Steroidal Alkaloids from the Leaves of *Sarcococca coriacea* of Nepalese Origin

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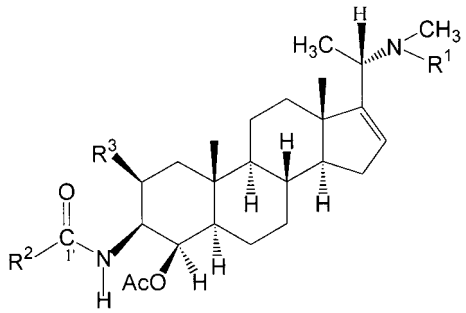
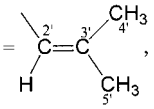
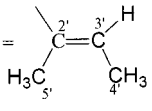
Two new steroidal alkaloids, (−)-vaganine D (**1**) [(20*S*)-20-(*N*,*N*-dimethylamino)-3*β*-(senecioylamino)-5α-pregn-16-en-4*β*-yl acetate], and (+)-nepapakistamine A (**2**) [(20*S*)-20-(*N*-methylamino)-3*β*-(tigloylamino)-5α-pregn-16-en-2*β*,4*β*-diacetate], were isolated from the leaves of *Sarcococca coriacea*. Their structures were elucidated on the basis of their spectral properties. Compounds **1** and **2** were found to be cholinesterase inhibitors.

Sarcococca species are rich in steroidal alkaloids, and some of these have interesting biological activities. There are four species of *Sarcococca* (i.e., *S. coriacea, S. hookeriana, S. saligna,* and *S. wallichii*) reported from different ecological zones of Nepal.[1] The extracts and compounds isolated from *Sarcococca* are reported to have antiacetylcholinesterase,[2] antibacterial,[3] antiulcer, and antitumor[4] activities. We have previously reported a number of steroidal alkaloids from various species of *Sarcococca*.[3,6,9−13] In our continuing studies on steroidal alkamines from this genus, we recently initiated work on *Sarcococca coriacea* (Hook. f.) Sweet. (Buxaceae), which is an evergreen shrub widely distributed in central Nepal. This study has resulted in the isolation of three new cholinesterase-inhibiting steroidal alkaloids, vaganine D (**1**) and nepapakistamine A (**2**). The structures of compounds **1** and **2** were determined by the combined use of different spectroscopic techniques.



1 $R^1 = CH_3$, $R^2 =$  , $R^3 = H$

2 $R^1 = H$, $R^2 =$  , $R^3 = OAc$

A methanolic extract of the aerial parts of the *S. coriacea* was evaporated, and the gummy extract was dissolved in distilled water and then partitioned with petroleum ether, chloroform, ethyl acetate, and butanol. The chloroform extract was subjected to column and thin-layer chromatography to obtain compounds **1** and **2**.

Compound **1** ($C_{30}H_{48}N_2O_3$, HREIMS *m*/*z* 484.3628) was isolated as a white amorphous solid. It showed UV absorptions at 206 and 197 nm. The IR spectrum displayed peaks at 3446, 3391 (NH), 1724 (ester C=O), and 1664 (amide C=O) cm^{-1}. The base peak at *m*/*z* 469 was due to the loss of a methyl group from the M^+ ion in the EIMS. The peak at *m*/*z* 72 represented the trimethyliminium cation, whereas the peaks at *m*/*z* 98, 83, and 55 suggested the presence of a 3′-methylbuten-2′-oyl amide functionality.[7,9] The ^1H NMR ($CDCl_3$) spectrum of **1** displayed two methyl singlets at *δ* 0.81 and 0.99 for the C-18 and C-19 tertiary methyl protons, respectively. A methyl doublet at *δ* 1.05 ($J_{21,20}$ = 6.5 Hz) was due to the C-21 secondary methyl protons. A second methyl doublet at *δ* 1.80 ($J_{5',2'}$ = 1.2 Hz) and a methyl triplet at *δ* 2.10 ($J_{4',2'}$ = 1.2 Hz) were assigned to the C-5′ and C-4′ methyl protons, respectively. A sharp methyl singlet at *δ* 2.08 was due to the acetoxy methyl protons attached to the C-4*β* position. The *N*,*N*-dimethyl protons resonated as a 6H singlet at *δ* 2.21. The C-20 methine proton appeared at *δ* 2.82 ($J_{20,21}$ = 6.4 Hz) as a quartet. A methine multiplet at *δ* 4.02 was assigned to the C-3α proton geminal to the amide group. A broad doublet at *δ* 5.16 ($J_{4,3}$ = 2.9 Hz) was assigned to the C-4 proton. The coupling constant (2.9 Hz) of the H-4 signal indicated its equatorial orientation. A broad doublet at *δ* 5.29 ($J_{NH,3}$ = 8.3 Hz) was assigned to the amide NH. An olefinic multiplet at *δ* 5.51 was assigned to the C-2′ proton, showing allylic coupling. Another olefinic broad singlet at *δ* 5.45 was due to the C-16 proton. The ^{13}C NMR spectrum (BB, DEPT) of compound **1** exhibited 30 signals with eight methyls, seven methylenes, nine methines, and six quaternary carbons (Table 1). Analyzing the spectroscopic parameters led to the assignment of the new compound **1** (vaginine D) as [(20*S*)-20-(*N*,*N*-dimethylamino)-3*β*-(senecioylamino)-5α-pregn-16-en-4*β*-yl acetate].

