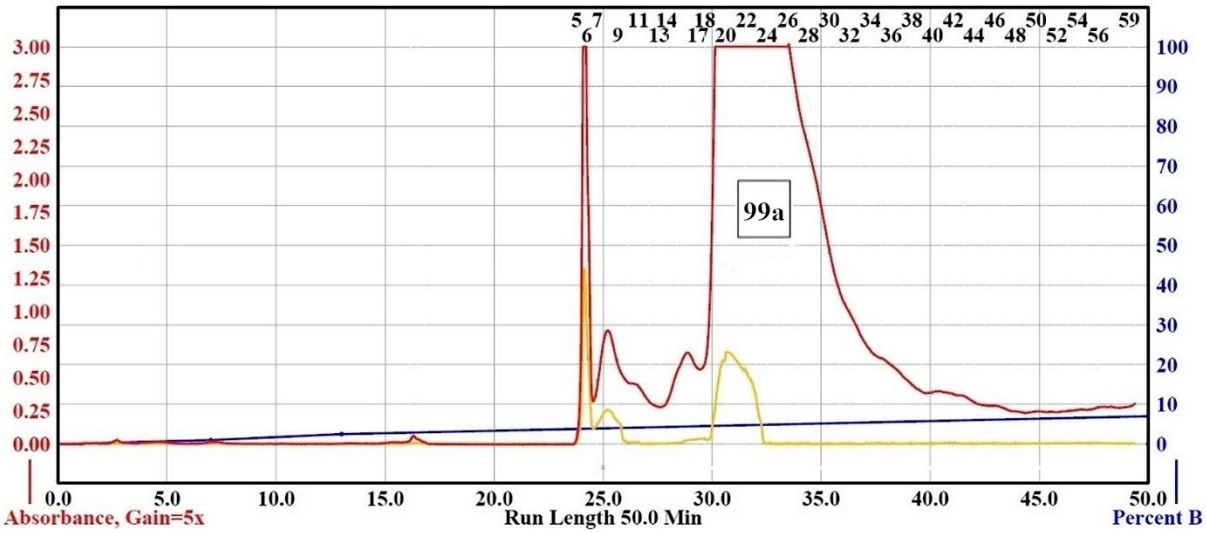
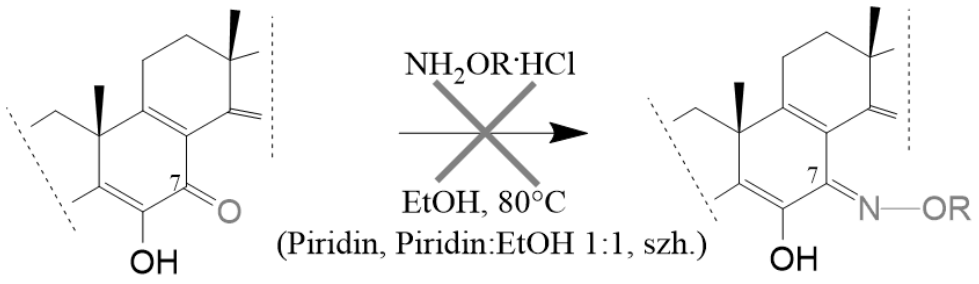


Figure 9 Chromatogram of **99a** purification by normal phase flash chromatography. UV detection was carried out on a wavelength of 248±4 nm (red).

IV.3.2 Experiment to produce the 7-oxime function on the B ring of calonysterone

Previous studies have revealed that poststerone's 6-enone function is comparatively less reactive to produce oximes than its C-17-oxo group [12].

Subsequently, our preliminary experiments to try to form an oxime at position C-7 on the B ring of calonysterone (**Scheme 2**) demonstrated that, after testing several reaction conditions, the desired product was not formed under the tested conditions. Among other reactions, the synthesis of oxime with hydroxylamine hydrochloride was tested using several solvents (ethanol, anhydrous methanol, pyridine, 1-butyl-3-methylimidazoline tetrafluoroborate, ethanol – pyridine (1:1, *v/v*). From these attempts we could therefore postulate that the oxo of a C-17-acetyl function, remaining after an oxidative side-chain cleavage, could be subjected to regioselective oxime formation similarly to the case of poststerone.

