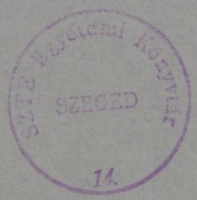
Investigation of the diterpene alkaloids of *Aconitum* species native to the Carpathian Basin



Summary of Ph.D. Thesis

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1. INTRODUCTION

For centuries, Ranunculaceae species have been used by various civilizations as sources of both poisons and medicines, due to their marked physiological effects. Preparations of certain *Aconitum* species native to Asia are indispensable materials in Eastern medicine, primarily as painkillers. In Europe, species of the *Aconitum* genus have played a less important role in therapy; the only important species was *Aconitum napellus*. After aconitine, the main alkaloid of the plant was discovered in 1833, the pure compound also became part of the medication. However, due to the high variability of the alkaloid content and the extreme toxicity of aconitine, both the herbal drug and the pure alkaloid have lost their significance in Western medicine.

In the second half of the 20th century, the structures of several diterpene alkaloids were elucidated. These compounds occur mainly in the Ranunculaceae family (genera *Aconitum*, *Delphinium* and *Consolida*). From the viewpoint of biogenesis, diterpene alkaloids belong into the class of the pseudoalkaloids, since they appear to be derivatives of the amination of nitrogen-free terpenes. Depending on the number of carbon atoms in the skeleton, the *compounds* can be divided into the major groups of C_{20}, C_{19} and C_{18} diterpene alkaloids.

A majority of the identified diterpene alkaloids were isolated from *Aconitum* species. *Aconitum* is a circumboreal arctic and alpine genus that extends into lower latitudes where there is suitable mesic habitat at high elevations along the north-south chains of mountains. The greatest distribution of *Aconitum* species is in Asia, with smaller groups in Europe and North America. In the flora of Europe, 7 species are to be found, 5 of which are native to the Carpathian Basin.

In view of their significant physiological effects, pharmacological studies on diterpene alkaloids have been carried out. The key points of the scientific research are the effects of the diterpene alkaloids on the central nervous system and the heart, activities which play important roles not only in the toxicity of the compounds, but also in their prospective medicinal use. Ranunculaceae alkaloids are the targets of structure-activity experiments, and regarded as lead compounds for the design and synthesis of new drug molecules. The hydrobromide salt of lappaconitine, a diterpene alkaloid of *A. septentrionale*, is currently on the market as an antiarrhythmic drug. In line with the growing pharmacological knowledge on the diterpene alkaloids, there is an increased demand for new compounds to find further perspective molecules for drug design and development.

2. AIMS OF THE STUDY

Several European Ranunculaceae species have been poorly examined phytochemically, or not at all. The increasing pharmacological significance of the diterpene alkaloids motivated us to investigate the alkaloids of certain *Aconitum* species native to the Carpathian Basin. In 2002, HOHMANN *et al.* (Department of Pharmacognosy, University of Szeged) initiated a research programme aimed at the phytochemical analysis of *Aconitum* and *Consolida* species in order to isolate diterpene alkaloids, the compounds most specific for this family. As part of this comprehensive work, the main goals of my study were to

- review the literature concerning the chemistry and pharmacology of diterpene alkaloids,
- collect plant material from certain *Aconitum* species native to the Carpathian Basin,
- examine the alkaloid contents of the collected species,
- select species with rich and complex alkaloid contents,
- carry out preparative work to isolate pure diterpene alkaloids from the selected species,
- elucidate the structures of the isolated compounds via NMR and HRMS techniques, provide characteristic spectral data on the isolated new compounds, and supplement missing NMR data on the already-known alkaloids,
- gain chemotaxonomically valuable information concerning the Ranunculaceae family,
- in the frame of cooperation, discover the pharmacological activities of the isolated compounds,
- identify diterpene alkaloids which are potential objects or tools for drug development.

