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Alkaloids of *Aconitum laeve* and their anti-inflammatory, antioxidant and tyrosinase inhibition activities

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Dedicated to Prof. Dr. Kurt Hostettmann on the occasion of his 60th birthday

Abstract

A lycoctonine-type norditerpenoid alkaloid, swatinine (**1**), along with four known norditerpenoid alkaloids, delphatine (**3**), lappaconitine (**4**), puberanine (**5**), and *N*-acetylsepaconitine (**6**), and were isolated from the aerial parts of *Aconitum laeve* Royle. Compound **2** has been isolated for the first time from a natural source. The structure of compound **1** was deduced on the basis of spectral data. The anti-inflammatory, antioxidant and tyrosinase inhibition studies on all six compounds have also been carried out.

Keywords: *Aconitum laeve*; Norditerpenoid alkaloids; Swatinine; Anti-inflammatory; Anti-oxidant; Tyrosinase inhibitor

1. Introduction

Plants of the genus *Aconitum* are a rich source of diterpenoid alkaloids, many of which exhibit a broad spectrum of activities. Some aconitine and mesaconitine derivatives possess potent analgesic and anti-inflammatory activities (Muroyama and Mori, 1993). Lycaconitine, obtained from several *Aconitum* species, was found to be effective against multi-drug resistant cancers (Kim et al., 1998). Previously, we have reported many diterpenoid and norditerpenoid alkaloids from *Aconitum* and *Delphinium* species (Atta-ur-Rahman et al., 1997, 2000). In the present paper, we describe the isolation and structure elucidation of a new norditerpenoid alkaloid, swatinine (**1**) and a benzene derivative 4-[2-(methoxycarbonyl) anilino]-4-oxobutanoic acid (**2**), along with four known alkaloids, delphatine (**3**), lappaconitine (**4**), puberanine (**5**), and *N*-acetylsepaconintine (**6**) from *Aconitum laeve* Royle. Compound **2** has been isolated for the first time from a natural source although it has previously been reported as a synthetic precursor of some heterocyclic compounds (Balasubramaniyan and Argade, 1988) as a substituent in many norditerpenoid alkaloids. The anti-inflammatory, anti-oxidant and tyrosinase inhibition studies on all isolated compounds were also carried out.

Neutrophils are essential elements for the host defense. The uncontrolled release of reactive oxygen species (ROS) is suspected to be responsible for certain pathological conditions such as heart attacks, septic shocks, rheumatoid arthritis and ischemia reperfusion injury (Bagchi et al., 1997). In these cases the administration of agents that can decrease the neutrophils accumulation

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in the inflamed area might be a remedy for these conditions. A cell-based in vitro bioassay (Tan and Berridge, 2000) was used in this study to examine the anti-inflammatory activity of compounds isolated from *A. laeve*.

Free radicals play an important role in carcinogenesis through their involvement in breaking of DNA strands (Pathak and Joshi, 1983). They are known to be involved in inflammation processes, cardiovascular disease (Hertog et al., 1993; Moure et al., 2001 and Hollman and Katan, 1999), rheumatoid arthritis, neurodegenerative disease and the aging process (Meyer et al., 1998 and Hunt et al., 2001). The harmful actions of free radicals can be blocked by anti-oxidants. During this study, the diterpenoid alkaloids isolated from *A. laeve* have been tested for their anti-oxidant activites in the present study.

Tyrosinase (EC 1.14.18.1), also known as polyphenol oxidase (PPO), is a multifunctional copper-containing enzyme widely distributed in plants and animals. Tyrosinase inhibitors may therefore be clinically useful for the treatment of some dermatological disorders associated with melanin hyperpigmentation and also important in cosmetics for whitening and depigmentation after sunburn (Shiino et al., 2001).

