Pregnane-Type Steroidal Alkaloids of *Sarcococca saligna:* a New Class of Cholinesterase Inhibitors

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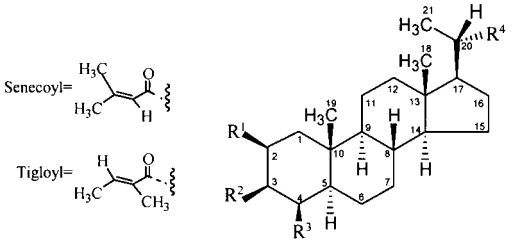
Phytochemical investigation of *Sarcococca saligna* by extensive bioassay-guided fractionation resulted in the isolation of the pregnane-type steroidal alkaloids **1**–**15**, *i.e.* of the five new compounds **1**–**5** and the ten known alkaloids **6**–**15**. The structures of the new alkaloids salignenamide C (**1**), salignenamide D (**2**), 2β-hydroxyepipachysamine D (**3**), salignenamide E (**4**), and salignenamide F (**5**) were elucidated with the help of modern spectroscopic techniques, while the known alkaloids axillarine C (**6**), axillarine F (**7**), sarcorine (**8**), N^3-demethylsaracodine (**9**), saligcinnamide (**10**), salignenamide A (**11**), vaganine A (**12**), axillaridine A (**13**), sarsalignone (**14**), and sarsalignenone (**15**) were identified by comparing their spectral data with those reported earlier. Inhibition of electric-eel acetylcholinesterase (EC 3.1.1.7) and horse-serum butyrylcholinesterase (EC 3.1.1.8) by alkaloids **1**–**15** were investigated. These new cholinesterase inhibitors may act as potential leads in the discovery of clinically useful inhibitors for nervous-system disorders, particularly by reducing memory deficiency in *Alzheimer*'s disease patients by potentiating and effecting the cholinergic transmission process. These compounds were found to inhibit both enzymes in a concentration-dependent fashion with the IC_{50} values ranging from 5.21–227.92 μM against acetylcholinesterase and 2.18–38.36 μM against butyrylcholinesterase.

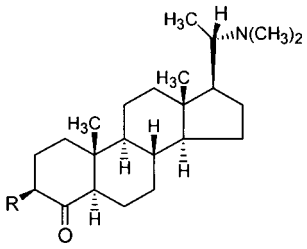
1. Introduction. – As part of our ongoing investigations on medicinal plants, we have studied the chemical constituents of the genus *Sarcococca*. The genus belongs to the family Buxaceae with sixteen to twenty species and is predominantly distributed from Afghanistan through the Indo-Pakistan subcontinent to Central China and Sri Lanka (Ceylon) to South-East Asia and the Philippine Islands [1]. The *Sarcococca* plants have been used since ancient times as folk remedies for ulcers, gastric disorders, and bacterial infections [2–4].

Our previous work on *S. saligna* (D. DON.) MUELL.-ARG. has resulted in the isolation of several steroidal alkaloids [5–9]. Recently, we focused our attention on the anticholinergic constituents of this plant. The aerial parts of the plant were collected, and its MeOH extract was subjected to an activity-guided fractionation by an *in vitro* high-throughput cholinesterase enzyme-inhibition assay. This resulted in the isolation of the five new steroidal alkaloids **1**–**5** and the ten known steroidal alkaloids **6**–**15**. Our interest in this study was mainly to develop a better understanding of the structure-activity relationship for this new class of cholinesterase inhibitors.

According to the cholinergic hypothesis, the memory impairment in the patients with senile dementia of the *Alzheimer* type results from a deficiency in cholinergic function in the brain [10][11]. Hence, the most promising therapeutic strategy for activating central cholinergic functions has been the use of cholinomimetic agents. The enzyme acetylcholinesterase (AChE) has long been an attractive target for the rational drug design and discovery of mechanism-based inhibitors, for the treatment of *Alzheimer*'s disease (AD). The aim of acetylcholinesterase inhibitors is to boost the



	R^1	R^2	R^3	R^4	Unsaturation
1	OH	NH-Tigloyl	AcO	Me_2N	$\Delta^{14,15}$
2	α–OH	NH-Tigloyl	H	Me_2N	$\Delta^{4,5}$ and $\Delta^{16,17}$
3	OH	NH-Benzoyl	H	Me_2N	
4	H	N(Me)COCH=C(Me)$CHMe_2$	H	Me_2N	$\Delta^{16,17}$
5	H	N(Me)COCH=C(Me)$CHMe_2$	H	Me_2N	
6	OH	NH-Benzoyl	AcO	Me_2N	
7	OH	NH-Tigloyl	AcO	Me_2N	
8	H	NHAc	H	Me_2N	
9	H	NHMe	H	AcN(Me)	
10	H	N(Me)-Cinnamoyl	H	Me_2N	
11	H	NHCOCH=C(Me)$CHMe_2$	H	Me_2N	
12	H	NH-Senecoyl	AcO	Me_2N	



	R	Unsaturation
13	NH-Benzoyl	$\Delta^{2,3}$
14	NH-Tigloyl	$\Delta^{5,6}$
15	NH-Tigloyl	$\Delta^{5,6}$ and $\Delta^{14,15}$

endogenous levels of acetylcholine in the brains of AD patients and, thereby, to boost cholinergic neurotransmission. Recently it has been found that butyrylcholinesterase (BChE) inhibition may also be an effective tool for the treatment of AD and related dementias [12].

The structures of the new alkaloids **1**–**5** were elucidated by NMR spectroscopic techniques (DEPT, broad-band decoupled ^{13}C-NMR, TOCSY, ROESY, HMQC, and HMBC) [13] and mass spectrometry.

