Cholinesterase-Inhibiting New Steroidal Alkaloids from *Sarcococca hookeriana* of Nepalese Origin

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Bioassay-guided phytochemical investigation of *Sarcococca hookeriana* has resulted in the isolation and structure elucidation of five new pregnane-type steroidal alkaloids: (−)-hookerianamide A (=(2β,3β,4β,20*S*)-20-(dimethylamino)-3-[(3-methylbut-2-enoyl)amino]-5α-pregn-16-ene-2,4-diol; **1**), (+)-hookerianamide B (=(2α,3β,4β,20*S*)-4-acetoxy-20-(dimethylamino)-3-[(3-methylbut-2-enoyl)amino]-5α-pregnan-2-ol; **2**), (−)-hookerianamide C (=(2β,3β,20*S*)-2-acetoxy-20-(dimethylamino)-3-[(3-methylbut-2-enoyl)amino]-5α-pregnane; **3**), (−)-hookerianamine A (=(3β,20*S*)-20-(dimethylamino)-3-(methylamino)-5α-pregn-14-ene; **4**), and (+)-phulchowkiamide A (=(3β,20*S*)-20-(methylamino)-3-[(2-methylbut-2-enoyl)amino]-5α-pregn-2-en-4-one; **5**). These compounds, as well as the two chemically derived acetyl derivatives **6** and **7**, displayed cholinesterase inhibition in a concentration-dependent manner.

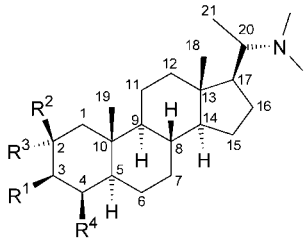
1. Introduction. – Acetylcholinesterase (AChE, EC 3.1.1.7) is a key component of cholinergic brain synapses and neuromuscular junctions. The main role of the enzyme is the termination of impulse transmission by rapid hydrolysis of the cationic neurotransmitter acetylcholine [1]. According to the cholinergic hypothesis, the memory impairment in patients with senile dementia due to *Alzheimer*'s disease (AD) results from a deficiency in cholinergic function in the brain [2]. Hence, the most promising therapeutic strategy for activating central cholinergic functions has been the use of cholinomimetic agents. The aim of acetylcholinesterase inhibitors is to boost the endogenous levels of acetylcholine in the brains of AD patients and, thereby, to boost cholinergic neurotransmission. Recently, it has been found that butyrylcholinesterase (BChE, EC 3.1.1.8) inhibition may also be an effective tool for the treatment of AD and related dementias [3]. Cholinesterase inhibitors may act as potential leads in the discovery of clinically useful treatments for nervous-system disorders.

In the flora of Nepal, the genus *Sarcococca* is represented by four species [4], which are important producers of steroidal alkaloids, and many of them have been reported to exhibit anticholinesterase [5], antibacterial [6], antitumor [7], and antiulcer [8] activities. Our ongoing studies on the constituents of this genus have led to the isolation of a number of pregnane-type steroidal alkaloids [5][6][9]. However, no phytochemical work has been reported on the constituents of *Sarcococca hookeriana* (BAILL.) HOOK. f. (Buxaceae). *S. hookeriana* is an evergreen shrub, widely distributed from Eastern to Western Nepal, North Assam, South Tibet, and Bhutan [10]. Rural communities in Nepal have been using the root extracts of this plant against gout [11].

Here, we report on the isolation and characterization of five new alkaloids from this plant. These natural compounds, as well as two acetylated derivatives, showed anticholinesterase properties. The structures of the new compounds were determined by modern spectroscopic techniques [12].

2. Results and Discussion. – During this study, compounds **1**–**5** were isolated from the alkaline $CHCl_3$ fraction obtained from the 80% aqueous MeOH extract of dried *S. hookeriana*. Column chromatography (CC) was used for the purification of compounds. The fractionation and purification of the active components was based on bioassay-guided screening, *i.e.*, cholinesterase-enzyme-inhibition assay.

Hookerianamide A (**1**) was isolated as white crystals, having the molecular formula $C_{28}H_{46}N_2O_3$, as established by HR-EI-MS (*m*/*z* 458.3447) and ^{13}C-NMR spectroscopy, indicating seven degrees of unsaturation. The characteristic mass-fragmentation pattern indicated the presence of a pregnane-type skeleton with two N-atoms at C(3) and C(20) [13]. The structure of compound **1** was similar to the previously isolated sarcovagenine A [7], differing at C(3).



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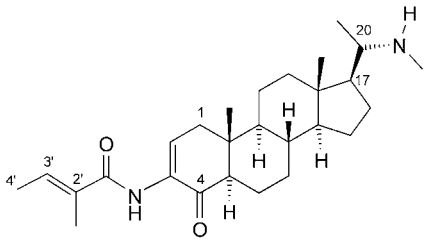
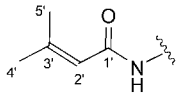


5

	R^1	R^2	R^3	R^4	Unsaturation
1	Sen	OH	H	OH	$\Delta^{16,17}$
2	Sen	H	OH	AcO	-
3	Sen	AcO	H	H	-
4	MeNH	H	H	H	$\Delta^{14,15}$
6	Sen	AcO	H	AcO	$\Delta^{16,17}$
7	Sen	H	AcO	AcO	-

