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Diploma Thesis

DERIVATIVES OF AMARYLLIDACEAE ALKALOIDS AS DRUGS

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Hradec Králové, 2019

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Hereby I declare, this thesis is my original copyrighted work. All literature and other sources that I used while processing are listed in bibliography and properly cited. To my knowledge, this thesis has not been submitted for obtaining the same or any other degree.

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Hradec Králové, August 2019 Aneta Ritomská

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TABLE OF CONTENTS

1) INTRODUCTION 6

2) THE AIM OF STUDY 8

3) THEORETICAL SECTION 9

3.1.) Amaryllidaceae family 9

3.2.) Amaryllidaceae alkaloids 9

3.2.1.) Biosynthesis of Amaryllidaceae alkaloids 11

3.2.2.) Structural types of Amaryllidaceae alkaloids - characteristics, occurrence, biological activity 13

3.2.2.1.) Norbelladine-type alkaloids 13

3.2.2.2.) Galanthamine-type alkaloids 14

3.2.2.3.) Tazettine-type alkaloids 16

3.2.2.4.) Lycorine-type alkaloids 17

3.2.2.5.) Homolycorine-type alkaloids 19

3.2.2.6.) Narciclasine-type alkaloids 20

3.2.2.7.) Montanine-type alkaloids 22

3.2.2.8.) Crinane-type alkaloids: haemanthamine and crinine structural types 23

3.3.) Ambelline 27

3.3.1.) Biological activity of ambelline 28

3.3.1.1.) Anticancer activity 28

3.3.1.2.) Inhibitory activity of AChE, BuChE 30

3.3.1.3.) Inhibitory activity of GSK-3β 31

3.3.1.4.) Inhibitory activity of POP 32

3.3.1.5) Antimicrobial activity 32

3.3.1.6) Antiprotozoal activity 33

3.4.) Derivatives of ambelline 34

3.4.1.) 11-*O*-Acetylambelline 35

4) EXPERIMENTAL SECTION 38

4.1.) Chemicals, materials and methods 38

4.1.1.) Ambelline 38

4.1.2.) Solvents, chemicals, detection reagents 38

4.1.3.) Material for preparative and analytical thin-layer chromatography (TLC) 39

4.1.4.) Mobile phases for preparative and analytical TLC 39

4.1.5.) Structural analysis of prepared derivatives 39

4.1.5.1.) GC-MS analysis 39

4.1.5.2.) ESI-MS analysis ... 39
4.1.5.3.) High resolution mass spectrometry (ESI-HRMS analysis) ... 40
4.1.5.4.) NMR analysis ... 40
4.1.5.5.) Optical rotation ... 40
4.1.6.) Methods for screening biological activities of prepared compounds ... 41
4.1.6.1.) Determination of anticancer activity of prepared derivatives ... 41
4.1.6.2.) Determination of AChE and BuChE inhibitory activity of prepared derivatives ... 41
4.1.6.3.) Determination of GSK-3β inhibitory activity of prepared derivatives ... 41

4.2.) Preparation of synthetic derivatives of ambelline and their structural identification ... 42
4.2.1.) Preparation of 11-*O*-acetylambelline (LC-85) ... 42
4.2.2.) Preparation of 11-*O*-propionylambelline (LC-86) ... 43
4.2.3.) Preparation of 11-*O*-pentanoylambelline (LC-87) ... 45
4.2.4.) Preparation of 11-*O*-isobutanoylambelline (LC-88) ... 47
4.2.5.) Preparation of 11-*O*-benzoylambelline (LC-92) ... 49
4.2.6.) Preparation of 11-*O*-(4-nitrobenzoyl)ambelline (LC-96) ... 51
4.2.7.) Preparation of 11-*O*-(4-methylbenzoyl)ambelline (LC-98) ... 53
4.2.8.) Preparation of 11-*O*-(3-nitrobenzoyl)ambelline (LC-100) ... 55
4.2.9.) Preparation of 11-*O*-(2-chloro-4-nitrobenzoyl)ambelline (LC-110) ... 57
4.2.10.) Preparation of 11-*O*-(4-methyl-3-nitrobenzoyl)ambelline (LC-119) ... 59
4.2.11.) Preparation of 11-*O*-(3-methoxybenzoyl)ambelline (LC-125) ... 61

5) RESULTS ... 63

5.1.) Preparation of semi-synthetic derivatives ... 63

5.2.) Screening of biological activities ... 64
5.2.1.) Screening of anticancer activity ... 64
5.2.2.) Screening of AChE, BuChE inhibitory activity ... 66
5.2.3.) Screening of GSK-3β inhibitory activity ... 67

6) DISCUSSION AND CONCLUSIONS ... 68

7) REFERENCES ... 71

8) LIST OF ABBREVIATIONS ... 83

9) LIST OF FIGURES ... 87

10) LIST OF TABLES ... 88

ABSTRACT ... 89

ABSTRAKT ... 91

1) INTRODUCTION

Plants and natural products have been part of human lives since ancient times. From the beginning they were used as a food source and their therapeutic, respectively toxic effects, were discovered gradually[1]. Various infusions, herbal teas, ointments and other home-made medicinal products were prepared from different parts of the plants. Many of them have retained their traditional use until today but now, in the modern age of technology, secondary metabolites of plants are gaining more and more attention.

Secondary metabolites are mostly the end products of specialised plant metabolism, and several hypotheses described their purpose, which is much more complex than it seems to be[2]. They are considered as an essential, reputable source of new potential drugs and drug leads. Although there are still more synthetic drugs in the pharmaceutical market than herbal medicine, about 60% of pharmaceuticals originate from natural products[3]. Plants possess great potential for diverse chemical structures, but there are still about 75% of plant species that are not sufficiently phytochemically described[2].

Alkaloids belong to secondary metabolites of plants and their biosynthesis is based on amino acids. They are natural organic substances that contain at least one atom of nitrogen in their structure. It is known that about 10-20% of all plants contain alkaloids and they have been discovered in over 4000 plant species so far. However, description of their importance for plants is not adequate. In the past they were considered as a waste material of plant metabolism. Another point of view is that they are plant defence agents against herbivores. Despite that, these conclusions do not apply to all types of alkaloids, because of significant diversity among them. Structural differences between alkaloids also cause various uses in the pharmaceutical field[4].

Amaryllidaceae family plants have been known for their medicinal and toxic properties. For these mentioned effects are responsible Amaryllidaceae alkaloids (AA) which include about 100 characteristic substances. Therapeutically important is galanthamine which is used for the symptomatic treatment of Alzheimer's disease (AD). No less essential is lycorine, known for its cytotoxic activity[4].

This work is focused on the substance of natural origin, ambelline, and preparation of its derivatives. At the Department of Pharmaceutical Botany the research team has long been

dealing with AA and, in recent years, with semi-syntheses of their derivatives. Ambelline itself has no significant biological activity, so it remained unnoticed for a long time. Only last year was proven a moderate antiplasmodial activity against the chloroquine-resistant Dd2 strain of *P. falciparum*[5]. Nevertheless, this diploma thesis mainly discusses derivative's results of anticancer activity, inhibition of acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) and inhibition of glycogen synthase kinase 3β (GSK 3-β), which are the first targets in biological activity testing at the Department of Pharmaceutical Botany in Hradec Králové.

2) THE AIM OF STUDY

Amaryllidaceae alkaloids belong to the main research topics solved at the Department of Pharmaceutical Botany. The preparation of semi-synthetic derivatives of ambelline is based on the previous experience with the preparation of haemanthamine derivatives and structural similarity between these two alkaloids.

Partial aims of the thesis were:

- elaboration of literature study on a preparation of AA derivatives and their biological activity,
- semi-syntheses of pilot ambelline derivatives (aliphatic and aromatic esters),
- structural analysis of prepared compounds,
- preparation of derivatives for screening of their biological activities,
- participation in biological testing of GSK-3β inhibition,
- analysis of results, writing a diploma thesis.

3) THEORETICAL SECTION

3.1.) Amaryllidaceae family

Plants from family Amaryllidaceae belong to the monocot order Asparagales. They are herbaceous, perennial and bulbous flowering plants. Most species of this family have large, showy, actinomorphic and highly scented flowers with variety of colours. As a consequence, plants of the family Amaryllidaceae were cultivated from the wild type as ornamental plants. Typical examples are: daffodils (*Narcissus*), snowdrops (*Galanthus*) and snowflakes (*Leucojum*)[6].

About 85 genera and 1000 species are described in the Amaryllidaceae family. Their occurrence is predominantly in tropical and subtropical areas around the world, especially South Africa and South America, but we can also find them in the temperate climate[7,8].

These plants have traditionally been used in folk medicine as a treatment for many health conditions[6]. The first documented use dates back in the 4th century AD when Hippocrates of Kos used a pessary prepared from narcissus oil for treatment of uterine cancer. The use of narcissus oil continued to Middle Ages in Chinese, South African, Arabian and Central American medicine[9]. In traditional Chinese medicine had found their use also *Lycoris radiata* Herb. and *Narcissus tazetta* L., which was used to treat skin cancer[10,11]. Another utilized plant, *Hymenocallis littoralis* Jacq., was used by Mayans as an antineoplastic agent[11]. In the past was also used the antineoplastic effect in South Africa thanks to bulbs of *Amaryllis belladonna* L., *Boophone disticha* Herb. and *Crinum delagoense* I. Verd[7]. Other examples of therapeutic use of these plants are decoctions of bulbs from *Nerine* species. Sotho and Zulu tribes in South Africa used them to treat coughs and colds, in renal and hepatic dysfunctions, to obtain relief from back pain, and as a remedy for infertility[12].

3.2.) Amaryllidaceae alkaloids

Plants belonging to the Amaryllidaceae family are manifested by a wide range of biological activities. Anticancer, antimalarial, antimicrobial, antiviral and others biological activities of AA (e.g. an ability to inhibit AChE and BuChE) are currently being reported in the literature[13]. Because of that medicinal properties, plants of the Amaryllidaceae family have been under the intense scrutiny. In these plants alkaloids are the most presented substances and these substances are responsible for all the above biological effects[14]. Plants

also contain other secondary metabolites, but only marginal attention has been paid to them[15].

AA are derived from norbelladine and more than 300 different alkaloids have presently been identified from species of this family[16]. Their common feature is a ring system composed of a C_6–C_1 unit derived from phenylalanine, and a N–C_2–C_6 unit derived from tyrosine. The alkaloids are classified according to their main skeleton structure and named after a representative alkaloid from the class[17]. Best known AA are: norbelladine, lycorine, homolycorine, crinine, narciclasine, haemanthamine, tazzetine, montanine and galanthamine (**Figure 1**). They belong to the isoquinoline alkaloids, which include also morphine, codeine, papaverine and others[4].

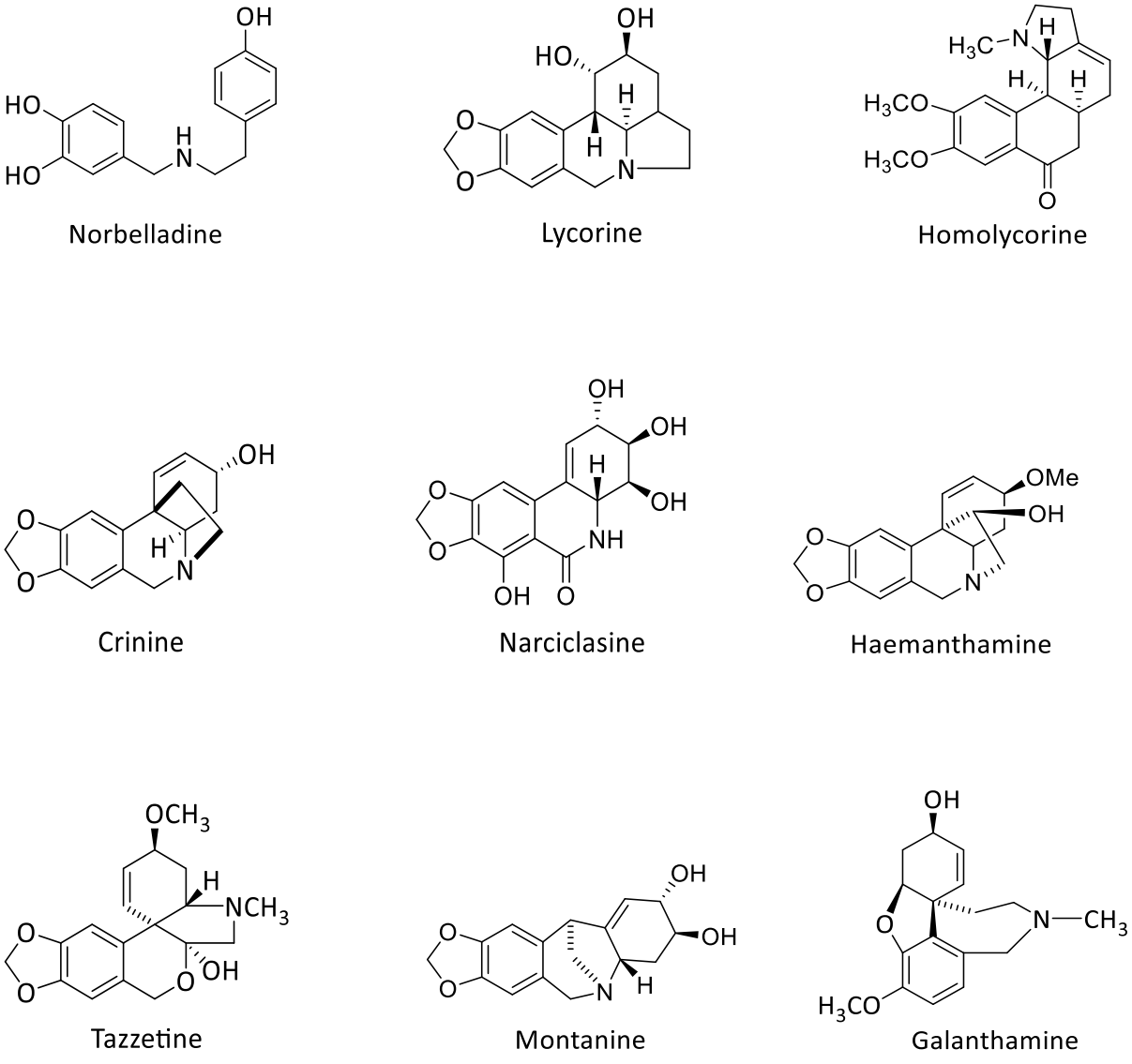


Figure 1: Amaryllidaceae alkaloids

3.2.1.) Biosynthesis of Amaryllidaceae alkaloids

The norbelladine pathway is a specific biosynthesis pathway for AA. This pathway was named by norbelladine because it is a key intermediate in this biosynthesis[15]. AA originate from aromatic amino acids L-tyrosine and L-phenylalanine. Tyramine is the result of changes in L-tyrosine. L-phenylalanine is converted to 3,4-dihydroxybenzaldehyde. These two intermediates react together and after several reactions 4'-*O*-methylnorbelladin is formed[18] (**Figure 2**).

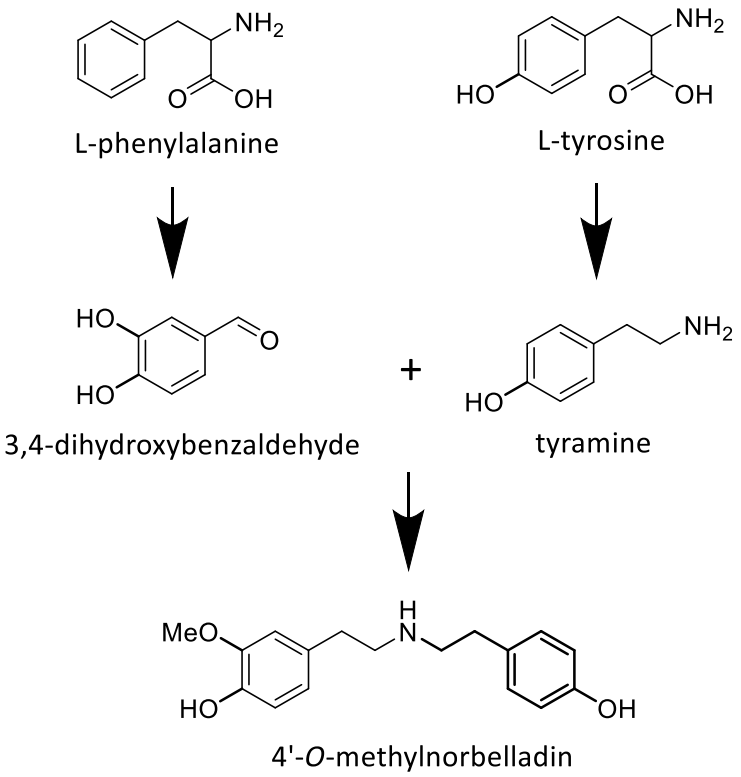


Figure 2: Simplified scheme of 4'-*O*-methylnorbelladin biosynthesis[19]

The next step is oxidative phenol coupling. The result is three elementary structural types[20]. According to the oxidative bond we distinguish 3 types of cyclization:

1. *para-ortho* (e.g. galanthamine),
2. *para-para* (e.g. marithidine, crinine, …),
3. *ortho-para* (e.g. lycorine) (**Figure 3**)[21].

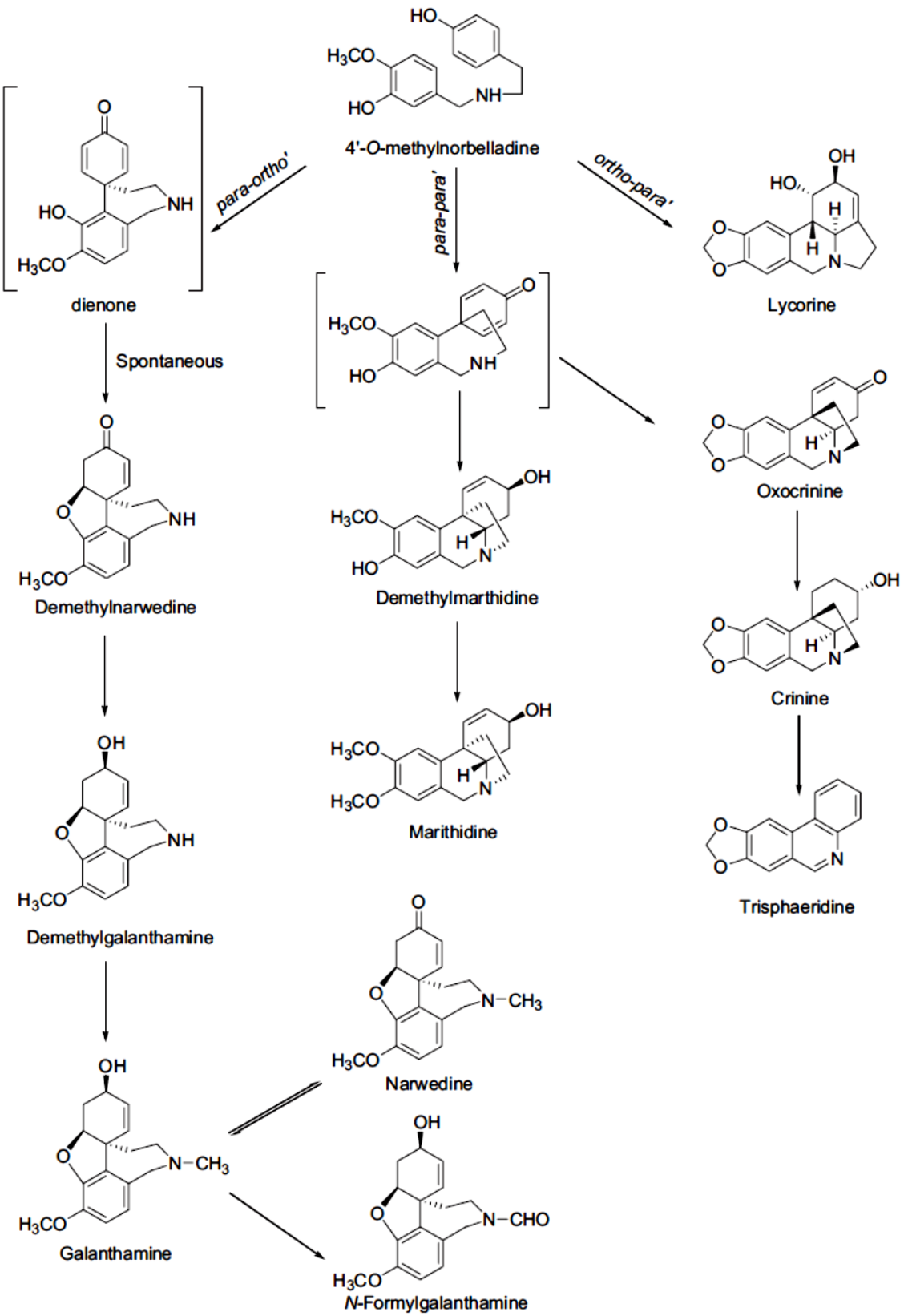


Figure 3: Postulated pathways of AA biosynthesis (reposted)21

3.2.2.) Structural types of Amaryllidaceae alkaloids - characteristics, occurrence, biological activity

Based on biosynthesis of AA we divide them into 9 main structural types. The most commonly used partition is: norbelladine, lycorine, homolycorine, crinine, haemanthamine, narciclasine, montanine, tazettine and galanthamine structure types. The name of each group relates to the major alkaloid that represents the whole group[16,17]. In this division is also counted norbelladine which is starting substance of AA biosynthesis, and montanine, although its biosynthetic pathway has not been fully understood[18].