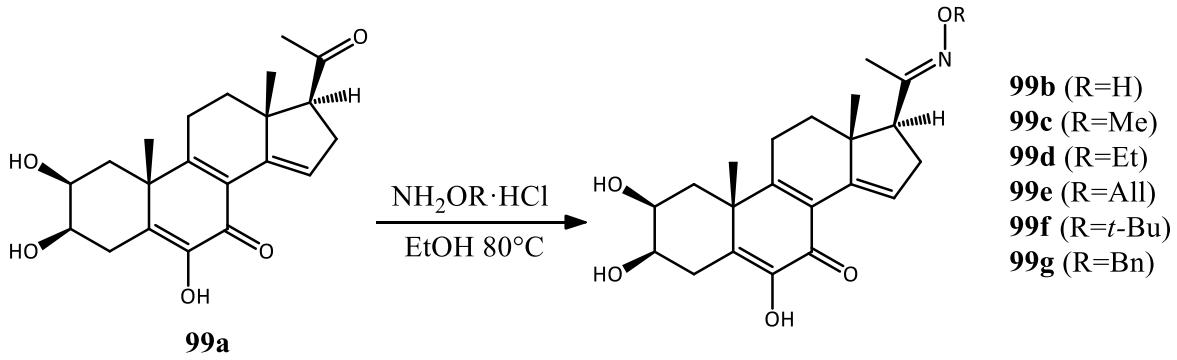


Scheme 2. Attempts of oxime function formation on the B-ring of the calonysterone.

IV.3.3 Regioselective preparation of oxime derivatives

As noted previously, the carbonyl group at position C-7 remains inert in an oxime-forming reaction under a variety of experimental conditions, so it was not expected that a product would be formed where the oxime function would be formed at two different sites. The absence of the C-14α-hydroxyl group typical of ecdysteroids in calonysterone was an advantage for us during reaction planning. This hydroxyl group is sensitive to both acid and alkali, and for those ecdysteroids where they are present, the synthetic formation of an oxime function in a pH medium other than neutral may be circumstantial.

After the reaction was initiated, TLC was performed every 10 min to monitor the process, and the resulting data showed that all derivatives reacted within 40 min (**Scheme 3**). We tried both pyridine and ethanol as solvents, and our observations revealed that the reaction proceeds similarly in either of them. Because of this, we opted for ethanol, as its lower boiling point makes its evaporation easier during the subsequent work-up. After quenching the reaction, the solvent was evaporated on a rotary vacuum evaporator, thus preparing the product for a liquid-liquid extraction with water and ethyl acetate (3 times) that separated the products from the salts remaining in the reaction mixture.



99a

Scheme 3. Synthesis of **99b**–**99g**.

After the extraction of the products, their HPLC chromatograms were recorded, and at the same time the mapping of the eluent systems for their preparative RP-HPLC purification was performed. To improve the solubility of the product mixtures, dimethyl sulfoxide (DMSO) was also used as a solvent prior to the purifications, thus all the materials were dissolved in a 3:7 (*v/v*) ratio solvent mixture of DMSO and acetonitrile.

After collection of the specified peaks, the solvent was removed using a rotary vacuum evaporator, and a small fraction of the material was dissolved in a 3:7 (*v/v*) ratio solvent mixture of DMSO and acetonitrile and the purity of the resulting product was determined by analytical HPLC. At the 254±4 nm wavelength we used for detection; all our purified material was found

to be at least 97% pure. During the chromatographic purity tests, tailing peaks were observed. To establish that the phenomenon was not due to the presence of another substance but to the poor solubility of our product, we examined the spectral purity of the peaks. The result was that the spectral purity of all our substances was above 99.9%, suggesting that the asymmetric chromatographic peaks were probably due to solubility issues.