Sen = (senecioyl)amino:



The UV spectrum of compound **1** displayed absorptions at 216 and 222 nm. The IR spectrum showed absorptions at 3300 (OH), 3280 (NH), 2922 (CH), 1640 (C=O), and 1624 (C=C) cm^{-1} [14]. The base peak at *m*/*z* 443 in the EI mass spectrum was due to the loss of a Me group from the molecular ion. The peaks at *m*/*z* 83 and 55 indicated the presence of a senecioylamino (= (3-methylbut-2-enoyl)amino) moiety [15], and the peak at *m*/*z* 72 suggested the loss of a Me_2N^+=C(H)Me group [16], which, however, had a lower abundance in comparison with the corresponding MS signals of common steroidal alkaloids with a 20-Me_2N group, in which this ion is always represented by the base peak. This may be due to the presence of the C(16)=C(17) bond [7].

The ^{1}H-NMR spectrum (*Table 1*) of **1** exhibited two *s* at δ 0.88 and 1.42, assigned to Me(18) and Me(19), respectively. The *d* resonating at δ 1.03 (^3J = 6.5 Hz, 3 H) was assigned to Me(21). The two *s* at δ 1.58 and 2.27 were ascribed to Me(5′) and Me(4′) of the senecioyl moiety. The *s* at δ 2.12 (6 H) was due to the Me_2N group. The *q* integrating for one H-atom at δ 2.90 (^3J = 6.2 Hz) was assigned to H–C(20), which supported the $\Delta^{16,17}$ unsaturation. The *dd* at δ 4.66 ($J(4\alpha,3\alpha)$ = 4.3, $J(4\alpha,5\alpha)$ = 4.2 Hz) was assigned to H_α–C(4), and the broad *s* at

δ 4.73 ($w_{1/2} = 7.2$ Hz) was assigned to H_α–C(2), suggesting β-orientation of the geminal 2- and 4-OH groups [17]. The broad *s* of H–C(2) might be due to the distortion of the chair conformation of ring *A* due to the 1,3-diaxial interactions of H–C(2) and H–C(4) with Me(19), and their steric repulsions from the 3β-senecioyl moiety. The *m* at δ 5.11 was assigned to H_α–C(3), and the *d* at δ 7.91 ($J(3\alpha,NH) = 7.2$ Hz) to the amide NH. The broad *s* at δ 5.54 and the *s* at 5.41 were assigned to H–C(16) and H–C(2′), respectively.

Table 1. *^{1}H-NMR Data of Compounds* **1**–**5**. All assignments were confirmed by ^{1}H,^{1}H-COSY, HMQC, and ^{13}C-NMR DEPT experiments. Conditions: 400 MHz, C_5D_5N (**1**); 500 MHz, $CDCl_3$ (**2**–**4**); and 300 MHz, $CDCl_3$ (**5**). Chemical shifts δ in ppm, coupling constants *J* in Hz

	1	**2**	**3**	**4**	**5**
CH_2(1)	1.62, 1.55	1.21, 1.92	1.13, 1.84	1.38, 1.55	1.21, 1.95
H–C(2) or CH_2(2)	4.73 (br. *s*, $w_{1/2} = 7.2$)	3.88 (br. *s*, $w_{1/2} = 18.1$)	5.11 (br. *s*, $w_{1/2} = 7.5$)	1.40, 1.75	7.62 (*dd*, $J = 6.7, 2.4$)
H–C(3)	5.11 (*m*)	4.03 (*m*)	4.01 (*m*)	2.66 (*m*)	–
H–C(4) or CH_2(4)	4.66 (*dd*, $J = 4.3, 4.3$)	4.09 (*dd*, $J = 4.0, 3.9$)	1.62, 1.70	1.35, 1.48	–
H–C(5)	1.79	1.49	1.39	1.58	2.29
CH_2(6)	2.42, 1.59	1.41, 1.66	1.15, 1.53	1.69, 1.87	1.38, 1.55
CH_2(7)	1.85, 1.21	1.65, 1.78	1.75, 1.86	1.21, 1.77	1.12, 1.75
H–C(8)	1.50	1.38	1.38	1.78	1.35
H–C(9)	0.88	1.42	1.08	1.67	1.11
CH_2(11)	2.39, 1.46	1.23, 1.81	1.01, 1.62	1.41, 1.44	1.32, 1.61
CH_2(12)	1.79, 1.32	1.82, 1.26	1.53, 1.78	1.30, 1.82	1.34, 1.92
H–C(14)	1.25	1.44	1.35	–	1.02
H–C(15) or CH_2(15)	1.60, 1.85	1.05, 1.66	1.61, 1.72	5.54 (br. *s*)	1.71, 1.92
H–C(16) or CH_2(16)	5.54 (br. *s*)	1.18, 1.41	1.31, 1.49	1.67, 1.81	1.25, 1.83
H–C(17)	–	1.13	1.20	2.00	1.18
Me(18)	0.88 (*s*)	0.63 (*s*)	0.61 (*s*)	0.77 (*s*)	0.65 (*s*)
Me(19)	1.42 (*s*)	1.20 (*s*)	0.94 (*s*)	0.79 (*s*)	0.85 (*s*)
H–C(20)	2.90 ($q, J = 6.2$)	2.67 (*m*)	2.42 (*m*)	2.76 (*m*)	2.48 (*m*)
Me(21)	1.03 ($d, J = 6.5$)	1.72 ($d, J = 6.7$)	0.95 ($d, J = 6.3$)	1.02 ($d, J = 6.5$)	1.80 ($d, J = 6.4$)
MeN	–	–	–	2.33 (*s*)	2.63 (*s*)
Me_2N	2.12 (*s*)	2.26 (*s*)	2.21 (*s*)	2.15 (*s*)	–
H–C(2′)	6.13 (*s*)	5.31 (*s*)	5.45 (*s*)	–	–
H–C(3′)	–	–	–	–	6.47 ($q, J = 6.2$)
Me(4′)	2.27 (*s*)	1.85 (*s*)	2.08 (*s*)	–	1.75 ($d, J = 6.9$)
Me(5′)	1.58 (*s*)	1.75 (*s*)	1.78 (*s*)	–	1.86 (*s*)
Ac	–	2.06 (*s*)	2.07 (*s*)	–	–