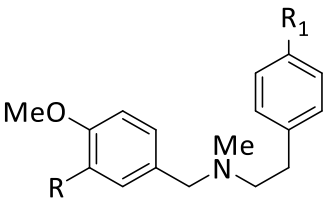
There are other different structural types of AA, but they have only been discovered in one plant genus or one plant species. There are also groups that are represented by only one alkaloid (e.g. gracilamine- and cherylline-type alkaloids).

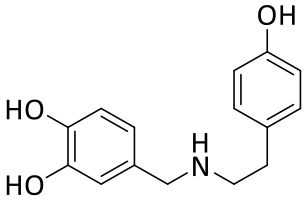
An interesting group is mesembrine-type alkaloids which were believed to belong to the crinine structural type of AA. However, more detailed phytochemical studies have shown that although the precursors for their biosynthesis are the same, the process of biosynthesis is different. This type of alkaloids was isolated only in 2 species of daffodils (*Narcissus triandrus* L. and *Narcissus pallidus* Garells), but the most important source of them is the species *Sceletium* L. (Aizoaceae)[6,22].

3.2.2.1.) Norbelladine-type alkaloids

Norbelladine, precursor for AA, can be found in several plants as *Galanthus nivalis*, *Leucojum aestivum*, *Narcissus tazetta*, *Nerine bowdenii* and *Pancratium maritimum*. Its biological activity was not tested on a larger scale, but there is a study from the year 2014 that suggests that norbelladine may be a compound able to provide significant antioxidant and anti-inflammatory effects via scavenging radicals and inhibiting COX enzymes, furthermore inhibiting NF-κB activation at relatively low concentrations[23].

It is believed that alkaloids of this type are based on simple methylation of norbelladine, although the order of methylation is not determined[24]. Except norbelladine, we can include here for instance belladine, 6-*O*-demethylbelladine and 4´-*O*-demethylbelladine (**Fig.4**).





Norbelladine

R = OMe	R_1 = OMe	Belladine
R = OMe	R_1 = OH	6-*O*-Demethylbelladine
R = OH	R_1 = OMe	4´-*O*-Demethylbelladine

Figure 4: Chemical structure of selected norbelladine-type alkaloids

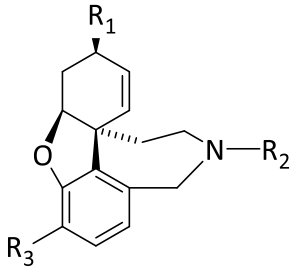
6-*O*-demethylbelladine and 4´-*O*-demethylbelladine were isolated in 2016 for the first time. Their cholinesterase and prolyloligopeptidase (POP) inhibitory activities were tested in the following study (**Tab.1**). Of all tested substances (22), 4´-*O*-demethylbelladine had one of the best BuChE and POP inhibitory activity[25].

Table 1: HuAChE, HuBuChE, and POP inhibitory activity of belladine, 6-*O*-demethylbelladine and 4´-*O*-demethylbelladine expressed as IC_{50} [25]

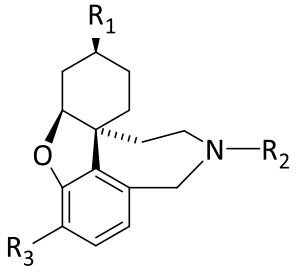
	HuAChE IC_{50} (µM)	**HuBuChE IC_{50} (µM)**	**POP IC_{50} (µM)**
Belladine	699.2 ± 19.4	315.3 ± 10.5	> 100
6-*O*-Demethylbelladine	223.2 ± 23.6	115.7 ± 10.1	0.66 ± 0.09
4´-*O*-Demethylbelladine	606.8 ± 74.2	30.7 ± 4.0	0.37 ± 0.03

3.2.2.2.) Galanthamine-type alkaloids

This group is the only group of AA with *para-ortho* cyclization. Galanthamine and other alkaloids with similar structure are mostly isolated from genus like *Crinum*, *Galanthus*, *Leucojum*, *Lycoris* and *Narcissus*[26,27,28]. Other alkaloids of this group are: norgalanthamine, narcisine, chlidantine, sanguinine, norlycoramine or lycoramine (**Fig.5**)[15].



R_1 = OH	R_2 = Me	R_3 = OMe	Galanthamine
R_1 = OH	R_2 = H	R_3 = OMe	Norgalanthamine
R_1 = OH	R_2 = Me	R_3 = OH	Sanguinine
R_1 = OH	R_2 = Ac	R_3 = OMe	Narcisine
R_1 = OMe	R_2 = Me	R_3 = OH	Chlidantine



R_1 = OH	R_2 = Me	R_3 = OMe	Lycoramine
R_1 = OH	R_2 = H	R_3 = OMe	Norlycoramine

Figure 5: Chemical structures of selected galanthamine-type alkaloids

Galanthamine causes bradycardia or atrioventricular disorders. In the CNS and in the periphery of the nervous system, galanthamine selectively and reversibly inhibit cholinesterases and allosterically modulates nicotinic receptors that promote cholinergic transmission[29]. Based on its properties it is the only one AA used in pharmacotherapy as symptomatic treatment of AD[4]. Its use in the treatment of AD has significantly increased interest in this type of AA and their potential use as cholinesterases inhibitors.

Chlidantine, a molecule that has also been connected to significant inhibition of cholinesterases. This assumption was based on a similar structure to galanthamine. In chlidantine structure there is a different position between hydroxy- and methoxy- groups unlike galanthamine. However, subsequent studies showed markedly lower AChE inhibitory activity compared to galanthamine[30].

Sanguinine had previously been shown to be more potent than galanthamine. Prior structure-activity relationship study indicated that properly placed hydrophilic groups on galanthamine contribute to its effective binding to the AChE molecule. Therefore, the extra hydroxyl group of sanguinine available for potential interaction with AChE can explain the strong inhibitory activity of this alkaloid[31].

Lycoramine and norlycoramine lack a double bond in a ring C and that does not allow these compounds to have the same spatial configuration as other galanthamine-type AA. This structural variation results in an undetected AChE inhibitory activity[32].

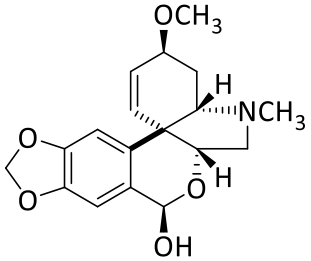
Table 2: An overview of AChE inhibitory activity of selected AA expressed as IC_{50} values[30,32]

	IC_{50} (μM)
Galanthamine	1.07 ± 0.18
Lycoramine	nd
Norlycoramine	nd
Sanguinine	0.10 ± 0.01
Chlidantine	24.10 ± 5.0

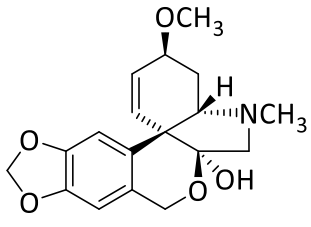
nd = not detected

3.2.2.3.) Tazettine-type alkaloids

These alkaloids are formed from norbelladine by *para-para* type cyclization. Their source is mainly *Eucharis*, *Crinum*, *Galanthus*, *Hippeastrum*, *Leucojum* genera[15,33,34]. The fundamental alkaloid of this group is tazettine, which is one of the most widespread AA. Its biosynthesis comes out from haemanthamine. Pretazettine, precursor of tazettine, is produced firstly (**Fig.6**)[35].



Pretazettine



Tazettine

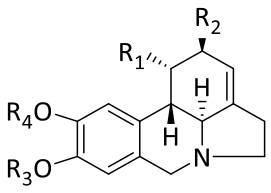
Figure 6: Chemical structure of tazettine and pretazettine

Biological activity of tazettine is not particularly interesting. Tazettine exhibits a negligible cytotoxic activity, has a weak hypotensive and antimalarial activity and interacts with DNA[36,37,38].

On the other hand, pretazettine shows a broad spectrum of cytotoxic activity: effectivity against fibroblastic LMTK cell lines, inhibition of HeLa cell growth, therapeutical effectivity against advanced Rauscher leukaemia, Ehrlich ascites carcinoma, spontaneous AKR lymphocytic leukaemia and Lewis lung carcinom[16,39-42]. Activity of pretazettine against MOLT-4 against selected RNAs viruses has been demonstrated in pretazettine – counteracts flavoviruses (Japanese encephalitis, yellow fewer and dengue) and bunyaviruses (Punta Toro and Rift Valley fever). Its activity was also confirmed in *Herpes simplex* virus type 1. This efficacy may just reflect the general ability to inhibit protein synthesis during viral replication[16,43]. Pretazettine is a chemically labile precursor of tazettine, but when it is rearranged to tazettine, a biological activity is almost inactivated[16].

3.2.2.4.) Lycorine-type alkaloids

This alkaloid set is formed by *ortho-para* cyclization of norbelladine. This large set of AA contains almost 200 structural varieties with a diverse array of biological activities. Characteristic representative is lycorine, which was the first described AA[44]. Other representatives include 1-*O*-acetyllycorine, pseudolycorine, galanthine, norpluviine, amarbellisine or lycorene (**Fig.7**)[45]. The most important genera containing the lycorine-type alkaloids are *Crinum*, *Galanthus*, *Leucojum*, *Lycoris* and *Narcissus*[28,46].



R_1 = OH	R_2 = OH	R_3 = -CH_2-	R_4 = -CH_2-	Lycorine
R_1 = OH	R_2 = OH	R_3 = Me	R_4 = H	Pseudolycorine
R_1 = OAc	R_2 = OH	R_3 = -CH_2-	R_4 = -CH_2-	1-*O*-Acetyllycorine
R_1 = OH	R_2 = OMe	R_3 = Me	R_4 =Me	Galanthine
R_1 = OH	R_2 = H	R_3 = Me	R_4 = H	Norpluviine
R_1 = OH	R_2 = H	R_3 = Me	R_4 = Me	Pluviine

Figure 7: Chemical structures of selected lycorine-type alkaloids

Lycorine has been studied for several years for its promising biological effects that have a wide range of activities. In addition to its antitumor effect, lycorine is able to inhibit the biosynthesis of ascorbic acid in plants, is effective against some viruses (e.g. poliovirus, SARS-associated coronavirus, varicella virus) or protozoa (*Trypanosoma brucei*) and acts antimycotically on *Saccharomyces cerevisiae*[47,48,49,50,51,52].

Many preclinical studies focusing on human cancer research are concerned with lycorine as it has cytotoxicity on cell lines resistant to apoptosis[53]. The antitumor activity of lycorine has been demonstrated *in vivo* in BL6 mouse melanoma cells as well as in Lewis lung carcinoma cells. *In vitro* studies were then performed on HeLa cells and other types of tumor cells (e.g. CEM, K562 or G-361) to proof the anticancer activity[54,55,56]. Further testing was carried out, for example, on human leukaemia cells HL-60, where cell cycle arrest was observed and also increased activation of caspases and induced apoptosis, which was independent of pro-apoptotic stimuli. Lycorine has also been studied in multiple myeloma KM-3. In this case, cell cycle had been arrested in the G_0/G_1 phase and repair of damaged DNA did not occur as a result of which apoptosis was initiated[57]. Study from the year 2010 compares effect of others lycorine-type alkaloids where were selected cell lines resistant to apoptosis. Ungeremine, amarbellisine or pseudolycorine were also active, but none of these alkaloids were more effective than lycorine itself (**Tab.3**)[54].

Table 3: Growth-Inhibitory Activity for Cancer Cell Lines of lycorine-type AA (reposted)[54]

cell line [*in vitro* IC_{50} (μM) growth-inhibitory values] ± SEM						
	A549	**OE21**	**Hs683**	**U373**	**SKMEL**	**B16F10**
Lycorine	4.2 ± 0.2	4.5 ± 0.7	6.9 ± 0.5	7.6 ± 0.4	8.4 ± 0.2	6.3 ± 0.2
Caranine	>10	>10	>10	>10	>10	>10
Pseudolycorine	7.4 ± 0.2	7.9 ± 0.3	7.9 ± 0.1	7.8 ± 0.1	>10	7.5 ± 0.1
Galanthine	>10	>10	>10	>10	>10	>10
Norpluvine	>10	>10	>10	>10	>10	>10
Amarbellisine	7.2 ± 0.3	6.7 ± 0.2	8.2 ± 0.1	7.2 ± 0.4	8.3 ± 0.1	6.7 ± 0.1
Ungeremine	>10	>10	>10	83 ± 1	>10	>10

A549 = human adenocarcinomic alveolar basal epithelial cells (human lung carcinoma), OE21 = oesophageal squamous cell carcinoma, Hs683 = human brain glioma, U373 = human glioblastoma astrocytoma, SKMEL = human skin malignant melanoma, B16F10 = mouse skin melanoma

Despite the fact lycorine alone does not show any activity against AChE, its derivatives (whether they are naturally occurring in the plants or those obtained by synthetic routes) are intensively investigated. The most well-known analogue is 1-*O*-acetyllycorine, which appears to be twice as effective as galanthamine. However, these results seem to be inconsistent, because the inhibitory activity varies according to what acetylcholinesterases are used in testing (human, eel)[53,58]. Some of these alkaloids have been tested for AChE inhibitory activity and the following table shows the results (**Tab.4**).

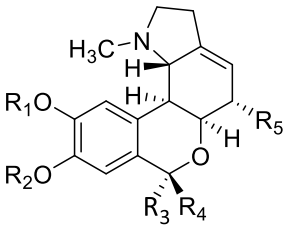
Table 4: AChE inhibitory activity of tested alkaloids expressed as IC_{50} values[32,53]

	IC_{50} (μM)
Oxoassoanine	47.21 ± 1.13
Assoanine	3.87 ± 0.24
Pseudolycorine	152.32 ± 32.06
Lycorine	nd
2-*O*-Acetylpseudolycorine	nd
1-*O*-Acetyllycorine	0.96 ± 0.04

nd = not detected

3.2.2.5.) Homolycorine-type alkaloids

Ortho-para cyclization of norbelladine formed homolycorine-type alkaloids. *Galanthus*, *Crinum*, *Narcissus*, *Leucojum* or *Hippeastrum*, these are the major plant genera in which these alkaloids occur[15,59,60,61]. The main representatives include lycorenine, hippeastrine, oduline, masonine and neronine (**Fig. 8**) [15].



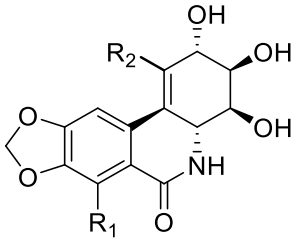
R_1 = Me	R_2 = Me	R_3 = O	R_4 = O	R_5 = H	Homolycorine
R_1 = -CH_2-	R_2 = -CH_2-	R_3 = O	R_4 = O	R_5 = OH	Hippeastrine
R_1 = Me	R_2 = Me	R_3 = OH	R_4 = H	R_5 = H	Lycorenine
R_1 = -CH_2-	R_2 = -CH_2-	R_3 = OH	R_4 = H	R_5 = H	Oduline
R_1 = -CH_2-	R_2 = -CH_2-	R_3 = O	R_4 = O	R_5 = H	Masonine

Figure 8: Chemical structure of selected homolycorine-type alkaloids

The bioactivity of most homolycorine-type alkaloids is largely unknown. Nevertheless, we know that homolycorine shows high antiretroviral activity and inhibits the growth of some tumor cell lines. Homolycorine was tested against the parasitic protozoa *Trypanosoma cruzi*, *Trypanosoma brucei rhodesiense*, *Leishmania donovani* and *Plasmodium falciparum,* but showed no significant activity. Another alkaloid is hippeastrine. Its examined effects are activity against *Herpes simplex* virus type 1, antifungal activity against *Candida albicans* and weak insect antifeedant activity[62]. Some of these alkaloids have also been tested for AChE inhibitory activity, but no considerable values have been reported[32].

3.2.2.6.) Narciclasine-type alkaloids

Narciclasine-type alkaloids could be derived by either lycorine or haemanthamine pathway because of their structural affinity to both structural types. But it has been proved that they proceed from the pathway similar with haemanthamine-type alkaloids. Type of cyclization is *para-para*[16]. First source of these alkaloids was genus *Narcissus*, but they are present also in different genera such as *Leucojum, Pancratium, Galanthus, Haemanthus*[63]. This group contains alkaloids like narciclasine, pancratistatin, lycoricidine, crinasiadine (**Fig.9**)[9].



R_1 = OH	R_2 = OH	Pancratistatin
R_1 = OH	R_2 = H	Narciclasine
R_1 = OCH_3	R_2 = H	Lycoricidine
R_1 = H	R_2 = H	Crinasiadine

Figure 9: Chemical structure of selected narciclasine-type alkaloids

One of the many biological effects of narciclasine is its antimitotic and anticancer action. This activity was firstly detected because of its very strong inhibitory effect on the growth of the radicles of wheat grains[64]. Narciclasine affects cell division at the metaphase stage and inhibits protein synthesis in eukaryotic ribosomes by interacting with the 60S subunit and inhibiting peptide bond formation[65]. Inhibition of calprotectin-induced cytotoxicity and retardation of DNA synthesis also belong to narciclasine range of biological activity[16,66]. This alkaloid inhibits HeLa cell growth, has antileukaemic properties (triggers apoptosis in Jurkat cells) and is active against a variety of tumor cells (human and murine lymphocytic leukaemia, larynx and cervix carcinomas and Ehrlich tumor cells). Narciclasine scale of activity contains also the activity against *Corynebacterium fascians* and inhibition of the pathogenic yeast *Cryptococcus neoformans*[16]. Gabrielsen et al. described an antiviral activity of narciclasine. It is active against flaviviruses and bunyaviruses[43].

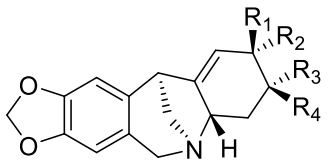
Another interesting substance in potential anticancer treatment is pancratistatin. The specificity of pancratistatin to cancer cells and the mechanism of its action remain unknown. Study of McLachlan et al. provides a detailed look at the effect of pancratistatin treatment on cancerous and normal cells. Their results indicate that pancratistatin induced apoptosis selectively in cancer cells and that the mitochondria may be the site of action of pancratistatin in cancer cells[67]. Siedlakowski et al. reported the ability of pancratistatin to induce apoptosis specifically in human breast cancer cell lines MCF7 and Hs-578-T compared to their non-cancerous counterparts. In cancer cells pancratistatin caused increased levels of reactive oxygen species (ROS), decreased adenosine triphosphate (ATP) and mitochondrial membrane permeabilization indicating the activation of the mitochondrial pathway of apoptosis. In combination with the anti-estrogen Tamoxifen, pancratistatin had a synergic effect[68]. Despite of that an article from the year 2004 states that narciclasine is more active in all tumor cell lines that were tested during that study (**Tab.5**)[69].

Table 5: Human Cancer Cell Line and Murine P-388 Lymphocytic Inhibitory Activities of pancratistatin and narciclasine (reposted)[69]

	ED_{50} (μg/mL)	GI_{50} (μg/mL)					
	leukemia P-388	**pancreas-a BXPC-3**	**breast MCF-7**	**CNS SF268**	**lung-NSC NCI-H460**	**colon KM20L2**	**prostate DU-145**
Pancratistatin	0.017	0.02	0.023	0.014	0.032	0.025	0.015
Narciclasine	0.013	0.0035	0.0032	0.0031	0.0084	0.0032	0.0032

3.2.2.7.) Montanine-type alkaloids

The montanine alkaloids belong to the group of alkaloids derived from norbelladine by the *para-para* oxidative cyclization[16]. By changing its configuration only at C-2 and C-3, the major alkaloids of the group are formed. Due to their low availability and the content of small amounts of these alkaloids in plants, attention is increasingly devoted to synthetic preparation of them from haemanthamine-type alkaloids. From plant sources we can name the genera *Narcissus, Hippeastrum, Rhodophiala* or *Pancratium*[70,71,72,73]. A typical representative of these alkaloids is montanine. Other representatives include pancracine, brunsvigine, coccinine and manthine (**Fig.10**)[28,74].