2. Results and discussion

The alkaloidal constituents of the aerial parts of *A. laeve* Royle, collected in flowering period (August 2001) from Loweri top near Ziarat village, Chitral district, NWFP Province of Pakistan, were studied. The known alkaloids, delphatine (Pelletier et al., 1980), lappaconitine (Ulubelen et al., 2002), puberanine (Yu quan De and Das, 1983), *N*-acetylsepaconitine (Tel'nov et al., 1988), a new lycoctonine-type norditerpenoid alkaloid swatinine (**1**) and a benzene derivative 4-[2-(methoxycarbonyl)anilino]-4-oxobutanoic acid (**2**) have been isolated from this plant.

In an early study, lappaconitine, lycaconitine, lapaconidine, lycoctonine, 14-demethyllycaconitine, and *N*-deethyllycaconitine-*N*-aldehyde have been isolated from this plant (Ulubelen et al., 2002).

Swatinine (**1**), an amorphous compound, had a molecular formula of $C_{25}H_{41}NO_8$ (*m/z* 483.2827, calcd. 483.2831) in the HREIMS. The IR spectrum of **1** showed absorption bands at 3492 (OH groups), and 1083 (simple ether bonds). The mass fragmentation of **1** was characteristic of alkaloids with a lycoctonine skeleton. The base peak was that of the $(M^+ - 31)$ ion which indicative of the presence of an α-methoxy group at C–1 (Nishanov et al., 1992).

The ^{1}H NMR spectrum of swatinine (**1**) exhibited signals for *N*-ethyl, four methoxy groups and several methine protons with geminal oxygen substituents. The overall spectral data of swatinine (**1**) was similar to that of lycoctonine (Atta-ur-Rahman, 1990), with an additional hydroxyl group at the C-10 position. The signal of H-14β in the ^{1}H NMR spectrum of swatinine (**1**) was shifted downfield by 0.34 ppm in comparison to lycoctonine, which indicated the presence of a hydroxyl group at C-10 (Sultankhodzhaev et al., 1980). The ^{13}C NMR spectrum (BB, DEPT) (Table 1) showed 25 signals, including one methyl, four methoxy, seven methylene, eight methine and five quaternary carbons. The ^{1}H–^{13}C correlations were determined by HMQC spectrum, while the long-range ^{1}H–^{13}C connectivities were obtained through the HMBC technique (Fig. 1). H-5 (δ 1.99) showed ^3J correlation with C-10 (δ 81.2), whereas H-9 (δ 2.69) and H-17 (δ 2.59) also exhibited ^2J correlations with C-10 thus confirming the presence of a hydroxyl group at C-10. Thus the structure of compound **1** was deduced as 7β,8β,10β,18-tetrahydroxy-1α,6β,14α,16β-tetramethoxy *N*-ethyl aconitane.

Compound **2** was obtained as an amorphous material, with a molecular formula of $C_{12}H_{13}NO_5$ by HREI-MS (*m/z* 251.0581, calcd. 251.0574). The IR spectrum of **2** showed absorption bands at 1693 (C=O), 1590, 1447 (aromatic) and 1090 (C–O–C). The ^{1}H NMR spectrum exhibited signals at δ 3.90 (3H, *s*) for methoxy, 2.76 (4H, *m*) for four methylene protons, δ 7.06–8.64 for four aromatic protons and δ 11.16 (1H, br. *s*) for an NH proton. The ^{13}C NMR spectrum (BB, DEPT) (Table 2) of compound **2** showed 12 signals, including one methoxy, two methylene, four methine and five quaternary carbons. The ^{1}H–^{13}C correlations were obtained with the help of an HMQC spectrum, while the long-range connectivities were determined

Table 1
^{13}C NMR data of swatinine (**1**) and lycoctonine (**7**) in $CDCl_3$

C. no.	Swatinine (**1**)	Lycoctonine (**7**)
1	77.4	84.2
2	25.9	26.1
3	31.2	31.6
4	38.3	38.6
5	45.3	43.3
6	91.1	90.6
7	87.6	88.3
8	75.7	77.5
9	53.6	49.7
10	81.2	38.0
11	54.4	48.9
12	39.2	28.8
13	38.0	46.1
14	82.0	84.0
15	34.6	33.7
16	82.4	82.7
17	65.1	64.8
18	67.7	67.6
19	52.6	52.9
N-CH_2	51.1	51.1
CH_3	14.0	14.1
OCH_3	55.5	55.7
OCH_3	58.0	58.0
OCH_3	57.8	57.7
OCH_3	56.1	56.2