The decoupled ^{13}C-NMR DEPT spectrum (*Table 2*) of **1** showed 28 signals: seven Me, six CH_2, ten CH, and five C_q resonances. The ^{1}H,^{1}H-COSY spectrum (45°) showed couplings between H–C(2) and H–C(3), H–C(3) and H–C(4), and between H–C(4′) and H–C(5′). The ^{1}H,^{13}C correlations were determined with the help of an HMQC spectrum (*Tables 1* and *2*), while the long-range ^{1}H,^{13}C connectivities were established through HMBC technique (*Fig. 1*). H–C(3) (δ 5.11) showed correlations with C(2) (δ 70.4) and C(4) (δ 66.5), while H–C(4) (δ 4.66) showed connectivity with C(5) (δ 47.9) in the HMBC spectrum. Similarly, H–C(16) (δ 5.54) showed correlations with C(17) (δ 158.0), C(15) (δ 31.9), and C(20) (δ 58.1). The Me(4′) (δ 2.27) and Me(5′) (δ 1.58) groups of the senecioyl moiety displayed correlations with C(2′) (δ 120.1), and H–C(2′) (δ 6.13) showed correlations with C(1′) (δ 168.5) and C(3′) (δ 149.0). The configuration of compound **1** was assigned on biogenetic grounds, as all known pregnane-type steroidal alkaloids are being synthesized from cholesterol *via* pregnenolone [18]. The assigned configuration was further supported by ROESY and NOESY experiments, and by chemical-shift comparison with reported data [5–9][12][19]. The NOESY interactions between H_α–C(3) (δ 5.11), H_α–C(2) (δ 4.73), and H_α–C(4) (δ 4.66) suggested β-orientations of the two OH and the

senecioylamino functionalities [19]. Hence, hookerianamide A (**1**) was identified as (2*β*,3*β*,4*β*,20*S*)-20-(dimethylamino)-3-[(3-methylbut-2-enoyl)amino]-5*α*-pregn-16-ene-2,4-diol.

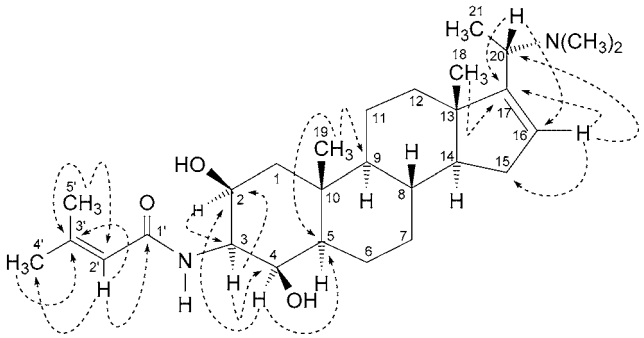


Fig. 1. *Selected HMBC interactions in compound* **1**

Table 2. *^{13}C-NMR Data of Compounds* **1**–**5**. All assignments were confirmed by ^{1}H,^{1}H-COSY, HMQC, and ^{13}C-NMR DEPT experiments. Conditions: 100 MHz, C_5D_5N (**1**); 125 MHz, $CDCl_3$ (**2**–**4**); and 100 MHz, $CDCl_3$ (**5**). Chemical shifts δ in ppm

	1	**2**	**3**	**4**	**5**
C(1)	40.1	39.6	39.3	32.4	39.3
C(2)	70.4	69.9	75.3	27.6	125.8
C(3)	57.3	51.5	49.6	54.9	131.4
C(4)	66.5	75.0	31.8	31.8	196.2
C(5)	47.9	49.7	42.0	39.8	54.7
C(6)	20.9	20.4	20.4	20.7	20.3
C(7)	31.0	31.5	27.5	31.0	30.3
C(8)	33.1	34.4	35.1	34.0	34.5
C(9)	56.1	53.1	56.2	54.5	54.7
C(10)	37.7	35.3	35.5	35.4	39.8
C(11)	22.7	21.5	24.1	20.5	20.7
C(12)	34.7	37.3	37.5	34.6	38.9
C(13)	46.7	44.3	42.6	48.7	42.7
C(14)	58.6	56.4	54.0	157.7	55.8
C(15)	31.9	24.2	24.3	123.3	24.1
C(16)	123.9	25.0	25.3	31.7	27.6
C(17)	158.0	55.9	56.1	57.6	56.1
Me(18)	15.9	12.1	12.2	15.7	12.1
Me(19)	16.3	13.9	14.1	16.0	12.3
C(20)	58.1	56.4	58.2	58.9	59.1
Me(21)	14.2	12.3	14.2	11.5	13.7
Me_2N	41.9	39.4	39.4	42.5	–
MeNH	–	–	–	34.1	34.4
C(1′)=O	168.5	168.7	166.0	–	167.6
C(2′)	120.1	118.1	118.4	–	132.1
C(3′)	149.0	151.4	150.9	–	131.8
Me(4′)	26.8	25.5	27.0	–	14.0
Me(5′)	19.7	16.7	19.7	–	13.2
MeCO_2	–	21.0	21.1	–	–
Me*C*O_2	–	169.0	170.6	–	–

