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New Butyrylcholinesterase Inhibitory Triterpenes from *Salvia santolinifolia*

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Slavins A (**1**) and B (**2**), the new amyrin type triterpenes, have been isolated from the chloroform soluble fraction of *Salvia santolinifolia* and assigned structures on the basis of spectral studies including 2D NMR. Both the compounds displayed inhibitory potential against the enzyme butyrylcholinesterase.

Key words: *Salvia santolinifolia*, Labiatae, Triterpenes, Butyrylcholinesterase inhibition

INTRODUCTION

The genus *Salvia* belongs to family Labiatae which comprises of 24 species. This is a genus of suffruticose, much branched herbs and under shrubs distributed in Pakistan, India and Afghanistan. *Salvia santolinifolia* is a branched, scabrid and hispid straggling under shrub occurring in rocky arid areas. In Pakistan it occurs in Peshawar, Baluchistan and Karachi (Ali & Nasir 1990). Various species of this genus are widely used for treatment of coronary heart diseases, particularly angina pectoris, myocardial infarction, amenorrhea, dismenorrhea and insomnia (Ryu *et al.*, 1997). These also possess antiseptic, carminative, diuretic, hemostatic and spasmolytic activities (Ulubelen *et al.*, 2000). The leaves and seeds of *S. santolinifolia* are used as demulcent in diarrhoea and haemorrhoids. The copious mucilage is frequently mixed and used with the seeds of *Plantago ovata* to produce "*Ispaghol*". The seeds are also said to be useful in removing foreign bodies from the eye (Krishnamurthi 1978). Literature survey of the genus *Salvia* revealed the presence of a variety of compounds including diterpenes, aromatic ethers, phenolic glycosides, abietane type pigments and triterpenes (Ryu *et al.*, 1997, Ulubelen *et al.*, 2000, Nagy *et al.*, 1998). However, no work has so far been carried out on *S. santolinifolia*. A methanolic extract of this plant revealed a strong toxicity in brine shrimp lethality test (Meyer *et al.*, 1982). Further pharmacological screening showed inhibitory activity against the enzyme butyrylcholinesterase which was most pronounced in the chloroform soluble fraction. This prompted us to carried out the isolation of bioactive compounds from the chloroform soluble fraction. These studies have now resulted in the isolation and structure elucidation of two new triterpenes of amyrin-class named as salvins A (**1**) and B (**2**), respectively. Both of these showed inhibitory potential against enzyme butyrylcholinesterase.

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MATERIALS AND METHODS

General experimental procedures

Column chromatography was carried out using silica gel 70-230 mesh and flash chromatography on silica gel 230-400 mesh. Aluminum sheets precoated with silica gel 60 F_{254} (20×20 cm, 0.2 mm thick; E-Merck) were used for TLC to check the purity and were visualized under UV light (254 and 366 nm) using ceric sulfate reagent. Optical rotations measured on a Jasco DIP-360 digital polarimeter. IR spectra were recorded on Shimadzu IR-460 spectrophotometer (ν in cm^{-1}). EIMS, FD, FABMS, and HRFABMS spectra were recorded on Jeol JMS-HX 110 spectrometer with data system. The ^{1}H- and ^{13}C-NMR spectra were recorded on Bruker AMX-400 and AMX-500 instruments, using TMS as an internal reference. The chemical shift values are reported in ppm (δ) units and the scalar

coupling constants (*J*) are in Hz.

Materials

The whole plant of *Salvia santolinifolia* Boiss (14 kg) was collected from Karachi (Pakistan) in July 2002 and identified by Dr. Surriya Khatoon, Plant Taxonomist, Department of Botany, University of Karachi, where a voucher specimen (LS 831) is deposited.

Extraction and isolation

The whole plant of *S. santolinifolia* were shade dried, ground and extracted with methanol (3×50 L). The combined methanolic extract (550 g) was partitioned between *n*-hexane and water. The aqueous fraction was further extracted with chloroform, ethylacetate and *n*-butanol. The $CHCl_3$ soluble fraction (65g) was subjected to column chromatography over silica gel eluting with *n*-hexane-$CHCl_3$, $CHCl_3$, $CHCl_3$-MeOH and MeOH in increasing order of polarity. The fraction (28 mg) obtained from $CHCl_3$-MeOH (9.9:0.1) was subjected to flash chromatography over silica gel using solvent system $CHCl_3$-MeOH (9.8:0.2) to afford compound **1** (15 mg). The fraction (36 mg) obtained from $CHCl_3$-MeOH (9.7:0.3) was subjected to flash chromatography over silica gel using solvent system $CHCl_3$-MeOH (9.6:0.4) to provide compound **2** (20 mg).