3. MATERIALS AND METHODS

The extracts of roots and above-ground parts of *A. moldavicum*, *A. anthora*, *A. toxicum*, *A. variegatum* subsp. *gracile*, *A. vulparia* and *A. vulparia* subsp. *lasianthum* were purified by solvent partitioning to yield fractions rich in alkaloids. The alkaloid contents of these fractions were investigated by TLC, followed by spraying with *Dragendorff* reagent. The isolation of the alkaloids was carried out by an extensive multi-step chromatographic separation procedure, including open column chromatography (CC), vacuum-liquid chromatography (VLC), gel filtration chromatography (GFC), centrifugal planar chromatography (CPC) and preparative layer chromatography (PLC). The structure determination of the isolated compounds was carried out by means of spectroscopic (NMR, HRMS, UV) experiments.

4. RESULTS

4.1. ISOLATION OF ALKALOIDS

The phytochemical work was started with the analysis of the alkaloid compositions of several endemic *Aconitum* species. The alkaloid content of the herbal samples was screened by using the classical method of alkaloid isolation, based on solvent partitioning at different pH, which allowed the separation and enrichment of alkaloids. Except for *A. moldavicum*, each sample contained a noteworthy amount of alkaloid. Due to the high alkaloid content and complex alkaloid composition, *A. vulparia* and *A. toxicum* were selected for further phytochemical analysis. With regard to the limited amount of the plant material, the above-ground parts and roots of *A. vulparia* were examined together. The roots of *A. toxicum* contained appreciably more alkaloid, than the above-ground parts did (without the presence of chlorophyll), and therefore the roots of the plant were processed.

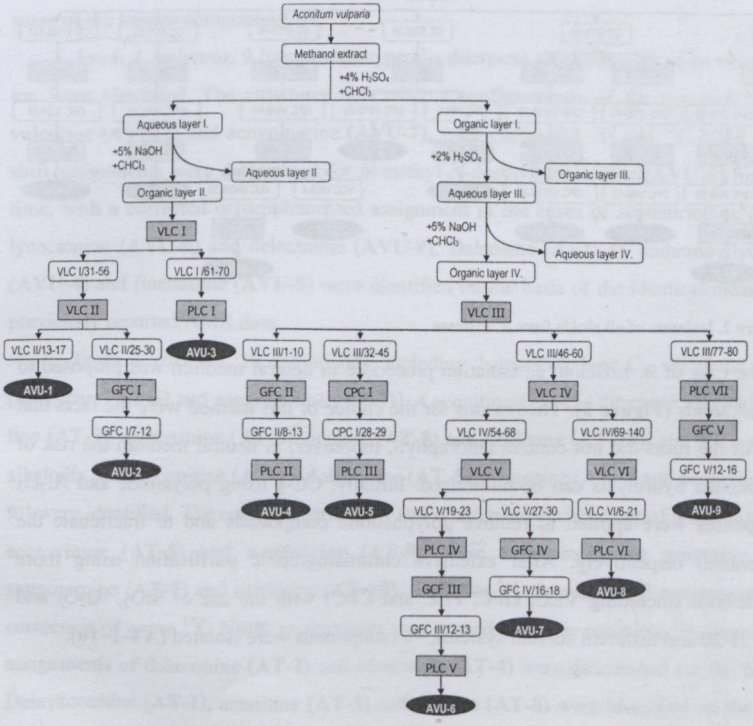


Figure 1. Isolation of alkaloids from *A. vulparia*

In the case of *A. vulparia*, classical alkaloid isolation based on solvent partitioning at different pH was applied (**Figure 1**). This method aims at the separation of alkaloids and neutral compounds. TLC examination revealed that organic layer I also contained alkaloids besides the bulk of the chlorophyll. On repetition of the phase-exchange process, two organic phases (II and IV) were obtained with different alkaloid compositions. Further multiple-step chromatographic separation (including VLC, GFC, PLC and CPC) on SiO_2, reversed-phase SiO_2, Al_2O_3 and Sephadex LH-20 resulted in the isolation of 9 pure compounds (**AVU-1–9**).