Hookerianamide B (**2**) was isolated as white crystals, with a molecular formula $C_{30}H_{50}N_2O_4$, as determined by HR-EI-MS (*m/z* 502.3716) and ^{13}C-NMR spectroscopy, indicating seven degrees of unsaturation. Compound **2** showed distinct resemblance with compound **1**. The differences included an α-OH group at C(2), a β-AcO group at C(4), and the absence of a C=C bond.

The UV spectrum of compound **2** displayed absorptions at 210 and 221 nm. The IR spectrum showed absorptions at 3562 and 3398 (NH), 3390 (OH), 2927 (CH), 1737 (ester C=O), 1632 (amide C=O), 1157 and 1056 (C–O) cm^{-1}. The base peak at *m/z* 72 in the EI mass spectrum was due to loss of Me_2N^+=C(H)Me. The peak at *m/z* 487 was due to the $[M-CH_3]^+$ ion. The ^{1}H-NMR spectrum (*Table 1*) showed a broad *s* at δ 3.88 ($w_{1/2}$ = 18.1 Hz) assigned to H_β–C(2) [17]. An additional *s* at δ 2.06 (3 H) indicated the presence of a 4-AcO functionality [20]. Its position was inferred as β because the H_α–C(4) resonance (δ_H 4.09) appeared as a *dd* ($J(4\alpha,5\alpha)$ = 4.0, $J(4\alpha,3\alpha)$ = 3.9 Hz). The α-orientation of H–C(4) was inferred from its NOESY interactions with H_α–C(3) and H_α–C(5) (*Fig. 2*). The ^{13}C-NMR DEPT spectrum (*Table 2*) of compound **2** showed 30 signals: eight Me, seven CH_2, ten CH, and five C_q resonances. The additional signals at δ 169.0 and 21.0 were due to the Ac group. On the basis of the above spectral analysis and literature comparison [20], hookerianamide B (**2**) was assigned the structure (2α,3β,4β,20*S*)-4-acetoxy-20-(dimethylamino)-3-[(3-methylbut-2-enoyl)amino]-5α-pregnan-2-ol.

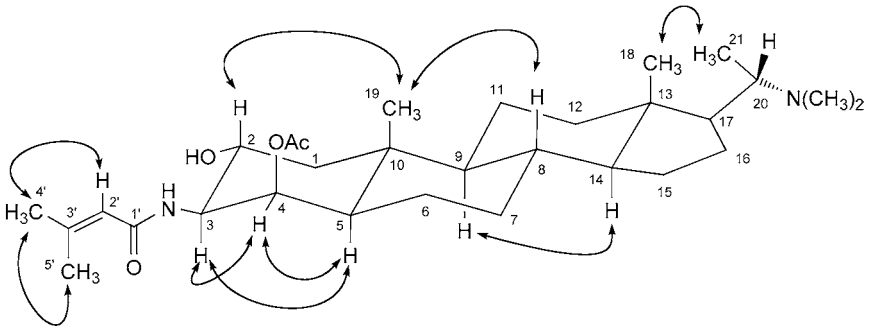


Fig. 2. *Selected NOESY interactions in compound* **2**

Hookerianamide C (**3**) was isolated as white crystals, with the molecular formula $C_{30}H_{50}N_2O_3$, as established by HR-EI-MS (*m/z* 486.3857) and ^{13}C-NMR spectroscopy, indicating seven degrees of unsaturation. Compound **3** also showed a distinct resemblance with compound **1**. The main differences included the presence of a 2-AcO functionality, lack of the 4-OH group, and absence of the C(16)=C(17) bond.

The UV spectrum of compound **3** displayed an absorption at 220 nm. The IR spectrum showed absorptions at 3304 (NH), 2934 (CH), 1737 (ester C=O), 1665 (amide C=O), 1182 and 1050 (C–O) cm^{-1}. The base peak at *m/z* 72 was due to loss of Me_2N^+=C(H)Me, and the peak at *m/z* 471 was assigned to the $[M-CH_3]^+$ ion. The ^{1}H-NMR spectrum (*Table 1*) showed a broad *s* at δ 5.11 ($w_{1/2}$ = 7.5 Hz) due to H_α–C(2) [17], which, in the NOESY spectrum, interacted with H_α–C(3) (δ 4.01). An additional *s* at δ 2.07 suggested the presence of a 2β-AcO functionality [21], interacting with Me(19) (δ 0.94). The ^{13}C-NMR DEPT spectrum (*Table 2*) of **3** showed 30 signals: eight Me, eight CH_2, nine CH, and five C_q resonances. The additional signals at δ 170.6 and 21.1 were due to an Ac group. On the basis of the above spectral analysis and literature comparison [21], hookerianamide C (**3**) was assigned the structure (2β,3β,20*S*)-2-acetoxy-20-(dimethylamino)-3-[(3-methylbut-2-enoyl)amino]-5α-pregnane.