The different side-chain products were all prepared starting from the same amount of 120 mg of **99a**. After purification, compounds were dried under nitrogen; calculated yields are presented in **Table 6**.

Table 6 Isolated yields and purities of **99b**–**99g**.

C20-NO*R*	Isolated product	Purity	Isolated yield
Comp. **99b** (R=H)	49.4 mg	98.1%	39.5%
Comp. **99c** (R=Me)	33.1 mg	99.1%	25.5%
Comp. **99d** (R=Et)	50.6 mg	98.0%	37.7%
Comp. **99e** (R=All)	52.1 mg	98.8%	37.7%
Comp. **99f** (R=tBu)	27.8 mg	97.4%	19.4%
Comp. **99g** (R=Bn)	54.9 mg	97.1%	35.3%

IV.3.4 Structure elucidation

Detailed structure elucidation description and results of **99b–99g** can be found in publication **No. I** and its supplementary information. The structure elucidation and complete ^{1}H and ^{13}C signal assignment of compound **99** and the sidechain-cleaved calonysterone derivative **99a** was reported previously [64]. The obtained HRMS and NMR data verified that in case of **99b–99g** (**Table 3** and **Table 7**), our synthetic oximation procedure was regioselective in each case, and the location and identity of the newly formed functions was determined by one- and two-dimensional NMR methods [101, 102]. ^{1}H NMR, ^{13}C DeptQ, edHSQC, HMBC, one-dimensional selective ROESY spectra were utilized to achieve complete ^{1}H and ^{13}C signal assignment, which were accomplished using general knowledge of chemical shift dispersion. ^{1}H and ^{13}C chemical shifts, multiplicities and coupling constants of compounds **99b–99g** are compiled in (**Table 7**).

Only one set of signals appeared in the ^{1}H and ^{13}C NMR spectra of each compound, indicating that the regioselective oximation led to the isolation of one stereoisomer for each. The measured Δδ 55 ppm diamagnetic change of δC-20 (211→156 ppm) supported the C═O→C═NOR conversion [88]. A series of selective ROESY experiments were utilized for

the differentiation of (*Z/E*)- isomers on the CH3-21 signals, and the detected steric responses unequivocally proved the (*E)*- configuration of the oxime moiety.

Table 7 ^{1}H and ^{13}C chemical shifts, multiplicities and coupling constants of compounds **99b–99g** in dmso-*d*6. Because the stereostructure of the steroid frame is identical within compounds **99b–99g** we described the multiplicity and *J* coupling constants only for **99b**.