Salvin A (1)

2α,3β,5α-Trihydroxyurs-12-en-28-oic acid. Colorless amorphous solid, IR ν_{max} KBr cm^{-1}: 3444, 1700, 1650. $[\alpha]_D^{26}$ -74.4° CH_3OH (c = 0.062); EIMS *m/z* (rel.int.) $[M]^+$ 488 (5), 442 (6), 369 (21), 248 (6), 240 (5), 203 (9), 145 (100); ^{1}H-NMR (400 MHz, CD_3OD) δ: 5.25 (1H, t, *J* = 3.4 Hz, H-12), 3.61 (1H, ddd, *J* = 10.8, 9.5, 4.0 Hz, H-2), 2.92 (1H, d, *J* = 9.5 Hz, H-3), 1.31 (3H, s, Me-27), 1.16 (3H, s, Me-25), 1.03 ((3H, s, Me-23), 1.00 (3H, d, *J* = 5.8 Hz, H-29), 0.99 (3H, s, Me-26), 0.91 (3H, d, *J* = 6.7 Hz, H-30), 0.78 (3H, s, Me-24), ^{13}C-NMR (100.6 MHz, CD_3OD) *d*: 181.0 (C-28), 138.2 (C-13), 128.5 (C-12), 83.4 (C-3), 72.9 (C-5), 68.4 (C-2), 55.0 (C-18), 53.1 (C-9), 47.5 (C-17), 47.0 (C-20), 46.1 (C-1), 41.1 (C-4), 41.0 (C-19), 39.8 (C-14), 39.0 (C-8), 37.9 (C-10), 37.4 (C-21), 32.0 (C-7), 28.3 (C-23), 28.0 (C-15), 26.9 (C-29), 25.9 (C-25), 25.3 (C-11), 25.2 (C-22), 24.1 (C-27), 23.5 (C-16), 18.3 (C-6), 16.4 (C-24), 16.2 (C-30), and 15.8 (C-26).

Salvin B (2)

3α,6α,24-Trihydroxyolean-12-en-28-oic acid, amorphous powder, IR ν_{max} KBr cm^{-1}: 3431, 2964, 1705, 1639. $[\alpha]_D^{26}$ -53.8° CH_3OH (c = 0.013); EIMS *m/z* (rel.int.) $[M]^+$ 488 (10), 442 (10), 248 (100), 240 (13), 203 (90); ^{1}H-NMR (500 MHz, CD_3OD) δ: 5.22 (1H, t, *J* = 3.4 Hz, H-12), 3. 91 (1H, m, $W_{1/2}$ = 23 Hz, H-6), 3.74 (1H, t, *J* = 3.4 Hz, H-3), 3.64 and 3.36 (1H each, AB d, *J* = 11.4 Hz, H-23), 2.83 (1H, dd, *J* = 14.0, 4.1 Hz, H-18), 1.16 (3H, s, Me-27), 1.08 (3H, s, Me-23), 0.93 (3H, s, Me-30), 0.92 (3H, s, Me-29), 0.91 (3H, s, Me-25), 0.80 (3H, s, Me-26), ^{13}C-NMR (125.6 MHz, CD_3OD) δ: 180.8 (C-28), 145.6 (C-13), 123.0 (C-12), 74.4 (C-3), 66.9 (C-6), 65.2 (C-24), 49.0 (C-5), 48.1 (C-9), 45.2 (C-17), 43.3 (C-4), 42.9 (C-18), 42.6 (C-19), 42.4 (C-7), 42.1 (C-14), 40.6 (C-8), 39.1 (C-10), 35.0 (C-21), 34.2 (C-22), 33.9 (C-30), 31.9 (C-1), 31.5 (C-20), 28.8 (C-15), 26.4 (C-27), 24.6 (C-2), 24.2 (C-16), 24.1 (C-11), 23.2 (C-29), 23.0 (C-23), 18.0 (C-26), and 17.5 (C-25).