Hookerianamine A (**4**) was obtained as a white powder, with a molecular formula $C_{24}H_{42}N_2$, as inferred from HR-EI-MS (*m/z* 358.3325) and ^{13}C-NMR spectroscopy,

indicating five degrees of unsaturation. Compound **4** showed a distinct resemblance with compound **1**. The main differences included the replacement of the 3-(senecioylamino) moiety by a MeNH group, the presence of a C(14)=C(15) bond, and the absence of the 2-OH and 4-OH groups of **1**.

The UV spectrum of compound **4** displayed an absorption at 211 nm. The IR spectrum showed absorptions at 3390 (NH), 2928 (CH), and 1651 (C=C) cm^{-1}. The EI mass spectrum showed the M^+ signal at *m/z* 358. The base peak at *m/z* 72 ($C_4H_{10}N$) indicated the loss of a $Me_2N^+=C(H)Me$ ion, and the peak at *m/z* 343 was assigned to the $[M - CH_3]^+$ fragment. The ^{1}H-NMR spectrum (*Table 1*) showed a *s* (3 H) at δ 2.33, which was assigned to the 3-MeNH substituent. A downfield broad *s* resonating at δ 5.54 was assigned to H–C(15), which showed HMBC connectivities with C(13) (δ 46.7), C(16) (δ 31.7), and C(17) (δ 57.6). Similarly, CH_2(16) (δ 1.67 and 1.81), H–C(17) (δ 2.00), and H–C(20) (δ 2.76) showed HMBC interactions with C(15) (δ 123.3). Also, H–C(8) (δ 1.78) and Me(18) (δ 0.77) showed connectivities with C(14) (δ 157.7). The ^{13}C-NMR DEPT spectrum (*Table 2*) of **4** showed 24 signals: six Me, eight CH_2, seven CH, and three C_q resonances. The signal at δ_C 34.1 was due to the MeNH C-atom at C(3). On the basis of spectral analysis and literature comparison [22], hookerianamine A (**4**) was assigned the structure (3*β*,20*S*)-20-(dimethylamino)-3-(methylamino)-5*α*-pregn-14-ene.

Phulchowkiamide A (**5**) was obtained as a white gum, having the molecular formula $C_{27}H_{42}N_2O_2$, as established by HR-EI-MS (*m/z* 426.3246) and ^{13}C-NMR spectroscopy, indicating eight degrees of unsaturation. Compound **5** also showed a distinct resemblance with compound **1**. The main differences included the presence of a tigloylamino (=(2-methylbut-2-enoyl)amino) moiety instead of senecioylamino moiety at C(3), a C(2)=C(3) bond, a C=O functionality at C(4), the lack of an olefinic bond between C(16)–C(17), and the absence of a 2-OH group.

The UV spectrum of **5** showed an absorption at 230 nm. The IR spectrum displayed absorptions at 3386 (NH), 2933 (CH), 1663 (C=O), and 1636 (C=C) cm^{-1}. The EI mass spectrum showed the M^+ signal at *m/z* 426. The base peak at *m/z* 58 was due to loss of $Me(H)N^+=C(H)Me$ [5], and the presence of a tigloylamino group was indicated by the peaks at *m/z* 98, 83, and 55. The ^{1}H-NMR spectrum (*Table 1*) showed a downfield *dd* at δ 7.62 ($J(2,1\alpha) = 6.7$, $J(2,1\beta) = 2.4$ Hz) assigned to H–C(2). The C=O moiety was placed at C(4) on the basis of the downfield chemical shift and splitting pattern of the conjugated C(2) vinylic H-atom [5]. A *s* resonating at δ 8.16 was assigned to the amide NH. A *d* at δ 1.75 ($J(4',3') = 6.9$ Hz) and a *s* at δ 1.86 was ascribed to Me(4′) and Me(5′) of the tigloyl group, respectively. A split *q* appeared at δ 6.47 ($J(3',4') = 6.2$ Hz), characteristic of the tigloyl H–C(3′) olefinic H-atom. A *s* at δ 2.63 was assigned to the *N*-Me group. Selected HMBC interactions in compound **5** are shown in *Fig. 3*. The ^{13}C-NMR DEPT spectrum (*Table 2*) showed 27 signals: eight Me, seven CH_2, six CH, and six C_q resonances.

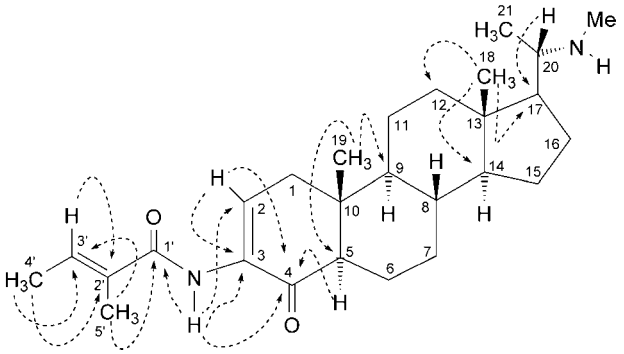


Fig. 3. *Selected HMBC interactions in compound* **5**

On the basis of the above spectral analysis and literature comparison [5], compound **5** was assigned the structure (3β,20*S*)-20-(methylamino)-3-[(2-methylbut-2-enoyl)amino]-5α-pregn-2-en-4-one.