Compound **2** ($C_{31}H_{48}N_2O_5$, HREIMS *m*/*z* 528.3550) was isolated as a colorless crystalline solid. It was found to have a structure distinctly similar to compound **1** with an additional acetyl group at C-2 and a tigloylamino group at C-3. The IR spectra displayed peaks at 3446, 3391 (NH), 1724 (ester C=O), and 1664 (amide C=O) cm^{-1}. EIMS showed the base peak at *m*/*z* 513 due to the loss of a methyl group from the M^+ ion. Other fragment peaks at *m*/*z* 98, 83, and 55 suggested the presence of a tigloyl amino

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Table 1. ^{13}C NMR ($CDCl_3$, 100 MHz) Chemical Shift Data (δ ppm) of Compounds **1** and **2**

carbon	1	2
1	37.1	40.6
2	31.1	71.6
3	49.7	49.8
4	75.5	74.1
5	49.3	48.8
6	20.3	25.0
7	25.5	30.9
8	33.9	33.4
9	55.6	54.0
10	35.8	35.0
11	24.4	20.5
12	31.8	31.6
13	46.6	46.4
14	57.4	56.2
15	34.4	35.0
16	123.6	122.0
17	157.2	158.0
18	14.2	16.3
19	15.8	15.3
20	59.0	56.8
21	16.0	22.4
$N(CH_3)_n$	42.5 ($n = 2$)	34.2 ($n = 1$)
1′ C=O	166.2	168.3
2′	118.5	131.4
3′	150.9	131.2
4′	27.1	12.2
5′	19.8	14.0
$OCOCH_3$		170.1, 21.0
$OCOCH_3$	170.6, 21.1	170.5, 21.3

moiety,[5] and the peak at *m*/*z* 58 showed the presence of monomethylaminoethane group at C-17.[6] The ^1H NMR ($CDCl_3$) spectrum of **2** showed a split quartet at δ 6.28 ($J_{3',4'}$ = 6.9 Hz, $J_{3',5'}$ = 1.2 Hz) characteristic of a tigloyl C-3′ olefinic proton showing vicinal and allylic couplings, a methyl double doublet at δ 1.68 ($J_{4',3'}$ = 6.9 Hz, $J_{4',5'}$ = 1.0 Hz), and a methyl triplet at δ 1.72 ($J_{5',3'}$ = 1.2 Hz), which were assigned to the C-4′ and C-5′ methyl protons of the tigloyl group, respectively. Two methyl singlets at δ 2.03 and 2.05 were due to the two acetoxy methyl protons attached at C-2 and C-4 of ring A. A multiplet at δ 4.27 integrated for one proton was assigned to the C-3α proton geminal to the amide group. A double doublet at δ 5.09 ($J_{2e,1a}$ = 6.5 Hz, $J_{2e,1e}$ = 3.1 Hz) and a broad doublet at δ 5.19 ($J_{4,3}$ = 3.7 Hz) were due to the C-2 and C-4 protons of ring A, which was supported by the COSY-45° spectrum. The ^{13}C NMR spectra (BB, DEPT) of compound **2** showed distinct resemblance with compound **1** with additional acetyl signals (δ 170.1 and 21.0) and characteristic carbon signals of tigloyl substituents (δ 168.3, 131.4, 131.2, 14.0, 12.2) at C-3[13] (Table 1). The β-orientation of the C-20 proton, the equatorial disposition of the C-3 tigloyl amino group, and the axial configuration of the acetoxy groups at C-2 and C-4 in compound **2** were inferred on the basis of coupling constants and literature comparison.[5−9] On the basis of its spectroscopic analysis, nepapakistamine A (**2**) was assigned the structure (20*S*)-20-(*N*-methylamino)-3β-(tigloylamino)-5α-pregn-16-en-2β,4β-diacetate.