2. Results and Discussion. – Our previous work on *Sarcococca* species has resulted in the isolation of several new steroidal alkaloids [5–9]. During this study, compounds

1–**15** were isolated from the MeOH extract of the dried whole plant of *S. saligna* by successive use of column chromatography and prep. TLC. The entire separation strategy was based on bioactivity screening in enzyme-inhibition assays.

Salignenamide C (**1**), obtained as a pale yellow gum, showed in the EI-MS a molecular ion at *m*/*z* 500, while the HR-EI-MS displayed the M^+ at *m*/*z* 500.3585 corresponding to the molecular formula $C_{30}H_{48}N_2O_4$ (calc. 500.3593) and indicating eight degrees of unsaturation. From the spectral data, it was inferred that compound **1** was closely related to the known base axillarine F (**7**), [15] with an additional double bond between C(14) and C(15). Consequently, the structure (*E*)-*N*-[(20*S*)-4β-(acetyloxy)-20-(dimethylamino)-2β-hydroxy-5α-pregn-14-en-3β-yl]-2-methylbut-2-enamide (**1**) was proposed for this new compound.

The characteristic mass fragments at *m*/*z* 72 and 83 of **1** helped in establishing the positions of the Me_2N and tiglamido moieties at the positions C(20) and C(3) of pregnane skeleton, respectively [14]. The IR spectrum of **1** showed absorptions at 3199–3400 (NH), 1750 (carbonyl), 1673, and 1630 (C=C) cm^{-1}. The UV spectrum displayed end absorption at 209 nm. The ^{1}H-NMR spectrum of **1** (*Table 1*) exhibited 2 *s* at δ 0.81 and 1.25 for Me(18) and Me(19). The *d* at δ 1.13 (J(21,20) = 6.5 Hz) was ascribed to Me(21). Another *s* at δ 2.07 was due to MeCO, while a 6 H *s* at δ 2.29 was ascribed to Me_2N. A *s* and a *d* at δ 1.79 and 1.73 (J(4′,3′) = 7.0 Hz) were attributed to Me–C(2′) and Me(4′) of the tiglamido moiety, respectively. A broad *s* at δ 4.04 ($w_{1/2}$ = 5.5 Hz) and a *ddd* at δ 4.09 (J(3α, NH) = 8.0, J(3α, 2α) = 4.0 and J(3α, 4α) = 4.0 Hz) were due to H–C(2) and H–C(3), respectively. A *dd* at δ 5.33 (proton geminal to the AcO group) was due to H–C(4), and this proton showed couplings with H–C(3) (δ 4.09) and H–C(2) (δ 4.04). A *q* at δ 6.37 (J3′,4′) = 7.0 Hz) was assigned to H–C(3′).

The ^{13}C-NMR spectrum (broad-band decoupled) of **1** (*Table 2*) showed signals for 30 C-atoms. The DEPT spectra indicated the presence of 8 Me, 6 CH_2, 10 CH, and (by difference from the broad-band spectrum) 6 quaternary C-atoms. The Me(4′) signal (δ 1.73) of the tiglamido moiety displayed HMBC correlations with C(3′) (δ 130.9), while Me–C(2′) (δ 1.79) showed correlations with C(1′) (δ 168.8). The positions of the OH and AcO groups were determined to be C(2) (δ 4.04, ($w_{1/2}$ = 5.5 Hz, H–C(2)) and C(4) (δ 5.33 (*dd*, J = 6.0, 5.7 Hz, H–C(4)), respectively, based on analysis of ^{1}H- and ^{13}C-NMR data. This was further confirmed by a COSY experiment, in which both H–C(2) (δ 4.04) and H–C(4) (δ 5.33) were found to be coupled with the H–C(3) (δ 4.09). In the HMBC spectrum, connectivities between δ(H) 1.25 (Me(19)) and δ(c) 69.8 (C(2)), 48.8 (C(5)), and 75.1 (C(4)) were also observed. The possible site of the C=C bond was determined by the HMBC experiment in which correlations between C(14) (δ(c) 156.7) and H–C(8) (δ(H) 1.58), and between Me(18) (δ(c) 15.8) and 2 H–C(15) (δ(H) 5.62) were observed. The configuration at C(2) and C(4) was suggested for biogenetic reasons, the coupling-constant values of H–C(2) and H–C(4) (δ(H) 4.04 (br. *s*, $w_{1/2}$ = 5.5 Hz) and δ(H) 5.33 (*dd*, J = 6.0, 5.7 Hz), resp., and the NOESY experiment. The NOESY interactions between H_α–C(3) (δ 4.09), H_α–C(2) (δ 4.04), and H_α–C(4) (δ 5.33) suggested β-orientations of the OH, tiglamido, and AcO functionalities (*Fig. 1*).

Salignenamide D (**2**) was isolated as colorless amorphous solid. The HR-EI-MS showed M^+ at *m*/*z* 440.3411, which corresponds to $C_{28}H_{44}N_2O_2$ (calc. 440.3402), indicating eight degrees of unsaturation in the molecule. Compound **2** had structural features similar to a known alkaloid salignenamide B, previously isolated by us [6]. The difference included the presence of a tiglamido group instead of a senecamido moiety, the α configuration of the OH group in ring A, and the presence of a C=C bond between C(16) and C(17) instead of C(14) and C(15) in ring D.

Thus, the structure (*E*)-*N*-[(20*S*)-20-(dimethylamino)-2α-hydroxypregna-4,16-dien-3β-yl]-2-methylbut-2-enamide (**2**) was deduced for this new compound.