	99b			**99c**		**99d**		**99e**		**99f**		**99g**	
No	**^{1}H**	***J* (Hz)**	**^{13}C**	**^{1}H**	**^{13}C**	**^{1}H**	**^{13}C**	**^{1}H**	**^{13}C**	**^{1}H**	**^{13}C**	**^{1}H**	**^{13}C**
1β	2.29	dd; 14.0;2.9	41.7	2.28	41.7	2.28	41.7	2.28	41.7	2.29	41.7	2.28	41.7
α	1.27	dd; 14.0;3.3		1.25		1.26		1.28		1.26		1.25	
2	3.84		68.3	3.84	68.4	3.83	68.3	3.83	68.3	3.83	68.3	3.83	68.3
3	3.33		72.2	3.33	72.3	3.33	72.2	3.35	72.2	3.33	72.2	3.35	72.2
4β	2.37	t; 12.1	27.0	2.37	27.1	2.37	27.0	2.37	27.0	2.37	27.0	2.37	27.0
α	2.92	ddd; 12.1;4.8;1.2		2.92		2.92		2.92		2.92		2.92	
5			133.1		133.2		133.1		133.1		133.1		133.2
6			142.8		142.9		142.8		142.8		142.8		142.8
7			179.6		179.6		179.5		179.5		179.5		179.5
8			123.1		123.2		123.1		123.1		123.1		123.1
9			164.3		164.4		164.3		164.3		164.2		164.3
10			41.1		41.2		41.1		41.1		41.1		41.1
11β	2.52		24.0	2.52	24.1	2.53	24.0	2.52	24.0	2.54	24.0	2.50	24.0
α	2.63	ddd; 19.0;5.0;~1		2.63		2.63		2.63		2.63		2.61	
12β	2.08		34.9	2.06	34.9	2.06	34.8	2.06	34.8	2.07	34.9	2.03	34.8
α	1.53	td; 12.5;5.0		1.51		1.52		1.52		1.53		1.51	
13			46.5		46.7		46.6		46.7		46.6		46.7
14			140.2		140.2		141.0		140.1		140.1		140.1
15	6.79	t; 2.7	126.3	6.78	126.2	6.78	126.2	6.78	126.1	6.78	126.2	6.77	126.1
16β	2.93		32.7	2.90	32.6	2.91	32.6	2.90	32.5	2.96	32.7	2.90	32.5
α	2.26	ddd; 16.9;7.5;~3		2.26		2.26		2.26		2.29		2.29	
17	2.57	dd; 10.6;7.5	55.9	2.57	55.5	2.57	55.5	2.58	55.5	2.58	55.9	2.58	55.5
18	0.68		18.3	0.68	17.3	0.69	17.3	0.63	17.3	0.68	17.3	0.63	17.3
19	1.40		27.3	1.38	27.3	1.40	27.2	1.39	27.2	1.40	27.2	1.39	27.2
20			154.8		156.6		156.1		156.7		154.2		157.1
21	1.81		15.1	1.82	15.7	1.83	15.7	1.88	15.7	1.81	15.7	1.88	15.9
22				3.76	61.1	4.01	68.27	4.51	73.7		77.2	5.05	74.8
23						1.17	14.9	5.95	135.2	1.23	27.7		138.6
24								5.24	116.9	1.23	27.7	7.34	128.0
25										1.23	27.7	7.34	128.4
26												7.27	127.7
27												7.34	128.4
28												7.34	128.0
HO-2		d; 2.9		3.84									
HO-3		d; 5.6		4.95									

IV.3.5 Biological evaluation of the products

The detailed results of the biological evaluation of the products are described in publication **No. I.** and its supplementary information. In collaboration with the research group of Prof. Mária Deli (Biological Barriers Research group, Biological Research Centre, Szeged, Hungary), we evaluated the effect of the compounds on the viability of hCMEC/D3 cells using impedance measurements at concentration ranges from 0.01–10 μM, and no notable changes in cell viability were observed, except for compounds **99b**, **99c** and **99g**. The onset of the effects for all compounds was at the 4-hour time point. For compound **99b** we could observe a significant cell index decrease for 10 μM concentration, however, for compound **99c** a significant increase for 1 μM concentration was observed. Notably, compound **99g** exhibited the highest and most significant activity. At concentrations of 0.01, 0.1, 1, and 10 μM, it demonstrated a positive effect on barrier integrity, thus it was investigated as a ROS anti-agent, caused artificially by 350 μM tert-butyl hydroperoxide (tBHP).

We could observe a significant decrease in cell viability by a total of ca. 60% in the presence of tBHP compared to the control group (**Fig. 10B and 10C**), indicating tBHP-induced oxidative damage on the cells, however 10 μM of compound **99g** was able to protect the cells efficiently from the harmful effects of tBHP. Notably, at smaller, 10 nM, 100 nM, and 1 μM concentrations a surprising opposite effect was observed, **99g** increased tBHP-induced toxicity, leading to a disruption of the cellular layer (**Fig. 10C**). **99c** and **99e** (3 and 10 μM) are also significantly increased oxidative. This method has been shown to be relevant to evaluate barrier integrity and the overall health of brain endothelial cells.

