NPC Natural Product Communications

2008
Vol. 3
No. 12
1985 - 1989

Diterpenoid Alkaloids and Phenol Glycosides from *Aconitum naviculare* (Brühl) Stapf.

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Received: June 20th, 2008; Accepted: October 28th, 2008

Phytochemical investigation of the aerial parts of *Aconitum naviculare*, a medicinal plant used in traditional Nepalese medicine, led to the isolation and characterization of two new diterpenoid alkaloids, navirine B (**1**), and navirine C (**2**), along with (+) chellespontine (**3**), kaempferol-7-*O*-β-D-glucopyranosyl(1→3)α-L-rhamnopyranoside (**4**), kaempferol-7-*O* α-L-rhamnopyranoside,3-*O*-β-D-glucopyranoside (**5**), *p*-coumaric-4-*O*-β-D-glucopyranoside acid (**6**), and ferulic-4-*O*-β-D-glucopyranoside acid (**7**). The structures of the isolated compounds were elucidated on the basis of extensive analyses of 1D and 2D NMR spectra (HMQC, HMBC, COSY, ROESY) and HR-MS data. The antiproliferative activity of alkaloids **1**-**3** against human tumor cell lines (LoVo and 2008) was also evaluated.

Keywords: *Aconitum naviculare*, diterpenoid alkaloids, antiproliferative activity, traditional medicine.

Aconitum species are well-known for their contents of C_{19} and C_{20} diterpenoid alkaloids. Some of these (e.g., aconitine) are highly toxic and some exhibit powerful biological activities, for example antiarrhythmic [1,2], analgesic [2], antiinflammatory [2], antiepileptic [2] and antiproliferative [3], making them potential new pharmaceutical entities. *Aconitum naviculare* (Brühl) Stapf (Ranunculaceae) is a biennial medicinal herb of Alpine grassland (>4000 m a.s.l.) found in the trans-Himalayan region of Nepal, i.e., the Manang, Mustang and Dolpa districts. The aerial parts are used in Nepalese and Tibetan folk medicine against cold, fever and headache, as well as for sedative and analgesic remedies [4,5]. In the Manang region, the aerial parts are collected during flowering. The local inhabitants usually dry the whole plant and prepare a bitter decoction, which is used for various medicinal purposes. Although Manangis living in and outside Manang commonly use *A. naviculare*, it has not yet become an item of trade. Due to its supposed high effectiveness in traditional healthcare, *A. naviculare* may become a potential source of income for mountain people. Little information is available regarding the phytochemical composition of *A. naviculare*. Gao et al. [4] reported the isolation of a new diterpenoid alkaloid and some atisine-like alkaloids. We recently reported three novel glycosylated flavonoids from this plant [6]. Here we report the isolation and characterization of two new diterpenoid alkaloids, navirine B (**1**) and navirine C (**2**), and five known compounds (**3**-**7**) from *A. naviculare.*

The isolated diterpenoid alkaloids **1**-**3** were also studied for their ability to affect tumor cell proliferation. In particular, antiproliferative activity against ovarian (2008 cells) and colon (LoVo cells) adenocarcinoma was tested.

Compound **1** had a molecular formula of $C_{30}H_{40}N_2O_3$ on the basis of the protonated molecular ion $[M+H]^+$ displayed at *m/z* 477.3122 in the HRAPITOFMS. The IR spectrum showed absorption bands supporting the presence of an imino group (1670 cm^{-1}).

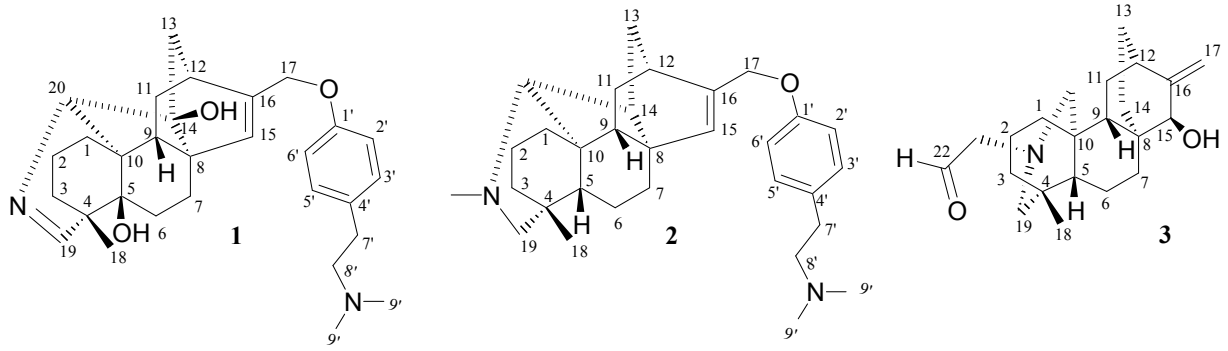

Figure 1: Structure of isolated compounds **1-3**.