The IR of **2** exhibited absorptions at 3602 (NH), 3349 (OH), 1641 (amide carbonyl), and 1611 (C=C) cm^{-1}, and UV absorptions at 254 and 231 nm. The ^{1}H-NMR spectrum (*Table 1*) exhibited 2 *s* at δ 0.87 and 1.03 assigned to Me(18) and Me(19). A *d* at δ 1.13 (J(21,20) = 6.6 Hz) was due to Me(21), while a 6 H *s* at δ 2.24 was ascribed to Me_2N. A *s* at δ 1.82 and a *d* at δ 1.74 (J(4′,3′) = 6.7 Hz) were attributed to Me–C(2′) and Me(4′),

Table 1. *^{1}H-NMR Data of the New Steroidal Alkaloids* **1**–**5**. δ in ppm, J in Hz[a])[b]).

	1	**2**	**3**	**4**	**5**
CH_2(1)	1.25, 2.18	1.61, 1.72	1.15, 1.88	1.88, 1.21	1.85, 1.13
H–C(2) or CH_2(2)	4.04 (br. *s*, $w_{1/2}=5.5$)	4.28 (*dddd*, $J=14.2$, 9.7, 5.0, 2.6)	4.01 (br. *s*, $W_{1/2}=6.6$)	1.80, 1.44	1.44, 1.80
H–C(3)	4.09 (*ddd*, $J=8.0$, 4.0, 4.0)	4.49 (*ddd*, $J=14.2$, 8.0, 5.0)	4.19 (*ddd*, $J=6.7$, 3.8, 2.9)	2.81 (*m*)	2.98 (*m*)
H–C(4) or CH_2(4)	5.33 (*dd*, $J=6.0$, 5.7)	5.70 (*dd*, $J=8.1$, 5.0)	1.25, 1.91	1.32, 1.58	1.32, 1.53
H–C(5)	1.39	–	1.4	1.25	2.15
CH_2(6)	1.45, 1.45	2.11, 2.30	1.15, 1.65	1.15, 1.65	1.01, 1.57
CH_2(7)	0.95, 1.68	1.32, 1.84	1.31, 2.10	1.81, 1.21	1.18, 1.21
H–C(8)	1.58	1.82	1.45	1.30	1.59
H–C(9)	0.71	0.81	1.04	1.59	1.58
CH_2(11)	1.45, 2.01	1.61, 1.42	1.31, 1.52	1.48, 1.05	1.21, 1.41
CH_2(12)	1.28, 1.77	1.36, 1.84	0.91, 1.75	0.91, 1.62	1.53, 0.84
H–C(14)	–	1.36	1.04	1.35	1.02
H–C(15) or CH_2(15)	5.62	2.01, 1.88	1.31, 2.13	1.25, 1.28	1.25, 1.28
CH_2(16) or H–C(16)	1.76, 2.01	5.51	1.71, 20.1	5.33 (br. *s*)	1.47, 1.79
H–C(17)	1.25	–	0.68	–	1.42
Me(18)	0.81 (*s*)	0.87 (*s*)	0.65 (*s*)	0.68 (*s*)	0.63 (*s*)
Me(19)	1.25 (*s*)	1.03 (*s*)	1.05 (*s*)	0.79 (*s*)	0.79 (*s*)
H–C(20)	2.93 (*m*)	2.90 (*q*, $J=6.6$)	3.84 (*m*)	2.80 (*q*, $J=6.8$)	3.15 (*m*)
Me(21)	1.13 (*d*, $J=6.5$)	1.13 (*d*, $J=6.6$)	0.86 (*d*, $J=6.7$)	1.03 (*d*, $J=6.8$)	0.86 (*d*, $J=6.5$)
MeN	–	–		2.83 (*s*)	2.83 (*s*)
Me_2N	2.29 (*s*)	2.24 (*s*)	2.50 (*s*)	2.39 (*s*)	2.16 (*s*)
H–C(2′)	–	–	–	5.71	5.7
H–C(3′) or Ph	6.37 (*q*, $J=7.0$)	6.40 (*q*, $J=6.7$)	7.91	–	–
Me(4′), Ph, or H–C(4′)	1.73 (*d*, $J=7.0$)	1.74 (*d*, $J=6.7$)	7.62	2.23	2.3 (*m*)
Me–C(2′), Pr, or Me(5′)	1.79 (*s*)	1.82	7.44	0.97 (*d*, $J=6.8$)	1.04 (*d*, $J=6.9$)
Ph or Me–C(4′)	–	–	7.62	0.97 (*d*, $J=6.7$)	1.06 (*d*, $J=6.8$)
Pr or Me–C(3′)	–	–	7.91	1.72	1.79
MeCOO	2.07				

[a]) All assignments were confirmed by ^{1}H,^{1}H COSY, HMQC, and DEPT techniques. [b]) $CDCl_3$.

respectively. A *q* at δ 6.40 ($J(3',4')=6.7$ Hz) was due to H–C(3′). Two signals at δ 4.28 (*dddd*, $J(2\beta,3\alpha)=14.2$, $J(2\beta,1\alpha)=9.7$, $J(2\beta,1\beta)=5.0$ and $J(2\beta,4)=2.6$ Hz) and 4.49 (*ddd*, $J(3\alpha,2\beta)=14.2$, $J(3\alpha,$ NH$)=8.0$ and $J(3\alpha,4)=4.0$ Hz) were due to H–C(2) and H–C(3), respectively. A *dd* at δ 5.70 ($J(4,2\beta)=8.1$ and $J(4,3\alpha)=5.0$ Hz) was due to the olefinic H–C(4).

