Bisnorditerpene, Norditerpene, and Lipo-alkaloids from *Aconitum toxicum*

Dezső Csupor,[†] Peter Forgo,[†] Eva Maria Wenzig,[‡] Rudolf Bauer,[‡] and Judit Hohmann*[,†]

Department of Pharmacognosy, University of Szeged, Eötvös u. 6, 6720 Szeged, Hungary, and Institute of Pharmaceutical Sciences, Department of Pharmacognosy, Karl-Franzens University Graz, Universitätsplatz 4, 8010 Graz, Austria

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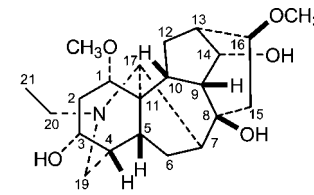
From the roots of *Aconitum toxicum*, acotoxicine (**1**), a new bisnorditerpene alkaloid, was isolated together with the structurally related delavaconitine, delavaconine, dolaconine, aconosine, and the norditerpene alkaloid neolinine. The structure of compound **1** was elucidated by spectroscopic data interpretation. Further, eight lipo-alkaloids (**2**−**9**) were detected in the plant material by LC-APCI-MS^n analysis. This is the first report of a C_{18} bisnorditerpene alkaloid and lipo-alkaloids from *A. toxicum*.

Aconitum species (Ranunculaceae) accumulate C_{20}, C_{19}, and C_{18} diterpene alkaloids. The C_{20} diterpene alkaloids are usually unesterified, but they sometimes occur in plants as monoesters of benzoic or acetic acid.[1] The C_{19} alkaloids can be divided into five main groups, depending on the degree of esterification and the esterifying agent, i.e., unesterified, monoester, diester, or triester diterpene alkaloids, and lipo-alkaloids. The C_{19} alkaloids are usually esterified with aromatic acids or acetic acid, with the exception that the lipo-alkaloids are esterified with fatty acids at C-8.[2−4] Members of the C_{18} bisnorditerpene alkaloid group are unesterified at C-4 or substituted with an ester group.[5]

The majority of the *Aconitum* alkaloids identified so far belong to the C_{20} and C_{19} monoester and diester diterpene alkaloid types. Physiologically, the most active compounds are the diester norditerpene alkaloids, which activate the voltage-gated Na^+ channel and therefore exert marked activities on the cardiovascular and central nervous systems. The monoester alkaloids deserve particular attention, since several such compounds block the voltage-gated Na^+ channel and therefore have antiarrhythmic activity.[6] The most active antiarrhythmic compounds are C_{18} bisnorditerpene alkaloids esterified with an acetylanthranilic or anthranilic acid at C-4 (e.g., lappaconitine).[7] Methyllycaconitine, a monoester norditerpene alkaloid, is one of the most selective antagonists of the brain α_7-type nAChRʼs identified so far.[8]

In Asia, the tubers and roots of *Aconitum* species are used as traditional medicines, characteristically after cautious processing in order to reduce their toxicity.[9] The role of lipo-alkaloids on the effect of traditional Chinese *Aconitum*-based medicines has not yet been studied thoroughly. These compounds are more stable than diester alkaloids, with the latter undergoing decomposition during the traditional processing of the roots. However, lipo-alkaloids can be detected in both crude and processed *Aconitum* roots.[10] The pharmacological interest in lipo-alkaloids is further enhanced by the fact that aconitine-type diester alkaloids can be converted to lipo-alkaloids by the human intestinal flora.[11]

We have described earlier the isolation of the norditerpene alkaloids acotoxinine, neoline, and aconitine, together with the C_{20} diterpene alkaloids songorine and songoramine, from *Aconitum toxicum* Rchb.[12] The present paper reports the isolation and structure determination of the bisnorditerpene alkaloids acotoxicine (**1**), delavaconitine, delavaconine, dolaconine, and aconosine, and an unesterified norditerpene alkaloid, neolinine, from the unprocessed roots of the plant. The structure of **1**, including its relative configuration, was elucidated by means of ^{1}H NMR, JMOD, ^{1}H−^{1}H COSY, NOESY, HMQC, and HMBC NMR and HRESIMS experiments. Also, eight lipo-alkaloids (**2**−**9**) were identified in the roots of *A. toxicum* using an LC-APCI-MS^n technique.