R_1 = H	R_2 = OMe	R_3 = H	R_4 = OH	Montanine
R_1 = H	R_2 = OH	R_3 = H	R_4 = OH	Pancracine
R_1 = H	R_2 = OMe	R_3 = H	R_4 = OMe	Manthine
R_1 = OMe	R_2 = H	R_3 = H	R_4 = OH	Coccinine
R_1 = H	R_2 = OH	R_3 = OH	R_4 = H	Brunsvigine

Figure 10: Chemical structures of selected montanine-type alkaloids

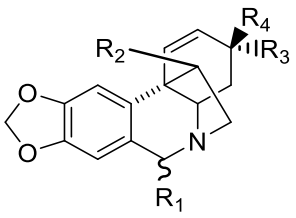
Spectrum of biological activity of montanine-type alkaloids is not well known. There are just few findings about pancracine, montanine and manthine. Pancracine is active against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*[75]. It shows also a weak activity against *Trypanosoma brucei rhodesiense, T. cruzi* and *Plasmodium falciparum*[70].

In article from Pagliosa et al. has been described acetylcholinesterase activity of montanine. The alkaloid montanine significantly inhibited AChE activity at concentrations 1mM, 500 μm and 100 μm[76].

Govindaraju et al. reported *in vitro* growth inhibitory effects of alkaloid manthine. Manthine showed inhibitory effects across the entire panel of cancer cell lines (resistant to apoptosis - A549, SKMEL-28, U373; sensitive to apoptosis - MCF7, Hs683, B16F10)[77].

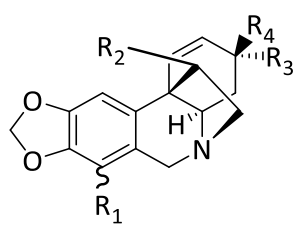
3.2.2.8.) Crinane-type alkaloids: haemanthamine and crinine structural types

The haemanthamine- and crinine-type alkaloids are formed by *para-para* oxidative cyclization of norbelladine. These two types differ only in the spatial configuration of the 5,10*b*-ethane bridge, which means that they are enantiomers[16]. Alkaloids of these types are one of the most widespread types from the family Amaryllidaceae. Plant sources are genera *Ammocharis*, *Crinum*, *Haemanthus*, *Pancratium*, *Rhodophiala*, *Zephyranthes*[28,78,79]. Haemanthamine-type alkaloids are α-stereoisomers and crinine-type alkaloids are β-stereoisomers. Haemanthamine, vitattine, haemanthidine, bulbispermine and crinamine belong to haemanthamine-type (**Fig.11**). Ambelline, crinine, buphanidrine, elwesine, powelline belong to crinine-type (**Fig.12**)[80].



R_1 = H	R_2 = OH	R_3 = H	R_4 = OMe	Haemanthamine
R_1 = H	R_2 = H	R_3 = H	R_4 = OH	Vitattine
R_1 = OH	R_2 = OH	R_3 = H	R_4 = OMe	Haemanthidine
R_1 = H	R_2 = OH	R_3 = H	R_4 = OH	Bulbispermine
R_1 = H	R_2 = OH	R_3 = OMe	R_4 = H	Crinamine

Figure 11: Chemical structure of selected haemanthamine-type alkaloids



R_1 = OCH_3	R_2 = OH	R_3 = OCH_3	R_4 = H	Ambelline
R_1 = H	R_2 = H	R_3 = H	R_4 = OH	Crinine
R_1 = H	R_2 = H	R_3 = H	R_4 = OMe	Buphanisine
R_1 = H	R_2 = H	R_3 = OH	R_4 = H	Elwesine
R_1 = OCH_3	R_2 = H	R_3 = OH	R_4 = H	Powelline

Figure 12: Chemical structure of selected crinine-type alkaloids

As early as 1958, the *in vivo* activity of some of those AA was investigated. These were crinine, haemanthamine, haemathidine, ambelline and buphanidrine. Effect of them on sarcoma 37 were negative[81]. Despite the first negative results, the research with those alkaloids continued. Many of these alkaloids have been tested to determine their biological activity. The antitumor activity of haemanthamine and haemanthidine was found when the anticancer activity (cancer cell lines A549, OE21, U373, Hs683, SKMEL and B16F10) of crinane group alkaloids was tested. Crinine alkaloids did not show any significant activity to any of the cancer cell lines[54]. Furthermore, according to results from the year 2015, haemanthamine and haemanthidine show antiproliferative effects on cancer cell lines such as human ovarian, lung, colon and breast tumors[82]. Both alkaloids were shown to be cytotoxic to p-53 mutated colorectal cancer cell lines (Caco-2 and HT-29) with minimal damage to healthy intestinal cells[83]. According to Weniger et al., haemanthamine and crinamine are effective against MOLT-4 lymphoma and non-tumoral fibroblastic LMTK cells. Ambelline, papyramine and buphanidrine were also tested, but they were inactive[37].

Also noteworthy is distichamine because of its cytotoxic effects (**Fig.13**). It belongs to crinine structure type. Despite not being a common occurring alkaloid, its biological activity has been tested several times. Study from 2012 described efficacy of distichamine against 5 cancer cell lines - CEM, K562, MCF7, HeLa and G-361. HeLa cancer cell line was the most sensitive one, but distichamine is not selective only for cancer cells and it also affects healthy cells[84]. This finding was also confirmed in 2013[85]. Important activity is not just anticancer but also antibacterial. Cheesman et al. revealed that distichamine is a novel, broad spectrum antibacterial agent with the best MIC (minimum inhibitory concentration) value set at 0.063 mg/ml. It is active against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumonia*[86].

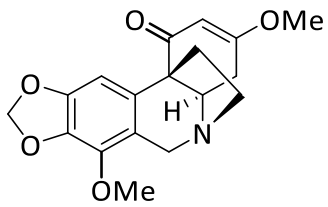


Figure 13: Chemical structure of distichamine

Table 6: Evaluation of the cytotoxic activity of haemanthamine, haemanthidine and distichamine[54,83,84,87]

	Haemanthamine	**Haemanthidine**	**Distichamine**
A-549 IC_{50} (µM)	4.5 ± 0.6	4.0 ± 0.4	nrf
OE21 IC_{50} (µM)	6.8 ± 0.7	3.7 ± 0.2	nrf
U373 IC_{50} (µM)	7.7 ± 0.5	3.8 ± 0.2	nrf
Hs683 IC_{50} (µM)	7.0 ± 0.3	4.3 ± 0.2	nrf
SKMEL IC_{50} (µM)	8.5 ± 0.2	4.2 ± 0.2	nrf
B16F10 IC_{50} (µM)	6.8 ± 0.2	3.1 ± 0.2	nrf
Caco2 IC_{50} (µM)	0.99 ± 0.14	3.3 ± 0.9	nrf
HT-29 IC_{50} (µM)	0.59 ± 0.01	1.7 ± 0.1	nrf
CEM IC_{50} (µM)	2.1 ± 0.4	nrf	4.5 ± 1.6
K562 IC_{50} (µM)	3.4 ± 1.6	nrf	4.1 ± 0.9
MCF7 IC_{50} (µM)	8.1 ± 3.3	1.8 ± 0.2	2.3 ± 0.8
HeLa IC_{50} (µM)	7.0 ± 2.2	1.6 ± 0.2	2.2 ± 0.1
G-631 IC_{50} (µM)	3.7 ± 0.4	nrf	14.7 ± 0.1

nrf = no results found

Crinane alkaloids have been shown to exhibit a range of biological activities, primarily cytotoxicity, but not AChE inhibitory activity. In 2013 study of AchE and BuChE inhibitory activity of AA took place at our department. 13 alkaloids from *Zephyranthes robusta* BAKER were tested and results of crinane alkaloids were negative[88]. 2 years later, 8-*O*-demethylmaritidine and undulatine (**Fig.14**) were tested for their inhibitory effects on AChE and predicted penetration across the blood-brain barrier (BBB). It has been demonstrated that undulatine, a crinine-type AA, has promising AChE inhibition (IC_{50} = 7.4 ± 0.03 µM) which acts via a mixed inhibition mechanism and on the basis of the PAMPA-BBB method is able to cross the BBB by passive permeation (Pe = 18.3 ± 0.2*10^{-6} cm sec^{-1} → CNS+)[89].

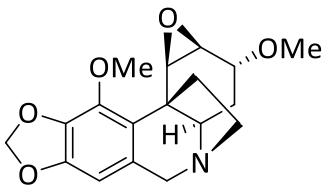


Figure 14: Chemical structure of undulatine

According to Herrera et al., haemanthidine showed some biological activity against *Trypanosoma brucei rhodesiense* with an IC_{50} of 1.1mg/ml. Maritidine, which was also tested, showed no activity. Haemanthidine also showed some activity against *Trypanosoma cruzi* with an IC_{50} of 1.4mg/ml[90]. Antimalarial activity of crinane-type alkaloids is summarized in the following table based on 2 studies. In line with the results of those studies we can say that 6-hydroxyhaemanthamine is the most potent antiplasmodial agent[91,92].

Table 7: Antiplasmodial activity of selected crinane-type alkaloids expressed as IC_{50} (µg/ml)[91,92]

	P. falciparum **(T9.96)**	***P. falciparum*** **(K1)**	***P. falciparum*** **(D10)**	***P. falciparum*** **(FAC8)**
Crinine	2.110	1.650		
Haemanthamine	0.703	0.433		
6-Hydroxyhaemanthamine	0.348	0.352		
3-Epihydroxybulbispermine	1.139	0.553		
Crinamine			2.8	3.4
Hamayne			15.6	18.2

Evidente et al. in 2004 published screening of antibacterial and antifungal properties of 6 alkaloids from bulbs of *A. belladonna* L. Vitattine showed an activity against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. 11-hydroxyvitattine was equally active, but unlike vitattine was not active against *E.coli*[75]. During phytochemical study of *Crinum jagus* in 1992 antimicrobial tests of isolated compounds had been conducted. None of the compounds showed any antifungal activity (*Trichophyton rnentagyrophytes* and *Aspergillus flavus*). Only crinamine exhibited the antibacterial activity with the MIC value

set at 10 μg/ml. Acetates of hamayne and crinamine demonstrated a similar antimicrobial activity (MIC = 10 μg/ml)[93]. In 2004 Szlávik et al. also confirmed the conclusion that haemanthamine inhibits HIV-1 replication *in vitro*[94].

3.3.) Ambelline

Ambelline is a colourless crystalline substance with lozenge-shaped crystals[95]. It belongs to crinine structure type of AA and that means β spatial orientation of the 5,10*b*-ethane bridge (**Fig.15**).

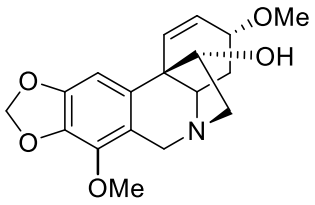


Figure 15: Chemical structure of ambelline

Botanical distribution of ambelline is in genera *Amaryllis*, *Ammocharis*, *Brunsvigia*, *Crinum*, *Hippeastrum*, *Nerine* and *Zephyranthes* and so on. Namely it is e.g. *Zephyranthes grandiflora*, *Amaryllis belladonna*, *Nerine bowdenii*, *Hippeastrum brachyandrum*, *Crinum laurentii*, *Ammocharis coranica*[96,97].

Chemical name for ambelline is (3α,11*S*)-1,2-Didehydro-3,7-dimethoxycrinan-11-ol. Molecular formula is $C_{18}H_{21}NO_5$ and the molecular weight 331,4 g/mol.

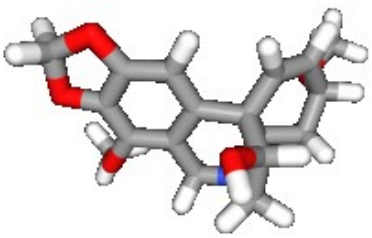


Figure 16: 3D model of ambelline[98]

3.3.1.) Biological activity of ambelline

3.3.1.1.) Anticancer activity

Cytotoxicity of ambelline has been tested across different cancer cell lines, but no interesting results appeared.

In 1976 the effect of several alkaloids on HeLa cell growth was investigated. Ambelline was one of them, but no inhibitory effect on HeLa cell growth was observed at concentrations up to 0.4 mM. α-crinane haemanthamine was the most active compound[65].

According to Petit et al., *Amaryllis belladonna* contains 2 crinine-type alkaloids – ambelline and undulatine. Of these, only ambelline was active in the murine P-388 lymphocytic leukemia assay (ED_{50} 1.6 μg/mL) indicating that the compound may be more amenable towards animal models of study as it was previously shown to be inactive in human adenocarcinoma (HeLa) cells[65,80,99].

In 1995 the cytotoxic evaluation arena was expanded via a study of 25 diverse alkaloid structures of the Amaryllidaceae against two human cancer cell lines (MOLT-4 and HepG2) and one murine non-tumoral cell line (LMTK). Interestingly, α-crinanes could be distinguished from their β-congeners based on their higher activities against the above tumors. Ambelline exhibited low inhibitory interaction (ED_{50} > 50 μg/mL) with both MOLT-4 and HepG2 cells[37].

In 1998 the activity of ambelline against BL-6 mouse melanoma cells was investigated. Even in this case ambelline was notably inactive (ED_{50} > 100 μg/mL)[100].

Evidente et al. evaluated 29 AA for their antiproliferative, apoptosis–inducing, and anti-invasive activities *in vitro*. Alkaloids were used in different concentrations (5 and 25μM for HeLa and Vero cell lines; 1 and 25μM for Jurkat cell line). Percentage of apoptosis in Jurkat cell lines after 24h of treatment with ambelline was at both concentrations just 4%. Percentage of cell viability after 48h of treatment with ambelline was higher than 90% (HeLa 5μM and Vero 5μM, 25μM). In case of HeLa 25μM was resulting value a little bit better - 79 ± 10%. However, haemanthamine seemed to be the most active from the crinane structure type alkaloids[45].

Fifteen AA were tested for their antiproliferative activities against 6 distinct cancer cell lines by van Goietsenoven et al. Ambelline exhibited low activities against both apoptosis-

resistant (A549, OE21, U373 and SKMEL) and apoptosis-sensitive (Hs683 and B16F10) cells. On the other hand, haemanthamine and haemanthidine were markedly active in both cell-forms[54].

During the phytochemical study of *Crinum zeylanicum*, ambelline was tested against other 4 cancer cell lines - SKW-3, HL-60, HI-60/Dox and MDA-MB-231. In all cases the IC_{50} for ambelline was greater than 100μM[101].

In 2012 appeared more detailed studies of crinane-type alkaloids. One of them is about bulbispermine. During this study was ambelline also tested and researchers ended with expected results. Ambelline's IC_{50} for all tested cell lines (T98G, U373, Hs683, HeLa, U87) were > 100μM[102].

Already in 2006 the study focused on selective apoptosis-inducing activity was taken. A group of crinane alkaloids was tested and SARs (structure-activity relationship) were described. Alkaloids ambelline and crinine possessing the β-5,10*b*-ethano bridge show just a little activity. The double bond in most active alkaloids (crinamine and haemanthamine) contributes a conformational role and that clearly indicates the requirement of the α-5,10*b*-ethano bridge as an important pharmacophoric element[103].

Nair et al. performed phytochemical research of *Boophone haemanthoides*. From their results is obvious that β-crinane compounds such as ambelline, all of which possess a C-1/C-2 double bond as well as polar substitution at C-3α, were inactive against both normal and cancerous cells – CEM, MCF7 and HeLa (IC_{50} > 50 μM)[85].

In 2017 our department participated in the testing of the antiproliferative effects of 22 alkaloids on 17 cancer cell lines. One of those alkaloids was also ambelline. Ambelline did not show any significant values. Following table (**Tab.8**) summarizes results of 3 most active alkaloids, ambelline and doxorubicine as a used standard[87].

Table 8: Cell proliferation after selected alkaloids treatment expressed as percentages of proliferation of untreated control cells[87]

	Ambelline	Haemanthamine	Haemanthidine	Lycorine	Doxorubicine
HL-60	111	4	4	2	2
Jurkat	99	18	23	5	4
MOLT-4	93	6	11	9	2
A549	95	33	38	27	46
H1299	91	20	28	18	28
COLO-201	97	12	14	14	46
HT-29	83	5	7	5	16
SW-480	92	21	25	20	29
AGS	100	21	33	22	5
PANC-1	94	37	38	36	63
A2780	96	38	52	39	45
HeLa	92	16l′	25	15	30
BT-549	92	17	27	25	14
MCF-7	98	17	24	27	52
MDA-MB-231	92	27	37	27	48
SAOS	96	34	35	27	23
NHDF	99	32	36	34	48

3.3.1.2.) Inhibitory activity of AChE, BuChE

This type of biological activity is tested because of its connection to AD. The main theories of AD are amyloid and cholinergic theories. Cholinergic theory is based on the lack of acetylcholine (ACh) and thus reduces cholinergic neurotransmission. It is synthesized in cholinergic neurons by cholin-acetyltransferase and decomposed to choline and acetate by cholinesterases. Cholinesterase is represented by AChE and BuChE in neurons and close to synapses. Levels of AChE and BuChE, by which ACh is decomposed, are increased during AD and thus is ACh more rapidly degraded. This is the reason why inhibitors of cholinesterases

are the widest group of compounds which are expected to provide a promising cognitive effect in the treatment of AD[104].

Phytochemical study of *Nerine bowdenii* W.Watson, which took place at our department, provided 22 isoquinoline alkaloids and among other things, testing of AChE and BuChE inhibition. Ambelline was also part of this testing with following results – the inhibitory activity of HuAChE IC_{50} = 169.2 ± 7.4 μM and HuBuChE IC_{50} = 985.4 ± 25.6 μM. Galanthamine was used as a standard with values for HuAChE IC_{50} = 1.7 ± 0.1 μM and HuBuChE IC_{50} = 42.3 ± 1.3 μM[25].

3.3.1.3.) Inhibitory activity of GSK-3β

Glycogen synthase kinase may be one of the most significant kinases from all because it deals with over 100 substrates in most of cells. GSK3 refers to two paralogs that are commonly referred to as isoforms, GSK-3α and GSK-3β[105]. GSK-3β is a ubiquitous pleiotropic serine/threonine kinase and one of its roles in human organism is the participation in the pathophysiology of AD. One of the characteristics of AD is the presence of neurofibrillary tangles (NFTs) that are formed by hyperphosphorylation of τ-protein. This hyperphosphorylation is dependent on GSK-3β and cyclin-dependent kinase 5 (CDK5)[106]. GSK-3β may also be involved in the formulation of amyloid β-protein, which is another important marker of AD. Aggregates of this amyloid in the brain are responsible for dysfunction of neurons, inflammation and oxidative stress[107].

Among other things, GSK-3β seems to have a role in cancer resistant to treatment, in contributing to neoplastic transformation and in exertion pro-proliferative effects in solid tumors[108,109,110].

Hulcová et al. recently published an article evaluating the inhibitory activity of GSK-3β for 28 AA. The following table shows the results of the alkaloids from crinane structural type of AA (**Tab.9**)[111].

Table 9: Screening of AA (50 μM) for their potency to inhibit GSK-3β[111]

structural type	alkaloid	% of inhibition
Haemanthamine	Epimaritidine	45.2 ± 1.1
	Haemanthamine	52.4 ± 0.1
	Haemanthidine	33.0 ± 2.2
	Hamayne	33.9 ± 0.1
	Seco-isopowellaminone	38.5 ± 0.8
Crinine	Ambelline	38.0 ± 0.8
	Crinine	39.6 ± 5.4
	Undulatine	43.3 ± 4.0
	Crinamidine	32.1 ± 7.9

3.3.1.4.) Inhibitory activity of POP

POP is a serine peptidase which digests small peptide-like hormones, neuroactive peptides, and various cellular factors. Therefore, this peptidase has been implicated in many physiological processes as well as in some psychiatric disorders[112].

In 1995 lower levels of POP were observed in people suffering from depression. In contrast, levels of POP are increased in patients with schizophrenia and mania[113]. Lower values of POP were described also in cases such as anorexia and bulimia nervosa[114]. Some evidence suggests that POP may be associated with symptomatology of AD. As early as 1997 POP inhibitors have been shown to suppress the formation of amyloid β-peptide and to prevent the deposition of these amyloid-like peptides in mouse models[115,116].

Ambeline as a POP inhibitor is not significant since its IC_{50} is greater than 100 mM. The standard for comparison is berberine, which IC_{50} is 0.14 ± 0.02 mM[25].

3.3.1.5) Antimicrobial activity

Antimicrobial activity of ambelline was investigated in 2015 by Ločárek et al. at our department. 25 alkaloids and 6 alkaloidal extracts from *Narcissus canaliculatus, Narcissus bulbocodium* var. *conspicuous, Galanthus elwesii, Leucojum aestivum, Narcissus jonquilla* cv. Baby Moon, *Narcissus poeticus* var. *recurvus* were tested against selected yeasts

and Gram-positive and Gram-negative bacteria. Ambelline acted against *Candida albicans* and *Lodderomyces elongiosporus.* Results of crinane alkaloids are summarized in the following table (**Tab.10**)[117].