The ^{13}C-NMR (broad-band decoupled and DEPT) spectra of **2** (*Table 2*) exhibited signals for 28 C-atoms (7 Me, 7 CH_2, 9 CH, and 5 quaternary C-atoms). The HMQC spectrum of **2** in conjunction with the COSY 45° spectrum was used to assign the chemical-shift values to all the C- and H-atoms which were further deduced from HMBC. The Me(4′) (δ 1.74) and Me–C(2′) (δ 1.82) displayed HMBC correlations with C(3′) (δ 130.3) and C(1′) (δ 168.7), respectively, and indicated the presence of a tiglamido moiety in pregnane alkaloid **2**. The

Table 2. *^{13}C-NMR Data* (δ in ppm) *of New Steroidal Alkaloids* **1**–**5**[a])[b])

	1	**2**	**3**	**4**	**5**
C(1)	44.4	33.3	40.6	39.6	39.6
C(2)	69.8	68.7	69.4	28.9	28.5
C(3)	51.5	43.7	51.2	48.5	45.5
C(4)	75.1	115.3	41.7	35.9	32.4
C(5)	48.8	151.8	53.6	45.9	45.5
C(6)	25.4	41.8	24.1	24.5	24.0
C(7)	31.6	34.4	28.7	27.6	37.6
C(8)	33.3	32.8	34.7	35.3	35.3
C(9)	56.8	54.5	56.4	53.9	54.0
C(10)	35.0	38.1	35.7	36.5	35.3
C(11)	20.4	21.3	20.8	21.0	21.0
C(12)	34.4	34.4	31.8	31.7	32.4
C(13)	46.7	46.8	42.7	42.1	39.5
C(14)	156.7	56.9	56.4	56.3	57.9
C(15)	124.1	31.2	28.1	29.7	29.7
C(16)	31.0	118.7	27.1	121.7	27.2
C(17)	57.2	151.8	55.4	152.7	54.1
Me(18)	15.8	15.9	11.45	12.1	11.9
Me(19)	19.4	19.4	14.29	12.2	12.1
C(20)	59.1	59.2	63.4	62.0	62.1
Me(21)	16.0	16.0	12.4	10.3	10.3
MeN	–	–	–	31.8	31.0
Me_2N	42.3	42.3	39.6	39.5	39.3
C(1′)=O	168.8	168.7	167.1	169.5	169.8
C(2′)	131.6	132.1	134.5	116.1	116.0
C(3′) or Ph	130.9	130.3	126.9	140.3	152.7
C(4′) or Ph	12.3	13.9	128.6	37.9	36.5
Me–C(2′), Ph, or Me(5′)	13.4	12.5	131.5	20.9	20.8
Ph or *Me*–C(4′)	–	–	128.6	20.9	20.9
Ph or *Me*–C(3′)	–	–	126.9	15.8	15.7
Me*C*OO	170.1	–	–	–	–
*Me*COO	20.7	–	–	–	–

[a]) All assignments were confirmed by ^{1}H,^{1}H COSY, HMQC, and DEPT techniques. [b]) $CDCl_3$.

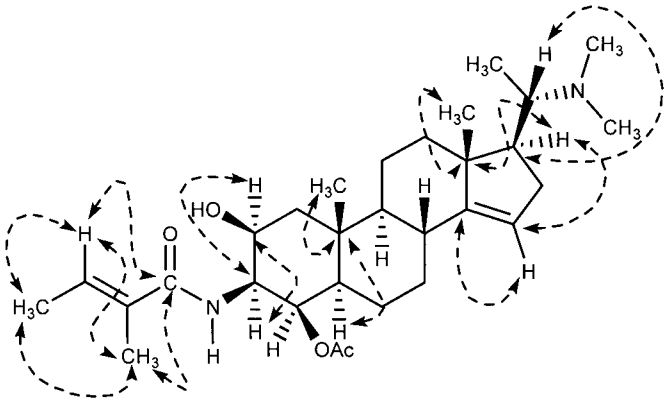


Fig. 1. *Selected HMBC interactions of compound* **1**

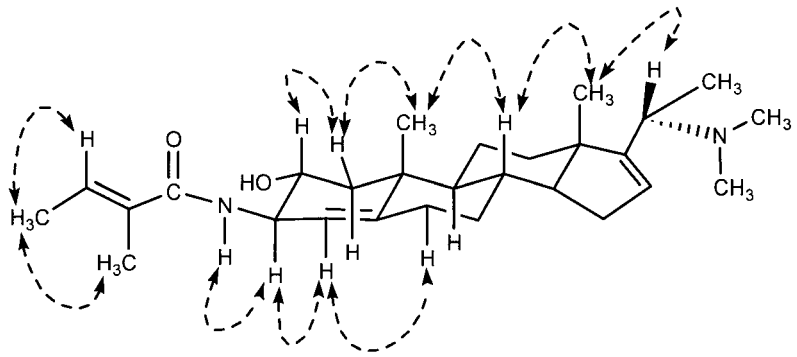


Fig. 2. *Selected ROESY interactions of compound* **2**

H–C(3) (δ 4.49) showed a COSY 45° coupling with H–C(4) (δ 5.70); furthermore, in the HMBC, the quaternary C(5) (δ 151.8) was coupled with Me(19) (δ 1.03), hence indicating the presence of a C=C bond between C(4) and C(5), while the other C=C bond was located at C(16) and C(17), since H–C(16) (δ 5.51) and Me(18) (δ 0.87) were correlated with C(13) (δ 46.8). Me(21) (δ 1.13) showed a correlation with C(17) (δ 151.8). Similarly, Me(19) (δ 1.03) exhibited a HMBC with C(10) (δ 38.1). The CH_2(12) (δ 1.36, 1.84) and Me(21) (δ 1.13) showed correlations with C(13) (δ 46.8) and C(17) (δ 151.8), respectively. The presence of an OH group in ring A was inferred from the HMBC couplings of H–C(2) (δ 4.28) with C(1) (δ 33.3), C(3) (δ 43.7) and C(10) (δ 38.1). The α configuration (equatorial) of OH–C(2) was deduced from the coupling constants (*dddd*, $J(2\beta,3) = 14.2$, $J(2\beta,1\alpha) = 9.7$, $J(2,1\beta) = 5.0$, and $J(2\beta,4) = 2.6$ Hz) and the ROESY interactions of H–C(2) with H–C(1) (δ 1.72) and Me(19) (δ 1.03). The configurations at C(2) and C(3) were further deduced from a 1D NOE experiment in which irradiation of the signals of H–C(2) and H–C(3) did not cause any enhancement on each other, which indicated the *trans* diaxial disposition of these protons, while irradiation of the H–C(2) signal resulted in the enhancement of the H_β–C(1) signal and, therefore, indicated the β orientation of this proton. The irradiation of the H_β–C(1) signal also resulted in the enhancement of the Me(19) signal (δ 1.03).