The present study provided evidence that treatment with tBHP resulted in brain endothelial damage, which was manifested by a decrease in cell and barrier integrity in certain concentrations of the compounds tested. However, co-treatment with compound **99g** significantly altered this effect, leading to the prevention or promotion of oxidative barrier damage. In the broader context, it may be worth stressing that the herein reported compounds are semi-synthetic ecdysteroids that contain oxime ether moieties in their side-chain. This functional group is not expectable to occur in natural ecdysteroids or their metabolites, therefore our results do not directly imply any risk connected to phytoecdysteroid consumption. In our previous study on minor phytoecdysteroids, only protective effects were observed.

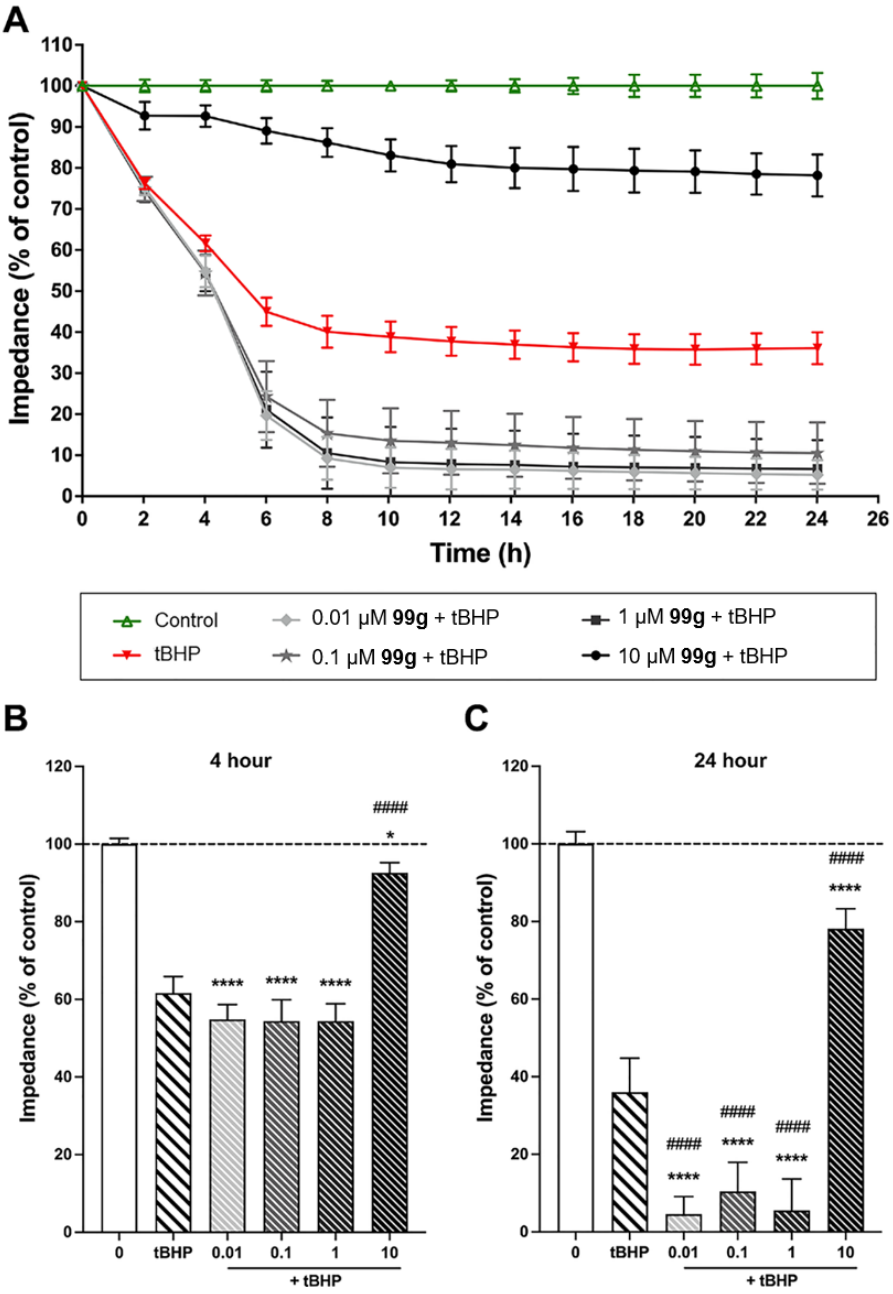


Figure 10 The effects of compound **99g** at concentrations of 0.01, 0.1, 1, and 10 µM treatment on human brain microvascular endothelial cells (hCMEC/D3) were evaluated using impedance-based assays to assess cell viability and barrier integrity in the absence and presence of oxidative stress promoted by *tert*-butyl hydroperoxide (tBHP). **A:** Time-dependent impact of **99g** on cell viability following co-treatment with tBHP (350 µM). **B:** Impact of **99g** on cell viability at 4 hours co-treatment with tBHP (350 µM). **C:** Impact of **99g** on cell viability at 24 hours co-treatment with tBHP (350 µM). The data are presented as the mean ± standard deviation (SD) and were obtained from a minimum