Table 10: The in vitro antimicrobial effect of crinane alkaloids[117]

	MIC_{80}/MFC (μg/mL)												MIC_{80} (μg/mL)	
	***C. a.*[A]**		***C. a. 1*[B]**		***C. a. 2*[B]**		***C. d.*[B]**		***C. g.*[B]**		***L. e.*[B]**		***E. c.*[A]**	***S. a.*[A]**
Haemanthamine	-	-	512	-	-	-	512	-	-	-	256	-	-	-
Haemanthidine	-	-	-	-	-	-	512	-	-	-	512	-	-	-
Hamayne	-	-	-	-	-	-	512	-	-	-	512	-	-	-
Ambelline	-	-	512	-	512	-	512	-	-	-	512	-	-	-
Crinine	-	-	512	-	512	-	512	-	512	-	256	512	-	-
Undulatine	-	-	512	-	-	-	512	-	512	-	512	512	-	-
1-*O*-Acetylbulbisine	-	-	-	-	-	-	512	-	-	-	512	-	-	-
Buphanamine	-	-	-	-	-	-	512	-	-	-	512	-	-	-
Buphanidrine	-	-	-	-	-	-	512	-	-	-	512	-	-	-
Buphanisine	512	-	512	-	512	-	512	-	512	-	256	-	1024	1024

MIC80 – minimum inhibitory concentration, MFC – minimum fungicidal concentration, NA – not active (in yeasts: >1024 μg/mL for extracts, > 512 μg/mL for pure compounds; in bacteria > 2048 μg/mL for both), nd – not determined, *C.a.* – *Candida albicans* (1- oral cavity, 2 – lower respiratory tract) , *C.d.* – *Candida dubliniensis*, *C.g.* – *Candida glabrata*, *L.e.* – *Lodderomyces elongiosporus*, *E.c.* – *Escherichia coli*, *S.a.* – *Staphylococcus aureus*, a – type strain, b – clinical isolate.

3.3.1.6) Antiprotozoal activity

In 1998 ambelline and other 7 alkaloids were isolated from *Crinum stuhlmannii* Baker. All of 8 isolated alkaloids were tested against *Entamoeba hystolytica* (strain HK9 and trophozoites stage), *Trypanosoma cruzi*, *Leishmania donovani* and *Plasmodium falciparum*. Lycorine had an IC_{50} of 0.23 μg/ml while crinamine had an IC_{50} of 0.53 μg/ml in case of *Entamoeba hystolytica*. Ambelline showed no activity in all cases[118].

Four known alkaloids were isolated from bulbs of *Brunsvigia littoralis*. The follow-up study of those alkaloids consisted of antimalarial screening. This activity was measured against the chloroquine-sensitive (D10) and chloroquine-resistant (FAC8) strains of cultured *Plasmodium falciparum*. Only lycorine and its diacetate showed moderate activity. IC_{50} for ambelline was higher than 20 μg/ml in both strains. Chloroquine as a standard has IC_{50} 0,01 (strain D10) and 0.09 (strain FAC8) μg/ml[100].

In 2018 an article was published that, however, attributed ambelline to antimalarial effects. It was the second most effective compound of all 6 tested substances (**Tab.11**). The activity for ambelline is in this instance noteworthy since it was previously shown to be ineffective against both resistant (FAC8) and sensitive (NF54 and D10) strains of *Plasmodium falciparum*[5].

Table 11: Antiplasmodial activities against P.falciparum Dd2 expressed as IC_{50} (reposted)[5]

	IC_{50} (µM)
1,4-Dihydroxy-3-methoxy Powellan	37 ± 3
Distichamine	> 50.0
11-*O*-Acetylambelline	35 ± 1.0
Ambelline	7.3 ± 0.3
Acetylcaranine	3.5 ± 0.3
Hippadine	na

na = not active

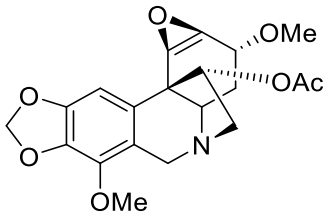
3.4.) Derivatives of ambelline

Whereas ambelline itself is not particularly biologically active, preparation of its synthetic derivatives did not appear in any way significantly interesting. In the literature 3 derivatives of ambelline are most commonly found: 11-*O*-acetylambelline, 1,2-β-epoxyambelline and 11-*O*-acetyl-1,2-β-epoxyambelline. These substances are either of natural origin or could be prepared synthetically[25,119,120].

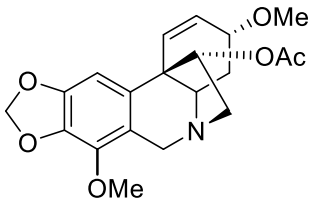
11-*O*-Acetylambelline is the most investigated for a biological activity. These results are described in the next subchapter. Of the other derivatives, only 11-*O*-acetyl-1,2-β-epoxyambelline is worth mentioning. In 1985 its immunoregulatory effects, which are compounded when combined with 11-*O*-acetylambelline in a 1:1 ratio, were described[121].

In 2005 Nair et al. isolated and described filifoline for the first time. Chemically it is 11-*O*-nicotinylambelline and during its phytochemical research it did not show any significant biological activity. Filifoline was not cytotoxic to myoblast (L6) cells and exhibited no anti-protozoal activity in an in vitro screen against four different parasitic protozoa -

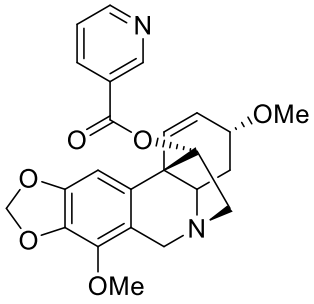
Trypanosoma brucei rhodesiense, *Trypanosoma cruzi*, *Leishmania donovani* and *Plasmodium falciparum*[12].



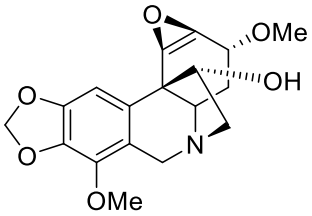
11-*O*-Acetyl-1,2-β-epoxyambelline



11-*O*-Acetylambelline



Filifoline



1,2-β-Epoxyambelline

Figure 17: Chemical structures of ambelline derivatives

3.4.1.) 11-*O*-Acetylambelline

11-*O*-Acetylambelline occurs most frequently in plant sources alongside with ambelline. From plant sources we could mention *Crinum latifolium*, *Crinum zeylanicum*, *Nerine bowdenii*, *Nerine filifolia*[12,25,101,119]. This derivative was also synthesised by Campbell et al.[100]. Of all the above-mentioned derivatives, for 11-*O*-acetylambelline there are the most available data relating to biological activity testing. Vaněčková et al. isolated 11-*O*-acetylambelline from *Nerine bowdenii* W. Watson during its phytochemical study. All of the isolated alkaloids were tested against cholinesterases and POP. IC_{50} for both human cholinesterases were higher than 1000 μM. IC_{50} for inhibitory activity of POP could not be correctly determined, but the published result is 0.79 mM[25]. We could say that from the viewpoint of AD's treatment, this derivative does not appear to be an active substance for any further research.

During that study 11-*O*-acetylambellin was also tested against 2 cancer cell lines (Caco-2, HT-29) and against normal cell lines to determine toxicity to non-cancerous cells. This

ambelline derivative is not toxic to any of the tested lines[25]. More comprehensive anticancer testing was conducted in 2017 when 11-*O*-acetylambelline was tested against 17 cell lines in a multiple alkaloid study. However, in almost all cases, the ambelline derivative was even less active than ambelline itself[87].

Table 12: Cell proliferation after ambelline and its acetylderivative treatment expressed as percentages of proliferation of untreated control cells[87]

	Ambelline	**11-*O*-Acetylambelline**
HL-60	111	98
Jurkat	99	115
MOLT-4	93	113
A549	95	106
H1299	91	94
COLO-201	97	98
HT-29	83	102
SW-480	92	105
AGS	100	103
PANC-1	94	103
A2780	96	95
HeLa	92	100
BT-549	92	88
MCF-7	98	110
MDA-MB-231	92	96
SAOS	96	105
NHDF	99	104

11-*O*-Acetylambelline has been tested several times for its antimalarial effects. In 1998 synthetic derivative underwent testing of an antimalarial activity with 2 strains of *Plasmodium falciparum.* Cytotoxicity against BL6 mouse melanoma cells was also the part of this study. Results for *P.falciparum* strain D10 and FAC8 were not interesting. IC_{50} for both

was higher than 20 μg/ml[100]. 20 years later it was isolated from *Amaryllis belladonna* Steud. This molecule, together with other 5 isolated alkaloids, was tested against chloroquine-resistant *P.falciparum* Dd2 strain. The most active compounds were acetylcaranine with IC_{50} 3.5 ± 0.3 μM and ambelline with IC_{50} 7.3 ± 0.3 μM. 11-*O*-acetylambelline had IC_{50} 35 ± 1 μM. According to that we could suggest, that oxy-substitution at C-11 of ambelline slightly decreased the inhibitory activity[5].

4) EXPERIMENTAL SECTION

4.1.) Chemicals, materials and methods

4.1.1.) Ambelline

Ambelline was isolated from *Nerine bowdenii* W. Watson at the Deparment of Pharmaceutical Botany of Pharmacy Faculty in Hradec Králové. More details about this work is in the article by Vaněčková et al.[25].

4.1.2.) Solvents, chemicals, detection reagents

- Acetic anhydride, ≥ 99% (Acros)
- Benzoyl chloride, ≥ 99% (Acros)
- Cyclohexane, p.a. (cHx) (Penta)
- Diethylamine, p.a. (Et_2NH) (Penta)
- Dimethylaminopyridine, p.a. (DMAP) (Penta)
- Dragendorff's reagent (prepared in the laboratory)
- Ethanol 95%, p.a. (EtOH) (Penta)
- Ethyl acetate, p.a. (EtOAc) (Penta)
- Chloroform, p.a. ($CHCl_3$) (Penta)
- 2-Chloro-4-nitrobenzoyl chloride, ≥ 99% (Acros)
- Isobutyric anhydride, ≥ 99% (Acros)
- 3-Methoxybenzoyl chloride, ≥ 99% (Acros)
- 4-Methyl-3-nitrobenzoyl chloride, ≥ 99% (Acros)
- 3-Nitrobenzoyl chloride, ≥ 99% (Acros)
- 4-Nitrobenzoyl chloride, ≥ 99% (Acros)
- Propionic anhydride, ≥ 99% (Acros)
- Pyridine, p.a. (Penta)
- Toluene, p.a. (To) (Penta)
- p-Toluoyl chloride, ≥ 99% (Acros)
- Valeric anhydride, ≥ 99% (Acros)

4.1.3.) Material for preparative and analytical thin-layer chromatography (TLC)

- Kiesel SiO_2 F_{254}, Merck, 20 cm × 20 cm, Aluminum TLC plate - silica gel coated with flourescent indicator F_{254} for thin-layer chromatography applications, layer 0.2 mm

4.1.4.) Mobile phases for preparative and analytical TLC

- S_1 – To:Et_2NH (9:1)
- S_2 - To:cHx:Et_2NH (60:40:5)
- S_3 – To:Et_2NH (95:5)

4.1.5.) Structural analysis of prepared derivatives

4.1.5.1.) GC-MS analysis

GC-MS analysis was used to determine the molecular weight of synthesised compounds. A gas chromatograph in conjunction with the mass spectrometer Agilent Technologies 7890 A GC 5975 inert MSD operating in EI mode at 70 eV was used for measurement. A DB-5 MS column (30 m × 0.25 mm × 0.25 µm) was used for separation. The temperature range was 100-300°C, with a temperature increase of 15°C per minute between 100-180°C. Upon reaching 180°C the temperature was maintained for one minute and then increased to 300°C at a rate of 5°C per minute and then maintained at that value for 40 minutes. The sample injection temperature was 280°C with a carrier gas (helium) flow rate of 0.8 ml per minute. Detection range m/35–600. The concentration of the injected sample in methanol was mostly 1 mg/ml. In some cases, injections with a lower concentration of 3µg/ml were required to show the derivative in the spectrum. Obtained known compounds were compared with data in the literature, structurally unwritten compounds were characterized for the first time.

4.1.5.2.) ESI-MS analysis

Substances difficult to ionize under EI spectra measurements were analysed to obtain Mr values by ESI ionization on LC/MS Thermo Finningan LCQDuo, ion trap, electrospray ionization in positive mode (ESI +). MS/MS spectra were measured at a collision energy of 40 eV. The substances for the experiments were dissolved in methanol (1 mg/ml).

4.1.5.3.) High resolution mass spectrometry (ESI-HRMS analysis)

The method was used to determine the exact value of newly prepared, not yet described compounds in the literature. Mass spectra were measured with a high-resolution UHPLC Waters Acquity I-Class with electrospray ionization in a positive mode and a quadrupole combined with a Waters Synapt G7-Si flight time analyser. Samples were measured in MeCN solution. Subsequently, the theoretical m/s was compared with the experimentally determined one.

4.1.5.4.) NMR analysis

NMR spectra were measured in $CDCl_3$ or CD_3OD solutions at 25°C on a Varian Iniova 500 spectrometer operating at 499.87 MHz for ^1H cores and 125.70 MHz for ^{13}C cores. OneNMR probe, a broadband dual-channel temperature-controlled gradient probe, was used to irradiate and detect the signal. Chemical shifts were measured as δ pars per million (ppm) and were inversely related to tetramethylsilane (TMS) as a standard by the residual solvent signal. The chemical shift values for $CDCl_3$ are ^1H δ = 7.26 ppm and ^{13}C δ = 77.0 ppm, for CD_3OD they are ^1H δ = 3.30 ppm and ^{13}C δ = 49.0 ppm. The measurements obtained are presented in the following order: chemical shift (δ), integrated intensity ^1H NMR spectra, multiplicity (s: singlet, d: doublet, t: triplet, q: quartet, dd: doublet of doublets, m: multiplet, bs: wide singlet) and integration constant (Hz).

4.1.5.5.) Optical rotation

Optical rotation was measured in alkaloid solutions at 20°C on an ADP 220 BS automatic polarimeter in methanol or ethanol. Then the specific rotation was calculated according to the formula: $[\alpha]_D^t = \frac{100 \times \alpha}{c \times l}$.

Explanatory notes:

t = measurement temperature,

D = line of sodium light → 589.3 nm,

α = measured rotation (°C),

c = concentration of measured alkaloid (g×100 ml^{-1}),

l = cell length (dm).

4.1.6.) Methods for screening biological activities of prepared compounds

4.1.6.1.) Determination of anticancer activity of prepared derivatives

This determination of cytotoxicity was possible thanks to cooperation with the Department of Medical Biochemistry, Faculty of Medicine in Hradec Králové, Charles University. It was performed by RNDr. Radim Havelek, Ph.D. and Mgr. Martina Seifertová. The cancer cell lines used during this study were: Jurkat, MOLT-4, A549, HT-29, PANC-1, A2780, HeLa, MCF-7, SAOS-2, MRC-5. xCELLigence system as described in the literature, was used during the assay[83].

4.1.6.2.) Determination of AChE and BuChE inhibitory activity of prepared derivatives

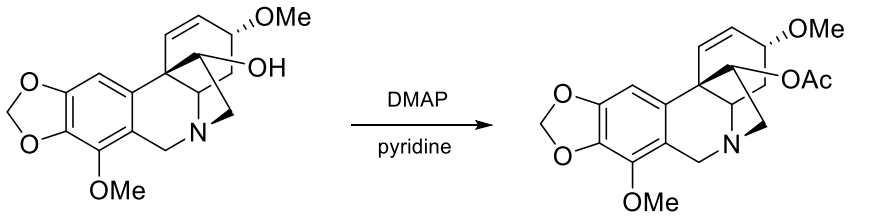
The inhibitory activity of those enzymes was measured at the Department of Pharmaceutical Botany, Faculty of Pharmacy in Hradec Králové. It was the Ellman's method and the measurement was performed by prof. RNDr. Lubomír Opletal, CSc. and PharmDr. Daniela Hulcová, Ph.D. A detailed description of this method is given in the following literature[122].

4.1.6.3.) Determination of GSK-3β inhibitory activity of prepared derivatives

Thanks to the Erasmus+ program I was allowed travel to Italy where I was part of the research team at Department for Life Quality Studies in Rimini. During 4 months I was working under the leadership of prof. Vincenza Andrisano, I learned the method and gradually determined the activity of the prepared derivatives. We worked according to the method that is summarized in the work of Baki et al.[123].

4.2.) Preparation of synthetic derivatives of ambelline and their structural identification

4.2.1.) Preparation of 11-*O*-acetylambelline (LC-85)



Chemical Formula: $C_{18}H_{21}NO_5$
Exact Mass: 331,14
Molecular Weight: 331,37

Chemical Formula: $C_{20}H_{23}NO_6$
Exact Mass: 373,15
Molecular Weight: 373,41

Figure 18: Preparation scheme of 11-*O*-acetylambelline

50 mg of ambelline (0.151 mmol) was dissolved in 2 ml of dry pyridine. After dissolution 800 µl of acetic anhydride was added. After that, a catalytic amount of DMAP was added to the reaction mixture. The mixture was stirred at the room temperature and after 2 hours the reaction was checked by TLC with developing mobile phase S_1. Subsequently, the mixture was evaporated to dryness. The residue was dissolved in $CHCl_3$ and applied gradually to the preparation plates. The preparation plates were developed in a mixture of S_1 and then the zone that represents our product was separated. The mixture from the preparation plates was washed with EtOH:$CHCl_3$. The obtained amount of 11-*O*-acetylambelline was 52.4 mg and it was a white powder. The yield of this reaction was 93%.

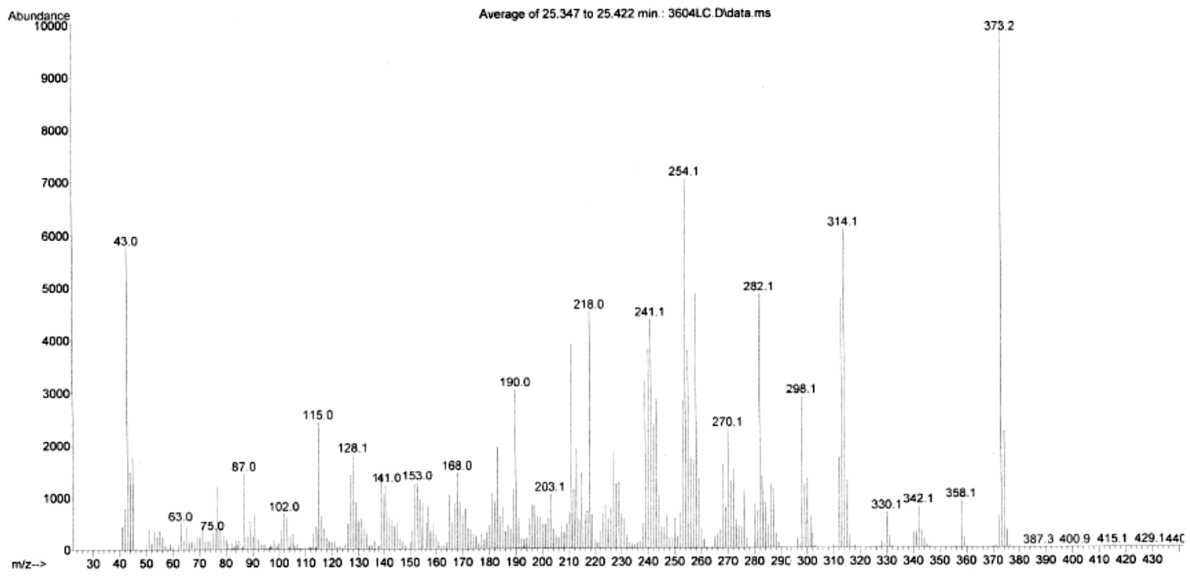
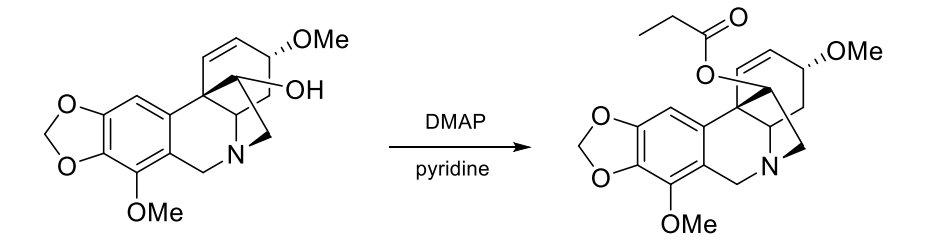


Figure 19: GC-MS analysis of 11-*O*-Acetylambelline

NMR study: in accordance with published data[124]

Optical rotation: in accordance with published data[124]

4.2.2.) Preparation of 11-*O*-propionylambelline (LC-86)



Chemical Formula: $C_{18}H_{21}NO_5$
Exact Mass: 331,14
Molecular Weight: 331,37

Chemical Formula: $C_{21}H_{25}NO_6$
Exact Mass: 387,17
Molecular Weight: 387,43

Figure 20: Preparation scheme of 11-*O*-propionylambelline

50 mg of ambelline (0.151 mmol) was dissolved in 2 ml of dry pyridine. After dissolution 100 µl of propic anhydride was added. After that, a catalytic amount of DMAP was added to the reaction mixture. The mixture was stirred at the room temperature and after 2 hours the reaction was checked by TLC with developing mobile phase S_1.