The ^{1}H NMR spectrum showed signals ascribable to one methyl group (δ 1.05 s, 3H), an *N*,*N*-dimethyl group (δ 2.40 s, 6H), and one olefinic proton (δ 5.67 brs, 1H). Two *ortho* coupled doublets (J = 8.0) in the aromatic region (δ 6.85 and 7.12, 2H each) also indicated the presence of an *o-p* disubstituted aromatic ring. The structure of compound **1**, a diterpenoid alkaloid, was obtained by exhaustive analysis of COSY, HMQC and HMBC data. Various spin systems were detected when COSY and HMQC data were compared, establishing the connectivity between CH_2 in positions 1,2,3 and 6,7 and between positions 9,11,12,13. Further connectivity between the highly deshielded CH-19 (δ_H 7.43 s, 1H; δ_C 169.9) and CH-20 (δ_H 3.57 brs, 1H; δ_C 80.6) and also between the olefinic CH-15 (δ_H 5.67 brs, 1H; δ_C 130.8) and CH_2-17 (δ_H 4.55 brs, 1H; δ_C 68.8) were seen in the COSY spectrum. Diagnostic long-range (HMBC) correlations were observed from the methyl group 18 and carbon resonances at δ 30.9 (C-3), 44.9 (C-4), 72.6 (C-5) and 169.9 (C-19). HMBC correlations, observed from the proton signal of CH-20 (δ 3.57) with carbon resonances C-19 and C-5, supported the presence of a six-member ring containing an imino group. Diagnostic long-range correlations were observed from the same proton signal (H-20) with C-1 (δ 27.9), C-9 (47.1), C-5 (72.6) and C-8 (43.8). HMBC correlations of the proton at δ 5.67 (H-15) with C-9 (δ 47.1), C-12 (δ 31.7) and C-17 (68.8) were also seen. These observations, as well as analysis of COSY and HMQC data, suggested two more hexa-atomic rings in the compound. Long-range correlations observed from proton signals at δ 1.91-1.56 (CH_2-13) with carbon resonances at δ 146.8 (C-16) and 80.6 (C-20) supported the linkage between positions 20 and 14. Long-range correlations observed from the aromatic doublet δ_H 7.12 (H-3' 5') with the carbon at δ_C 33.3 (C-7') and from the methyl group at δ_H 2.59 (CH_3-9') with the carbon at δ_C 52.5 (C-8') suggested the presence of one hordenine moiety in the molecule.

NOESY data and comparisons with the spectral data of Gao *et al*. [4] for navirine evidenced the relative stereochemistry of the molecule, supporting an α orientation for groups at positions 10, 8 and 12. NOESY cross-peaks were also obtained between the exchangeable proton signal at δ 3.48 (OH-5) and H-9 (δ 1.64), which were assigned as β on the basis of previous references [4,7], suggesting a β orientation for the hydroxy group. With all this evidence, compound **1** was established as a new alkaloid, called navirine B (5β-hydroxy navirine).

The HRAPITOFMS of compound **2** displayed a protonated molecular ion $[M+H]^+$ at *m/z* 461.3532, corresponding to a molecular formula of $C_{31}H_{45}N_2O$. The ^{1}H NMR spectrum of compound **2** was quite similar to that of compound **1**, but there were some differences. It lacked the signal for the imino proton, and the proton signal of H-20 was shifted downfield to δ 2.53 (δ 3.57 for **1**). In addition, a singlet signal integrating for nine protons was observed at δ 2.50, supporting the presence of three nitrogen linked methyl groups. Extensive analyses of COSY, HMQC and HMBC data revealed a similar diterpenoid skeleton, as well as the same hordenine moiety as found in compound **1**. In the HMBC spectrum, long-range correlations were observed between the methyl group at δ 1.00 (CH_3-18) and carbon resonances at δ 34.1 (C-3), 41.7 (C-4), 53.5 (C-5) and 58.0 (C-19), which supported the presence of amino-linked CH_2 at position 19 (δ_H 2.30; δ_C 58.0), as well as a CH group at position 5 (δH 1.26; δC 53.5). The β configuration for H-5 was established on the basis of the NOESY correlation observed from the methyl group at δ 1.00 and the proton signal at δ 1.26 (H-5). Complete analyses of 1D and 2D NMR data afforded a novel structure, called navirine C.

