Two Ceramides from *Tanacetum artemesioides*

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Two new ceramides have been isolated from *Tanacetum artemisioides*, besides the known constituents β-sitosterol and β-sitosterol glycoside. The structure elucidation of the isolated new compounds was based primarily on 1D and 2D NMR analysis, including COSY, HMQC, HMBC correlations. The compound **1** and **2** showed inhibitory activity against acetylcholinesterase.

Key words: Tanacetum artemisioides, Compositae, Ceramides, Acetylcholinesterase

Introduction

Tanacetum artemisioides, a pale green annual shrub with several branches from the woody rootstock, grows in different parts of Pakistan [1]. The genus *Tanacetum* has several important medicinal applications. Some of its species such as *T. parthenium* have been used traditionally in the treatment of migraine and arthritis; *T. vulgare* has beneficial effects in gastroduodenal diseases while *T. microphyllum* is used in Spanish folk medicine for ulcer and inflammatory conditions [2]. These medicinal properties prompted us to carry out a phytochemical investigation of *Tanacetum artemisioides*. Our current study has led to the isolation of two new ceramides **1** and **2** which have also been found to be promising acetylcholinesterase (AChE) inhibitors. The enzyme acetylcholinesterase (acetycholine degrading enzyme) has long been an attractive target for rational drug therapy for *Alzheimer's* disease (AD). It is believed that AD develops due to decreased level of acetylcholine in the brain [3 – 4]. The inhibition of its metabolizing enzyme (AChE) boosts the endogenous levels of acetylcholine in the brain and thereby, enhances cholinergic neurotransmission. The AChE inhibiting properties of the compounds **1** and **2** could make them good candidates for the development of AD therapeutics.

Results and Discussion

Tanacetamide A (**1**) and tanacetamide B (**2**) were isolated by column chromatography of the chloroform soluble fraction over silica gel, as described in Experimental Section. Tanacetamide A (**1**) was obtained as a colorless oil and showed the molecular ion peak in HREIMS at *m/z* 663.6157 corresponding to molecular formula $C_{42}H_{81}NO_4$ (calcd. for $C_{42}H_{81}NO_4$ 663.6144). The IR spectrum showed an absorption band at 3605 cm^{-1} (hydroxyl), a strong absorption band at 1630 cm^{-1} indicating the presence of a secondary amide group [5], and at 2940, 2850 and 1455 cm^{-1} (aliphatic) suggesting it to be a fatty acid amide. A very strong signal at $\delta = 1.24$ in the ^{1}H NMR spectrum and the lack of upfield methine signals in the ^{13}C NMR spectrum revealed that

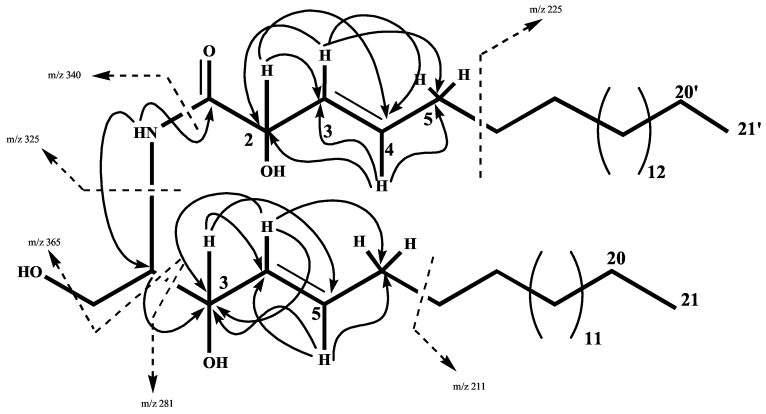

Fig. 1. Mass fragmentation pattern and important HMBC correlations of compound **1**.

1 must be derived from a long-chain fatty acid precursor [5].