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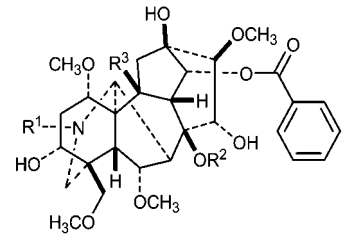

	R^1	R^2	R^3
2	CH_3	linn	H
3	CH_3	lin	H
4	CH_3	pal	H
5	CH_3	pen	H
6	CH_3	myr	H
7	C_2H_5	non	H
8	C_2H_5	lin	H
9	CH_3	lin	OH

pen: pentadecenoate; myr: myristate; non: nonadecenoate
linn: linolenate; lin: linoleate; pal: palmitate

The $CHCl_3$−MeOH extract of the roots of *A. toxicum* was subjected to multiple chromatographic purification steps, yielding the new alkaloid **1**, along with five known compounds.

Compound **1** was obtained as an amorphous solid, and its molecular formula was determined by HRMS as $C_{22}H_{35}NO_5$ with the M^+ ion at *m*/*z* 393.2512 (calcd *m*/*z* 393.2515). The ^{1}H NMR spectrum of **1** (Table 1) revealed the presence of an *N*-ethyl (δ_H 1.08 t (3H), 2.48 brm (2H)) and two methoxy groups (δ_H 3.27 s, 3.34 s). The ^{13}C NMR spectrum confirmed the above substituents and showed the presence of a C_{18} bisnorditerpene skeleton. Analysis of the HSQC spectrum led to the assignment of the protonated carbons: the bisnorditerpene core is composed of five methylenes (δ_C 27.3, 28.5, 35.2, 39.3, 43.8), four *O*-substituted (δ_C 70.7, 75.5, 82.1, 84.3) and seven alkyl-substituted methines (δ_C 38.1, 43.0, 44.2, 45.2, 45.8, 47.0, 62.9), and two quaternary carbons (δ_C 47.9,

* Corresponding author. Tel: +36-62-546453. Fax: +36-62-545704. E-mail: hohmann@pharm.u-szeged.hu.
[†] University of Szeged.
[‡] Karl-Franzens University Graz.

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Table 1. NMR Spectroscopic Data of Acotoxicine (**1**) [500 MHz (^{1}H), 125 MHz (^{13}C), $CDCl_3$, δ (ppm) (J = Hz)]

position	^{1}H	^{13}C	HMBC (C→H)	^{1}H−^{1}H COSY	NOESY
1	3.15 dt (9.9, 6.9)	84.3	OCH_3-1, 2, 10	2	2, 3, 5, 10
2	2.22 m (2H)	35.2	1	1, 3	1, 3, 1-OCH_3
3	3.70 brd (10.7)	70.7	2, 19a, 19b	2, 4	1, 2, 4, 5
4	1.74 brs	44.2	2, 6α, 6β, 19a, 19b	3, 5, 19b	3, 6β, 19a, 19b
5	1.79 m	43.0	6α, 6β, 17, 19a	4, 17[a], 6	1, 3
6α	2.21 m	28.5	17	6β, 5	17, 15α
6β	1.41 dd (14.3, 7.6)			6α	4
7	2.20 m	45.8	5, 6β, 15α	17	
8		73.1	6α, 6β, 7, 9, 10, 14, 15α, 15β		
9	2.28 m	47.0	7, 10, 12, 14, 15α	10, 14	14
10	1.67 dd (13.8, 8.3)	45.2	1, 5, 9, 17	9, 12	1, 14
11		47.9	1, 2, 6β, 6α, 7, 10, 12, 17		
12	1.83 m (2H)	27.3	9, 10	13	16, 1-OCH_3, 17
13	2.34 brs	38.1	9, 12, 15α	12, 14, 16	14, 16-OCH_3
14	4.15 t (4.0)	75.5	9, 12	9, 13, 16[a]	9, 10, 13
15α	2.43 dd (17.1, 8.6)	39.3	7, 9	15β, 16	16, 17
15β	2.07 d (17.1)			15α, 16	16-OCH_3
16	3.40 d (8.6)	82.1	12, 14, 15α, 15β, OCH_3-16	13, 14[a], 15α, 15β	12, 15α
17	3.08 s	62.9	1, 5, 6α, 7, 10, 19a, 20	5[a], 7	6α, 12, 16, 15α, 20, 21
19a	2.99 d (12.0)	43.8	3, 17	19b	4, 21
19b	2.30 m			19a, 4	4
20	2.48 brm (2H)	49.6	17, 19b, 21	21	17, 21
21	1.08 t (6.9)	13.5	20	20	17, 20, 19a
OCH_3-1	3.27 s	56.4	1		2, 12
OCH_3-16	3.34 s	56.4	16		13, 16, 15β

[a] $^4J_{H,H}$ couplings.