of two independent experiments (n = 2−3) with 3−9 technical replicates. Data analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. The results were statistically significant with $^{*}p < 0.05$, $^{****}p < 0.0001$, compared to the control group, and $^{\#\#\#\#}p < 0.0001$, compared to the tBHP group.

To complete the data, ROS production was also measured in human brain endothelial cells after 0.01–10 μM compound **99g** treatment alone or in combination with tBHP for 4 hours. It was confirmed that 350 μM tBHP significantly increased the ROS production, four times the level of the control group (**Fig 11**). Addition of 10 μM of compound **99g** significantly decreased the tBHP-induced ROS production compared to tBHP treatment alone, although it was still 3 times higher than the control. Treatment with compound **99g** alone did not affect ROS production.

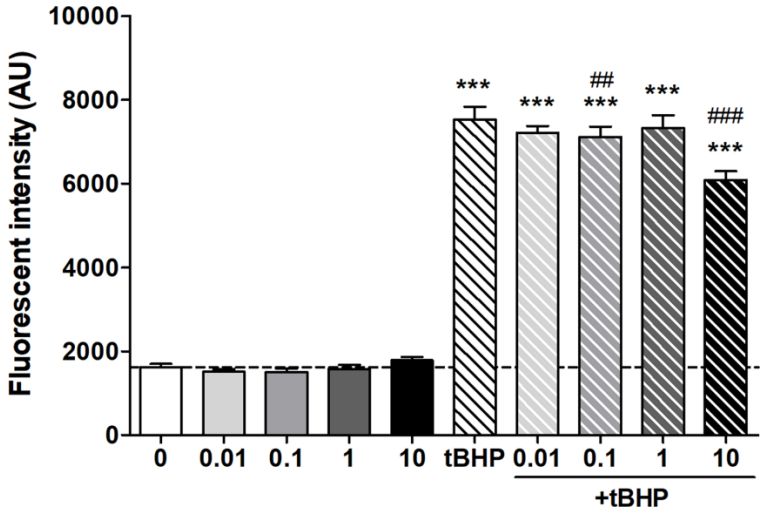


Figure 11 ROS measurement in human brain microvascular endothelial cells (hCMEC/D3) after 4-hour treatment with 0.01–10 μM compound **99g** in the absence and presence of oxidative stress promoted by *tert*-butyl hydroperoxide (tBHP, 350 μM). Data is given in fluorescent intensity corresponding to ROS amount produced intracellularly measured by DCFDA assay. Data are presented as mean ± standard deviation (SD; n = 7–8). Data analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. Statistical significance: $^{***}p < 0.001$, compared to the control group, $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$, compared to the tBHP group.