The ^{1}H NMR spectrum in C_5D_5N exhibited four exchangeable proton signals due to NH [$\delta = 8.56$ (d, $J = 8.7$ Hz)] and three OH [7.62 (d, $J = 3.6$ Hz, 6.70 br s and 6.24 br s)] [6]. Compound **1** also showed the presence of two primary methyls (Me-21 and 21') which appeared as triplets of six protons at $\delta = 0.86$ (t, $J = 6.1$ Hz), a methylene group at $\delta = 4.33$ (d, $J = 5.5$, 10.7 Hz, H-1a), 4.42 (dd, $J = 3.8$, 10.0 Hz, H-1b), two methine groups [$\delta = 4.60$ m, H-2'), [$\delta = 4.52$ m , H-3), and a signal at low field at $\delta = 5.11$ (m) which was identified as a methine proton vicinal to the nitrogen atom of the amide group in the ^{1}H NMR spectrum [7]. The ^{1}H NMR spectrum also showed four disubstituted olefinic protons $\delta = 5.44$ (1H, H-3'), 5.57 (1H, H-4'), 5.36 (1H, H-4), 5.52 (1H, H-5). The usual methylenes associated with the chain appeared as a broad singlet at $\delta = 1.25$. The ^{13}C NMR spectrum showed characteristic signals appearing to be due to an amide carbonyl at $\delta = 175.2$, a methine carbon linked to amide nitrogen at $\delta = 53.0$ [8], and two other methines at $\delta = 73.0$ (CHOH) and 72.5 (CHOH). A downfield methylene signal bearing the hydroxyl function appeared at $\delta = 62.1$. The remaining methylenes of the chain showed their signals in the carbon spectrum at their normal positions [9 – 10]. The ^{1}H-^{1}H- and ^{1}H-^{13}C connectivities were supported by the ^{1}H-^{1}H-COSY and HMQC spectra. These spectral data and the molecular formula suggest that compound **1** is a ceramide. The position of the double bond between 3' and 4' in fatty acid were confirmed by HMBC (Fig. 1). The important cross peaks between H-2' with C-3' and C-4'. H-3' with C-2', C-4' and C-5', H-4' with C-2', C-3' and C-5' and H-5' with C-3' and C-4' in HMBC spectrum confirm the position of double bond in C-3' and C-4' in the fatty acid.

Cross peaks between H-3 to C-4 and C-5, H-4 to C-3, C-5 and C-6, H-5 to C-3, C-4 and C-6 and H-6 to C-4 and C-5 in HMBC spectrum (Fig. 1) confirm the position of the double bond at C-4 and C-5 in the long chain base.

The length of the fatty acid was determined by the characteristic ions at *m/z* 323 $[CH_3(CH_2)_{16}$ CH=CHCH(OH)CO$]^+$, 340 $[CH_3(CH_2)_{16}$CH=CH CH(OH)CONH$_2$+H$]^+$ in the EIMS [8]. The length of the long chain base also determined by the characteristic ions at 382 [M-$CH_3(CH_2)_{15}$CH=CHCH(OH)$]^+$ and 365 [M-$CH_3(CH_2)_{15}$CH=CHCH(OH)$_2$$]^+$ in the EIMS [8]. The formula of these fragments were also confirmed with the aid of HREIMS. Its structure could be established through characteristic fragment ion in EIMS (Fig. 1). Thus, the long chain base and fatty acid of **1** must be 2-amino-4-henicosene-1, 3-diol and 2'-hydroxy-3'-henicosenoic acid, respectively.

The geometry of the double bond at C-3' and C-4 was deduced to be *E* from the ^{1}H-^{1}H coupling constant ($J = 15.5$, 15.1 Hz) between H-3' and H-4 respectively. The absolute stereochemistry at C-2, C-2' and C-3 was determined to be 2*S*, 3*R* by comparing the optical rotation values [5, 7, 8, 11] of **1** ($[\alpha]_D^{23} - 6.00$) and **3** ($[\alpha]_D^{23} - 13.3$) [8]. On the basis of this evidence, the structure of **1** was determined to be (2*S*, 2′*R*, 3*R*, 3′*E*, 4*E*)-*N*-[2'-hydroxy-3'-henicosenoyl]-2-amino-4-henicosene-1,3-diol.

Tanacetamide B (**2**) was isolated as a colorless oil. The molecular formula was determined to be $C_{23}H_{45}NO_3$ by HREIMS. The IR spectrum presented hydroxyl band at 3340 cm^{-1}, and bands 1620 and 1540 cm^{-1} due to the amide group. The ^{1}H NMR spec-

Table. 1. *In vitro* quantitative inhibition of acetylcholinesterase by compound **1** and **2**.

S.No.	Compound	*AChE* $IC_{50}(\mu M) \pm SEM)$[a]
1	**1**	67.1 ± 1.5
2	**2**	74.1 ± 5.0
3	Galanthamine[b]	8.5 ± 0.0001

[a] Standard mean error of five assays; [b] standard inhibitor of acetylcholinesterase.