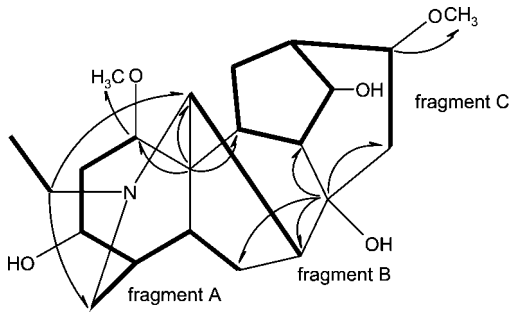

Figure 1. Selected ^{1}H−^{1}H COSY (—) and HMBC (C→H) correlations for acotoxicine (**1**).

73.1). Analysis of the proton−proton connectivities in the ^{1}H−^{1}H COSY spectrum (Table 1) resulted in the identification of three structural fragments [−CH(OR)−CH_2−CH(OR)−C(CH_2)−CH−CH_2− (fragment A), −CH−CH− (fragment B), and −CH(OR)−CH−CH−CH_2−CH−CH(OR)−CH_2− (fragment C) (R = H or CH_3] and one ethyl group (Figure 1). The long-range HMBC correlations of the structural elements and quaternary carbons C-8 and C-11 allowed identification of the bisnorditerpene core. The HMBC correlations between C-11 and H-1, H-10, and H-17 and between C-8 and H-6a, H-6b, H-7, H-9, and H-15 were highly informative during the structure elucidation process (Figure 1). Further, this spectrum provided information on the positions of the methoxy groups in **1**. The long-range correlations of the carbon atoms of the skeleton at δ_C 84.3 (C-1) and 82.1 (C-16) with the protons at δ_H 3.27 and 3.34 (both 3H) revealed the positions of two methoxy groups on C-1 and C-16. In view of the molecular formula of **1** and the ^{13}C NMR chemical shifts of C-3 (δ_C 70.7), C-8 (δ_C 73.1), and C-14 (δ_C 75.5), the presence of two secondary and one tertiary hydroxy group was concluded, situated at C-3, C-8, and C-14. The correlation of the methylene carbon at δ_C 49.6 (C-20) with the protons at δ_H 3.08 (H-17) and 2.30 (H-19b) confirmed the position of the *N*-ethyl group. The relative configurations of the stereogenic centers were studied by means of NOESY experiments (Table 1). As a reference point, the configuration of H-5 was considered to be β, as is characteristic for C_{19} and C_{18} diterpene alkaloids.[3] In the NOESY spectrum, Overhauser effects were detected between H-5 and H-3; H-3 and H-1; H-3 and H-4; H-4 and H-6β; H-1 and H-10; H-10 and H-14; H-14 and H-9; and H-14 and H-13, indicating the β-orientation of these protons. The NOESY cross-peaks between H-6α and H-15α; H-15α and H-16; and H-16 and H-17 confirmed the α-orientation of the listed protons. As a result of the above NMR study, the structure of compound **1** was established as shown. This compound is a new natural product, for which the trivial name acotoxicine was given.

By the comparison of their spectroscopic data with literature data, five known alkaloids also isolated in this study were identified as delavaconitine,[13] delavaconine,[13] dolaconine,[13] aconosine,[13] and neolinine.[14] For the last four of these compounds, complete and unambiguous assignments of the chemical shifts of all protons and carbons were achieved. The previously published ^{1}H NMR data[13,14] were supplemented (Table S1, Supporting Information). Further, the results of 2D NMR studies showed that the previously published assignments of the ^{13}C NMR signals of C-10 and C-13 must be reversed for neolinine, as listed in the Supporting Information (S1).

In the lipophilic fractions of *A. toxicum*, further alkaloid-type compounds were detected, but their very low quantities in the plant material meant that their preparative isolation was not successful. Previous studies have indicated that lipo-alkaloids can be identified by ESIMS on the basis of their protonated molecular ions $[M + H]^+$ and their fragmentation patterns.[10,15−17] In the course of our investigations, we analyzed the MeOH extract of the roots of *A. toxicum*, applying an APCI-MS^n method in the positive-ion mode. In the APCI full MS chromatogram of the extract, the ions observed in the range *m/z* 800−900 corresponded to the protonated lipo-alkaloid molecules. These compounds were detected with retention times of 35−40 min. Further, MS^n analysis provided diagnostic information facilitating the determination of the structures of these alkaloids. Lipo-alkaloids identified to date from *Aconitum* species are esterified by long-chain fatty acids at C-8.[2] Fatty acids are relatively easily eliminated by electrospray or chemical ionization. C-8 is the active site in the molecules, and therefore the major fragment ion in the MS^2 spectra of these alkaloids is $[M + H - RCOOH]^+$.[16] The neutral loss denotes the fatty acid; the major fragment ion determines the diterpene core of the molecule (Table 2). The MS^3 spectra of the daughter ions at *m/z* 572, 586, and 588