The stereochemical assignments of the asymmetric centers in compounds **1** and **2** were made on the basis of NOESY interactions, chemical shift comparison, and biogenetic considerations, keeping in view the fact that all the pregnane-type steroidal alkaloids are biosynthesized from cholesterol via pregnenolone.[14] In compound **2** the *cis* configuration of the tigloyl methyl groups at C-2′ and C-3′ were inferred by NOESY interactions between the C-3′ olefinic proton with the C-4′ methyl group.

Table 2. In Vitro Quantitative Inhibition of AChE and BChE by Compounds **1** and **2**

compound	AChE IC_{50} (μM)	BChE IC_{50} (μM)
vaganine D (**1**)	46.89 ± 1.94	10 ± 0.12
nepapakistamine A (**2**)	50.1 ± 1.35	25 ± 0.79
eserine	0.041 ± 0.001	0.857 ± 0.008

Compounds **1** and **2** were screened for their cholinesterase inhibitory activity. Two forms of cholinesterase coexist throughout the body, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Inhibition of brain acetylcholinesterase (AChE) can provide relief from the cognitive disorders associated with Alzheimer's disease (AD). A number of steroidal alkaloids isolated from the *Sarcococca* genus have shown potent cholinesterase inhibitory properties.[2] The concentrations of compounds **1** and **2** which inhibited the enzymes by 50% (IC_{50}) are presented in Table 2. Eserine (physostigmine) was used as the standard inhibitor. As shown in Table 2, compounds **1** and **2** showed greater selectivity toward BChE.

Experimental Section

General Experimental Procedures. Melting points are uncorrected and were recorded on a Buchi 535 melting point apparatus. Optical rotations were measured on a JASCO DIP-360 digital polarimeter. UV spectra were recorded on a Hitachi UV 3200 spectrophotometer. IR spectra were recorded on a JASCO 302-A spectrophotometer. The ^1H NMR spectra were recorded on a Bruker AM-400 instrument operating at 400 MHz, while the ^{13}C NMR spectra were recorded on the same instrument at 100 MHz. The chemical shift (δ) values are reported in ppm units, and coupling constants (J) are given in Hz. The EIMS were recorded on a Varian MAT 311A mass spectrometer, while high-resolution EIMS measurements were carried out on a JEOL-JMS 110 mass spectrometer. Precoated TLC plates (G 254) were used to check the purity of the compounds.

Plant Material. The fresh plant material was collected from Hattian in the Kathmandu valley, Nepal, during August and September 1999. The plant was identified by Prof. R. P. Choudhary, Taxonomist, Central Department of Botany, Kirtipur, Kathmandu, Nepal. A voucher specimen (SK 2057) was deposited in the Central Department of Botany, Tribhuvan University.

Extraction and Isolation. The air-dried aerial parts (14 kg) of *S. coriacea* were extracted with methanol (35 L). The concentrated methanolic extract (968 g) was dissolved in cold distilled water (3 L) and defatted with petroleum ether (10 L). The aqueous layer was then extracted with chloroform to afford a reddish gummy material (32.0 g), which was adsorbed on an equal amount of Si gel and loaded onto a Si gel (500 g, E. Merck, type 60, 70−230 mesh) packed column and eluted with gradients of petroleum ether, chloroform, and methanol to obtain a number of fractions. A fraction (F_3, 3.95 g), eluted with 5% MeOH in $CHCl_3$, was further chromatographed on a Si gel (flash, 240−300 mesh, 50 g) column, and elution was carried out with petroleum ether, acetone, and diethylamine. Subfraction S_2 (0.62 g) was eluted with petroleum ether−acetone−diethylamine (90:9:1), to afford pure compound **1** (11.4 mg, % yield 8.1×10^{-5}).

Fraction F_4 (15 g), eluted from the initial column with 10% MeOH in $CHCl_3$, was subjected to repeated column chromatography to afford a number of subfractions. Subfraction F (0.56 g) was loaded on a column and eluted with petroleum ether−acetone−diethylamine (87:12:1) to afford the crystalline compound **2** (23 mg, % yield 1.6×10^{-4}).