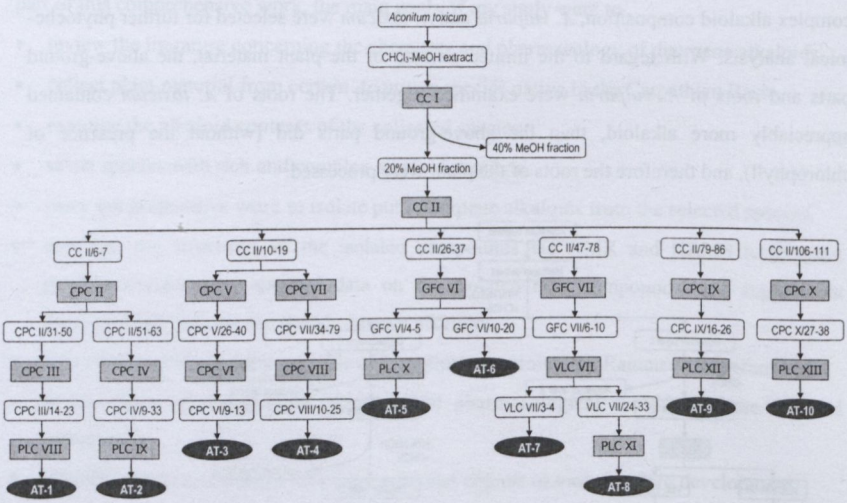


Figure 2. Isolation of alkaloids from *A. toxicum*

In the case of *A. toxicum*, an isolation procedure in neutral medium was proposed to obtain the alkaloids (**Figure 2**). The reasons for the choice of this method were the facts that the extract of the roots did not contain chlorophyll, moreover, in neutral medium the risk of acidic or alkaline hydrolysis can be minimized. Initially, CC-s using polyamide and Al_2O_3 stationary phases were applied to remove polyphenolic compounds and to fractionate the alkaloid content, respectively. After extensive chromatographic purification using more selective methods (including VLC, GFC, PLC and CPC) with the use of SiO_2, Al_2O_3 and Sephadex LH-20 and different solvent systems, 10 compounds were isolated (**AT-1–10**).

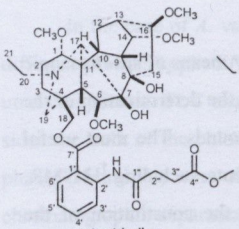
4.2. STRUCTURE ELUCIDATION OF THE ISOLATED COMPOUNDS

The structures of the isolated compounds were determined by means of spectroscopic methods. High-resolution mass spectrometric measurements allowed the determination of the exact molecular weights and molecular compositions of the compounds. The most useful methods in the structure elucidation were 1D and 2D NMR experiments, including ^{1}H NMR, JMOD, ^{1}H,^{1}H-COSY, HSQC, HMBC measurements, from which the constitution of the compounds were elucidated. By means of NOESY experiments, the relative configurations were determined. UV spectroscopy provided information on the structures only when aromatic ester groups were present in the molecules.

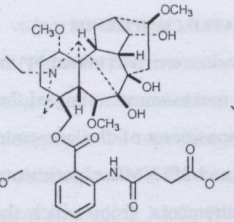
The already known compounds were identified by comparing their NMR data with those in the literature. The structures of the new compounds were elucidated by detailed analysis of the NMR spectra, supplemented with mass spectrometric experiments. Complete ^{1}H and ^{13}C NMR chemical shift assignments were made for the new compounds, and also for some of the known compounds.

From *A. vulparia*, 9 lycoctonine-type C_{19} diterpene alkaloids with an aconitane skeleton were identified. The structures and relative configurations of the two new alkaloids, vulparine (**AVU-2**) and acovulparine (**AVU-7**), were elucidated. ^{1}H and ^{13}C NMR chemical shift assignments were determined for *N*-methyl-*N*-deethyllycoctonine (**AVU-8**) for the first time, with a corrected or supplemented assignment in the cases of septentriodine (**AVU-1**), lycoctonine (**AVU-6**) and delectinine (**AVU-9**). Delcosine (**AVU-3**), anthranoyllycoctonine (**AVU-4**) and finetiadine (**AVU-5**) were identified on the basis of the identical measured and previously reported NMR data.