Subsequently, the mixture was evaporated to dryness. The residue was dissolved in $CHCl_3$ and applied gradually to the preparation plates. The preparation plates were developed in a mixture of S_1 and then the zone that represents our product was separated. The mixture from the preparation plates was washed with EtOH:$CHCl_3$. 55.7 mg of 11-*O*-propionylambelline was obtained as a white powder. The yield of this reaction was 95%.

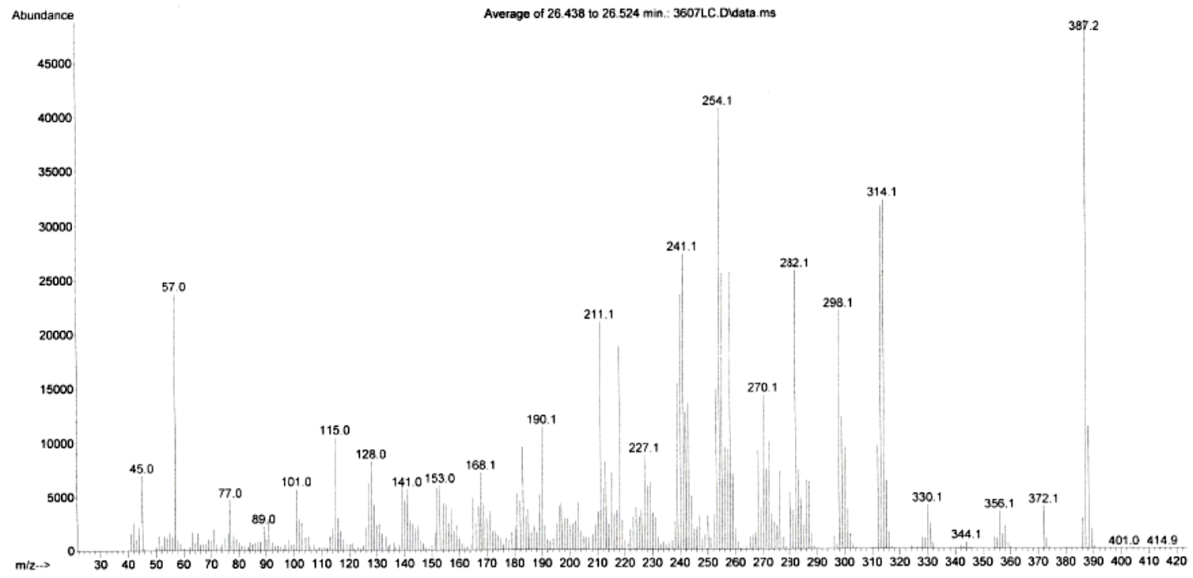


Figure 21: GC-MS of 11-*O*-propionylambelline

NMR study:

^{1}H NMR (500MHz, $CDCl_3$) δ: 6.55 (1H, d, *J* = 10.0 Hz), 6.43 (1H, s), 6.04–6.00 (1H, m), 5.84 (1H, overlapped, d, *J* = 14.2 Hz), 5.83 (1H, overlapped, d, *J* = 14.2 Hz), 5.10 (1H, dd, *J* = 8.3 Hz, *J* = 3.9 Hz), 4.31 (1H, d, *J* = 17.6 Hz), 3.96 (3H, s), 3.86–3.82 (1H, overlapped, m), 3.85 (1H, overlapped, d, *J* = 17.6 Hz), 3.73 (1H, dd, *J* = 14.2 Hz, *J* = 8.3 Hz), 3.41 (1H, dd, *J* = 13.5 Hz, *J* = 3.9 Hz), 3.33 (3H, s), 2.72 (1H, ddd, *J* = 14.2 Hz, *J* = 3.9 Hz, *J* = 1.9 Hz), 2.14–1.98 (3H, m), 1.71 (1H, td, *J* = 13.5 Hz, *J* = 3.9 Hz), 0.92 (3H, t, *J* = 7.6 Hz)

^{13}C NMR (125MHz, $CDCl_3$) δ: 174.2, 147.6, 140.5, 133.8, 133.6, 131.9, 126.0, 117.4, 100.5, 99.2, 87.1, 72.2, 63.5, 59.5, 59.0, 58.6, 56.4, 47.5, 28.8, 27.4, 8.8

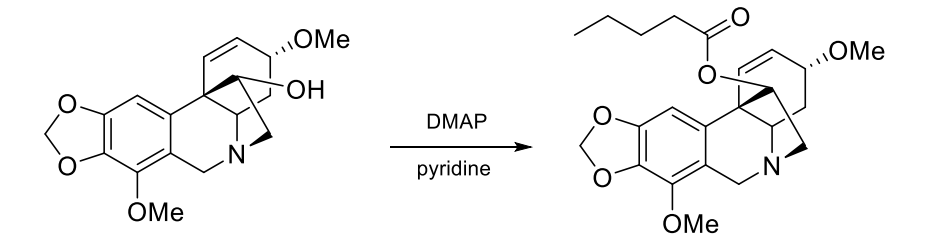
Optical rotation:

$[\alpha]^{24}_{D}$= - 20 (*c* = 0,165; $CHCl_3$)

ESI-HRMS study:

ESI-HRMS *m/z* calcd for $C_{21}H_{26}NO_6$ $[M+H]^+$ 388.1760 found 388.1757

4.2.3.) Preparation of 11-*O*-pentanoylambelline (LC-87)



Chemical Formula: $C_{18}H_{21}NO_5$
Exact Mass: 331,14
Molecular Weight: 331,37

Chemical Formula: $C_{23}H_{29}NO_6$
Exact Mass: 415,20
Molecular Weight: 415,49

Figure 22: Preparation scheme of 11-*O*-pentanoylambelline

50 mg of ambelline (0.151 mmol) was dissolved in 2 ml of dry pyridine. After dissolution 100 µl of valeric anhydride was added. After that, a catalytic amount of DMAP was added to the reaction mixture. The mixture was stirred at the room temperature and after 24 hours the reaction was checked by TLC with developing mobile phase S_1. Subsequently, the mixture was evaporated to dryness. The residue was dissolved in $CHCl_3$ and applied gradually to the preparation plates. The preparation plates were developed in a mixture of S_1. The preparation plates were developed in a mixture of S_1 and then the zone that represents our product was separated. The mixture from the preparation plates was washed with EtOH:$CHCl_3$. 50.5 mg of 11-*O*-pentanoylambelline was obtained as a pale yellow oil. The yield of this reaction was 80%.

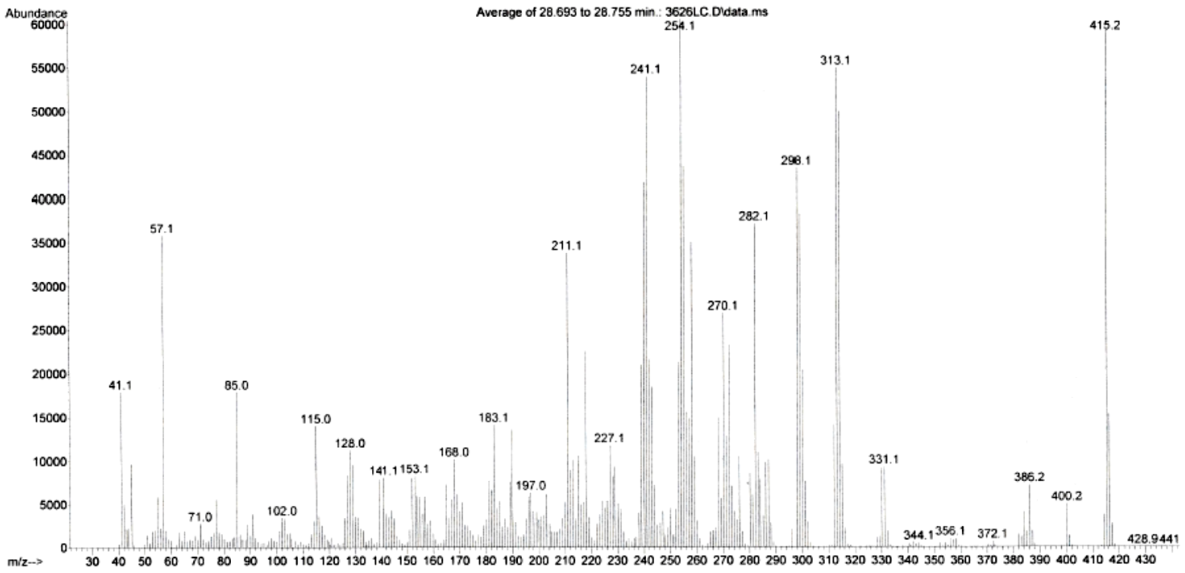


Figure 23: GC-MS of 11-*O*-pentanoylambelline

NMR study:

^{1}H NMR (500MHz, $CDCl_3$) δ: 6.55 (1H, d, *J* = 10.2 Hz), 6.44 (1H, s), 6.02 (1H, dd, *J* = 10.2 Hz, *J* = 4.4 Hz), 5.84 (1H, overlapped, d, *J* = 7.3 Hz), 5.84 (1H, overlapped, d, *J* = 7.3 Hz), 5.09 (1H, dd, *J* = 8.3 Hz, *J* = 3.9 Hz), 4.31 (1H, d, *J* = 17.6 Hz), 3.97 (3H, s), 3.86–3.82 (1H, overlapped, m), 3.85 (1H, overlapped, d, *J* = 17.6 Hz), 3.75 (1H, dd, *J* = 14.2 Hz, *J* = 8.3 Hz), 3.42 (1H, dd, *J* = 13.7 Hz, *J* = 3.9 Hz), 3.33 (3H, s), 2.71 (1H, ddd, *J* = 14.2 Hz, *J* = 3.9 Hz, *J* = 1.9 Hz), 2.14–1.98 (3H, m), 1.71 (1H, td, *J* = 13.7 Hz, *J* = 3.9 Hz), 1.39–1.31(2H, m), 1.25–1.15 (2H, m), 0.83 (3H, t, *J* = 7.3 Hz).

^{13}C NMR (125MHz, $CDCl_3$) δ: 173.5, 147.7, 140.5, 133.8, 133.6, 131.8, 126.1, 117.3, 100.5, 99.3, 87.1, 72.2, 63.4, 59.6, 59.1, 58.6, 56.5, 47.5, 33.8, 28.7, 26.8, 22.1, 13.6.

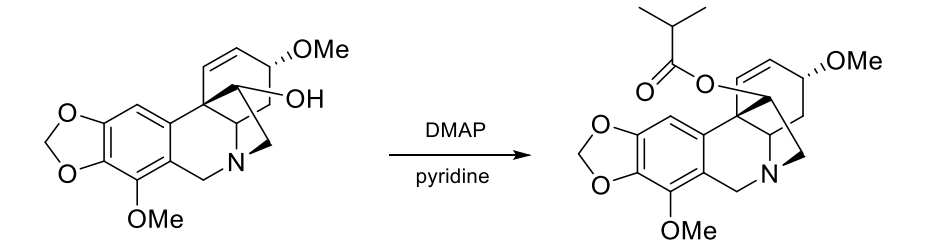
Optical rotation:

$[\alpha]^{24}_{D}$= - 44.9 (*c* = 0.241; $CHCl_3$)

ESI-HRMS study:

ESI-HRMS *m/z* calcd for $C_{23}H_{30}NO_6$ $[M+H]^+$ 416.2073 found 416.2071

4.2.4.) Preparation of 11-*O*-isobutanoylambelline (LC-88)



Chemical Formula: $C_{18}H_{21}NO_5$
Exact Mass: 331,14
Molecular Weight: 331,37

Chemical Formula: $C_{22}H_{27}NO_6$
Exact Mass: 401,18
Molecular Weight: 401,46

Figure 24: Preparation scheme of 11-*O*-isobutanoylambelline

50 mg of ambelline (0.151 mmol) was dissolved in 2 ml of dry pyridine. After dissolution 100 µl of isobutyric anhydride was added. After that, a catalytic amount of DMAP was added to the reaction mixture. The mixture was stirred at the room temperature and after 2 hours the reaction was checked by TLC with developing mobile phase S_1. Subsequently, the mixture was evaporated to dryness. The residue was dissolved in $CHCl_3$ and applied gradually to the preparation plates. The preparation plates were developed in a mixture of S_1 and then the zone that represents our product was separated. The mixture from the preparation plates was washed with EtOH:$CHCl_3$. 61 mg of 11-*O*-isobutanoylambelline was obtained as a pale yellow oil. The yield of this reaction was 100%.

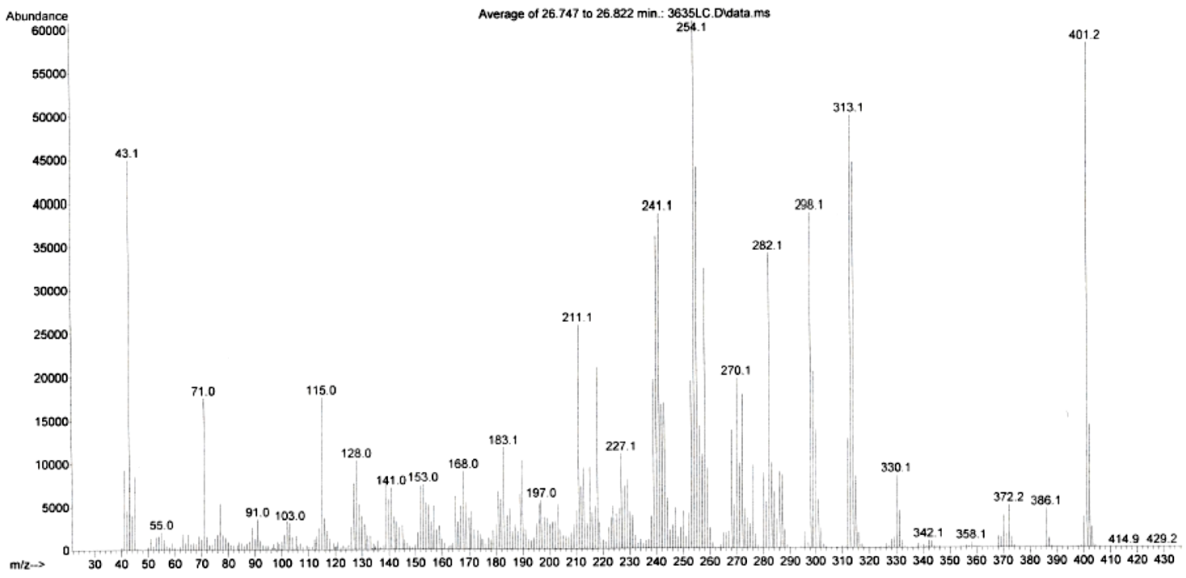


Figure 25: GC-MS of 11-*O*-isobutanoylambelline

NMR study:

^{1}H NMR (500MHz, $CDCl_3$) δ: 6.57 (1H, d, *J* = 10.2 Hz), 6.42 (1H, s), 6.03 (1H, dd, *J* = 10.2 Hz, *J* = 4.9 Hz), 5.85–5.80 (2H, m), 5.06 (1H, dd, *J* = 8.3 Hz, *J* = 3.9 Hz), 4.32 (1H, d, *J* = 17.6 Hz), 3.96 (3H, s), 3.86 (1H, overlapped, d, *J* = 17.6 Hz), 3.86–3.82 (1H, overlapped, m), 3.74 (1H, dd, *J* = 14.2 Hz, *J* = 8.3 Hz), 3.43 (1H, dd, *J* = 13.2 Hz, *J* = 4.0 Hz), 3.33 (3H, s), 2.77–2.68 (1H, m), 2.32–2.22 (1H, m), 2.11 (1H, dd, *J* = 14.2 Hz, *J* = 4.0 Hz), 1.71 (1H, td, *J* = 13.2 Hz, *J* = 4.0 Hz), 0. 93 (3H, d, *J* = 6.8 Hz), 0.89 (3H, d, *J* = 6.8 Hz).

^{13}C NMR (125MHz, $CDCl_3$) δ: 176.8, 147.6, 140.5, 133.8, 133.5, 131.9, 126.0, 117.4, 100.5, 99.5, 87.0, 72.2, 63.5, 59.7, 59.1, 58.6, 56.5, 47.6, 33.7, 28.8, 18.7, 18.5.

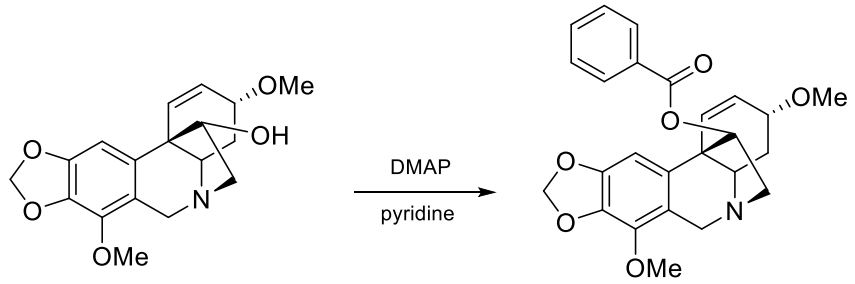
Optical rotation:

$[\alpha]^{24}_{D}$= - 9.0 (*c* = 0.134; $CHCl_3$)

ESI-HRMS study:

ESI-HRMS *m/z* calcd for $C_{22}H_{28}NO_6$ $[M+H]^+$ 402.1917 found 402.1913

4.2.5.) Preparation of 11-*O*-benzoylambelline (LC-92)



Chemical Formula: $C_{18}H_{21}NO_5$
Exact Mass: 331,14
Molecular Weight: 331,37

Chemical Formula: $C_{25}H_{25}NO_6$
Exact Mass: 435,17
Molecular Weight: 435,48

Figure 26: Preparation scheme of 11-*O*-benzoylambelline

50 mg of ambelline (0.151 mmol) was dissolved in 2 ml of dry pyridine. After dissolution 100 µl of isobutyric anhydride was added. After that, a catalytic amount of DMAP was added to the reaction mixture. The mixture was stirred at 50°C and after 2 hours the reaction was checked by TLC with developing mobile phase S_1. Confirmation of reaction progress after spraying of TLC plate with Dragendorff's reagent. Subsequently, the mixture was evaporated to dryness. The residue was dissolved in $CHCl_3$ and applied gradually to the preparation plates. The preparation plates were developed in a mixture of S_1 and then the zone that represents our product was separated. The mixture from the preparation plates was washed with EtOH:$CHCl_3$. 72.3 mg of 11-*O*-benzoylambelline was obtained as a pale yellow oil. The yield of this reaction was 100%.

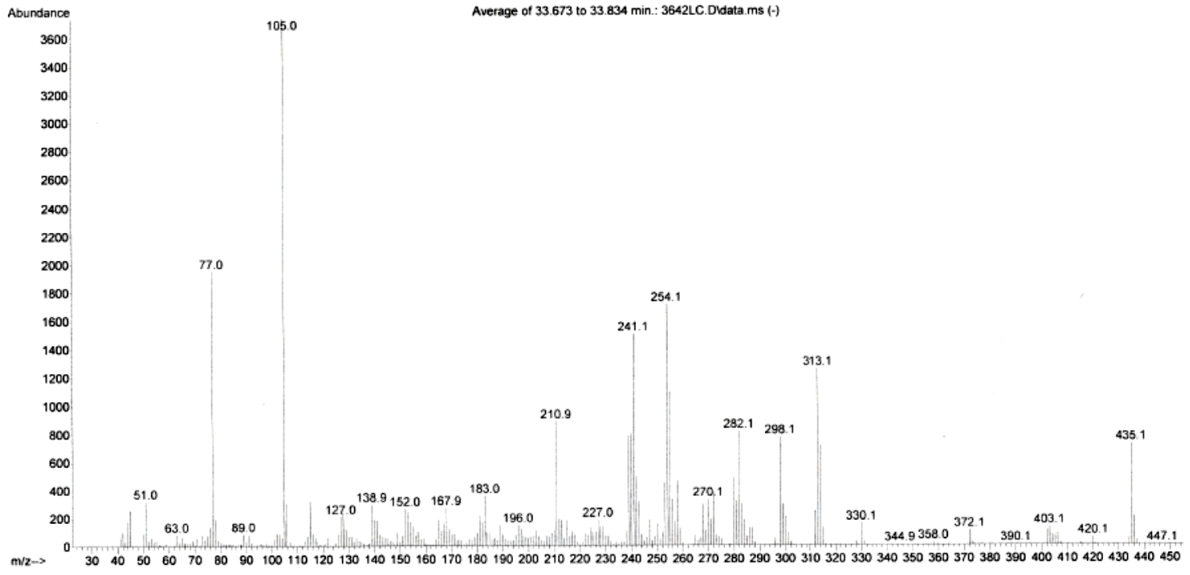


Figure 27: GC-MS of 11-*O*-benzoylambelline

NMR study:

^{1}H NMR (500MHz, $CDCl_3$) δ: 7.64 (2H, d, *J* = 7.6 Hz), 7.48 (1H, t, *J* = 7.6 Hz), 7.32 (2H, t, *J* = 7.6 Hz), 6.59 (1H, d, *J* = 10.0 Hz), 6.54 (1H, s), 6.07 (1H, dd, *J* = 10.0 Hz, *J* = 4.9 Hz), 5.84-5.81 (1H, m), 5.77–5.75 (1H, m), 5.34 (1H, dd, *J* = 7.8 Hz, *J* = 3.8 Hz), 4.38 (1H, d, *J* = 17.7 Hz), 3.98 (3H, s), 3.97–3.90 (2H, m), 3.90–3.85 (1H, m), 3.54 (1H, dd, *J* = 13.7 Hz, *J* = 3.8 Hz), 3.35 (3H, s), 2.85 (1H, dd, *J* = 13.7 Hz, *J* = 3.8 Hz), 2.22 (1H, dd, *J* = 13.7 Hz, *J* = 3.8 Hz), 1.79 (1H, td, *J* = 13.7 Hz, *J* = 3.8 Hz).