Acetylation of compound **1** using Ac_2O in pyridine afforded the diacetate **6** ($C_{32}H_{50}N_2O_5$; M^+ at *m*/*z* 542). The ^{1}H-NMR spectrum of **6** showed downfield shifts of H_α–C(2) from δ 4.73 (**1**) to 5.07 (**6**), and from δ 4.66 (**1**) to 5.26 (**6**), as well as two new singlets at δ 2.00 and 2.04, representing the Ac groups.

Acetylation of compound **2** afforded the diacetate **7** ($C_{32}H_{52}N_2O_5$; M^+ at *m*/*z* 544). The ^{1}H-NMR spectrum of **7** showed downfield shifts of H_β–C(2) from δ_H 3.88 (**2**) to 4.31 (**7**), and a new singlets at δ 2.06, representing, the 2-Ac group.

Biological Evaluation. All compounds were found to be selective inhibitors of butyrylcholinesterase, except **1** and the standard inhibitor galanthamine, which is selective towards acetylcholinesterase (*Table 3*). Interestingly, the cholinesterase-inhibitory activity of compound **5** was considerably higher than of the OH-substituted congeners **1**–**4**. Similarly, the acetylated derivatives **6** and **7** were found to be more inhibiting than their parent compounds **1** and **2**, respectively.

Table 3. In vitro *Inhibition of Acetyl-* (AChE) *and Butyrylcholinesterase* (BChE) *by Compounds* **1**–**7**. Galanthamine was used as a standard inhibitor (for details, see the *Exper. Part*)

	IC_{50} [μM] ± SEM[a])		Selectivity	
	AChE	BChE	AChE	BChE
1	82.7 ± 4.6	200 ± 0.4	2.4	
2	26.4 ± 3.5	0.76 ± 0.002		34.7
3	23.2 ± 1.7	0.64 ± 0.001		36.2
4	18.9 ± 0.48	0.90 ± 0.05		21.0
5	0.50 ± 0.0002	0.39 ± 0.0001		1.28
6	35.8 ± 3.1	2.62 ± 0.02		13.6
7	33.25 ± 2.0	6.0± 0.2		5.5
Galanthamine	0.5 ± 0.0001	8.1 ± 0.85	16.2	

[a]) Standard error of the mean of five assays.

Experimental Part

General. All reagents used were of anal. grade. TLC: precoated SiO_2 plates (*Merck GF-254*), visualization with *Dragendorff* spraying reagent. M.p.: *Yanaco MP-S3* micro-melting-point apparatus. Optical rotation: *Jasco DIP-360* digital polarimeter; in MeOH. UV Spectra: *Hitachi UV-3200* spectrophotometer; in MeOH; λ_{max} in nm, (log ε). IR Spectra: *Jasco A-302* IR spectrophotometer; as discs in $CHCl_3$; cm^{-1}. Mass spectra: double-focusing *Varian MAT-311-A* mass spectrometer, peak matching; field-desorption (FD) and high-resolution electron-ionization (HR-EI) mass spectra: *Jeol HX-110* mass spectrometer; in *m*/*z* (rel. %). NMR Spectra: *Bruker Avance AMX-500*, *AM-400*, and *AC-300* spectrometers; chemical shifts δ in ppm rel. to $SiMe_4$ (=0 ppm) as internal standard, coupling constants *J* in Hz.

Plant Material. Whole plants of *S. hookeriana* BAILL. were collected at an altitude of 8000 feet from Phulchowki, Lalitpur District, Nepal, during April 2002. The plants were identified by a taxonomist at the National Herbarium and Plant Laboratories Section, Department of Plant Resources, Ministry of Forests and Soil Conservation, Godawari, Nepal, where a voucher specimen (No. 101/2002) was deposited.

Extraction and Isolation. The air-dried whole plant (31.0 kg) of *S. hookeriana* was extracted with 80% aq. MeOH (120 l). The conc. extract (2.8 kg) was dissolved in cold distilled H_2O (12 l) and defatted with petroleum ether (30 l) to afford, after concentration, fraction *SH-A* (304.3 g). The aq. layer was then extracted with CH_2Cl_2