Compound **3** showed a protonated molecular ion $[M+H]^+$ at *m/z* 344.2590, establishing its molecular formula as $C_{22}H_{33}NO_2$. The ^1H NMR spectrum of **3** displayed one aldehydic proton signal (8.71 s, 1H), one exocyclic methylene (δ 5.02 d, 5.12 d, 1H each; *J* = 1.0 Hz), one methyne signal linked to an electronegative atom (δ 3.70 m, 1H), and one methyl group (δ 1.06 s, 3H). Its COSY, HMQC and HMBC data revealed a diterpenoid skeleton. Compound **3** possessed a hydroxyl group at position 15, as supported by the HMBC correlation between H-17 (δ 5.05 and 5.12) and the carbon resonance at δ 74.7 (C-15). The β orientation of the OH group at position 15 was deduced on the basis of NOESY cross-peaks observed between H-15 and H-13 and H-14. The proton and carbon resonances at position 15 were at δ_H 3.70 and δ_C 74.7 respectively, due to the β-OH group. The H 9 configuration in compound **3** was established by observing the NOESY correlations from the methyl group 18 (δ 1.06) and H-5 (δ 1.38), and from this latter and the H-9 signal (δ 2.19). On the basis of the spectral data, compound **3** was established as chellespontine [8].

To our knowledge alkaloid **3**, flavonol glycosides **4** and **5**, and phenylpropanoid glycosides **6** and **7** have been isolated and characterized from *A. naviculare* for the first time.

Recently some *Aconitum* alkaloids have been evaluated for their antiproliferative activity against A172 malignant cells [3]. For this reason, we decided to study this activity for the isolated diterpenoid alkaloids **1-3**.

Table 1: Antiproliferative activity of compounds **1-3**.

Compound	LoVo cells[a] IC_{50} (confidence limits) μM	2008 cells[a] IC_{50} (confidence limits) μM
1	22 (19-25)	33 (30-37)
2	nd	nd
3	38 (33-41)	nd
Cisplatin[b]	33.3 (28.7-36.1)	13.8 (11.5-!6.5)

[a]LoVo: colon cell line; 2008: ovarian cell line.
[b]Cisplatin was used as a reference compound.
nd: IC_{50} is not determined.

Compounds **1** and **3** showed a significant antiproliferative activity, whereas compound **2** was inactive. The capacity of the compounds to affect tumor cell growth revealed a dose-dependent effect. In particular, colon cell line LoVo was more sensitive than ovarian cells 2008. In fact, compounds **1** and **3** were able to decrease cell proliferation in LoVo cells, whereas, in 2008 cells, only compound **1** induced a marked inhibition of cell growth, as shown in Table 1 as IC_{50} values.

Our study on the phytochemical composition of *A. naviculare* may be useful in improving scientific knowledge of this Nepalese medicinal plant.

Experimental

Optical rotations were measured on a Jasco P2000 digital polarimeter, IR spectra on a Perkin Elmer 1600 FT-IR spectrometer, and NMR spectra, in $CDCl_3$ or CD_3OD, on a Bruker AMX-300 spectrometer, operating at 300.13 MHz for ^1H NMR and 75.03 MHz for ^{13}C NMR. 2D experiments, ^1H-^1H DQF-COSY, and inverse-detected ^1H-^{13}C HMQC and HMBC spectra were performed with UXNMR software.

HRMS were obtained on an API-TOF spectrometer (Mariner Biosystems). Samples were diluted in a mixture of H_2O-AcCN (1:1), with 0.1% formic acid for positive ion mode, and directly injected at a flow rate of 10 μL/min. Sephadex LH 20 and silica gel 60 were used for column chromatography. Silica gel plates were used for preparative and analytical TLC (Merck cat. 5717 and 5715). Compounds on TLC were detected with a UV lamp (254 nm) and by treating plates with Dragendorff's reagent.

Semi-preparative HPLC was performed on a Gilson series 305 liquid chromatograph equipped with a LiChrosphere 100 RP-18 column (particle size 10 μm, 250 x 10 mm ID, E. Merck).

Plant material: Aerial parts, including stems, leaves, flowers and immature fruits of *Aconitum naviculare* were collected from Ladtar (4100 m a.s.l., upper Manang) during the last week of September 2004. A voucher specimen was collected during a field survey and deposited at Tribhuvan University Central Herbarium (TUCH; n° ANV904); identification was confirmed by Prof. Ram Prasad Chaudhary of the Central Department of Botany, Tribhuvan University, Kathmandu.