^{13}C NMR (125MHz, $CDCl_3$) δ: 166.1, 147.9, 140.6, 133.8, 133.5, 132.9, 131.1, 129.7, 129.3, 128.1, 126.6, 116.8, 100.5, 99.5, 87.3, 72.0, 63.2, 59.6, 59.1, 58.5, 56.5, 47.4, 28.5.

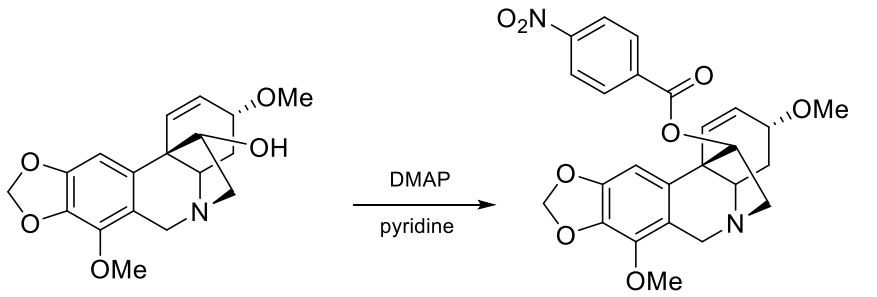
Optical rotation:

$[\alpha]^{24}_{D}$= + 4.2 (*c* = 0.263; $CHCl_3$)

ESI-HRMS study:

ESI-HRMS *m/z* calcd for $C_{25}H_{26}NO_6$ $[M+H]^+$ 436.1760 found 436.1756

4.2.6.) Preparation of 11-*O*-(4-nitrobenzoyl)ambelline (LC-96)



Chemical Formula: $C_{18}H_{21}NO_5$
Exact Mass: 331,14
Molecular Weight: 331,37

Chemical Formula: $C_{25}H_{24}N_2O_8$
Exact Mass: 480,15
Molecular Weight: 480,47

Figure 28: Preparation scheme of 11-*O*-(4-nitrobenzoyl)ambelline

50 mg of ambelline (0.151 mmol) was dissolved in 2 ml of dry pyridine. After dissolution 50 mg of 4-nitrobenzoyl chloride was added. After that, a catalytic amount of DMAP was added to the reaction mixture. The mixture was stirred at 60°C on oil bath and after 3 hours the reaction was checked by TLC with developing mobile phase S_2. Subsequently, the mixture was evaporated to dryness. The residue was dissolved in $CHCl_3$ and applied gradually to the preparation plates. The preparation plates were developed in a mixture of S_2 and then the zone that represents our product was separated. The mixture from the preparation plates was washed with EtOH:$CHCl_3$. The obtained amount of 11-*O*-(4-nitrobenzoyl)ambelline was 65.6 mg and it was a pale yellow oil. The yield of this reaction was 90%.

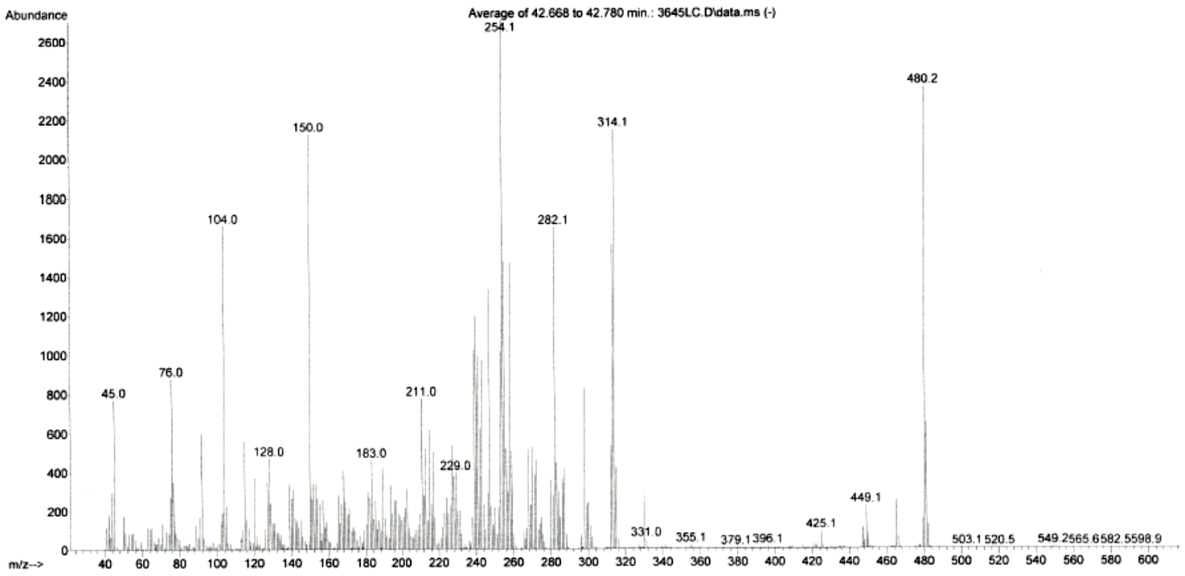


Figure 29: GC-MS of 11-*O*-(4-nitrobenzoyl)ambelline

NMR study:

^{1}H NMR (500MHz, $CDCl_3$) δ: 8.17–8.13 (2H, m, AA´BB´), 7.78–7.75 (2H, m, AA´BB´), 6.57 (1H, d, *J* = 9.8 Hz), 6.48 (1H, s), 6.09 (1H, dd, *J* = 9.8 Hz, *J* = 4.9 Hz), 5.82–5.80 (1H, m), 5.79–5.78 (1H, m), 5.36 (1H, dd, *J* = 7.8 Hz, *J* = 3.9 Hz), 4.36 (1H, d, *J* = 17.6 Hz), 3.98 (3H, s), 3.92 (1H, overlapped, d, *J* = 17.6 Hz), 3.93–3.86 (2H, overlapped, m), 3.52 (1H, dd, *J* = 13.8 Hz, *J* = 3.9 Hz), 3.35 (3H, s), 2.85 (1H, ddd, *J* = 13.8 Hz, *J* = 3.9 Hz, *J* = 1.5 Hz), 2.18 (1H, dd, *J* = 13.8 Hz, *J* = 3.9 Hz), 1.76 (1H, td, *J* = 13.8 Hz, *J* = 3.9 Hz).

^{13}C NMR (125MHz, $CDCl_3$) δ: 164.3, 150.4, 147.9, 140.7, 135.1, 133.8, 133.4, 130.9, 130.4, 126.9, 123.3, 117.1, 100.5, 99.1, 88.5, 72.0, 63.2, 59.5, 59.1, 58.6, 56.5, 47.6, 28.6

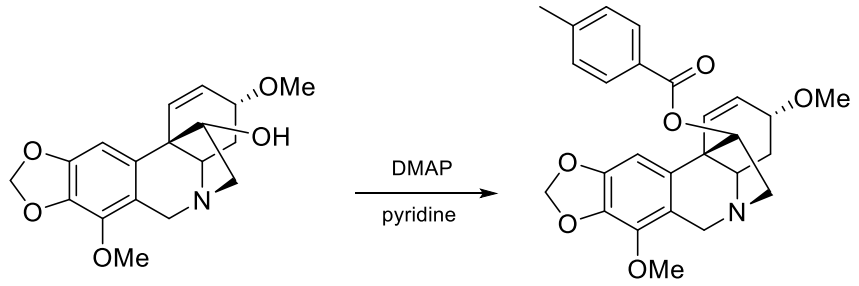
Optical rotation:

$[\alpha]^{24}_{D}$= + 39.6 (*c* = 0.100; $CHCl_3$)

ESI-HRMS study:

ESI-HRMS *m/z* calcd for $C_{25}H_{24}N_2O_8$ $[M+H]^+$ 481.1611 found 481.1618

4.2.7.) Preparation of 11-*O*-(4-methylbenzoyl)ambelline (LC-98)



Chemical Formula: $C_{18}H_{21}NO_5$
Exact Mass: 331,14
Molecular Weight: 331,37

Chemical Formula: $C_{26}H_{27}NO_6$
Exact Mass: 449,18
Molecular Weight: 449,50

Figure 30: Preparation scheme of 11-*O*-(4-methylbenzoyl)ambelline

50 mg of ambelline (0.151 mmol) was dissolved in 2 ml of dry pyridine. After dissolution 100 µl of 4-methylbenzoyl chloride was added. After that, a catalytic amount of DMAP was added to the reaction mixture. The mixture was stirred at 60°C on oil bath and after 48 hours the reaction was checked by TLC with developing mobile phase S_2. Subsequently, the mixture was evaporated to dryness. The residue was dissolved in $CHCl_3$ and applied gradually to the preparation plates. The preparation plates were developed in a mixture of S_2 and then 2 zones were separated, because it was not sure which one represented our product. Both mixtures from the preparation plates were washed with EtOH:$CHCl_3$. We analysed both samples for GC-MS. Our derivative was in the first zone. 39.5 mg of 11-*O*-(4-methylbenzoyl)ambelline was obtained as a pale yellow oil. The yield of this reaction was 58%.

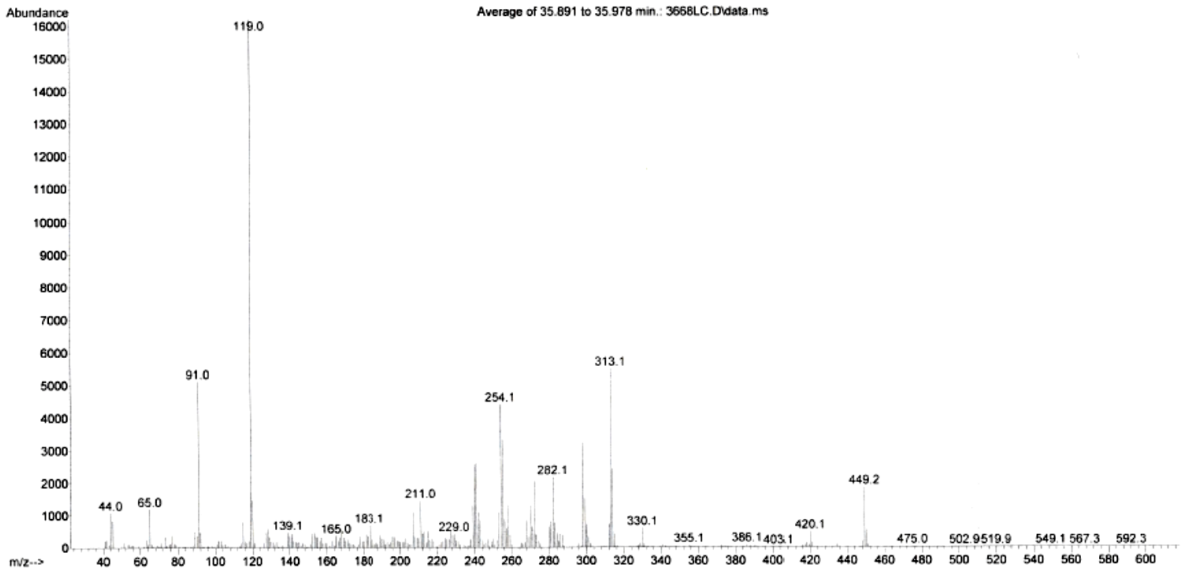


Figure 31: GC-MS of 11-*O*-(4-methylbenzoyl)ambelline

NMR study:

^{1}H NMR (500MHz, $CDCl_3$) δ: 7.56–7.51 (2H, m, AA´BB´), 7.15–7.11 (2H, m, AA´BB´), 6.60 (1H, d, *J* = 10.0 Hz), 6.55 (1H, s), 6.07 (1H, dd, *J* = 10.0 Hz, *J* = 5.4 Hz), 5.81 (1H, overlapped, d, *J* = 25.4 Hz), 5.81 (1H, overlapped, d, *J* = 25.4 Hz), 5.34 (1H, dd, *J* = 7.8 Hz, *J* = 3.9 Hz), 4.37 (1H, d, *J* = 17.1 Hz), 3.99 (3H, s), 3.95–3.86 (3H, m), 3.51 (1H, dd, *J* = 13.6 Hz, *J* = 3.9 Hz), 3.36 (3H, s), 2.88–2.78 (1H, m), 2.36 (3H, s), 2.18 (1H, dd, *J* = 13.6 Hz, *J* = 3.9 Hz), 1.79 (1H, td, *J* = 13.6 Hz, *J* = 3.9 Hz).

^{13}C NMR (125MHz, $CDCl_3$) δ: 166.3, 147.8, 143.6, 140.6, 133.8, 133.7, 131.4, 129.4, 128.8, 127.1, 126.5, 117.2, 100.4, 99.6, 87.5, 72.2, 63.2, 59.8, 59.1, 58.7, 56.5, 47.4, 28.7, 21.6.

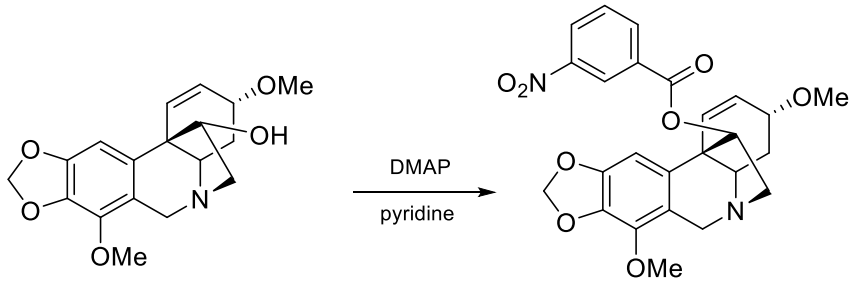
Optical rotation:

$[\alpha]^{24}_{D}$= + 18.5 (*c* = 0.275; $CHCl_3$)

ESI-HRMS study:

ESI-HRMS *m/z* calcd for $C_{26}H_{28}NO_6$ $[M+H]^+$ 450.1917 found 450.1925

4.2.8.) Preparation of 11-*O*-(3-nitrobenzoyl)ambelline (LC-100)



Chemical Formula: $C_{18}H_{21}NO_5$
Exact Mass: 331,14
Molecular Weight: 331,37

Chemical Formula: $C_{25}H_{24}N_2O_8$
Exact Mass: 480,15
Molecular Weight: 480,47

Figure 32: Preparation scheme of 11-*O*-(3-nitrobenzoyl)ambelline

50 mg of ambelline (0.151 mmol) was dissolved in 2 ml of dry pyridine. After dissolution 50 mg of 3-nitrobenzoyl chloride was added. After that, a catalytic amount of DMAP was added to the reaction mixture. The mixture was stirred at 100°C on water bath and after 24 hours the reaction was checked by TLC with developing mobile phase S_2. Subsequently, the mixture was evaporated to dryness. The residue was dissolved in $CHCl_3$ and applied gradually to the preparation plates. The preparation plates were developed in a mixture of S_2 and then the zone that represents our product was separated. The mixture from the preparation plates was washed with EtOH:$CHCl_3$. 47.6 mg of 11-*O*-(3-nitrobenzoyl)ambelline was obtained as a pale yellow oil. The yield of this reaction was 66%.

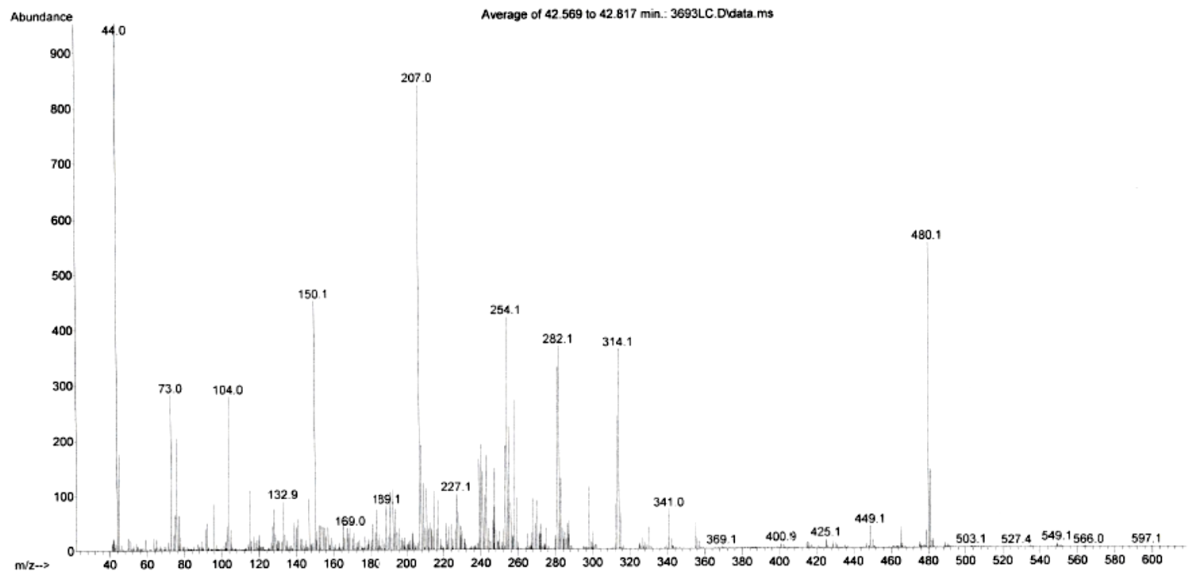


Figure 33: GC-MS of 11-*O*-(3-nitrobenzoyl)ambelline

NMR study:

^{1}H NMR (500MHz, $CDCl_3$) δ: 8.36–8.31 (1H, m), 8.21–8.18 (1H, m), 8.10–8.06 (1H, m), 7.56 (1H, t, *J* = 7.8 Hz), 6.56 (1H, d, *J* = 10.2 Hz), 6.51 (1H, s), 6.09 (1H, dd, *J* = 10.2 Hz, *J* = 5.1 Hz), 5.86 (1H, overlapped, d, *J* = 19.0 Hz), 5.86 (1H, overlapped, d, *J* = 19.0 Hz), 5.40 (1H, dd, *J* = 7.8 Hz, *J* = 3.4 Hz), 4.37 (1H, d, *J* = 17.2 Hz), 4.01 (3H, s), 3.93 (1H, overlapped, d, *J* = 17.2 Hz), 3.94–3.85 (2H, overlapped, m), 3.54 (1H, dd, *J* = 13.6 Hz, *J* = 3.9 Hz), 3.36 (3H, s), 2.84 (1H, dd, *J* = 13.6 Hz, *J* = 3.4 Hz), 2.18 (1H, dd, *J* = 13.6 Hz, *J* = 3.9 Hz), 1.76 (1H, td, *J* = 13.6 Hz, *J* = 3.9 Hz). **^{13}C NMR** (125MHz, $CDCl_3$) δ: 164.1, 148.1, 148.0, 140.7, 135.0, 134.1, 133.2, 131.6, 130.8, 126.4, 127.3, 127.0, 124.4, 117.0, 100.8, 98.9, 88.4, 72.1, 63.2, 59.8, 59.1, 58.6, 56.5, 47.5, 28.7.