In vitro enzyme inhibition activity

Butyrylcholinesterse inhibition assay

Horse-serum butyrylcholinesterse (E.C. 3.1.1.8), butyrylthiocholine chloride, 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB) and galanthanline were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade. Butyrylcholinesterase inhibiting activities was measured by slightly modified the spectrophotometric method developed by (Ellman *et al.*, 1961). Butyrylthiocholine chloride was used as substrates to assay butyrylcholinesterase, respectively. The reaction mixture contained 150 µL of (100 mM) sodium phosphate buffer (pH 8.0), 10 µL of DTNB, 10 µL of test-compound solution and 20 µL of butyrylcholinesterase solution which were mixed and incubated for 15 minutes (25°C). The reaction was then initiated by the addition of 10 µL butyrylthiocholine, respectively. The hydrolysis of butyrylthiocholine was monitored by the formation of yellow 5-thio-2-nitro-benzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of butyrylthiocholine, respectively 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 microplate SpectraMax 340 (Molecular Devices, U.S.A.). The percentage (%) inhibition was calculated as follows (E - S)/E × 100, where E is the activity of the enzyme without test compound and S is the activity of enzyme with test compound.

Estimation of IC_{50} values

The concentrations of test compounds that inhibited the hydrolysis of substrates (butyrylthiocholine) by 50% (IC_{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 using the EZ-Fit Enzyme Kinetics program *(Perrella Scientific Inc., Amherst,* U.S.A.).

RESULTS AND DISCUSSION

Salvins A (**1**) and B (**2**) were isolated from the chloroform soluble fraction through column and flash chromatographic techniques. Both of these gave positive color reactions of

a triterpenes and brisk effervesence with a dilute solution of sodium bicarbonate revealing the presence of free carboxyl group. The IR spectrum showed the absorptions due to hydroxyl group (3444 cm^{-1}), acid carbonyl (1700 cm^{-1}) and double bond (1650 cm^{-1}). The HREIMS showed $[M]^+$ peak at *m/z* 488.3496 corresponding to molecular formula $C_{30}H_{48}O_5$ (calcd. for $C_{30}H_{48}O_5$, 488.3501). It was also confirmed by broad band and DEPT ^{13}C-NMR spectra which disclosed thirty carbon signals including seven methyl, eight meyhylene, seven methine and eight quaternary carbons. The downfield signals at δ 181.0 could be assigned to the acid carbonyl while the signals of olefinic carbons were observed at δ 138.2 and 128.5, respectively. Two oxymethine carbons were observed at δ 83.4 and 68.4 while a downfield quaternary carbon at δ 72.9 indicated the presence of tertiary hydroxyl group. The ^{1}H-NMR spectrum showed trisubstituted double bond at δ 5.25 (t, *J* = 3.4 Hz) and two oxymethine protons at ä 3.61 (1H, ddd, *J* = 10.8, 9.5, 4.0 Hz) and 2.92 (1H, d, *J* = 9.5 Hz). In addition five tertiary methyls were observed as singlets at δ 1.31, 1.16, 1.03, 0.99, 0.78 while doublets of two further secondary methyls were observed at δ 1.00 (3H, d, *J* = 5.8 Hz) and 0.91 (3H, d, *J* = 6.7 Hz). The above data was consistant with an ursane type triterpenes carrying a double bond at $^{12}\Delta$. This was further confirmed by formation of characteristic RDA fragments at *m/z* 248.1768 ($C_{16}H_{24}O_2$, 248.1776) and 240.1720 ($C_{14}H_{24}O_3$, 240.1725), respectively. This confirmed the presence of two oxygen functionalities in rings D/E and the remaining three oxygens functionalites in rings A/B. The fragment at *m/z* 248 being common to that of ursolic acid, allowed us to assign the carboxylic group at the angular C-17 position (Riaz *et al.*, 2003). This was further authenticated by a peak at *m/z* 203 due to the loss of carboxylic group from the fragment ion at *m/z* 248. The oxymethine proton at δ 2.92 showed ^{1}H-^{1}H correlations to the oxymethine proton at δ3.61, while the latter also showed further ^{1}H-^{1}H correlations with two other protons. This allowed us to assign the hydroxyl groups to C-2 and C-3 positions. The larger coupling constants of the respective oxymethine protons allowed us to assign the equatorial configuration to both the hydroxyl groups. The remaining problem was to assign the position of tertiary hydroxyl group. The upfield shift of H-3 (*d* 2.92) indicated the presence of hydroxyl group at C-5 which could further be confirmed by HMBC correlations (Fig. 2) in which the oxymethine protons at *d* 2.92 showed ^2J correlations with C-4 (δ41.1), C-2 (δ 68.4) and ^3J *correlations* with C-5 (δ 72.9), C-23 (δ 28.3) and C-24 (δ 16.4). The methyl protons at C-25 at *d* 1.16 showed ^3J correlation with C-5 (δ 72.9), while methyl protons of both the methyl groups at C-23 and C-24 (δ 1.03 & 0.78) showed ^3J correlation with C-5 (δ 72.9). Rerecording of ^{1}H-NMR spectrum of **1** in pyridine-d_5 did not induce downfield shift of methyl protons of Me-24 and Me-25, therefore, α-configuration could be inferred for the hydroxyl group at C-5 (Nawaz *et al.*, 2000). The stereochemistry at C-2 and C-3 position was finally confirmed through 2D-NOESY, in which H-2 (δ 3.61) showed correlation with Me-24 (δ0.78), Me-25 (δ1.16) with Me-26 (δ 0.99) and H-3 (δ 2.92) with Me-23 (δ 1.03). Thus the structure of salvin A (**1**) could be assigned as 2α,3β,5α-trihydroxyurs-12-en-28-oic acid.