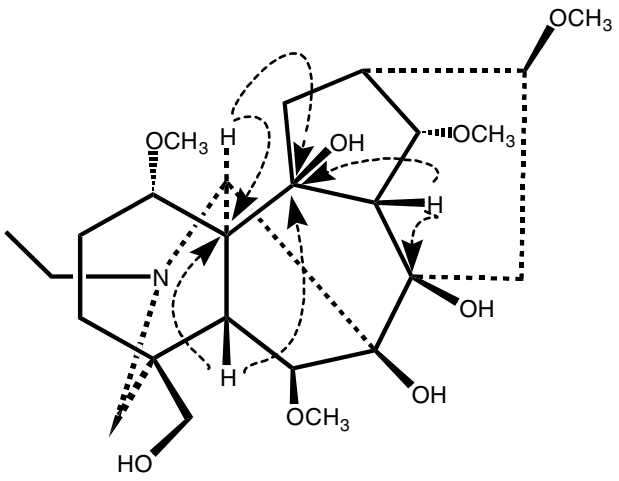


Fig. 1. Key HMBC interactions in compound **1**.

through an HMBC spectrum (Fig. 2). On the basis of spectral data, the structure of compound **2** was deduced as 4-[2-(methoxycarbonyl) anilino]-4-oxobutanoic acid which has been found as a substituent in many norditerpenoid alkaloids (Pelletier et al., 1979, 1986).

Anti-inflammatory activities of all alkaloids have also been determined in an in vitro assay. Only compounds **4** and **5** exhibited significant anti-inflammatory activity (Table 3).

Tyrosinase inhibition studies on all alkaloids (**1**, **3**–**6**) have been carried. Only compounds **4** and **5** exhibited mild inhibition against the enzyme (Table 4). The IC_{50} values of compounds lappaconitine (**4**) and puberanine (**5**) were found to be 93.33 and 205.21 μM, respectively, whereas the reference L-mimosine (IC_{50} = 3.68 μM) and kojic acid (IC_{50} = 16.67 μM) were used as reference compounds.

The proton-radical scavenging action is known as an important mechanism of anti-oxidation. Compounds **1**–**6** were screened for their anti-oxidant activities using DPPH radicals (Table 5). Among all the diterpenoidal alkaloids screened, only swatinine (**1**) (54.1%) and delphatine (**3**) (55.4%) scavenged DPPH radicals, while standard anti-oxidant BHA was active at a level of 92.1% at 1 mM.

Table 2
^{13}C NMR data of compound **2** in $CDCl_3$

C. no.	Chemical shift	Multiplicity
1′	114.9	C
2′	141.2	C
3′	120.4	CH
4′	134.6	CH
5′	122.6	CH
6′	130.8	CH
1	176.6	C
2	32.4	CH_2
3	29.1	CH_2
4	170.4	C
–C(=O)-OCH3	168.7	C
–C(=O)-OCH3	52.4	CH_3

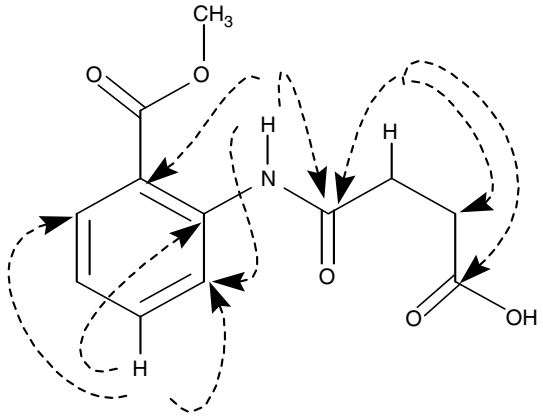
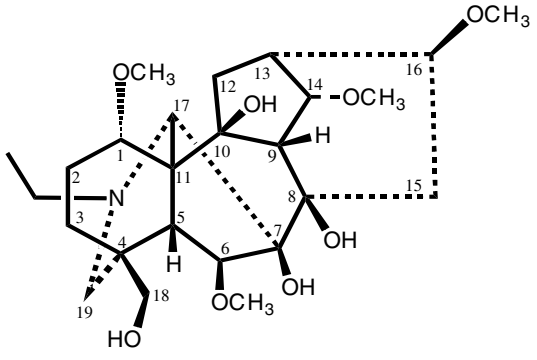