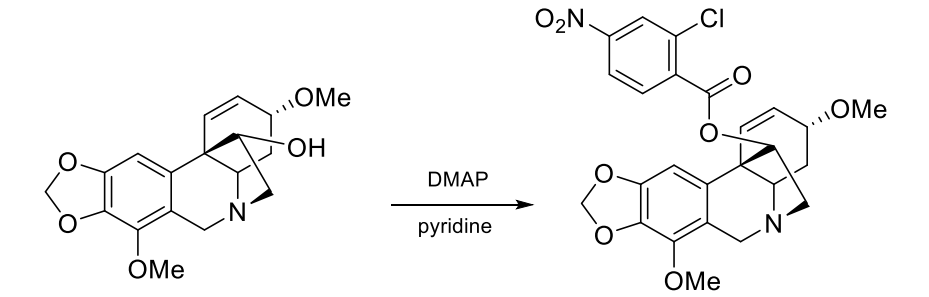
Optical rotation:

$[\alpha]^{24}_{D}$= + 41.9 (*c* = 0.105; $CHCl_3$)

ESI-HRMS study:

ESI-HRMS *m/z* calcd for $C_{25}H_{24}N_2O_8$ $[M+H]^+$ 481.1611 found 481.1613

4.2.9.) Preparation of 11-*O*-(2-chloro-4-nitrobenzoyl)ambelline (LC-110)



Chemical Formula: $C_{18}H_{21}NO_5$
Exact Mass: 331,14
Molecular Weight: 331,37

Chemical Formula: $C_{25}H_{23}ClN_2O_8$
Exact Mass: 514,11
Molecular Weight: 514,92

Figure 34: Preparation scheme of 11-*O*-(2-chloro-4-nitrobenzoyl)ambelline

50 mg of ambelline (0.151 mmol) was dissolved in 2 ml of dry pyridine. After dissolution 60 µl of 2-chloro-4-nitrobenzoyl chloride was added. After that, a catalytic amount of DMAP was added to the reaction mixture. The mixture was stirred at 80°C on oil bath and after 24 hours the reaction was checked by TLC with developing mobile phase S_2. Subsequently, the mixture was evaporated to dryness. The residue was dissolved in $CHCl_3$ and applied gradually to the preparation plates. The preparation plates were developed in a mixture of S_2 and then the zone that represents our product was separated. The mixture from the preparation plates was washed with EtOH:$CHCl_3$. 91 mg of 11-*O*-(2-chloro-4-nitrobenzoyl)ambelline was obtained. The yield of this reaction was 100%.

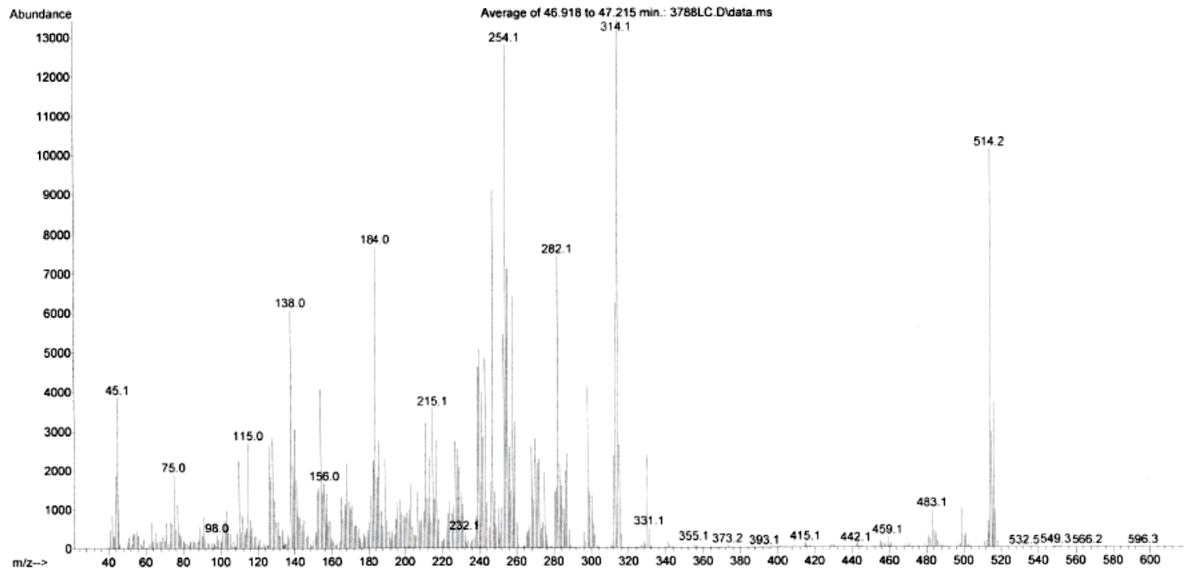


Figure 35: GC-MS of 11-*O*-(2-chloro-4-nitrobenzoyl)ambelline

NMR study:

^{1}H NMR (500MHz, CDCl3) δ: 8.21 (1H, d, J = 2.1 Hz), 7.99 (1H, dd, J = 8.3 Hz, J = 2.1 Hz), 7.29 (1H, d, J = 8.3 Hz), 6.65 (1H, d, J = 10.0 Hz), 6.47 (1H, s), 6.10 (1H, dd, J = 9.8 Hz, J = 5.4 Hz), 5.82–5.79 (2H, m), 5.34 (1H, dd, J = 7.8 Hz, J = 3.5 Hz), 4.36 (1H, d, J = 17.6 Hz), 3.98 (3H, s), 3.94–3.85 (3H, m), 3.51 (1H, dd, J = 13.6 Hz, J = 3.5 Hz), 3.36 (3H, s), 2.94–2.87 (1H, m), 2.20–2.12 (1H, m), 1.75 (1H, td, J = 13.6 Hz, J = 3.5 Hz).

^{13}C NMR (125MHz, CDCl3) δ: 163.6, 149.3, 147.9, 140.7, 135.0, 134.9, 134.0, 133.3, 132.1, 131.3, 126.8, 125.9, 121.0, 117.6, 100.6, 99.2, 89.5, 72.1, 63.6, 59.6, 59.1, 58.5, 56.5, 47.9, 28.8

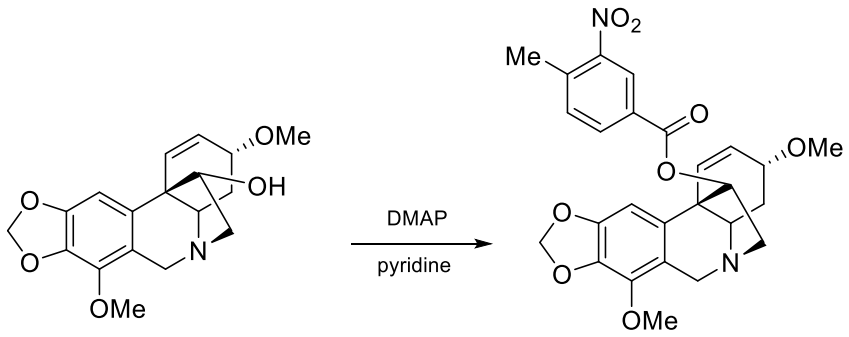
Optical rotation:

In the measurement process.

ESI-HRMS study:

ESI-HRMS *m/z* calcd for $C_{22}H_{28}NO_6$ $[M+H]^+$ 515.1230 found 515.1216

4.2.10.) Preparation of 11-*O*-(4-methyl-3-nitrobenzoyl)ambelline (LC-119)



Chemical Formula: $C_{18}H_{21}NO_5$
Exact Mass: 331,14
Molecular Weight: 331,37

Chemical Formula: $C_{26}H_{26}N_2O_8$
Exact Mass: 494,17
Molecular Weight: 494,50

Figure 36: Preparation scheme of 11-*O*-(4-methyl-3-nitrobenzoyl)ambelline

50 mg of ambelline (0.151 mmol) was dissolved in 2 ml of dry pyridine. After dissolution 100 µl of 4-methyl-3-nitrobenzoyl chloride was added. After that, a catalytic amount of DMAP was added to the reaction mixture. The mixture was stirred at 80°C on oil bath and after 24 hours the reaction was checked by TLC with developing mobile phase S_2. Subsequently, the mixture was evaporated to dryness. The residue was dissolved in $CHCl_3$ and applied gradually to the preparation plates. The preparation plates were developed in a mixture of S_2 and then the zone that represents our product was separated. The mixture from the preparation plates was washed with EtOH:$CHCl_3$. 76.4 mg of 11-*O*-(4-methyl-3-nitrobenzoyl)ambelline was obtained. The yield of this reaction was 100%.

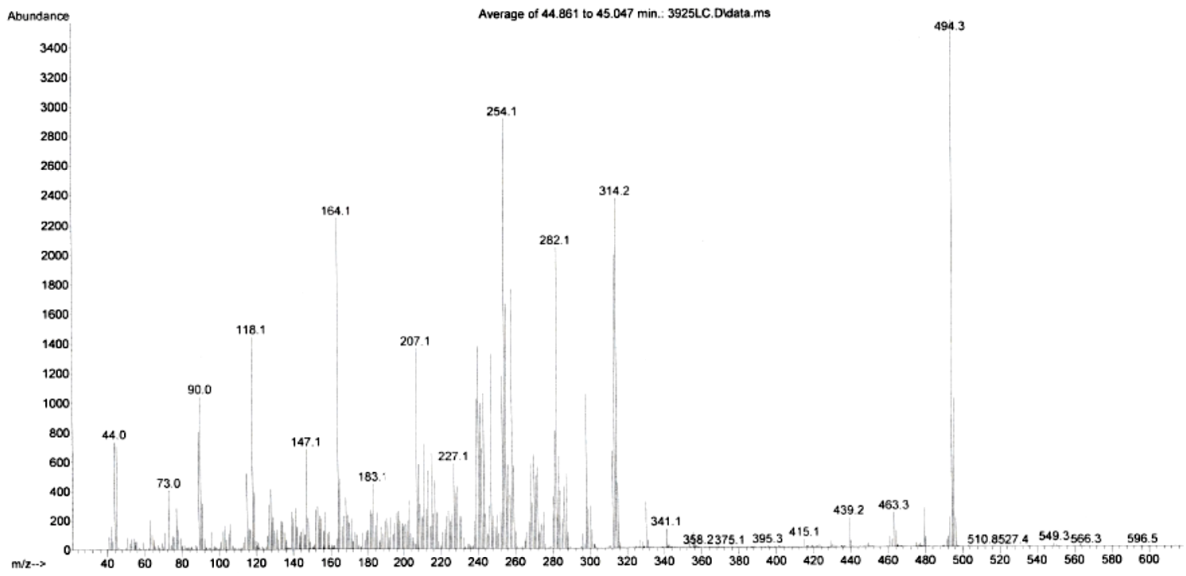


Figure 37: GC-MS of 11-*O*-(4-methyl-3-nitrobenzoyl)ambelline

NMR study:

^{1}H NMR (500MHz, CDCl3) δ: 7.97–7.95 (1H, m), 7.88 (1H, dd, J = 7.8 Hz, J = 1.5 Hz), 7.36 (1H, d, J = 7.8 Hz), 6.56 (1H, d, J = 10.0 Hz), 6.50 (1H, s), 6.09 (1H, dd, J = 10.0 Hz, J = 5.2 Hz), 5.91–5.89 (1H, m), 5.87–5.86 (1H, m), 5.40 (1H, dd, J = 8.0 Hz, J = 3.7 Hz), 4.38 (1H, d, J = 17.5 Hz), 4.02 (3H, s), 3.94 (1H, overlapped, d, J = 17.5 Hz), 3.94–3.88 (2H, overlapped, m), 3.55 (1H, dd, J = 13.8 Hz, J = 3.9 Hz), 3.37 (3H, s), 2.87–2.81 (1H, m), 2.62 (3H, s), 2.23–2.16 (1H, m), 1.78 (1H, td, J = 13.8 Hz, J = 3.9 Hz).

^{13}C NMR (125MHz, CDCl3) δ: 164.2, 149.0, 148.1, 140.7, 138.5, 134.1, 133.29, 133.25, 132.9, 130.9, 129.2, 126.9, 125.7, 117.0, 100.8, 99.0, 88.1, 72.1, 63.2, 59.8, 59.2, 58.6, 56.6, 47.6, 28.8, 20.6.

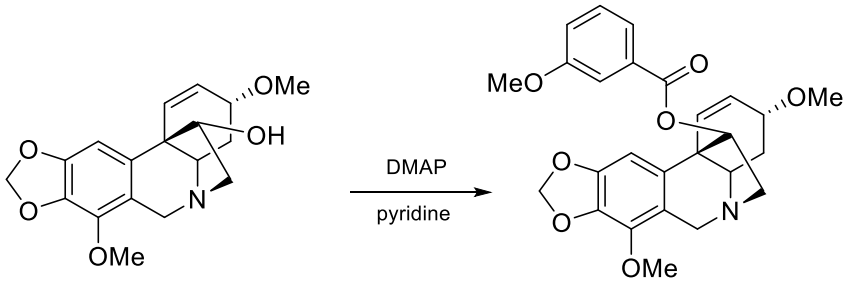
Optical rotation:

In the measurement process.

ESI-HRMS study:

ESI-HRMS *m/z* calcd for $C_{22}H_{28}NO_6$ $[M+H]^+$ 495.1770 found 495.1762

4.2.11.) Preparation of 11-*O*-(3-methoxybenzoyl)ambelline (LC-125)



Chemical Formula: $C_{18}H_{21}NO_5$
Exact Mass: 331,14
Molecular Weight: 331,37

Chemical Formula: $C_{26}H_{27}NO_7$
Exact Mass: 465,18
Molecular Weight: 465,50

Figure 38: Preparation scheme of 11-*O*-(3-methoxybenzoyl)ambelline

50 mg of ambelline (0.151 mmol) was dissolved in 2 ml of dry pyridine. After dissolution 100 µl of 3-metxhoxybenzoyl chloride was added. After that, a catalytic amount of DMAP was added to the reaction mixture. The mixture was stirred at 100°C on oil bath and after 2 hours the reaction was checked by TLC with developing mobile phase S_3. Subsequently, the mixture was evaporated to dryness. The residue was dissolved in $CHCl_3$ and applied gradually to the preparation plates. The preparation plates were developed in a mixture of S_3 and then the zone that represents our product was separated. The mixture from the preparation plates was washed with EtOH:$CHCl_3$. 55.5 mg of 11-*O*-(3-methoxybenzoyl)ambelline was obtained as a pale yellow oil. The yield of this reaction was 79%.

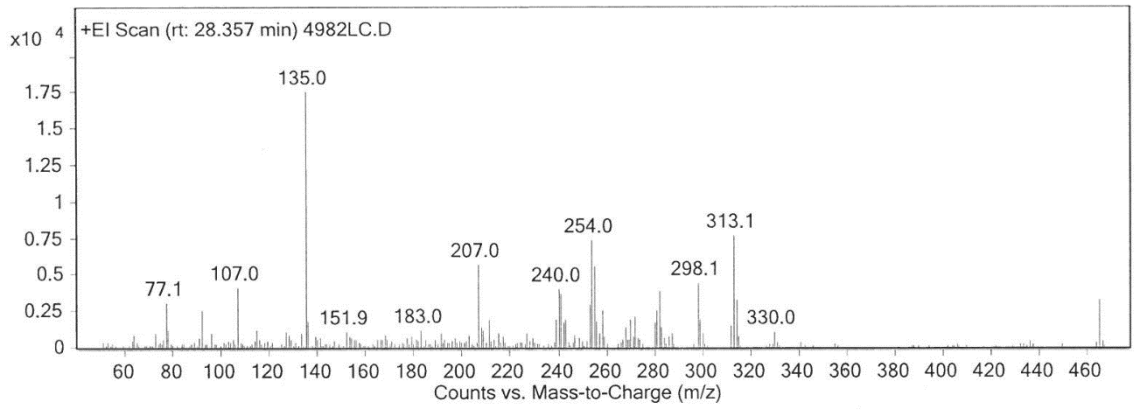


Figure 39: GC-MS of 11-*O*-(3-methoxybenzoyl)ambelline

NMR study:

^{1}H NMR (500MHz, $CDCl_3$) δ: 7.32–7.29 (1H, m), 7.24 (1H, t, *J* = 7.8 Hz), 7.12–7.10 (1H, m), 7.05–7.01 (1H, m), 6.60 (1H, d, *J* = 10.0 Hz), 6.57 (1H, s), 6.08 (1H, dd, *J* = 10.0 Hz, *J* = 5.4 Hz), 5.81 (1H, overlapped, d, *J* = 23.9 Hz), 5.80 (1H, overlapped, d, *J* = 23.9 Hz), 5.33 (1H, dd, *J* = 7.8 Hz, *J* = 3.9 Hz), 4.36 (1H, d, *J* = 17.6 Hz), 3.98 (3H, s), 3.94–3.86 (3H, m), 3.76 (3H, s), 3.50 (1H, dd, *J* = 13.7 Hz, *J* = 4.0 Hz), 3.36 (3H, s), 2.84–2.77 (1H, m), 2.21–2.14 (1H, m), 1.79 (1H, td, *J* = 13.7 Hz, *J* = 4.0 Hz)

^{13}C NMR (125MHz, $CDCl_3$) δ: 166.0, 159.3, 147.7, 140.6, 133.9, 133.8, 131.2, 131.0, 129.2, 126.7, 121.9, 120.0, 117.4, 113.1, 100.4, 99.4, 87.9, 72.2, 63.1, 59.9, 59.1, 58.7, 56.5, 55.1, 47.3, 28.7

Optical rotation:

$[\alpha]^{24}_{D}$= + 74.6 (*c* = 0.140; $CHCl_3$)

ESI-HRMS study:

ESI-HRMS *m/z* calcd for $C_{26}H_{28}NO_7$ $[M+H]^+$ 466.1860 found 466.1865

5) RESULTS

5.1.) Preparation of semi-synthetic derivatives

Table 13: Prepared compounds and their percentual yields

Compound	Chemical name of compound	Yield (%)
LC-85	11-*O*-Acetylambelline	93
LC-86	11-*O*-Propionylambelline	95
LC-87	11-*O*-Pentanoylambelline	80
LC-88	11-*O*-Isobutanoylambelline	100
LC-92	11-*O*-benzoylambelline	100
LC-96	11-*O*-(4-Nitrobenzoyl)ambelline	90
LC-98	11-*O*-(4-Methylbenzoyl)ambelline	58
LC-100	11-*O*-(3-Nitrobenzoyl)ambelline	66
LC-110	11-*O*-(2-Chloro-4-nitrobenzoyl)ambelline	100
LC-119	11-*O*-(4-Methyl-3-nitrobenzoyl)ambelline	100
LC-125	11-*O*-(3-Methoxybenzoyl)ambelline	79

5.2.) Screening of biological activities

5.2.1.) Screening of anticancer activity

Prepared derivatives were tested against 10 different cell lines – Jurkat (acute T-cell leukemia), MOLT-4 (acute lymphoblastic leukemia), A549 (lung cancer), HT-29 (colorectal adenocarcinoma), PANC-1 (epitheloid pancreatic cancer), A2780 (ovarian cancer), HeLa (cervical adenocarcinoma), MCF-7 (breast adenocarcinoma), SAOS-2 (osteosarcoma), MRC-5 (pulmonary human fibroblast). As a standard for comparison of an anticancer activity was used doxorubicine, a cancer medicament. All our results are summarised in the following tables (**Tab.14**, **Tab.15**). Unfortunately, all derivatives are not interesting for the further development as anticancer candidates.