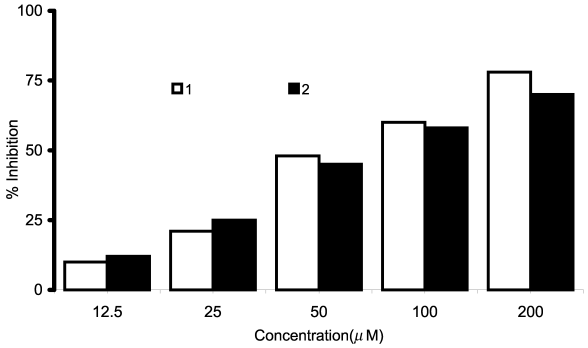

Fig. 2. Inhibition [%] of acetylcholinesterase enzyme at various concentrations of compounds **1** and **2.**

trum in $CDCl_3$ confirmed the presence of an amide with a proton signal at $\delta = 7.42$ (d, $J = 8.6$ Hz), It possessed three characteristic signals of geminal protons to hydroxyl groups at $\delta = 3.65$ (dd, $J = 4.8$, 11.7, H-1a), 3.72 (dd, $J = 4.1$, 11.7 Hz, H1b) and 4.50 (m, H-3). A fourth signal was present at $\delta = 4.00$ (m, H-2) and identified as a methine proton vicinal to the nitrogen atom of the amide group. Compound **2** also showed two geminal olefinic protons [$\delta = 5.35$ (1H, H-5), 5.27 (1H, H-4), one terminal methyl at $\delta = 0.80$, and methylenes at $\delta = 1.23$ (br. s, CH_2 chain). ^{13}C NMR spectrum showed two methines at $\delta = 51.3$ (CHNH) and 71.9 (CHOH), and a methylene group at $\delta = 60.8$ (CH_2OH). The lengths of the long chain base and fatty acid were determined by EIMS, which showed significant fragment ion peaks at 281 $[CH_3(CH_2)_{15}CH{=}CHCH(OH)]^+$ and 298 $[CH_3(CH_2)_{15}CH{=}CHCH(OH)_2]^+$, indicating that the long chain base (2-amino-4-henicosene-1, 3-diol) was the same as that of **1**, and the fatty acid is only an acetate group instead of 2'-hydroxy-3'-henicosenoic acid. In the ^{1}H NMR the presence of the singlet of three protons at $\delta = 2.03$ for NHAc and in ^{13}C NMR one quaternary carbon at $\delta = 171.0$ (C=O) and 24.8 q ($NHCOCH_3$) was compatible with the proposed structure. Its structure could be established through characteristic fragment ion in EIMS (Fig. 1) and be further confirmed through ^{1}H-^{1}H COSY, HMQC and HMBC correlations. The optical rotation values of **2** ($[\alpha]_D^{23} - 6.67$) and **1** ($[\alpha]_D^{23} - 6.00$) suggest that **2** has the same absolute configuration as that of **1** for the C-2 and C-3 parts [8]. Therefore, the structure of **2** was determined to be (2*S*, 3*R*, 4*E*)-*N*-[acetoxy]-2-amino-4-henicosene-1,3-diol.

In addition to the new compounds **1** and **2**, some known constituents such as β-sitosterol [12 – 13] and β-sitosterol glucoside [14] have been isolated for the first time from this plant. Their structures were established by comparing their spectral data and physical constants.

Both the compounds **1** and **2** were found to be promising acetylcholinesterase inhibitors. These 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. The compounds were found to be inhibit enzyme in a concentration-dependent fashion with the IC_{50} values 67.1 and 74.0 μM respectively against acetylcholinesterase.

Experimental Section

General experimental procedure

Column chromatography (CC): silica gel, 70 – 230 mesh. Flash chromatography (FC): silica gel 230 – 400 mesh. TLC: pre-coated silica gel G-25-UV_{254} plates: detection at 254 nm, and by ceric sulphate reagent. Optical rotations: Jasco-DIP-360 digital polarimeter. UV and IR Spectra: Hitachi-UV-3200 and Jasco-320-A spectrophotometer, respectively. ^{1}H- and ^{13}C NMR, COSY, HMQC and HMBC Spectra: Bruker spectrometers operating at 500 and 400 MHz; chemical shifts δ in ppm and coupling constants in Hz. EI-, CI MS: JMS-HX-110 with a data system.