Fig. 2. Key HMBC interactions in compound **2**.

3. Experimental

3.1. Equipment

Optical rotations were measured on a JASCO DIP 360 polarimeter. IR spectra were recorded on a JASCO 302-A spectrophotometer. EI-MS and HREI-MS were recorded on JMS HX 110 with a data system and on



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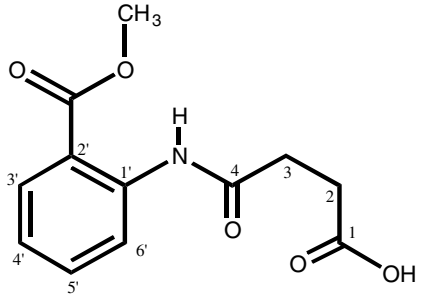

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Table 3
Anti-inflammatory activities of compounds **1–6**

Compound	% Inhibition (at 100 μg/mL)
Swatinine (**1**)	22.82
4-[2-(Methoxycarbonyl) anilino]-4-oxobutanoic acid (2)	ND
Delphatine (**3**)	17.39
Lappaconitine(**4**)	29.34
Puberanine (**5**)	33.69
N-Acetylsepaconitine (**6**)	25.00
Indomethacin	42.02

Table 4
Tyrosinase inhibitory activities of the compounds **1–6**

Compound	IC_{50} (mean ± SEM[a]) (in μM)
Swatinine (**1**)	NA[c]
4-[2-(Methoxycarbonyl) anilino]-4-oxo butanoic acid (**2**)	ND[d]
Delphatine (**3**)	NA[c]
Lappaconitine (**4**)	93.33 ± 0.1638
Puberanine (**5**)	205.21 ± 0.19645
N-Acetylsepaconitine (**6**)	NA[c]
Kojic acid[b]	16.67 ± 0.5190
L-Mimosine[b]	3.68 ± 0.02234

[a] SEM is the standard error of the mean.
[b] The standard inhibitors (KA and LM) of the enzyme tyrosinase.
[c] Is not active against Tyrosinase.
[d] Not done due to insufficient quantities.

Table 5
Anti-oxidant activities of the compounds **1–6**

Compounds	DPPH radical % scavenging activity (at 1 mM)
Swatinine (**1**)	54.1
4-[2-(Methoxycarbonyl) anilino]-4-oxobutanoic acid (**2**)	13.4
Delphatine (**3**)	55.4
Lappaconitine (**4**)	12.0
Puberanine (**5**)	12.2
N-Acetylsepaconitine (**6**)	38.1
3-*t*-Butyl-4-Hydroxyanisole	92.5

JMS-DA 500 mass spectrometers. The ^{1}H- and ^{13}C NMR spectra were recorded on Bruker NMR spectrometers operating at 500 and 400 MHz, (100 and 125 MHz). The chemical shifts values are reported in ppm (δ) units and the coupling constants (J) are given in Hz.

3.2. Chromatographic conditions

For TLC, precoated aluminium sheets (silica gel 60F-254, E. Merck) were used. Visualization of the TLC plates was achieved under UV light at 254 and 366 nm and by spraying with the Dragendorff's reagent. Solvent system *n*-hexane–acetone–diethylamine (7:3:5 drops) was used.

3.3. Plant material

The aerial parts (5 kg dry wt) of *A. laeve* Royle were collected from Loweri top, near Ziarat village of the Chitral district of Pakistan at an elevation of 1200 m in August 2001 and identified by Mr. Habib Ahmad (Assistant Professor) Department of Botany, Jahan Zeb Post Graduate College, Saidu Sharif, Swat, NWFP, Pakistan. The voucher specimen (RA-01) is deposited in the herbarium of the Botany Department.