Table 14: Tested derivatives on selected cell lines and their viability values in % (cell proliferation after 48 hours, concentration 10 μM)

	LC-85	**LC-86**	**LC-87**	**LC-88**	**LC-92**	**LC-96**	**Doxorubicine**
Jurkat	106 ± 4	105 ± 3	102 ± 5	119 ± 4	74 ± 4	75 ± 2	1 ± 0
MOLT-4	95 ± 5	101 ± 5	100 ± 7	100 ± 3	94 ± 4	87 ± 11	1 ± 2
A549	100 ± 3	104 ± 4	108 ± 4	105 ± 2	110 ± 5	99 ± 3	17 ± 6
HT-29	96 ± 6	100 ± 8	89 ± 4	104 ± 4	109 ± 4	94 ± 8	57 ± 4
PANC-1	104 ± 8	101 ± 4	109 ± 3	108 ± 6	93 ± 6	91 ± 5	67 ± 7
A2780	99 ± 5	94 ± 11	100 ± 4	108 ± 6	95 ± 7	88 ± 3	11 ± 2
HeLa	97 ± 5	94 ± 4	94 ± 2	96 ± 3	98 ± 7	75 ± 2	25 ± 11
MCF-7	104 ± 3	105 ± 5	109 ± 7	105 ± 12	106 ± 6	97 ± 10	36 ± 3
SAOS-2	112 ± 8	106 ± 7	120 ± 110	115 ± 14	102 ± 10	102 ± 7	27 ± 5
MRC-5	101 ± 7	111 ± 16	110 ± 5	114 ± 5	98 ± 5	94 ± 6	52 ± 7

Table 15: Tested derivatives on selected cell lines and their viability values in % (cell proliferation after 48 hours, concentration 10 µM)

	LC-98	LC-100	LC-110	LC-119	LC-125	Doxorubicine
Jurkat	54 ± 0	88 ± 9	78 ± 11	58 ± 4	27 ± 8	1 ± 0
MOLT-4	78 ± 13	86 ± 9	80 ± 9	65 ± 8	57 ± 12	1 ± 2
A549	95 ± 7	98 ± 12	95 ± 5	105 ± 10	53 ± 9	17 ± 6
HT-29	89 ± 7	100 ± 5	106 ± 5	100 ± 4	113 ± 14	57 ± 4
PANC-1	71 ± 6	96 ± 11	112 ± 3	78 ± 5	34 ± 4	67 ± 7
A2780	65 ± 6	87 ± 4	96 ± 1	60 ± 11	33 ± 16	11 ± 2
HeLa	80 ± 6	96 ± 11	91 ± 4	93 ± 11	73 ± 18	25 ± 11
MCF-7	83 ± 4	94 ± 13	106 ± 4	73 ± 15	53 ± 5	36 ± 3
SAOS-2	72 ± 6	104 ± 10	93 ± 13	70 ± 9	21 ± 2	27 ± 5
MRC-5	98 ± 8	102 ± 18	111 ± 5	92 ± 14	57 ±15	52 ± 7

5.2.2.) Screening of AChE, BuChE inhibitory activity

At the Department of Pharmaceutical Botany, substances of both natural and semi-synthetic origin have been tested for a long time for their inhibitory activity against erythrocyte acetylcholinesterase and serum butyrylcholinesterase. The following table (**Tab.16**) summarizes the results of inhibitory activity against AchE and BuchE prepared ambelline derivatives. LC-92 and LC-125 appear to be the most interesting of the tested substances. We also calculated log BB in order to understand how our derivatives penetrate BBB. Compounds with logBB > 0.3 can readily penetrate the BBB, compounds with values between 0.3 > logBB > -1 can still pass the BBB while compounds having logBB <−1.0 are only poorly distributed to the brain[125].

Table 16: Inhibitory activity of prepared derivatives and their IC_{50} for human cholinesterases

	% of AChE inhibition	**AChE IC_{50} (µM)**	**% of BuChE Inhibition**	**BuChE IC_{50} (µM)**	**log BB[a]**
LC-85	4 ± 0	>100	28 ± 1	>100	n.c.
LC-86	15 ± 1	>100	16 ± 2	>100	n.c.
LC-87	7 ± 1	>100	39 ± 1	>100	n.c.
LC-88	3 ± 0	>100	12 ± 1	>100	n.c.
LC-92	25 ± 3	>100	97 ± 3	3.83 ± 0.07	0.27
LC-96	31 ± 7	>100	37 ± 2	>100	0.03
LC-98	28 ± 3	>100	68 ± 1	28 ± 1	0.23
LC-100	58 ± 5	48 ± 2	53 ± 1	81 ± 2	0.03
LC-110	34 ± 3	>100	16 ± 1	>100	n.c.
LC-119	54 ± 4	53.67 ± 4.37	41 ± 2	>100	n.c.
LC-125	25 ± 3	>100	91 ± 1	6.2 ± 0.4	0.03

n.c. = not calculated, [a] calculated at http://www.way2drug.com/geb/

5.2.3.) Screening of GSK-3β inhibitory activity

All derivatives were tested for GSK-3β inhibitory activity. Of the derivatives tested, aliphatic ambelline derivatives had the best results. LC-125 also showed an interesting activity, for which we also determined IC_{50}. All results are in the following table (**Tab.17**). IC_{50} was determined only for LC-125 due to the lack of enzyme required for testing assay. Determination of other derivatives is planned in the future.

Table 17: Percentage of GSK-3β inhibitory activity synthesised derivatives

	% of GSK-3β inhibition	**IC_{50} (μM)**
LC-85	87.70 ± 0.87	n.t.
LC-86	87.02 ± 3.55	n.t.
LC-87	99.58 ± 0.42	n.t.
LC-88	83.86 ± 1.10	n.t.
LC-92	56.21 ± 3.85	n.t.
LC-96	40.24 ± 0.39	n.t.
LC-98	41.74 ± 0.12	n.t.
LC-100	42.28 ± 1.87	n.t.
LC-110	31.79 ± 0.06	n.t.
LC-119	54.34 ± 1.51	n.t.
LC-125	78.78 ± 0	93.32 ± 0

n.t. = not tested

6) DISCUSSION AND CONCLUSIONS

At the Faculty of Pharmaceutical Botany, Associate Professor Cahlíková has long been devoted to the preparation of AA derivatives. She has been involved in preparation of lycorine and haemanthamine derivatives, that showed an interesting biological activity even on their own[126,127]. Ambelline itself is, on the contrary, insignificant. During the phytochemical study of *Nerine bowdenii* at our department, a sufficient amount of ambelline was isolated and we decided to prepare a pilot series of ambelline derivatives.

Ambelline is a structural analogue of haemanthamine, from which about 70 derivatives have been prepared at our department. Some of them exhibit an interesting biological activity, and we wondered what activity the same ambelline derivatives would have since ambelline and haemanthamine differ by the presence of the methoxy group at the C7-position and they have opposite spatial arrangement of the 5,10*b*-ethane bridge.

This diploma thesis deals with the preparation of ambelline derivatives. In the literature there are mainly derivatives that naturally occur in plants as secondary metabolites, and work that would concentrate solely on the preparation of ambelline derivatives has not yet occurred. 11 derivatives - 4 aliphatic esters and 7 aromatic esters of ambelline are discussed in this work.

11-*O*-Acetylambelin (LC-85) occurs naturally in plants and other derivatives have been prepared for the first time. The yield of all reactions ranged from 55-100%, so there was no major problem with their preparation. All syntheses were carried out in anhydrous pyridine in the presence of DMAP as a catalyst. Various anhydrides were used for the preparation of aliphatic esters and differently substituted benzoyl chlorides for the aromatic esters. Preparative TLC was used to obtain and purify synthesised derivatives. Prepared compounds were identified by MS, NMR and optical rotation. Some derivatives were obtained in the form of white to yellow crystals and some of them were obtained as a yellow pale oil.

All prepared derivatives were studied in terms of their cytotoxic effect. A panel of nine cancer cell lines (Jurkat, MOLT-4, A549, HT-29, PANC-1, A2780, HeLa, MCF-7, SAOS-2) and one healthy cell line (MRC-5) were used for this study. Unfortunately, none of the derivatives showed a significant cytotoxic activity. These results were not particularly surprising since it

has been concluded in the past that for the cytotoxic activity in crinane-type AA is an α-orientation of 5,10*b*-ethane bridge essential[103].

All derivatives were studied for their potential inhibitory activity of human cholinesterases. Effective inhibition of cholinesterases is associated with a possible treatment of AD[128]. All substances were first screened at 100 μM concentration. Derivatives showing inhibitions >45% to AChE and >50% to BuChE were further selected for IC_{50} determination. Galanthamine was used as a standard for this assay. The most interesting derivative is LC-92 with IC_{50} = 3.83 ± 0.07 μM and then LC-125 with IC_{50} = 6.2 ± 0.4 μM for BuChE inhibition. These 2 compounds seem to be selective inhibitors of BuChE, which plays significant role in later stages of AD[129]. From our results we could conclude that aliphatic esters are not interesting for the further investigation. On the contrary, aromatic esters seem to be quite interesting because of their selective inhibition of BuChE. Aromatic esters will be studied in our upcoming experiments for their potency to act against other therapeutically relevant targets in AD (inhibition of BACE-1, POP). Furthermore, another possible substitution in aromatic ester region will be inspected to provide more SAR.

Derivatives were studied also in terms of their GSK-3β inhibitory potential due to its connection to AD[105]. The strongest inhibitory activity had LC-87 with 99.58 ± 0.42 % of inhibition. LC-87 as well as all other aliphatic esters showed a very interesting inhibitory activity that will be investigated in the future. Unfortunately, we could not determine IC_{50} for these substances during our Erasmus+ project due to the lack of GSK-3β enzyme. Based on our results we could summarize that aromatic derivatives did not show such a significant activity against GSK-3β. Of our aromatic derivatives, LC-125 (methoxy- derivative) had the best result (78.78 % of GSK-3β inhibition). This substance showed interesting values also in the inhibition of BuChE, and that is why we decided to determine the IC_{50} value for this substance (93.32 μM).

Currently, an antimalarial activity of prepared derivatives is being studied and an antimicrobial activity is also planned.

During our work at the Department of Pharmaceutical Botany we prepared 20 derivatives in total, but this thesis only deals with 11 of them. Some of them showed

an interesting cytotoxic activity and we are thinking about a patent application, some of them have not yet been investigated for their biological activities and are therefore omitted from this diploma thesis. As part of my Ph.D. study we plan to test all prepared substances and publish our results to complement the characteristics of them. In the future we would also like to evaluate SAR in more depth in order to understand the difference between ambelline and haemanthamine derivatives in case of their biological activity. A fine outline of this study of comparing the same ambelline and haemanthamine derivatives was presented at the Student Scientific Conference in 2018[130].

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8) LIST OF ABBREVIATIONS

AA	Amaryllidaceae alkaloids
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ATP	Adenosine triphosphate
BACE-1	Beta-secretase 1
BBB	Blood-brain barrier
BuChE	Butyrylcholinesterase
$CDCl_3$	Deuterated chloroform
CDK5	Cyclin-dependent kinase 5
CD_3OD	Deuterated methanol
$CHCl_3$	Chloroform
cHx	Cyclohexane
CNS	Central nervous system
COX	Cyclooxygenase
DMAP	Dimethylaminopyridine
DNA	Deoxyribonucleic acid
ED_{50}	The median effective dose
ESI	Electrospray ionisation
ESI-HRMS	High resolution electrospray ionization mass spectrometry
ESI-MS	Electrospray ionisation mass spectrometry
EtOAc	Ethyl acetate
EtOH	Ethanol
Et_2NH	Diethylamine
GC-MS	Gas chromatography–mass spectrometry
GSK-3β	Glycogen synthase kinase 3β
HIV	Human immunodeficiency virus

HuAChE	Human acetylcholinesterase
HuBuChE	Human butyrylcholinesterase
IC_{50}	The half maximal inhibitory concentration
MeCN	Acetonitrile
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
NF-κB	Nuclear factor kappa B
NFTs	Neurofibrillary tangles
NMR	Nuclear magnetic resonance
POP	Prolyloligopeptidase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAR	Structure-activity relationship
SARS	Severe Acute Respiratory Syndrome
TLC	Thin-layer chromatography
TMS	Tetramethylsilane
To	Toluene
UHPLC	Ultra-high-performance liquid chromatography

Table 18: Cell lines used for determination of anticancer activity

Abbreviation	Meaning	Abbreviation	Meaning
A2780	Human ovarian cancer cells	**K562**	Myelogenous leukemia cell line
A549	Adenocarcinomic human alveolar cells	**KM20L2**	Human colon adenocarcinoma cells
AGS	Human gastric adenocarcinoma cells	**KM-3**	Human plasma cell myeloma
AKR	Cell line capable to produce murine leukaemia virus	**MCF-7**	Human breast cancer cell line
B16F10	Murine melanoma cell line	**MDA-MB-231**	Human breast cancer cell line
BL-6	Murine melanoma cell line	**MOLT-4**	Acute lymphoblastic leukemia cell
BT549	Human breast cancer cell line	**NCI-H460**	Large cell lung cancer
BxPC-3	Human pancreatic cancer cell line	**OE21**	Human oesophageal squamous cell carcinoma
Caco-2	Heterogeneous human epithelial colorectal adenocarcinoma cells	**P-388**	Murine leukaemia cells
CEM	Human acute lymphoblastic leukaemia	**PANC-1**	Human pancreatic cancer cell line
COLO-201	Human colon adenocarcinoma cells	**SAOS-2**	Human osteosarcoma cells
DU-145	Human prostate cancer cell line	**SF268**	Human glioma and astrocytoma cells
G-361	Human malignant melanoma	**SKMEL**	Malignant melanoma cells
H1299	Human non-small cell lung carcinoma cell line	**SKW-3**	Acute lymphoblastic leukemia (T-ALL) cell lines
HeLa	Human cervical cancer cell line	**SW480**	Colon adenocarcinoma cell line
HepG2	Human liver cancer cell line	**U373**	Human glioblastoma astrocytoma
HL-60	Human leukemia cell line	**U87**	Human primary glioblastoma cell line
HL-60/DOX	Multi-drug resistant cells of acute myeloblastic leukemia	**T98G**	Glioblastoma cell line
HS578T	Human breast cancer cell line	LMTK	Non-cancerous murine fibroblasts
Hs-683	Human brain cancer cell line	MRC-5	Non-cancerous human lung fibroblasts

HT-29	Human colon cancer cell line	NHSF	Non-cancerous human skin fibroblasts
Jurkat	Human acute T cell leukemia	Vero	Non-cancerous monkey kidney cells

9) LIST OF FIGURES

Figure 1: Amaryllidaceae alkaloids 10
Figure 2: Simplified scheme of 4'-O-methylnorbelladin biosynthesis[19] 11
Figure 3: Postulated pathways of AA biosynthesis (reposted)21 12
Figure 5: Chemical structure of selected norbelladine-type alkaloids 14
Figure 6: Chemical structures of selected galanthamine-type alkaloids 15
Figure 7: Chemical structure of tazettine and pretazettine 16
Figure 8: Chemical structures of selected lycorine-type alkaloids 17
Figure 9: Chemical structure of selected homolycorine-type alkaloids 20
Figure 10: Chemical structure of selected narciclasine-type alkaloids 20
Figure 11: Chemical structures of selected montanine-type alkaloids 22
Figure 12: Chemical structure of selected haemanthamine-type alkaloids 23
Figure 13: Chemical structure of selected crinine-type alkaloids 23
Figure 14: Chemical structure of distichamine 24
Figure 15: Chemical structure of undulatine 26
Figure 16: Chemical structure of ambelline 27
Figure 17: 3D model of ambelline[98] 27
Figure 18: Chemical structures of ambelline derivatives 35
Figure 19: Preparation scheme of 11-*O*-acetylambelline 42
Figure 20: GC-MS analysis of 11-*O*-Acetylambelline 43
Figure 21: Preparation scheme of 11-*O*-propionylambelline 43
Figure 22: GC-MS of 11-*O*-propionylambelline 44
Figure 23: Preparation scheme of 11-*O*-pentanoylambelline 45
Figure 24: GC-MS of 11-*O*-pentanoylambelline 46
Figure 25: Preparation scheme of 11-*O*-isobutanoylambelline 47
Figure 26: GC-MS of 11-*O*-isobutanoylambelline 48
Figure 27: Preparation scheme of 11-*O*-benzoylambelline 49
Figure 28: GC-MS of 11-*O*-benzoylambelline 50
Figure 29: Preparation scheme of 11-*O*-(4-nitrobenzoyl)ambelline 51
Figure 30: GC-MS of 11-*O*-(4-nitrobenzoyl)ambelline 52
Figure 31: Preparation scheme of 11-*O*-(4-methylbenzoyl)ambelline 53
Figure 32: GC-MS of 11-*O*-(4-methylbenzoyl)ambelline 54
Figure 33: Preparation scheme of 11-*O*-(3-nitrobenzoyl)ambelline 55
Figure 34: GC-MS of 11-*O*-(3-nitrobenzoyl)ambelline 56
Figure 35: Preparation scheme of 11-*O*-(2-chloro-4-nitrobenzoyl)ambelline 57
Figure 36: GC-MS of 11-*O*-(2-chloro-4-nitrobenzoyl)ambelline 58
Figure 37: Preparation scheme of 11-*O*-(4-methyl-3-nitrobenzoyl)ambelline 59
Figure 38: GC-MS of 11-*O*-(4-methyl-3-nitrobenzoyl)ambelline 60
Figure 39: Preparation scheme of 11-*O*-(3-methoxybenzoyl)ambelline 61

10) LIST OF TABLES

Table 1: HuAChE, HuBuChE, and POP inhibitory activity of belladine, 6-*O*-demethylbelladine and 4´-*O*-demethylbelladine expressed as IC_{50} [25] 14
Table 2: An overview of AChE inhibitory activity of selected AA expressed as IC_{50} values[30,32] 16
Table 3: Growth-Inhibitory Activity for Cancer Cell Lines of lycorine-type AA (reposted)[54] 18
Table 4: AChE inhibitory activity of tested alkaloids expressed as IC_{50} values[32,53] 19
Table 5: Human Cancer Cell Line and Murine P-388 Lymphocytic Inhibitory Activities of pancratistatin and narciclasine (reposted)[69] 22
Table 6: Evaluation of the cytotoxic activity of haemanthamine, haemanthidine and distichamine[54,83,84,87] 25
Table 7: Antiplasmodial activity of selected crinane-type alkaloids expressed as IC_{50} (µg/ml)[91,92] 26
Table 8: Cell proliferation after selected alkaloids treatment expressed as percentages of proliferation of untreated control cells[87] 30
Table 9: Screening of AA (50 µM) for their potency to inhibit GSK-3β[111] 32
Table 10: The in vitro antimicrobial effect of crinane alkaloids[117] 33
Table 11: Antiplasmodial activities against P.falciparum Dd2 expressed as IC_{50} (reposted)[5] 34
Table 12: Cell proliferation after ambelline and its acetylderivative treatment expressed as percentages of proliferation of untreated control cells[87] 36
Table 13: Prepared compounds and their percentual yields 63
Table 14: Tested derivatives on selected cell lines and their viability values in % (cell proliferation after 48 hours, concentration 10 µM) 64
Table 15: Tested derivatives on selected cell lines and their viability values in % (cell proliferation after 48 hours, concentration 10 µM) 65
Table 16: Inhibitory activity of prepared derivatives and their IC_{50} for human cholinesterases 66
Table 17: Percentage of GSK-3β inhibitory activity synthesised derivatives 67
Table 18: Cell lines used for determination of anticancer activity 85

ABSTRACT

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Title of diploma thesis: Derivatives of Amaryllidaceae Alkaloids as Drugs

Plants of the family Amaryllidaceae belong to the widespread species. They contain a large amount of Amaryllidaceae alkaloids (AA) which are known for their biological activity. AA possess a broad spectrum of biological activities including an antiviral, antimalarial, antitumor, cholinesterase's inhibitory activity and others.

An interesting AA is ambelline which occurs mainly in plants of the genus *Crinum* and *Nerine*. So far the biological activity of this compound has been studied only marginally. In the studies conducted, this substance appears to be less interesting.

A series of aliphatic and aromatic derivatives of ambelline has been prepared in the framework of this thesis. Subsequently, their cholinesterase inhibitory activity and GSK-3β inhibitory activity were studied. Cytotoxic activity on a panel of selected tumor and resting cell lines was also screened.

Of the prepared derivatives, LC-125 (3-methoxybenzoylambellin) had an interesting biological activity. This substance showed a promising activity in all biological studies. GSK-3β inhibitory activity is characterized by an IC_{50} = 93.32 µM. In addition, LC-125 appears to be a selective butyrylcholinesterase inhibitor with an IC_{50} = 6.2 ± 0.4 µM. This derivative also had a mild cytotoxic activity.

Keywords: ambelline, derivatives, Amaryllidaceae family, alkaloids, antitumor activity, Alzheimer's disease

ABSTRAKT

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Rostliny čeledi Amaryllidaceae patří k široce rozšířeným druhům. Obsahují velké množství Amaryllidaceae alkaloidů (AA), které jsou známé pro svou biologickou aktivitu. AA disponují širokým spektrem biologických aktivit mezi které patří antivirová, antimalarická, protinádorová, inhibiční aktivita vůči cholinesterázám a další.

Zajímavým AA je ambellin, který se vyskytuje především v rostlinách rodu *Crinum* a *Nerine*. Biologická aktivita této sloučeniny byla doposud studována pouze okrajově. V rámci provedených studií se tato látka jeví jako méně zajímavá.

V rámci diplomové práce byla připravena série alifatických a aromatických derivátů ambellinu. Následně byla studována jejich inhibiční aktivita vůči cholinesterázám a inhibiční aktivita vůči GSK-3β. Screeningově byla studována i cytotoxická aktivita na panelu vybraných nádorových a klidových buněčných linií.

Z připravených derivátů, zajímavou biologickou aktivitou disponovala látka označená jako LC-125 (3-methoxybenzoylambellin). Tato látka vykazovala slibnou aktivitu ve všech biologických studií. Inhibiční aktivita GSK-3β je charakterizována IC_{50} = 93,32 μM. Navíc se LC-125 jeví jako selektivní inhibitor butyrylcholinesterázy s IC_{50} = 6,2 ± 0,4 μM. Tento derivát také disponoval mírnou cytotoxickou aktivitou.

Klíčová slova: ambellin, deriváty, čeleď Amaryllidaceae, alkaloidy, protinádorová aktivita, Alzheimerova choroba