Salvin B (**2**) was obtained as an amorphous powder. The molecular formula $C_{30}H_{48}O_5$ was determined through HREIMS, which showed molecular ion peak at *m/z* 488.3495 (calcd for $C_{30}H_{48}O_5$, 488.3501). The IR and mass spectral fragmentation pattern was similar to those of **1**. The ^{13}C-NMR spectrum showed thirty carbon signals comprising of six methyl, ten methylene, six methine and eight quaternary carbons. Contrary to **1** it showed two oxymethine carbons at δ 74.4 and δ 66.9 and an oxymethylene carbon at δ 65.2. The signal of one of the methyl group at C-4 was also missing. The ^{1}H-NMR showed two oxymethine protons at δ 3.91 (1H, m, $W_{1/2}$ = 23 Hz) and 3.74 (1H, t, *J* = 3.4 Hz). In addition it showed signals of six tertiary methyls at 1.16, 1.08, 0.93, 0.92, 0.91 and 0.80, consistant with an oleane type skeleton. The oxymethine proton which resonated at δ3.74 showed ^{1}H-^{1}H correlation with two other protons and hence could be assigned to the usual C-3 position. The smaller coupling allowed us to assign *a* and axial configuration to

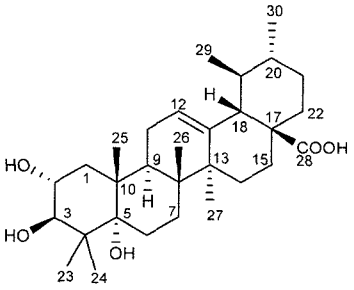

Fig. 1. Structure of salvin A (**1**)

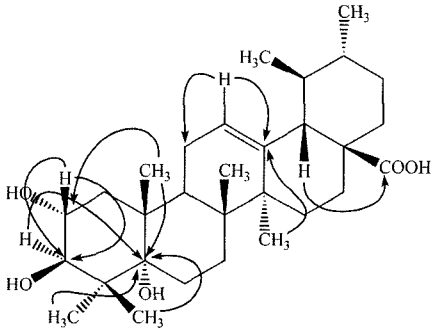

Fig. 2. Important HMBC correlatins of **1**

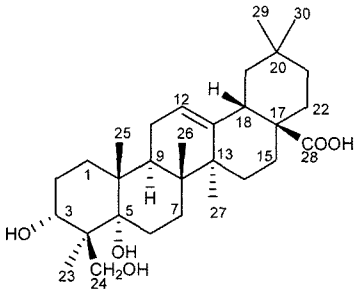

Fig. 3. Structure of salvin B (**2**)

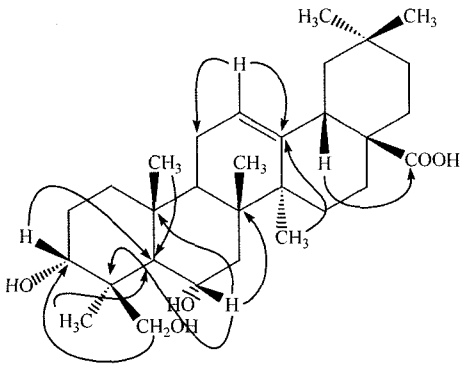

Fig. 4. Important HMBC correlatins of **2**

the hydroxyl group (Riaz *et al.*, 2002). The other oxymethine proton at δ 3.91 showed ^{1}H-^{1}H correlations with three other protons limiting its possible position to either C-2 or C-6. The latter position and the position of hydroxymethyl group at C-4 could be authenticated through HMBC correlations (Fig. 4). The position of oxymethylene was deduced through ^{13}C-NMR. In case of C-24 oxidation, the CH_3-23 resonates at δ 23-25 ppm while in case of oxidation of C-23 it is observed at δ 14-16. In case of 2 the methyl group resonated at δ 23.0 in confirmity to its presence at C-4 in *a* configuration (Cheung *et al.*, 1969). This was also supported by NOESY spectrum showing the interactions between H-3 (δ 3.74) with CH_2-24 (δ 3.64 and 3.36), CH_2-24 (δ 3.64 and 3.36) with CH_3-25 (δ 0.91) and CH_3-25 (δ 0.91) with CH_3-26 (δ 0.80) which is in accordance with the assigned structure of **2** as 3α,6α,24-trihydroxyolean-12-en-28-oic acid.