3.4. Extraction and isolation

The air-dried powdered plant material (5 kg) was first exhaustively extracted with *n*-hexane and 11.5 g of gummy residue was obtained. The remaining plant material was extracted with 90% ethanol. The ethanolic extract was concentrated and acidified with 0.5 N H_2SO_4 and extracted with $CHCl_3$. The acidic aqueous solution was basified with 10% KOH solution and extracted with $CHCl_3$ to obtain 10.3 g of a crude alkaloidal mixture. This crude alkaloidal mixture was fractionated by VLC using a column packed with basic alumina (200 g Al_2O_3 EM 1085) and eluted with petroleum ether, and gradients of CH_2Cl_2 and MeOH. A fraction obtained on elution with 2% MeOH–CH_2Cl_2 was fractionated further by silica gel column chromatography, using an isocratic solvent system of 5% acetone in *n*-hexane with 5-drops of diethyl amine per 100 mL. Fractions 5–14 (0.6 g) were combined and again subjected to column chromatography to obtain delphatine (15 mg), lappaconitine (25 mg), and puberanine (33 mg). Fractions 15–30 (1 g) contained swatinine (**1**) (23 mg), ranaconitine (16 mg), and compound **2** (10 mg). The known compounds were identified by comparing their spectral data and physical constants with the reported values.

3.4.1. Swatinine (*1*)

Amorphous powder (23 mg); $[\alpha]_D^{30}$ 12.5 ($c = 2$, $CHCl_3$); IR ν_{max} $CHCl_3$ 3492 (OH), 1083 (simple ether bonds); ^{1}H NMR (500 MHz, DMSO): δ 3.92 (1H, *t*, $J = 4.5$ Hz, H-14), 3.85 (1H, *br. s*, H-6), 3.51 (1H, *t*, $J = 8.3$ Hz, H-1), 2.97 (1H, *t*, $J = 8.0$ Hz, H-16), 2.69 (1H, *d*, $J = 4.6$ Hz, H-9), 2.59 (1H, *s*, H-17), 1.99 (1H, *br*, *s*, H-5), 3.32 (3H, *s*, OMe), 3.22 (3H, *s*, OMe), 3.13 (3H, *s*, OMe), 3.15 (3H, *s*, OMe). ^{13}C NMR (Table 1); EI-MS; (rel. int.) *m/z*: 483 $[M]^+$ (3), 467 $[M\text{-}Me]^+$ (9), 452 $[M^+\text{-}Me\text{-}OMe]^+$ (49), 359 (6), 238 (2), 164 (7), 85 (33), 58 (100). HREI-MS: 483.2827 $C_{25}H_{41}NO_8$ (calcd. 483.2832).

3.4.2. 4-[2-(Methoxycarbonyl)anilino]-4-oxobutanoic acid (*2*)

Amorphous powder (10 mg), IR ν_{max} $CHCl_3$ 1693 cm^{-1} (C=O), 1590, 1447 cm^{-1} (aromatic), 1090 cm^{-1} (simple ether bonds); ^{1}H NMR (400 MHz,

$CHCl_3$): δ 3.90 (3H, *br. s*, OCH_3), 2.76 (4H, *br. s*, H-2′,H-3′), 7.06 (1H, *t*, $J = 7.5$ Hz, H-5), 7.52 (1H, *t*, $J = 7.4$ Hz, H-4), 7.96 (1H, *d*, $J = 7.9$ Hz, H-6), 8.64 (1H, *d*, $J = 8.4$ Hz, H-3). ^{13}C NMR (Table 2); EI-MS; (rel. int.) *m/z*: 251 $[M]^+$ (10.10), 202 (2.13), 174 (5.21), 151 (52.43), 119 (56.53), 73 (43.67), 58 (100). HREI-MS: *m/z* 251.0581 ($C_{12}H_{13}NO_5$, calcd. 251.0574).