Extraction and isolation: Air-dried powdered aerial parts (100 g) were exhaustively extracted in a Soxhlet apparatus with MeOH. The solvent was evaporated under reduced pressure (230–250 mbar) and a semisolid MeOH extract was obtained (8 g). About

5.0 g of this extract was suspended in a mixture of 9:1 H_2O–MeOH (200 mL), and the pH was adjusted to 2 with 5% aq HCl. The solution was then partitioned first with $CHCl_3$ (5 x 50 mL) and then with EtOAc (5 x 50 mL). Solvents were removed under vacuum, yielding the $CHCl_3$ fraction CL-I (850 mg) and EtOAc fraction EA-I (330 mg). The pH of the residual aqueous layer was then adjusted to 8 with diluted NH_3. It was then partitioned with $CHCl_3$ (5 x 50 mL) and EtOAc (5 x 50 mL). Solvents were removed under vacuum, yielding the $CHCl_3$ fraction CL-II (250 mg) and EtOAc fraction EA-II (133 mg). The pH of the aqueous layer was then adjusted to 7.0, and the solvent was removed by freeze-drying, giving fraction AQ (3.3 g).

TLCs of the fractions CL-II and EA-II in several solvents (toluene/diethylamine/EtOAc 80:5:20; v/v $CHCl_3$/MeOH/NH_3 85:15:0.5; v/v) showed spots that gave a positive reaction to Dragendorf reagent. Fraction CL-II was repeatedly subjected to preparative thin layer chromatography (PTLC) with (toluene/diethylamine/EtOAc 80:5:20 v/v, $CHCl_3$/MeOH/NH_3 85:15:0.5 v/v). as eluents, yielding compounds **1** (10.1 mg) and **2** (6.5 mg). Fraction EA-II was subjected to silica plate chromatography using chloroform/methanol 4:1 as eluent. Further purification was achieved by semi-preparative HPLC with aqueous 0.1% HCOOH (A) and AcCN (B) as eluents. Gradient elution was used from 90% (A) to 85% (A) in 4 min, and then to 40% (A) in 22 min, yielding compound **3** (5.2 mg).

The AQ fraction was subjected to several semi-preparative HPLC steps (Spherisorb C-18, 1 x 300 mm, 10 μm) with aqueous 0.1% HCOOH (A) and methanol (B) as eluents. Gradient elution used for the isolation of compounds **4**-**7** was as follows: from 90% (A) to 85% (A) in 10 min, and then to 70% (A) in 15 min, yielding compounds **4** (11.0 mg) and **5** (13.0 mg); from 75% (A) to 45 % A in 25 min for the isolation of compounds **6** (8.0 mg) and **7** (6.5 mg).

Navirine B (**1**)

amorphous solid.
$[\alpha]_D$: +12.0 (*c* 0.083, CH_3OH).
IR (KBr): 3350, 3030, 3020, 1670, 1650, 1610, 1508, 820 cm^{-1}.
^1H NMR (300 MHz, $CDCl_3$): 1.65-1.78 m (H-1), 1.30 m (H-2), 1.75-1.98 m (H-3), 1.87-2.02 m (H-6), 1.26-1.58 m (H-7), 1.64 m (H-9), 1.42-1.88 m (H-11), 2.54 m (H-12), 1.56-1.91 m (H-13), 5.67 brs (H-15), 4.55 brs (H-17), 1.05 s (H-18), 7.43 brs (H-19), 3.57 brs (H-20), 6.85 d (8.0) (H-2'/6'), 7.12 d (8.0) (H-3'/5'), 2.99 brt (H-7'), 3.02 brt (H-8'), 2.40 s N-$(CH_3)_2$-9'.
^{13}C NMR (75.03 MHz, $CDCl_3$): 27.9 (C-1), 30.1 (C-2), 30.9 (C-3), 44.9 (C-4), 72.6 (C-5), 30.9 (C-6), 20.9 (C-7), 43.8 (C-8), 47.1 (C-9), 41.0 (C-10), 43.2 (C-11), 31.7 (C-12), 43.5 (C-13), 69.8 (C-14), 130.8 (C-15), 146.8 (C-16), 68.8 (C-17), 19.1 (C-18), 169.9 (C-19), 80.6 (C-20), 158.2 (C-1'), 114.9 (C-2'/6'), 129.2 (C-3'/5'), 125.0 (C-4'), 33.3 (C-7'), 52.5 (C-8'), 34.3 (N-$(CH_3)_2$-9').
HRAPITOFMS: *m/z* [M + H^+] calcd for $C_{30}H_{40}N_2O_3$: 477.3117; found: 477.3122.