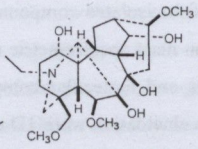
From *A. toxicum*, 10 compounds, including 2 napelline-type C_{20} diterpene alkaloids [songorine (**AT-6**) and songoramine (**AT-7**)], 4 aconitine-type C_{19} diterpene alkaloids [aconitine (**AT-3**), acotoxinine (**AT-5**), neoline (**AT-8**) and neolinine (**AT-10**)] and 4 C_{18} diterpene alkaloids [delavaconitine (**AT-1**), dolaconine (**AT-2**), aconosine (**AT-4**) and acotoxicine (**AT-9**)] were identified. The complete structures and relative stereochemistry of the new alkaloids, acotoxinine (**AT-5**) and acotoxicine (**AT-9**), were determined. For songorine (**AT-6**), songoramine (**AT-7**) and neolinine (**AT-10**), complete ^{1}H chemical shift assignment and the correction of some ^{13}C NMR assignments was carried out. The complete ^{1}H chemical shift assignments of dolaconine (**AT-2**) and aconosine (**AT-4**) were determined for the first time. Delavaconitine (**AT-1**), aconitine (**AT-3**) and neoline (**AT-8**) were identified on the basis of the good agreement of the measured and literature NMR data.



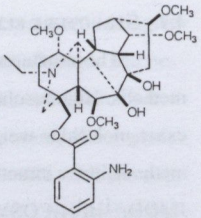
septentriodine
(AVU-1)



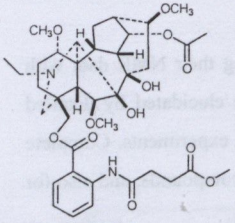
vulparine
(AVU-2)



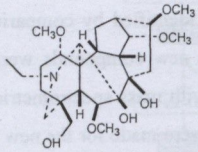
delcosine
(AVU-3)



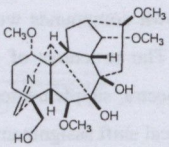
anthranoyllycoctonine
(AVU-4)



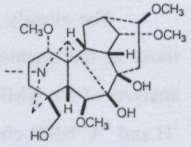
finetiadine
(AVU-5)



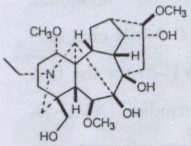
lycoctonine
(AVU-6)



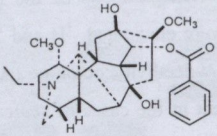
acovulparine
(AVU-7)



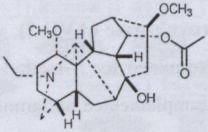
N-methyl-*N*-deethyllycoctonine
(AVU-8)



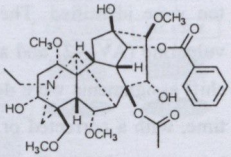
delectinine
(AVU-9)



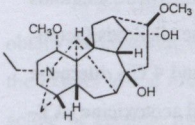
delavaconitine
(AT-1)



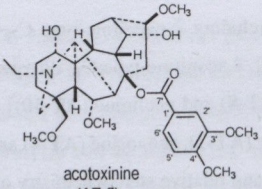
dolaconine
(AT-2)



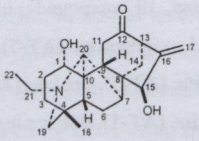
aconitine
(AT-3)



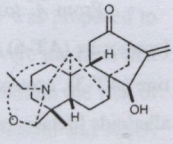
aconosine
(AT-4)



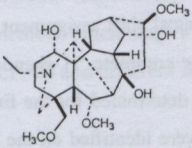
acotoxinine
(AT-5)



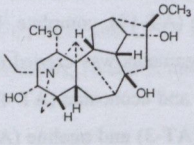
songorine
(AT-6)



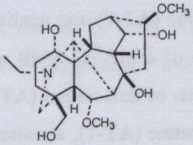
songoramine
(AT-7)



neoline
(AT-8)



acotoxicine
(AT-9)



neolinine
(AT-10)