3.5. Tyrosinase inhibition assay

Tyrosinase inhibition assay was performed in a 96-well microplate format using a SpectraMax 340 microplate reader (Molecular Devices, CA, USA) according to the method developed by Hearing (1987). Briefly, first the compounds were screened for the *o*-diphenolase inhibitory activity of tyrosinase using L-DOPA as substrate. All the active inhibitors from the preliminary screening were subjected to IC_{50} studies. Compounds were dissolved in methanol to a concentration of 2.5%. Thirty units of mushroom tyrosinase (28 nM from Sigma Chemical Co., USA) was first pre incubated with the test compounds in 50 nM Na-phosphate buffer (pH 6.8) for 10 min at 25 °C. Then the L-DOPA (0.5 mM) was added to the reaction mixture and the enzyme reaction was monitored by measuring the change in absorbance at 475 nm (at 37 °C) due to the formation of the DOPAchrome for 10 min. The percent inhibition of the enzyme was calculated as follows, by using MS Excel®™ 2000 (Microsoft Corp., USA) based program developed for this purpose:

$$\text{Percent inhibition} = [B - S/B] \times 100.$$

Here the B and S are the absorbances for the blank and samples, respectively. After screening of the compounds, median inhibitory concentrations (IC_{50}) were also calculated. All the studies have been carried out at least in triplicates and the results represents the mean ± SEM (standard error of the mean). Kojic acid and L-mimosine were used as standard inhibitors for the tyrosinase and were purchased from Sigma Chem. Co., USA.

3.6. Anti-oxidant assays: DPPH (1,1-diphenyl-2-picryl hydrazyl) free radical scavenging activity

The reaction mixture containing 5 μL of test sample (1 mM in DMSO) and 95 μL of DPPH (Sigma, 300 μM) in ethanol was taken in a 96-well microtiter plate and incubated in Elisa (multiple reader spectra Max-3400) at 37 °C for 30 min. The absorbance was measured at 515 nm. Percent radical scavenging activity was determined by comparison with a DMSO containing control (Table 5). IC_{50} values represent concentration of compounds to scavenge 50% of DPPH radicals. BHA (3-*t*-butyl-4-hydroxyanisole) was used as a positive control. All the chemicals used were of analytical grade (Sigma, USA).

3.7. Anti-inflammatory assay

Heparinized fresh venous blood was drawn from healthy volunteers in a local blood bank and neutrophils were isolated by the method of Siddiqui et al. (1995) (Daeseok et al., 1995). Briefly whole blood was mixed with Ficoll or Dextran (6%) with the ratio of 1:3 and allowed to settle down. Buffy coat was collected and a layer on the bed of the Ficoll (3 mL) was centrifuged at 1500 rpm for 30 min. The pellets were collected and washed with PBS Buffer (pH 7.4). The RBCs were lysed with ammonium chloride solution and then centrifuged. The pellets were then washed with PBS and resuspended with the same buffer at the concentration of 1×10^6 cells/mL.

To determine anti-inflammatory activity of a compound, the modified assay of Berridge et al. was used (Tan and Berridge, 2000). This in vitro assay is based on the reduction of a highly water soluble tetrazolium salt WST-1 in presence of activated neutrophils. Anti-inflammatory activity was determined in a total volume of 200 μL PBS (pH 7.4) containing $0.5–1.0 \times 10^4$ neutrophils/mL, 750 μM WST-1, and various concentrations of test compounds. The control only contained buffer, neutrophils and WST-1. All compounds were equilibrated at 37 °C for 10 min and the reaction was initiated by adding Zymosan Activated Serum (ZAS), prepared as described previously (Daeseok et al., 1995). Indomethacin was used as positive control. Absorbance was measured at 450 nm using a SpectraMAX 340 microplate reader for 30 min. Each value is the mean of reactions in six wells for a single compound placed in a 96-well plate. The IC_{50} was calculated by comparing with DMSO as a blank and expressed as % inhibition of the superoxide produced.

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