(30 l) to obtain a 'neutral' fraction (107.8 g; *SH-B*). The aq. fraction was then acidified with AcOH to pH 3–4, and extracted with CH_2Cl_2 (30 l) to obtain an 'acidic' fraction (85.0 g; *SH-C*). The aq. acidic fraction was made alkaline (pH 9–10) by adding aq. NH_3 soln., and extracted again with CH_2Cl_2 (30 l) to obtain an 'alkaline' fraction (24.7 g; *SH-D*). The crude MeOH/H_2O extract was subjected to an anti-esterase assay. It inhibited AChE (acetylcholinesterase) and BChE (butyrylcholinesterase) by 81 and 87%, resp. Similarly, the fractions *SH-B*, *SH-C*, and *SH-D* showed 85 and 96, 68 and 87, and 88 and 97% inhibition of AChE and BChE, resp. Based on these results, fraction *SH-D* was selected for bio-assay-directed fractionation through repeated column chromatography (CC). Thus, the 'alkaline' fraction was adsorbed on SiO_2 (28 g; *Merck* silica gel *60*, 70–230 mesh) and eluted with different gradients of petroleum ether, acetone, and EtOH. Elution with acetone/petroleum ether 1:5 afforded subfraction *E* (2.5 g), which was further purified by FC (3.0 g SiO_2, 240–300 mesh) to afford the sub-subfractions E_1–E_{15}. Thereby, E_2–E_{10} were found to exhibit significant cholinesterase-inhibition activity. Compound **1** was obtained by elution of fraction E_9 (98.00 mg) with petroleum ether/acetone/Et_2NH 20:79:1. Similarly, compounds **2**, **3**, **4**, and **5** were obtained after elution of fractions E_8 (70.1 mg), E_3 (60.2 mg), E_{10} (70.0 mg), and E_1 (100.0 mg) with petroleum ether/acetone/Et_2NH 75:24:1, 94:5:1, 70:29:1, and 98:1:1, resp.

*(2β,3β,4β,20*S*)-20-(Dimethylamino)-3-[(3-methylbut-2-enoyl)amino]-5α-pregn-16-ene-2,4-diol* (*Hookerianamide A*; **1**). Yield: 37.41 mg (1.2×10^{-4}%). White crystalline solid. M.p. 212–214°. $[\alpha]_D^{24} = -53$ ($c = 0.060$, MeOH). UV (MeOH): 216 (4.1), 222 (4.2). IR ($CHCl_3$): 3300 (OH), 3280 (NH), 2922 (CH), 1640 (C=O), 1624 (C=C). ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*, resp. EI-MS: 458 (3, M^+), 443 (100), 258 (2), 149 (4), 100 (11), 83 (41), 72 (47), 55 (12). HR-EI-MS: 458.3447 (M^+, $C_{28}H_{46}N_2O_3^+$; calc. 458.3508).

*(2α,3β,4β,20*S*)-4-Acetoxy-20-(dimethylamino)-3-[(3-methylbut-2-enoyl)amino]-5α-pregnan-2-ol* (*Hookerianamide B*; **2**). Yield: 17.65 mg (5.6×10^{-5}%). White crystalline solid. M.p. 224–226°. $[\alpha]_D^{24} = +19.2$ ($c = 0.050$, MeOH). UV (MeOH): 210 (4.8), 221 (4.4). IR ($CHCl_3$): 3562, 3398 (NH); 3390 (OH); 2927 (CH); 1737 (ester C=O); 1632 (amide C=O); 1624 (C=C); 1157, 1056 (C–O). ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*, resp. EI-MS: 502 (2, M^+), 487 (4), 100 (12), 83 (34), 72 (100), 55 (34). HR-EI-MS: 502.3716 (M^+, $C_{30}H_{50}N_2O_4^+$; calc. 502.3685).

*(2β,3β,20*S*)-2-Acetoxy-20-(dimethylamino)-3-[(3-methylbut-2-enoyl)amino]-5α-pregnane* (*Hookerianamide C*; **3**). Yield: 14.22 mg (4.5×10^{-5}%). White crystalline solid. M.p. 240–242°. $[\alpha]_D^{24} = -136$ ($c = 0.10$, MeOH). UV (MeOH): 220 (3.6). IR ($CHCl_3$): 3304 (NH); 2934 (CH); 1737 (ester C=O); 1665 (amide C=O); 1630 (C=C); 1182, 1050 (C–O). ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*, resp. EI-MS: 486 (21, M^+), 471 (4), 100 (3), 83 (34), 72 (100), 55 (12). HR-EI-MS: 486.3857 (M^+, $C_{30}H_{50}N_2O_3^+$; calc. 486.3821).

*(3β,20*S*)-20-(Dimethylamino)-3-(methylamino)-5α-pregn-14-ene* (*Hookerianamine A*; **4**). Yield: 17.00 mg (5.4×10^{-5}%). White powder. $[\alpha]_D^{24} = -60$ ($c = 0.04$, MeOH). UV (MeOH): 211 (3.6). IR ($CHCl_3$): 3390 (NH), 2928 (CH), 1651 (C=C). ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*, resp. EI-MS: 358 (2, M^+), 343 (32), 72 (100), 71 (8), 57 (14). HR-EI-MS: 358.3325 (M^+, $C_{24}H_{42}N_2^+$; calc. 358.3263).

*(3β,20*S*)-20-(Methylamino)-3-[(2-methylbut-2-enoyl)amino]-5α-pregn-2-en-4-one* (*Phulchowkiamide A*; **5**). Yield: 27.00 mg (8.7×10^{-5}%). White gum. $[\alpha]_D^{24} = +48$ ($c = 0.10$, MeOH). UV (MeOH): 230 (3.9). IR ($CHCl_3$): 3386 (NH), 2933 (CH), 1663 (C=O), 1636 (C=C). ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*, resp. EI-MS: 426 (3, M^+), 411 (5), 98 (7), 83 (68), 58 (100), 55 (59). HR-EI-MS: 426.3246 (M^+, $C_{27}H_{42}N_2O_2^+$; calc. 426.3158).