4.3. BIOGENETIC AND CHEMOTAXONOMICAL ASPECTS

The alkaloids isolated from *A. vulparia* represent a series of structurally close compounds, which points to a common biogenetic pathway. The alkaloids are highly oxygenated, all containing 7 oxygen functions. The biogenesis of the skeletons, including the introductions of the oxygen functions in the cases of the 9 lycoctonine-type alkaloids, may be identical, followed by dissimilar methylation and esterification patterns. The methylation may proceed similarly to the sequence hypothesized for aconitine-type alkaloids (C-16 → C-6 → C-8 → C-1 → C-14). In each compound C-16 and C-6 are substituted with methoxy groups, while methylation on C-1 is lacking in 1 alkaloid [delcosine (**AVU-3**)] and on C-14 in 4 compounds [vulparine (**AVU-2**), delcosine (**AVU-3**), finetiadine (**AVU-5**) and delectinine (**AVU-9**)]. Interestingly, none of the compounds is methoxylated at C-8. In 3 alkaloids, 1 oxygen function, and in 1 compound, 2 hydroxy groups are esterified. The esterifying acids were acetic acid [finetiadine (**AVU-5**)], anthranilic acid [anthranoyllycoctonine (**AVU-4**)] and 2-[(4-methoxy-4-oxobutanoyl)amino]benzoic acid [septentriodine (**AVU-1**), vulparine (**AVU-2**) and finetiadine (**AVU-5**)]. Lycoctonine (**AVU-6**), acovulparine (**AVU-7**) and *N*-methyl-*N*-deethyllycoctonine (**AVU-8**) are closely related, since they differ only in the *N*-ethyl/*N*-azomethine/*N*-methyl functions.

The chemotaxonomical analysis of the isolated compounds provides valuable information. Structurally, the identified alkaloids are in accordance with the earlier described alkaloids from Swiss populations of *A. lycoctonum*, since similarly to the majority of the compounds in the literature (7 of 9), they are poorly esterified lycoctonine-type C_{19} diterpene alkaloids. The compounds described from a Moroccan sample of *A. vulparia* subsp. *neapolitanum* contained 3 lycoctonine-type alkaloids, however, from a Spanish sample of the same subspecies, 4 lycoctonine- and 4 aconitine-type alkaloids were identified. One of them, lamarckinine, similarly to acovulparine (**AVU-7**) contains the *N*-azomethine group, which is very rare among the diterpene alkaloids. Lycoctonine (**AVU-6**) was detected in the Spanish and Swiss samples and by ourselves too. Delcosine (**AVU-3**) was also present in the Spanish sample.

Our results demonstrate that the alkaloid contents of these taxonomically very close (sub)species (frequently regarded as one species) are similar, and can be characterized mainly by the presence of lycoctonine-type alkaloids. However, the alkaloid composition depends on the habitat and genetic differences. To differentiate the phenotypically hardly distinguishable (sub)species or plants of different geographical origin on a chemotaxonomic basis, further studies are required.

The compounds isolated from *A. toxicum* indicate complex biogenetic pathways, since they belong in the groups of C_{20}, C_{19} and C_{18} diterpene alkaloids. The napelline-type C_{20} diterpene alkaloids [songorine (**AT-6**) and songoramine (**AT-7**)] are closely related to each other; songoramine (**AT-7**) can be regarded as the product of epoxy ring formation in songorine (**AT-6**). Aconitine (**AT-3**), acotoxinine (**AT-5**), neoline (**AT-8**) and neolinine (**AT-10**) are based on the aconitane skeleton. These aconitine-type C_{19} diterpene alkaloids differ only in the presence and positions of ester, methoxy and hydroxy groups. Neoline (**AT-8**) and neolinine (**AT-10**) are unesterified alkaloids, and putatively represent an early stage of biogenesis. Delavaconitine (**AT-1**) contains a benzoyl group, aconitine (**AT-3**) bears acetyl and benzoyl groups, whilst acotoxinine (**AT-5**) is esterified with veratric acid, a relatively rare esterifying agent of diterpene alkaloids. Delavaconitine (=13-β-hydroxy-14-benzoylaconosine) (**AT-1**), dolaconine (=14-acetylaconosine) (**AT-2**), aconosine (**AT-4**) and acotoxicine (=3-α-hydroxyaconosine) (**AT-9**) represent a series of biogenetically related C_{18} diterpene alkaloids. Dolaconine (**AT-2**) and aconosine (**AT-4**) contain only 4 oxygen functions, which is very rare among C_{18} diterpene alkaloids.