Table 2. Lipo-alkaloids Identified by LCMS in the Roots of *A. toxicum*

compound	parent ion $[M + H]^+$ (*m*/*z*)	neutral loss (Da)	daughter ion $[M + H]^+$ (*m*/*z*)
14-benzoylmesaconine-8-linolenate (**2**)	850	278	572
14-benzoylmesaconine-8-linoleate (**3**)	852	280	572
14-benzoylmesaconine-8-palmitate (**4**)	828	256	572
14-benzoylmesaconine-8-pentadecenoate (**5**)	812	240	572
14-benzoylmesaconine-8-myristate (**6**)	800	228	572
14-benzoylaconine-8-nonadecenoate (**7**)	882	296	586
14-benzoylaconine-8-linoleate (**8**)	866	280	586
10-hydroxy-14-benzoylmesaconine-8-linoleate (**9**)	868	280	588

are identical to those in the literature and indicate a 14-benzoylmesaconine, a 14-benzoylaconine, and a 10-hydroxy-14-benzoylmesaconine structure in the molecules, respectively.[10,16] The major fragment ions at *m*/*z* 512, 526, and 528, respectively, in the MS^3 spectra result from the loss of CO plus CH_3OH.[17] Our investigations therefore identified eight lipo-alkaloids from the roots of *A. toxicum*: 14-benzoylmesaconine-8-linolenate (**2**), 14-benzoylmesaconine-8-linoleate (**3**), 14-benzoylmesaconine-8-palmitate (**4**), 14-benzoylmesaconine-8-pentadecenoate (**5**), 14-benzoylmesaconine-8-myristate (**6**), 14-benzoylaconine-8-nonadecenoate (**7**), 14-benzoylaconine-8-linoleate (**8**), and 10-hydroxy-14-benzoylmesaconine-8-linoleate (**9**).

The lipo-alkaloids, the diterpene alkaloid **1**, and five known compounds were identified for the first time in *A. toxicum*. Acotoxicine (**1**), delavaconine, aconosine, and neolinine are unesterified alkaloids and are putative products of an early stage of biogenesis. Dolaconine and aconosine contain only four oxygen functions, which is very rare among C_{18} diterpene alkaloids. The bisnorditerpene alkaloids, representing a series of biogenetically related compounds, are of chemotaxonomic value, in view of the relative scarcity of these types of compounds. For the identification of lipo-alkaloids, the APCI-MS^n technique was applied for the first time. In contrast with the alkaloids isolated from the plant here, the lipo-alkaloids **2**−**6** and **9** contain an *N*-methyl group instead of an *N*-ethyl. This fact, together with the presence of a hydroxy group at C-10 in **9**, illustrates the rich structural diversity of lipo-alkaloids.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Perkin-Elmer 341 polarimeter. NMR spectra were recorded in $CDCl_3$ on a Bruker Avance DRX 500 spectrometer at 500 MHz (^1H) or 125 MHz (^{13}C); the signals of the deuterated solvent were taken as the reference. 2D data were acquired and processed with standard Bruker software. In the $^1H-^1H$ COSY, NOESY, HSQC, and HMBC experiments, gradient-enhanced versions were used. HRMS measurements were performed on a VG ZAB SEQ hybrid mass spectrometer equipped with a Cs SIMS ion source. The resolution was 10 000. Samples were dissolved in MeOH, and this solution was measured by using glycerol as matrix. The glycerol cluster peak at *m*/*z* 645 was used in peak-matching experiments. For column chromatography, polyamide (ICN Polyamide for Column Chromatography) and Al_2O_3 (Aluminiumoxid neutral, Brockmann II, Reanal) were used. Preparative TLC was carried out on 20 × 20 cm silica gel (Kieselgel 60 F_{254}, Merck) plates. Compounds were eluted from the scraped adsorbent with $CHCl_3$−MeOH (9:1). Centrifugal planar chromatography (CPC) was performed with a Chromatotron (model 8924, Harrison Research) on manually prepared silica gel layers, 2 mm in thickness (Kieselgel 60 GF_{254}, Merck), and Al_2O_3 (Aluminiumoxid G (Typ E), Merck) plates. Chromatographic fractions were monitored by TLC on silica gel (Merck 5715) and visualized by spraying with Dragendorff reagent or concentrated H_2SO_4, followed by heating.