The 2β-hydroxyepipachysamine D (**3**) of formula $C_{30}H_{46}N_2O_2$ (HR-EI-MS: *m*/*z* 466.3550) was also a new metabolite. Extensive spectral studies revealed that the compound was distinctly similar to the known alkaloid epipachysamine D [7], except

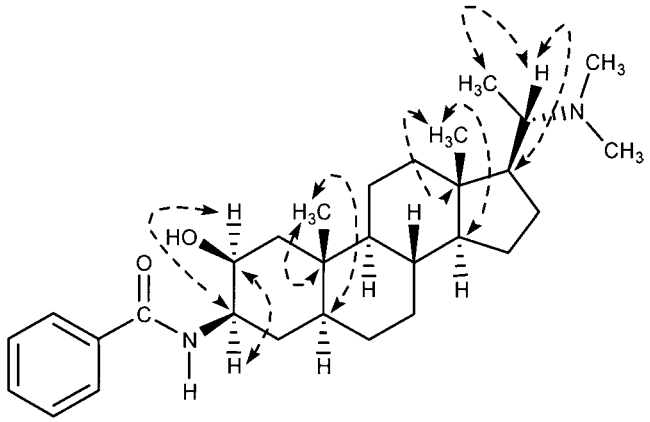


Fig. 3. *Selected HMBC interactions of compound* **3**

for the presence of an additional β-OH group in ring A. Therefore, the structure (*E*)-*N*-[(20*S*)-20-(dimethylamino)-2β-hydroxy-5α-pregnan-3β-yl]benzamide (**3**) was deduced.

Alkaloid **3** exhibited IR absorptions at 3620 (NH) and 3425 (OH) cm^{-1}. The ion at *m*/*z* 451 resulted from the loss of Me(21) from M^+. The base peak at *m*/*z* 72 indicated the presence of an (dimethylamino)ethyl substituent at C(17), while the ion at *m*/*z* 105 suggested the presence of a benzamide group at C(3) [14]. The ^{1}H-NMR spectrum (*Table 1*) exhibited two *s* at δ 0.65 and 1.05 assigned to Me(18) and Me(19). A *d* at δ 0.86 (J(21,20) = 6.7 Hz) was due to Me(21), while a 6 H *s* at δ 2.50 was ascribed to Me_2N. Three *m* at δ 7.91 (2 H), 7.62 (2 H), and 7.44 (1 H) indicated the presence of a monosubstituted benzene ring. A broad *s* at δ 4.01 ($W_{1/2}$ = 6.6 Hz) and a *ddd* at δ 4.19 were assigned to H–C(2) and H–C(3), respectively. In the HMBC spectrum of **3**, H–C(2) (δ 4.01) showed correlations with C(1) (δ 40.6), C(3) (δ 51.2), and C(10) (δ 35.7), which indicated that an OH group was present at C(2) of the pregnane skeleton. The β configuration of OH–C(2) was deduced from the coupling constant ($W_{1/2}$ = 6.6 Hz) and the ROESY interactions of H–C(2) (δ 4.01) with H–C(3) (δ 4.19) and H_a–C(1) (δ 1.15).

Salignenamide E (**4**) was found to have the molecular formula $C_{31}H_{52}N_2O$ (HR-EI-MS *m*/*z* 468.4085). The spectral data (see also *Tables 1* and *2*) led to the assignment of the structure (*E*)-*N*-[(20*S*)-20-(dimethylamino)-5α-pregn-16-en-3β-yl)-*N*,3,4-trimethylpent-2-enamide (**4**) for this new compound.