The C_{19} alkaloids isolated from *A. toxicum* are structurally analogous to takaosamine, the only diterpene alkaloid previously identified from the plant. Aconitine (**AT-3**), similarly as for many *Aconitum* species, is the main alkaloid of *A. toxicum*. The 4 C_{18} diterpene alkaloids are of great chemotaxonomical value, due to the relative scarcity of these types of compounds.

4.4. PHARMACOLOGICAL PERSPECTIVES

The compounds bearing a 2-[(4-methoxy-4-oxobutanoyl)amino]benzoyl ester side-chain [septentriodine (**AVU-1**), vulparine (**AVU-2**) and finetiadine (**AVU-5**)] are structurally closely related to the potent nicotinic acetylcholine receptor (nAChR) ligand methyllycaconitine; nevertheless, in the alkaloids isolated from *A. vulparia*, the succinyl group does not constitute a 5-membered ring through an imide bond formation. The effects of such compounds on the nAChRs have not yet been studied.

Anthranoyllycoctonine (**AVU-4**) has a similar constitution to that of lappaconitine, a potent antiarrhythmic drug currently on the market, and bears all the substituents related to the effect. The cardiovascular effects of anthranoyllycoctonine (**AVU-4**), a C_{19} diterpene alkaloid analogue of the antiarrhythmic C_{18} diterpene alkaloid *N*-deacetyllappaconitine has not yet been examined.

In recent years, it has been demonstrated that certain diterpene alkaloids exert a cytotoxic effect against different tumour cell lines. On the basis of these results, cytotoxic assays of the effects of septentriodine (**AVU-1**), vulparine (**AVU-2**), anthranoyllycoctonine (**AVU-4**) and finetiadine (**AVU-5**) on breast adenocarcinoma (MCF-7) and cervix adenocarcinoma (HeLa) were tested in co-operation with the Department of Pharmacodynamics and Biopharmacy, University of Szeged, using an MTT assay. Finetiadine (**AVU-5**) exhibited the highest cytotoxicity (39.48±3.42% and 25.59±1.87% inhibition on MCF-7 and HeLa cells, respectively, at 30 μg/ml). Septentriodine (**AVU-1**) and anthranoyllycoctonine (**AVU-4**) displayed marginal tumour cell inhibitory activity, while vulparine (**AVU-2**) was found to be inactive. The tested anthranoyl-substituted diterpene alkaloids possessed weak cytotoxic potencies against human tumour cells, and therefore the activities of further compounds were not investigated.

Previous studies on songorine (**AT-6**) suggested that this compound acts as a non-competitive antagonist at the $GABA_A$ receptor in rat hippocampal slices. As part of a research programme in co-operation with the Department of Experimental Zoology and Neurobiology, University of Pécs, the *in vivo* effects of the isolated compounds on neocortical, hippocampal and thalamic neurons of rats has been investigated. Songorine (**AT-6**) and various other bioactive compounds were administered simultaneously by means of microiontophoresis through a multi-barrrel electrode. The statistical analysis of 367 drug administration trials revealed that songorine (**AT-6**) exhibited a potent inhibitory effect on the spontaneous, maintained firing of the recorded neurons. Moreover, the songorine-elicited inhibition was successfully mimicked by GABA, and attenuated by the $GABA_A$ receptor antagonist picrotoxin. Overall, we found that songorine (**AT-6**) acts predominantly as a potent $GABA_A$ agonist, and may be a promising target for further pharmacological research. Interestingly, our *in vivo* experiments demonstrated an opposite effect of songorine to that reported earlier on the basis of *in vitro* measurements. The examination of further diterpene alkaloids in the same test system, and primarily songoramine (**AT-7**), a close structural relative of songorine (**AT-6**), is projected.

In light of the pharmacological properties of the diterpene alkaloids that have been elucidated so far, investigation of the cardiovascular and central nervous effects of the compounds and their mechanisms of action seems to be promising. The diverse, though in many features analogous structures of the compounds isolated from *A. vulparia* and *A. toxicum* offer the possibility of a deeper understanding of the structure-activity relationships.

ACKNOWLEDGEMENTS

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LIST OF PUBLICATIONS RELATED TO THE THESIS

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1. **D. Csupor**, P. Forgo, I. Máthé, J. Hohmann:
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