Navirine C (**2**)

amorphous solid.
$[\alpha]_D$: +7.5 (*c* 0.070, CH_3OH).
IR (KBr): 3350, 3030, 3018, 1650, 1605, 1508, 820 cm^{-1}.
^1H NMR (300 MHz, $CDCl_3$): 1.74-2.08 m (H-1), 1.62 m (H-2), 1.25-1.78 m (H-3), 1.26 m (H-5), 1.83-2.14 m (H-6), 1.80 m (H-7), 1.34-1.68 m (H-11), 2.87 m (H-12), 1.33 m (H-13), 5.63 brs (H-15), 4.52 brs (H-17), 1.00 s (H-18), 2.30 m (H-19), 2.53 m (H-20), 6.84 d (8.0) (H-2'/6'), 7.10 d (8.0) (H-3'/5'), 2.89 m (H-7'), 2.89 m (H-8'), 2.55 s (N-$(CH_3)_2$-9').
^{13}C NMR (75.03 MHz, $CDCl_3$): 21.8 (C-1), 28.5 (C-2), 34.1 (C-3), 41.7 (C-4), 53.5 (C-5), 33.9 (C-6), 34.1 (C-7), 44.4 (C-8), 44.5 (C-9), 44.0 (C-10), 28.1 (C-11), 31.1 (C-12), 44.1 (C-13), 45.0 (C-14), 132.4 (C-15), 145.8 (C-16), 68.7 (C-17), 27.8 (C-18), 58.0 (C-19), 76.7 (C-20), 158.8 (C-1'), 115.1 (C-2'/6'), 129.7 (C-3'/5'), 128.0 (C-4'), 33.2 (C-7'), 60.5 (C-8'), 44.3 (N-$(CH_3)_2$-9').
HRAPITOFMS: *m/z* [M + H^+] calcd for $C_{31}H_{44}N_2O$: 461.3532; found: 461.3512.

Chellespontine (**3**)

amorphous solid.
$[\alpha]^{20}_D$: +5.5 (*c* 0.052, $CHCl_3$).
IR (KBr) ν_{max}: 2915, 1730, 990, 892 cm^{-1}.
^1H NMR (300 MHz, $CDCl_3$): 1.68 m (H-1), 1.31 m (H-2), 1.48-1.74 m (H-3), 1.38 m (H-5), 1.70 m (H-6), 1.61-2.05 (H-7), 2.19 m (H-9), 1.16-1.93 m (H-11), 2.40 m (H-12), 1.80-1.97 m (H-13), 1.30-1.93 m (H-14), 3.70 m (H-15), 5.05-5.12 d (*J* = 1.0) (H-17), 1.06 s (H-18), 3.77 m (H-19), 4.00 m (H-20), 3.75 m (H-21), 8.71 s (H-22).
^{13}C NMR (75.03 MHz, $CDCl_3$): 25.7 (C-1), 19.6 (C-2), 40.5 (C-3), 33.5 (C-4), 45.6 (C-5), 19.7 (C-6), 34.2 (C-7), 37.2 (C-8), 39.8 (C-9), 46.0 (C-10), 27.9 (C-11), 36.1 (C-12), 25.3 (C-13), 27.9 (C-14), 74.7

(C-15), 154.8 (C-16), 109.7 (C-17), 24.0 (C-18), 59.5 (C-19), 56.5 (C-20), 65.0 (C-21), 183.3 (C-22). HRAPITOFMS: *m/z* [M + H^+] calcd. for $C_{22}H_{33}NO_2$: 344.2590; found: 344.2550.

Compounds **4-7** were characterized on the basis of reported data [9-11].

Antiproliferative activity*:* Human ovarian carcinoma (2008) and human intestinal adenocarcinoma(LoVo) cell lines were used. The 2008 cells were maintained in RPMI 1640, and LoVo cells in Ham's F 12, in both cases supplemented with 10% heat-inactivated FCS, 1% antibiotics and 1% 200 mM L-glutamine (all products of Biochrom KG Seromed). Cells were seeded on 96-well tissue plates (Falcon) at 5 x 10^4 cells/mL, and treated 24 h later with various concentrations of the compounds **1-3**.

Cell growth was determined by the MTT reduction assay [12] after 72 h of incubation. Briefly, 20 μL of MTT solution (5 mg/mL in PBS) was added to each well and plates were incubated for 4 h at 37°C. DMSO (150 μL) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. Absorbance was measured on a micro-culture plate reader (Titertek Multiscan) at a test wavelength of 570 nm and a reference wavelength of 630 nm. Experiments were performed at least in triplicate, and results were statistically evaluated using Student's t-test [13]. IC_{50} 95% confidence limits were estimated with the Litchfield and Wilcoxon method.

Acknowledgment - The authors are grateful to MIUR for financial support.

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