Vaganine D (1): white amorphous solid ($CHCl_3$); mp 220 °C; $[\alpha]^{23}_D$ −21.4° (*c* 0.14, $CHCl_3$); UV λ_{max} (MeOH 206 nm (log ϵ 4.2); IR ν_{max} ($CHCl_3$) 3446, 3391, 1724, 1664 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.81 (3H, s, CH_3-18), 0.99 (3H, s, CH_3-19), 1.05 (3H, d, $J_{21,20}$ = 6.5 Hz, CH_3-21), 1.80 (3H, d, $J_{5',2'}$ =

1.2 Hz, CH_3-5′), 2.08 (3H, s, $OCOCH_3$), 2.10 (3H, t, $J_{4',2'} = 1.2$ Hz, CH_3-4′), 2.21 [6H, s, $N_b(CH_3)_2$], 2.82 (1H, q, $J_{20,21} = 6.4$ Hz, H-20), 4.02 (1H, m, H-3), 5.16 (1H, bd, $J_{4,3} = 2.9$ Hz, H-4*eq*), 5.29 (1H, bd, $J_{NH,3} = 8.3$ Hz, NH), 5.45 (1H, bs, H-16), 5.51 (1H, bs, H-2′); ^{13}C NMR ($CDCl_3$), 100 MHz, see Table 1; EIMS *m*/*z* $[M]^+$ 484 (6), 469 (100), 98 (2), 83 (36), 72 (30), 55 (2); HREIMS *m*/*z* 484.3628 (calcd for $C_{30}H_{48}N_2O_3$, 484.3664).

Nepapakistamine A (2): colorless crystalline solid ($CHCl_3$); mp 162−163 °C; $[\alpha]^{23}_D$ +18.7° (*c* 0.092, $CHCl_3$); UV λ_{max} (MeOH) 203 nm (log ϵ 4.8); IR ν_{max} ($CHCl_3$) 3446, 3391, 1724, 1664 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.73 (3H, s, CH_3-18), 1.12 (3H, s, CH_3-19), 1.09 (3H, d, $J_{21,20} = 6.5$ Hz, CH_3-21), 1.68 (3H, dd, $J_{4',3'} = 6.9$ Hz, $J_{4',5'} = 1.0$ Hz, CH_3-4′), 1.72 (3H, t, $J_{5',3'} = 1.2$ Hz, CH_3-5′), 2.03 (3H, s, $OCOCH_3$), 2.05 (3H, s, $OCOCH_3$), 2.27 [3H, s, N_b (CH_3)], 3.0 (1H, q, $J_{20,21} = 6.4$ Hz, H-20), 4.27 (1H, m, H-3 *ax*), 5.09 (1H, dd, $J_{2e,1a} = 6.5$ Hz, $J_{2e,1e} = 3.1$ Hz, H-2 *eq*), 5.19 (1H, bd, $J_{4,3} = 3.7$ Hz, H-4 *eq*), 5.39 (1H, bs, H-16), 5.94 (1H, bd, $J_{NH,3} = 8.7$ Hz, NH), 6.28 (1H, dd, $J_{3',4'} = 6.9$ Hz, 1.2 Hz, H-3′); ^{13}C NMR, ($CDCl_3$) 100 MHz, see Table 1; EIMS $[M]^+$ 528 (29), 513 (100), 98 (20), 83 (85), 58 (25); HREIMS *m*/*z* 528.3550 (calcd for $C_{31}H_{48}N_2O_5$, 528.3563).

Cholinesterase Inhibition Assay. Electric eel acetylcholinesterase (EC 3.1.1.7), horse butyrylcholinesterase (EC 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine chloride, 5,5′-thiobis-2-nitrobenzoic acid (DTNB), and eserine [(−)-physostigmine] were purchased from Sigma (St. Louis, MO). Buffers and other chemicals were of extrapure analytical grade. Acetylcholinesterase inhibition was determined spectrophotometrically using acetylthiocholine as substrate by modifying the method of Ellman et al.[16] The butyrylthiocholine chloride was used as a substrate to assay butyrylcholinesterase enzyme, while all the other reagents and conditions were the same as described previously.[17] The hydrolysis of acetylthiocholine or butyrylthiocholine was determined by monitoring the formation of the yellow 5-thio-2-nitrobenzoate anion (as a result of the reaction of 5,5′-dithiobis-2-nitrobenzoic acid with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine or butyrylthiocholine) at a wavelength of 412 nm.

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