*(2β,3β,4β,20*S*)-2,4-Bis(acetoxy)-20-(dimethylamino)-3-[(3-methylbut-2-enoyl)amino]-5α-pregn-16-ene* (**6**). Compound **1** (8.00 mg) was treated with Ac_2O in anh. pyridine at r.t. for 4 h: 8.11 mg of **6**. M.p. 236–238°. $[\alpha]_D^{28} = +80$ ($c = 0.040$, MeOH). UV (MeOH): 207 (4.4), 220 (4.2). IR ($CHCl_3$): 3335 (NH), 2930 (CH), 1737 (ester C=O), 1669 (amide C=O), 1640 (C=C). ^{1}H-NMR (400 MHz, $CDCl_3$): 0.79 (*s*, Me(18)); 1.02 (*s*, Me(19)); 1.18 (*d*, $J(21,20) = 6.1$, Me(21)); 1.83 (*s*, Me(5′)); 2.11 (*s*, Me(4′)); 2.34 (*s*, Me_2N); 2.00 (*s*, Ac); 2.04 (*s*, Ac); 2.91 (*q*, $J(20,21) = 6.4$, H–C(20)); 5.26 (*dd*, $J(4\alpha,5\alpha) = 4.0$, $J(4\alpha,3\alpha) = 3.9$, H_α–C(4)); 5.07 (br. *s*, $w_{1/2} = 7.2$, H_α–C(2)); 4.39 (*m*, H_α–C(3)); 5.68 (br. *s*, H–C(16)); 5.59 (*s*, H–C(2′)); 5.35 (*d*, $J(3\alpha,NH) = 7.5$, NH). EI-MS: 542 (19, M^+), 527 (70), 100 (30), 83 (97), 72 (100), 55 (72).

*(2α,3β,4β,20*S*)-2,4-Bis(acetoxy)-20-(dimethylamino)-3-[(3-methylbut-2-enoyl)amino]-5α-pregnane* (**7**). Compound **2** (8.00 mg) was treated with Ac_2O in anh. pyridine at r.t. for 3 h: 8.22 mg of **7**. M.p. 249–251°. $[\alpha]_D^{28} = +40$ ($c = 0.12$, MeOH). UV (MeOH): 208 (4.1), 216 (4.3). IR ($CHCl_3$): 3279 (NH), 2931 (CH), 1728 (ester C=O), 1641 (amide C=O), 1635 (C=C). ^{1}H-NMR (300 MHz, $CDCl_3$): 0.63 (*s*, Me(18)); 0.76 (*s*, Me(19)); 1.24 (*d*, $J(21,20) = 7.5$, Me(21)); 1.89 (*s*, Me(5′)); 1.97 (*s*, Me(4′)); 2.42 (*s*, Me_2N); 2.03 (*s*, Ac); 2.06 (*s*, Ac); 4.04 (*dd*, $J(4\alpha,5\alpha) = 4.0$, $J(4\alpha,3\alpha) = 3.9$, H_α–C(4)); 4.31 (br. *s*, $w_{1/2} = 18.7$, H_β–C(2)); 5.74 (*s*, H–C(2′)); 5.53 (*d*, $J(3\alpha,NH) = 8.0$, NH). EI-MS: 544 (4, M^+), 529 (5), 100 (15), 72 (100), 55 (97).

In vitro *Cholinesterase-Inhibition Assay.* Acetylcholinesterase (AChE; Electric-eel EC 3.1.1.7), butyrylcholinesterase (BChE; horse-serum E.C 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine chloride, 5,5′-dithiobis[2-nitrobenzoic-acid] (DTNB), and galanthamine were purchased from *Sigma* (St. Louis, MO, USA). Buffer and other chemicals were of anal. grade. AChE- and BChE activity-inhibiting activities were measured by a slightly modified spectrophotometric method developed by *Ellman et al.* [23]. Acetylthiocholine iodide and butyrylthiocholine chloride were used as substrates to assay AChE and BChE activity, resp. DTNB was used for the measurement of cholinesterase activity. Sodium phosphate buffer (pH 8.0; 140 µl of 100 mM soln.), DTNB (10 µl), test-compound soln. (20 µl), and AChE (20 µl) or BChE soln. (20 µl) were mixed and incubated for 15 min at 25°. The reaction was initiated by addition of acetylthiocholine (10 µl) or butyrylthiocholine (10 µl), resp. The hydrolyses of acetylthiocholine and butyrylthiocholine were monitored by the formation of the yellow 2-nitro-5-sulfidobenzenecarboxylate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine and butyrylthiocholine, resp., at a wavelength of 412 nm (15 min). Test compounds and the control were dissolved in EtOH. All the reactions were performed in triplicate in 96-well micro-plates (*SpectraMax 340*; *Molecular Devices*, USA).

Estimation of IC_{50} *Values.* The concentrations (IC_{50}) of test compounds that inhibited the hydrolysis of substrates (acetylthiocholine and butyrylthiocholine) by 50% were determined by monitoring the effect of increasing concentrations of these compounds in the assays on the inhibition values. The IC_{50} values were then calculated with the *EZ-Fit Enzyme Kinetics* program (*Perrella Scientific, Inc.*, Amherst, USA).

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