Barrier integrity tests. To confirm the protective effect of compound **99g** at 10 μM concentration against tBHP-induced ROS damage, transendothelial electrical resistance (TEER), permeability assay with paracellular permeability marker 4 kDa FITC-dextran (FD4) and Evans blue labeled albumin (EBA) immunocytochemistry visualization were used (**Fig. 12**). Results showed that tBHP decreased the TEER and increased the FD4 permeability of the brain endothelial cell monolayer. Treatment with tBHP alone or in combination with compound **99g** had no significant effect on the EBA permeability. Compound **99g** alone decreased the permeability of the BBB model both for FD4 and EBA compared to the control indicating a barrier tightening effect. The morphology of brain endothelial cells treated with compound **99g** was similar to the control. The barrier integrity decreasing effect of tBHP was also visible on the cellular morphology: irregular cell borders and discontinuity in the staining could be

observed. Co-treatment with compound **99g** resulted in the recovery of the brain endothelial cell morphology (**Fig. 12**).

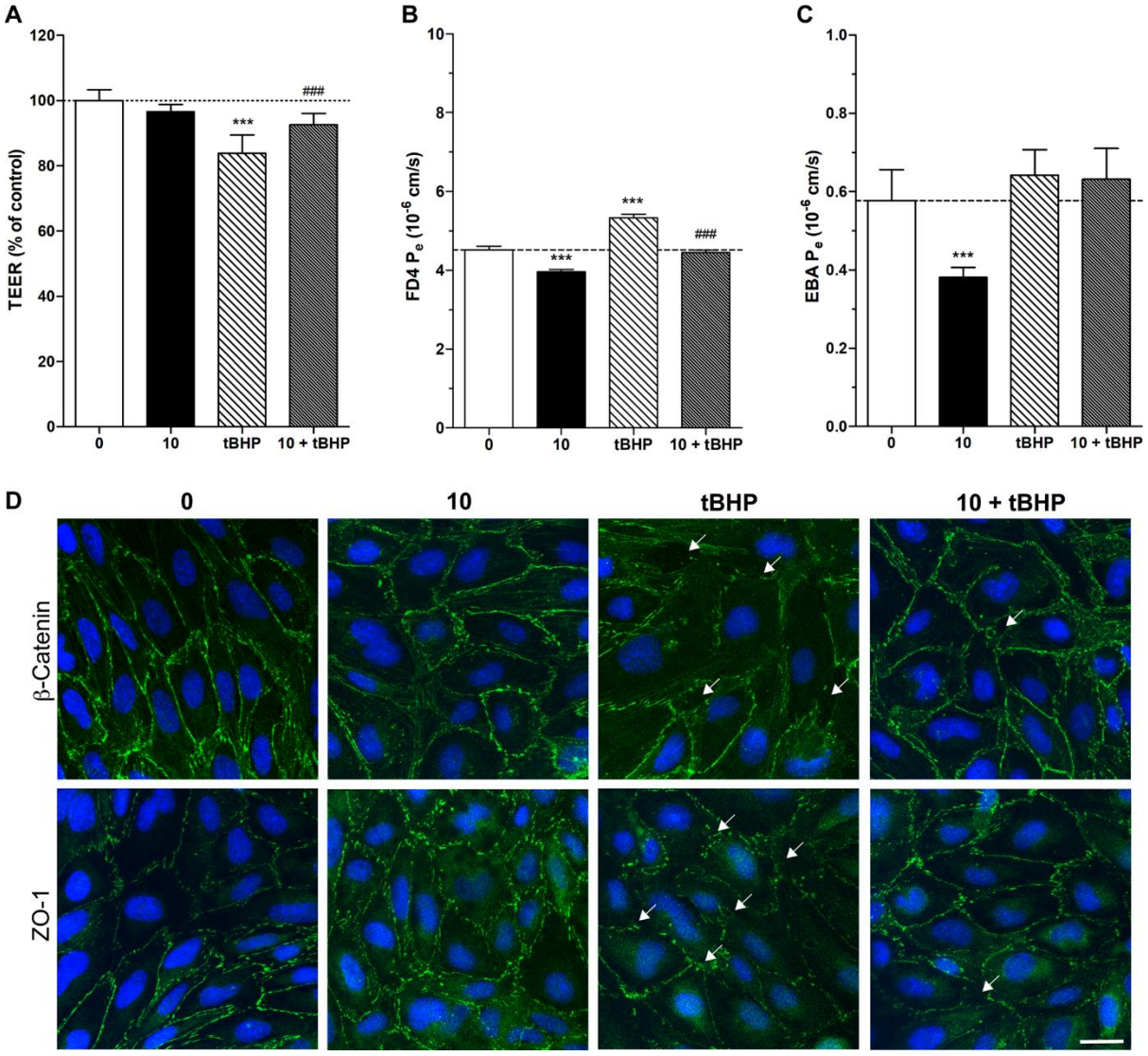


Figure 12 Effects of compound **99g** (10 µM) on the barrier integrity of human brain microvascular endothelial cells (hCMEC/D3) in the absence and presence of oxidative stress promoted by *tert*-butyl hydroperoxide (tBHP, 350 µM). All treatments were performed for 4 hours. **A:** Transendothelial electrical resistance (TEER) measurement. **B:** Permeability measurement for the paracellular marker molecule FITC-dextran 4 kDa (FD4). **C:** Permeability measurement for the transcellular marker molecule Evans blue labeled albumin (EBA, 67 kDa). Data are presented as mean ± standard deviation (SD; n = 4). Data analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. Statistical significance: $^{***}p < 0.001$, compared to the control group, and $^{###}p < 0.001$, compared to the tBHP group. **D:** Immunocytochemistry for β-catenin and zonula occludens-1 (ZO-1) junctional associated molecules. Green: junctional staining. Blue: cell nuclei. Bar: 20 µm.

V. CONCLUSIONS

In this study, through extensive analytical investigation of industrial *Cyanotis arachnoidea* extracts, prepared and processed by unknown methods, we revealed the presence of a wide range of oxidized ecdysteroids that are likely artifacts. This may pose both threats and opportunities. On the one hand, the intensively growing food supplement market and the ever-wide distribution of plant extracts is an explicit cause for concern, as practically nothing is