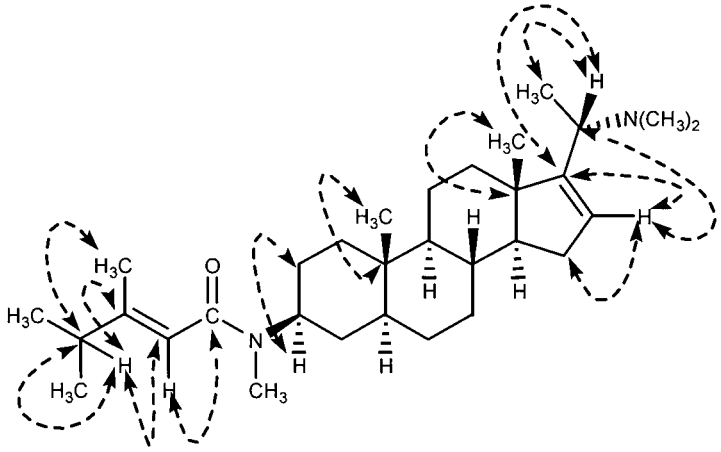


Fig. 4. *Selected HMBC interactions of compound* **4**

The IR spectrum of **4** showed absorptions at 3400 (NH), 2850 and 1695 cm^{-1}. The peak at *m*/*z* 453 resulted from loss of a Me group from the M^+. The ion at *m*/*z* 72 represented the *N*,*N*-dimethylethanaminium ion, while the ions at *m*/*z* 111 and 142 indicated the presence of *N*,3′,4′-trimethylpentenamido moiety at C(3) [14]. The ^{1}H-NMR spectrum of **4** (*Table 1*) showed the presence of six tertiary Me groups resonating at δ 0.68 (*s*, Me(18)), 0.79 (*s*, Me(19)), 1.72 (*s*, Me–C(3′)), 2.39 (br. *s*, Me_2N), and 2.83 (3 br. *s*, MeN). H–C(3) resonated as a *m* at δ 2.81, which showed *W* coupling with H–C(5) (δ 1.25) in the TOCSY and COSY spectra. A downfield-shifted olefinic proton at δ 5.33 (br. *s* was ascribed to H–C(16)). The position of a C=C bond between C(16) and C(17) was based on the HMBC interaction of H–C(20) (δ 2.80) and H–C(16) (δ 5.33) with the olefinic C(17) (δ 152.7). In addition to this, EI-MS also gave strong evidence that a C=C bond is present between C(16) and C(17) because of the lower intensity of the *N*,*N*-dimethylethanaminium ion [14]. The HMBC spectrum indicated the presence of a *N*,3′,4′-trimethylpentenamido moiety at C(3) by the interactions of olefinic H–C(2′) (δ 5.71) with C(1′) (δ 169.5), C(3′) (δ 140.3), and C(4′) (δ 37.9). The (2*E*) configuration of the

N,3′,4′-trimethylpentenamido group was inferred from the ROESY correlations of H–C(2′) (δ 5.71) with H–C(4′) (δ 2.23) and Me(5′) (δ 0.97), which suggested that both H–C(2′) and the Me_2CH group were in close proximity.

Salignenamide F (**5**) was isolated as a white powder. HR-EI-MS Analysis established the molecular formula $C_{31}H_{54}N_2O$ (*m*/*z* 470.4241) with six degrees of unsaturation. With the help of HMBC and TOCSY experiments, the structure (*E*)-*N*-[(20*S*)-20-(dimethylamino)-5α-pregnan-3β-yl)-*N*,3,4-trimethylpent-2-enamide (**5**) was deduced for this new alkaloid.

The mass fragmentation pattern, IR, and ^{1}H- and ^{13}C-NMR data of **5** (*Tables 1* and *2*) were distinctly similar to those of compound **4**, except for the absence of the C(16)=C(17) bond in ring D of the steroidal skeleton. Absence of the C=C bond was also supported by the appearance of *N*,*N*-dimethylethanaminium cation as the base peak at *m*/*z* 72 in the EI-MS mass fragmentation [14].

The configurations assigned to compounds **1**–**5** is relative and based on spectroscopic evidences. Biogenetic considerations about the pregnane series showed the latter to arise in nature from cholesterol, and, thus, the assigned configuration in the cyclopentenophenanthrene moiety is based thereupon [16].

The steroidal alkaloids axillarine C (**6**) [15], axillarine F (**7**) [15], and axillaridine A (**13**), [18] were also isolated for the first time from *S. saligna*. These compounds were previously reported from *Pachysandra axillaris*. The UV, IR, NMR, and mass spectra of these compounds were identical to those reported in the literature.

Seven known compounds, sarcorine (**8**) [5], N^3-demethylsaracodine (**9**) [9], saligcinnamide (**10**) [7], salignenamide A (**11**) [6], vagenine A (**12**) [8], sarsalignone (**14**) [8], and sarsalignenone (**15**) [8] were also isolated from this plant and structurally identified by comparison with literature data. The anticholinesterase activity of these compounds is reported here for the first time.

Biological Evaluation. The alkaloids **1**–**15** were found to be nontoxic in the brine-shrimp lithality assay ($LD_{50} > 1000$ μg/ml). *Table 3* illustrates the inhibitory activities of compounds **1**–**15** against the enzymes AChE and BChE. All these compounds were more selective toward BChE, whereas eserine was about 21 times more selective towards AChE. Since most of the steroidal alkaloids **1**–**15** described here displayed cholinesterase inhibition, structure-activity-relationship (SAR) studies were also carried out, indicating that the sarsalignone (**14**) and sarsalignenone (**15**) are the most potent in this series of compounds. Apparently, the anticholinesterase activity may be associated with the presence of an α,β-unsaturated amide functionality at C(3) of ring A. In addition to this, the presence of an α,β-unsaturated carbonyl moiety between rings A and B is probably also contributing to the enhancement of the activity in many manifolds. The presence of an electron-rich functionality in ring A apparently decreases the anticholinesterase activity of the concerned compounds, *e.g.*, compounds **1**–**3**, **6**, and **7** have high IC_{50} values (*Table 3*) as compared to those of compounds **14** and **15**. Another factor that probably contributes to the anticholinesterase activity of these compounds is the presence of dimethylamino moiety at C(20). The presence of a basic N-atom can also be linked to the possibility of active transport of the drug into the cell.