Cholinesterase is implicated as key biological players in Alzheimer's disease (AD), which makes them logical targets for inhibitory therapeutics. According to the cholinergic hypothesis, the memory impairment in the patients with senile dementia of *Alzheimer's* type results from a deficiency in cholinergic function in the brain. The role of BChE in normal aging and brain diseases is still elusive. It has been found that BChE is found in significantly higher quantities in Alzheimer's plaques than that of normal age-related non-demented brains (Choudhary *et al.*, 2005). Both **1** and **2** showed significant inhibitory potential against the enzyme butyrylcholinesterase (Table I). No inhibitory activity could be observed with acetylcholinesterase.

Table I. In vitro quantitative inhibition of and BChE by compounds **1** and **2**

Compounds	IC_{50} ± S.E.M.[a] [μM]
	BChE
1	12.5 ± 0.08
2	65.5 ± 0.01
Galanthamine[b]	8.7 ± 0.01

[a]Standard mean error of five assays
[b]Standard inhibitor of the butyrylcholinesterase

REFERENCES

Ali, S. I. and Nasir, Y. J., "*Flora of Pakistan*," Vol. 192, 200-202 (1990).

Cheung, H. T. and Williamson D. G., NMR signals of methyl groups of triterpenes with oxygen functions at positions 2, 3 and 23. *Tetrahedron*, 25, 119-128 (1969).

Choudhary, M. I., Nawaz, S. A., Zaheer-ul-Haq, Lodhi, M. A., Ghayur, N., Jalil, S., Riaz, N., Yousaf, S., Malik, A., Gilani, A. H. and Atta-ur-Rahman. Withanolides, a new class of natural cholinesterase inhibitors with calcium antagonistic properties. *Biochem. Biophys. Res. Commun.*, 234, 276-287 (2005).

Ellman, G. L., Courtney, K. D., Andres, V. and Featherstone, R. M., A new and rapid colorimetric determination of acetyl-cholinesterase activity. *Biochem. Pharmacol.*, 7, 88-95 (1961).

Krishnamurthi, A., "*The Wealth of India*," CSIR, New Delhi, Vol. IX, 195-198 (1978).

Meyer, B. N., Ferrigni, N. R., Putnam, J. E., Jacobsen, L. B., Nichols, D. E., and McLaughlin, J. L., Brine shrimp, a convenient general bioassay for active plant constituents. *Planta Med.*, 45, 31-34 (1982).

Nagy, G., Dobos, A., Gunther, G., Yang, M., Blunden, G., Crabb, T. A., and Mathe I., Abietane diterpenoids from the roots of *Salvia pratensis*. *Planta med.*, 64, 288-289 (1998).

Nawaz, H. R., Malik, A., Khan, P. M., Shujaat, S. and Atta-ur-Rehman, Novel β-glucuronidase inhibiting triterpenoid from *Paeonia emodi*. *Chem. Pharm. Bull.*, 48, 1771-1773 (2000).

Riaz, N., Anis, I., Aziz-ur-Rehman, Malik, A., Ahmed, Z., Muhammad, P., Shujaat, S., and Atta-ur-Rahman, Emodinol, a new β-glucoronidase inhibiting triterpenes from *Paeonia emodi*. *Nat. Prod. Res.*, 17, 247-251 (2003).

Riaz, N., Anis, I., Muhammad, P., Shah R., and Malik, A., Alysinol, a new triterpenes from *Alysicarpus monolifer*. *Nat. Prod. Lett.*, 16, 415-418 (2002).

Ryu, S. Y., No, Z., Kim, S. H., and Ahn J. W., Two novel abietane diterpenes from *Salvia miltiorrhiza*. *Planta Med.*, 63, 44-46 (1997).

Ulubelen, A., Oksuz, S., Kolak U., Birman H., and Voelter W., Cardioactive terpenoids and a new rearranged diterpene from *Salvia syriaca*. *Planta Med.*, 66, 627-629 (2000).