Plant Material. The roots of *Aconitum toxicum* were gathered near Maroshévíz (Romania) in August 2002 and were identified by Károly Csedő (University of Medicine and Pharmacy Târgu Mureş, Târgu Mureş, Romania). The roots of the plants were dried, ground, and stored at room temperature until processed. A voucher specimen (No. 655) has been deposited in the Herbarium of the Department of Pharmacognosy, University of Szeged, Szeged, Hungary.

Extraction and Isolation. The crushed, dry roots (950 g) were extracted with a mixture of $CHCl_3$−MeOH 9:1 (15 L) at room temperature. After evaporation, the oily residue was purified via polyamide column chromatography (CC I), with 20% and 40% MeOH as eluents. Elution with 20% MeOH resulted in a fraction rich in alkaloids; hence, this fraction was further processed after evaporation. The dry residue was subjected to column chromatography on Al_2O_3 (CC II), with mixtures of cyclohexane−$CHCl_3$−MeOH of increasing polarity as eluents.

The fractions from CC II eluted with cyclohexane−$CHCl_3$ (5:2) were subjected to multiple step CPC on Al_2O_3 and silica gel, using a gradient eluent system of cyclohexane−EtOAc−EtOH, which afforded aconosine (15.0 mg). Subsequent preparative TLC (silica gel; mobile phase: toluene−acetone−EtOH−conc. NH_3, 80:20:7:3) of the CPC fractions resulted in the isolation of pure delavaconitine (25.3 mg) and dolaconine (30.0 mg). The fractions eluted from CC II with cyclohexane−$CHCl_3$ (5:3) were purified by CPC on silica gel (mobile phase: cyclohexane−EtOAc−EtOH gradient), followed by preparative TLC (silica gel; mobile phase: toluene−acetone−EtOH−conc. NH_3, 70:50:16:4.5), which resulted in the isolation of acotoxicine (8.2 mg). The fractions from CC II eluted with cyclohexane−$CHCl_3$−MeOH (50:30:1) were subjected to CPC on a silica gel stationary phase. Gradient elution was carried out by using cyclohexane−EtOAc−EtOH mixtures with increasing polarity. The fractions rich in alkaloids were purified via preparative TLC (silica gel; mobile phase: toluene−acetone−EtOH−conc. NH_3, 70:50:16:4.5), which resulted in the isolation of delavaconine (2.0 mg) and neolinine (15.1 mg).

Acotoxicine (1): amorphous solid; $[\alpha]_D^{26.5}$ +7 (*c* 0.05, $CHCl_3$); ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 393.2512 (M^+ calcd for $C_{22}H_{35}NO_5$, 393.2515).

LC-APCI-MS^n Analysis of Lipo-alkaloids. Milled *A. toxicum* roots (1.0 g) were extracted at room temperature three times (15 min each) in an ultrasonic bath with 3 × 20 mL of MeOH. The extracts were filtered, concentrated, and diluted to 2 mL. From the extract, 20 μL was injected. Chromatographic separation was performed on an Agilent Zorbax Eclipse XDB-C8 (4.6 × 150 mm, 5 μm) column using a gradient system of a 10 mM NH_4OAc buffer (pH 8.9) and MeOH (0 min: buffer−MeOH, 60:40; 35 min: buffer−MeOH, 5:95; 36 min: buffer−MeOH, 5:95; 42 min: buffer−MeOH, 60:40; 45 min: buffer−MeOH, 60:40) at a flow rate of 1 mL/min. LC-MS analysis was carried out on a Thermo Finnigan Surveyor liquid chromatograph interfaced with an LCQ Deca XP^{PLUS} mass detector in the APCI positive mode. The vaporizer temperature was 400 °C, the capillary temperature 350 °C, the discharge current 5 μA, the capillary voltage 25 V, and the tube lens offset −15 V. The sheath gas flow was 55, and the auxiliary gas flow 15 arbitrary units.

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Supporting Information Available: Physical and NMR spectroscopic data for delavaconitine, delavaconine, dolaconine, aconosine, and neolinine. This information is available free of charge via the Internet at http://pubs.acs.org.

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