All the known acetylcholinesterase-inhibiting drugs used in *Alzheimer*'s disease suffer from several major drawbacks such as high toxicity, short duration of biological

Table 3. In vitro *Anticholinesterase Activities of Compounds* **1**–**15**

	Acetylcholinesterase	Butyrylcholinesterase	Selectivity	
	IC_{50} [μM] (mean ± s.e.m. (n)[a]))	IC_{50} [μM] (mean ± s.e.m. (n)[a]))	AChE	BChE
1	61.3 ± 2.02 (5)	38.36 ± 0.745 (3)		7.79
2	185.2 ± 7.66 (3)	23.78 ± 0.157 (3)		1.6
3	78.2 ± 2.325 (5)	28.96 ± 0.007 (3)		2.7
4	6.21 ± 0.234 (5)	3.65 ± 0.023 (3)		3.1
5	6.357 ± 0.224 (3)	4.07 ± 0.108 (3)		1.56
6	227.92 ± 8.677 (3)	17.99 ± 0.222 (3)		12.67
7	182.4 ± 5.542 (3)	18.24 ± 0.015 (3)		10
8	69.99 ± 0.056 (3)	10.33 ± 0.024 (3)		6.778
9	204.2 ± 4.951 (5)	16.55 ± 0.20 (3)		12.33
10	19.99 ± 0.123 (5)	4.84 ± 0.122 (3)		4.13
11	50.64 ± 0.930 (5)	4.63 ± 0.073 (3)		10.94
12	8.59 ± 0.155 (5)	2.32 ± 0.06 (3)		3.7
13	5.21 ± 0.105 (5)	2.49 ± 0.063 (3)		2.09
14	7.02 ± 0.007 (5)	2.18 ± 0.035 (3)		3.22
15	5.83 ± 0.070 (5)	4.29 ± 0.029 (3)		1.36
Eserine	0.041 ± 0.001	0.857 ± 0.008	20.9	

[a]) n = Number of independent experiments.

action, low bioavailability, and narrow therapeutic windows. These new cholinesterase inhibitors may act as potential leads in the discovery of clinically useful inhibitors for nervous-system disorders, particularly by reducing memory deficiency in *Alzheimer*'s disease patients by potentiating and effecting the cholinergic transmission process [19].

Experimental Part

General. All reagents used were of anal.-grade. TLC: pre-coated aluminium foils *60* F_{254} of *E. Merck*, *Dragendorff* spraying reagent. $[\alpha]_D$: a *Jasco* digital polarimeter, model *DIP-360*, *International Co. Ltd.*, Japan; in MeOH or $CHCl_3$. UV Spectra: anal.-grade MeOH; *Hitachi* spectrophotometer model *U-3199*; λ_{max} (log ε) in nm. IR Spectra: *Shimadzu IR-460*-spectrometer (*Shimadzu Corporation*, Japan); as discs in KBr, *Jasco A-302-IR* spectrophotometer; in cm^{-1}. NMR Spectra: *Bruker AM-400-MHz* and *-AMX-500-MHz* spectrometers; chemical shifts δ in ppm rel. to $SiMe_4$ (= 0 ppm) as internal standard, coupling constants J in Hz. Mass spectra: double-focusing mass spectrometers *MAT 112S* or *Finnigan MAT 312*; peak matching, field-desorption (FD), and fast-atom-bombardment (FAB) mass measurements with a *Varian-MAT-312* mass spectrometer; accurate mass measurements with the FAB source and glycerol as an internal standard; high-resolution electron impact (HR-EI) with a *Jeol-JMS-HX-110* mass spectrometer; in m/z (rel. %).

Extraction and Isolation. Whole plants of *Sarcococca saligna* (50 kg) were collected in October 1999 from the District Bagh of Azad Kashmir, Pakistan. The air-dried plant material (14 kg) was crushed and soaked in MeOH (50 l) for 15 days. The MeOH extract was evaporated and the residue (1.25 kg) dissolved in distilled H_2O (5 l) and extracted with petroleum ether (251.0 g; *A*), $CHCl_3$ at pH 3 (6.0 g; *B*), pH 7 (220.0 g; *C*), and pH 9 (25.0 g; *D*), and finally with AcOEt (45.0 g; *E*). The crude MeOH extract of *S. saligna* exhibited promising anticholinesterase activity (100%, 45 mg/ml). The $CHCl_3$ *Fr. B* and *D* also showed 100% inhibition against the enzyme (25 mg/ml). Based on these results, *Fr. B* and *D* were subjected to bioassay-guided repeated column chromatography (silica gel) and prep. TLC to give five new and ten known compounds. *Fr. B* was loaded on a column prepacked with silica gel (230–240 mesh). Elution was carried out with increasing polarities of petroleum ether/acetone/Et_2NH to give eight subfractions (*Fr. B1*–*B8*). *Fr. B2*–*B5* exhibited significant cholinesterase inhibition at 20 mg/ml. *Fr. B2*–*B5* were individually subjected to flash column chromatography (silica gel, increasing polarities of petroleum ether/acetone/Et_3NH) to give impure subfractions that were finally

purified by prep. TLC (precoated silica-gel glass plates, acetone/petroleum ether/Et_2NH 1.5 : 8.3 : 0.2): two new compounds **3** and **4** and two known compounds **6** and **11**.

Fr. D was also subjected to column chromatography and eluted with increasing polarities of petroleum ether/acetone to afford 11 (*Fr. D1 – D11*). *Fr. D2 – D10* showed significant inhibitory activity against cholinesterase (20 mg/ml) and were subjected to flash column chromatography (silica gel, type *60*, PF_{254} (No. 7749 *Merck*), petroleum ether/acetone/Et_2NH). The resulting fractions were purified by prep. TLC (precoated silica-gel glass plates acetone/petroleum ether/Et_2NH 2.0 : 8.0 : 0.2): three new compounds **1**, **2**, and **5** and eight known compounds **7** – **10** and **12** – **15**.

The anti-cholinesterase activities of all the pure compounds were determined (see *Table 3*).