known about the toxicological characteristics of these new, chemically diverse process-related artifacts. On the other hand, some of these newly revealed artifacts may serve as potential leads themselves, and their drug discovery value could be exploited by a suitable and well-designed industrial processing strategy. The implications for our findings highlight the need for further studies on the safety and efficacy of minor ecdysteroids that are unintentionally taken by people worldwide, including, but not limited to, bodybuilders and athletes.

Based on our expanding scientific knowledge of minor ecdysteroids, it is clear that their structural diversity translates to similarly diverse biological profiles. To expand this knowledge by relevant in vivo and possibly even clinical studies, simple, economic, and scalable semi-synthesis, and/or isolation strategy is required. We have previously found several promising bioactivities for calonysterone. Within the framework of this thesis, we have successfully developed a semi-synthetic production method using 20E as starting material, which is an inexpensive and widely available substance. Following the synthesis, we have successfully developed a scalable centrifugal partition chromatographic purification method that fully adapts to both the organic and inorganic impurity profile of the crude reaction mixture. This will ensure the basis for wide availability of the compound at near industrial scale.

For the reason of confirmed neuroprotective activity of 20E, the aim of this thesis was also to prepare new ecdysteroid derivatives with potential BBB protecting activity. Inspired by our preliminary results, we aimed at a regioselective modification of the side-chain of calonysterone to prepare structurally diverse oxime and oxime ether derivatives. After the optimization of reaction conditions, the oxidative cleavage of the side-chain was achieved at C-17, followed by regioselective oxime ether formation at the C-20 position. In the framework of this thesis, we have successfully prepared 7 new ecdysteroid derivatives and tested their BBB protective potential. While at high concentrations, **99g** acted as a neurovascular protective agent, at low concentrations it increased stress-induced cellular damage. This indicates that certain ecdysteroid derivatives may have unexpected and unwanted harmful (side) effects. Our

results highlight the importance of further detailed studies of semi-synthetic ecdysteroid derivatives through the example of this unexpected bioactivity profile change.

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