*Salignenamide C (=(*E*)-*N*-[(20*S*)-4β-(Acetyloxy)20-(dimethylamino)-2β-hydroxy-5α-pregn-14-en-3β-yl]-2-methylbut-2-enamide*; **1**)]: Pale yellow amorphous gum (19.7 mg, $1.41 \cdot 10^{-4}$%). $[\alpha]_D^{25} = 90$ ($c = 0.1$, $CHCl_3$). UV (MeOH): 209 (3.1). IR ($CHCl_3$): 3199 – 3400 (NH), 2854, 2911, 1750, 1673, 1630, 1501, 1384, 956. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*, resp.). HR-EI-MS: 500.3585 (15, M^+, $C_{30}H_{48}N_2O_4^+$; calc. 500.3593), 485.3420 (54, $C_{29}H_{45}N_2O_4^+$), 83.0504 (68, $C_5H_7O^+$), 72.0803 (100, $C_4H_{10}N^+$), 55.0564 (55, $C_4H_7^+$).

*Salignenamide D (=(*E*)-*N*-[(20*S*)-20-(Dimethylamino)-2α-hydroxypregna-4,16-dien-3β-yl]-2-methylbut-2-enamide*; **2**): Colorless amorphous solid (9.9 mg, $7.07 \cdot 10^{-5}$%). $[\alpha]_D^{25} = 8$ ($c = 0.3$, $CHCl_3$). UV (MeOH): 254 (3.66), 231 (3.63). IR: 3602 (NH), 3349 (OH), 1641 (C=O), 1611 (C=C). HR-EI-MS: 440.3411 (6, $C_{28}H_{44}N_2O_2^+$, M^+; calc. 440.3402), 425.3167 (100, $[M - Me]^+$, $C_{27}H_{41}N_2O_2^+$), 83.0517 (47, $C_5H_7O^+$), 72.0803 (85, $C_4H_{10}N^+$), 55.0499 (36, $C_3H_5N^+$).

*2β-Hydroxyepipachysamine D (=(*E*)-*N*-[(20*S*)-20-(Dimethylamino)-2β-hydroxy-5α-pregnane-3β-yl]benzamide*; **3**): Colorless amorphous solid (9.8 mg, $7.00 \cdot 10^{-5}$%). $[\alpha]_D^{25} = 52$ ($c = 0.21$, $CHCl_3$. UV (MeOH): 269 (3.78), 221 (2.7). IR: 3620 (NH), 3425 (OH), 1665 (benzamide). ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*, resp. HR-EI-MS: 466.3550 (1, $C_{30}H_{46}N_2O_2^+$, M^+; calc. 466.3559), 451.3270 (6, $C_{29}H_{43}N_2O_2^+$), 105.0405 (42, $C_7H_5O^+$), 72.0811 (100, $C_4H_{10}N^+$).

*Salignenamide E (=(*E*)-*N*-[(20*S*)-20-(Dimethylamino)-5α-pregn-16-en-3β-yl]-N,3,4-trimethylpent-2-enamide*; **4**): Colorless amorphous solid (13.25 mg, $9.46 \cdot 10^{-5}$%). UV (MeOH): 270 (3.52), 219 (3.7). IR: 3400 (NH), 1695 (C=O), 1595 (C=C). ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*, resp. HR-EI-MS: 468.4085 (10, $C_{31}H_{52}N_2O^+$, M^+; calc. 468.4079), 453.3835 (100, $C_{30}H_{49}N_2O^+$), 142.1212 (9, $C_8H_{16}ON^+$), 111.0792 (30, $C_7H_{11}O^+$), 84.0822 (12, $C_5H_{10}N^+$), 72.0825 (68, $C_4N_{10}N^+$).

*Salignenamide F (=(*E*)-*N*-[(20*S*)-20-(Dimethylamino)-5α-pregnan-3β-yl]-N,3,4-trimethylpent-2-enamide*; **5**): Colorless amorphous solid (12.52 mg, $8.94 \cdot 10^{-5}$%). UV (MeOH): 279 (3.78), 266 (2.7). IR: 3402 (NH), 1690 (C=O), 1600 (C=C). ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*, resp. HR-EI-MS: 470.4241 (11, M^+, $C_{31}H_{54}N_2O^+$; calc. 470.4236), 455.3960 (13, $C_{30}H_{51}N_2O^+$), 142.1212 (10, $C_8H_{16}NO^+$), 111.0792 (36, $C_7O_{11}O^+$), 84.0822 (14, $C_5H_{10}N^+$), 72.0825 (100, $C_4H_{10}N^+$).

In vitro *Cholinesterase Inhibition Assay.* Acetylcholinesterase- and butyrylcholinesterase-inhibiting activities were measured by slightly modifying the spectrophotometric method developed by *Ellman et al.* [20]. Electric-eel AChE (type VI-S, *Sigma*), and horse-serum BChE (*Sigma*) were used, while acetylthiocholine iodide and butyrylthiocholine chloride (*Sigma*), resp., were used as substrates of the reaction. The 5,5′-dithiobis[2-nitrobenzoic-acid] (DTNB, *Sigma*) was used for the measurement of the cholinesterase activity. All the other reagents and conditions were the same as described previously [21]. In this procedure, 140 µl of 0.1 mM sodium phosphate buffer (pH 8.0), 10 µl of DTNB, 20 µl of test-compound soln., and 20 µl of acetylcholinesterase or butyrylcholinesterase soln. were mixed and incubated for 15 min at 25°. The reaction was then initiated by the addition of 10 µl of acetylthiocholine or butyrylthiocholine, resp. The hydrolysis of acetylthiocholine and butyrylthiocholine was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine or butyrylthiocholine, resp. at a wavelength of 412 nm. Test compounds and control were dissolved in 5% EtOH. All the reactions were performed in triplicate.

Estimation of IC_{50} *Values.* The concentrations of test compounds that inhibited the hydrolysis of substrates (acetylthiocholine and butyrylcholine) by 50% (IC_{50} values) 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 using with the EZ-Fit Enzyme Kinetics Program (*Perrella Scientific Inc.*, Amherst, U.S.A.).

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