Plant material

The plant *Tanacetum artemisioides* (Compositae) was collected at Parachinar Kurram Agency, N.W.F.P Pakistan, in 2001, and identified by Dr. Jahandar Shah (plant taxonomist) at the Department of Botany, Islamia College, University of Peshawar. A voucher specimen (no. 16) has been deposited at the herbarium of the Botany Department, Islamia College, University of Peshawar.

Extraction and purification

The whole plant, including roots (9.5 kg) of *T. artemisioides* was crushed and extracted $\times 3$ with methanol (20 l

each) at r. t.. The resulting methanol extract (304 g) was suspended in water and successively portioned to provide *n*-hexane (50 g), chloroform (68 g), ethyl acetate (85 g), and *n*-butanol (45 g) fractions. The chloroform (68 g) was subjected to silica gel column (90 × 850 mm, 500 ml each part). The column was eluted with solvent of increasing polarity [hexane-$CHCl_3$ (3:1, 3:2, 1:1, 1:2, and 1:4), $CHCl_3$, $CHCl_3$-MeOH (19:1, 9:1, 4:1) and MeOH] to give ten fractions (J1-J10). The J7 fraction (350 mg) was then subjected to silica gel (flash silica 230 – 400 mesh) column chromatography using MeOH-$CHCl_3$ (1:9) to afford **1** (7.5 mg) and **2** (3.5 mg) respectively. Both the compounds were found ceric sulfate active.

Tanacetumide A (**1**): Colorless oil (7.5 mg): $[\alpha]_D^{23} - 6.00$ ($c = 0.01$, $CHCl_3$ + MeOH); IR ν_{max} (C_5D_5N): 3605, 2940, 2850, 1630, 1455 cm^{-1} ^1H NMR (C_5D_5N): $\delta = 0.86$ (6H, t, $J = 6.1$ Hz, H-21 and H-21'), 1.25 (br. s, CH_2 chain), 2.05 (2H, m, H-5'), 2.19 (2H, m, H-6), 4.33 (1H, dd, $J = 5.5$, 10.7 Hz, H-1a), 4.42 (1H, dd, $J = 3.8$, 10.0 Hz, H-1b), 4.52 (1H, m, H-3), 4.60 (1H, m, H-2'), 5.10 (1H, m, H-2), 5.36 (1H, dt, $J = 6.1$, 15.1 Hz, H-4), 5.44 (1H, dt, $J = 6.2$, 15.5 Hz, H-4'), 5.52 (1H, dt, $J = 5.8$, 15.1 Hz, H-5), 5.57 (1H, dt, $J = 6.0$, 15.3 Hz, H-3'), 6.24 (1H, br.s, OH), 6.70 (1H, br.s, OH), 7.62 (1H, d, $J = 3.6$ Hz, OH), 8.56 (1H, d, $J = 8.7$ Hz, N*H*), ^{13}C NMR (C_5D_5N): $\delta = 14.2$ (C-21 and C-21'), 22.9 (C-20 and C-20'), 25.8 (C-19 and C-19'), 32.1 (C-6), 32.9 (C-5'), 53.0 (C-2), 62.1 (C-1), 72.5 (C-3), 73.0 (C-2'), 129.1 (C-4), 129.5 (C-3'), 130.2 (C-5), 130.8 (C-4'), 175.2 (C-1'), HREIMS: *m/z* 663.6157 (calcd. *m/z* 663.6144 for $C_{42}H_{81}NO_4$), 382.3311 (calcd. *m/z* 382.3321 for $C_{23}H_{44}NO_3$), 365.3282 (calcd. *m/z* 365.3293 for $C_{23}H_{43}NO_2$), 340.3229 (calcd. *m/z* 340.3215 for $C_{21}H_{42}NO_2$), 323.2936 (calcd. *m/z* 323.2949 for $C_{21}H_{39}NO_2$); EIMS *m/z* (rel. int.): 663 $[M]^+$(1), 382 [M-$CH_3(CH_2)_{15}CH$=CHCH(OH)]$^+$, 365 [M-$CH_3(CH_2)_{15}CH$=CHCH$(OH)_2$], 340 (56), 325 (54), 323 $[CH_3(CH_2)_{16}CH$=CHCH(OH)CO]$^+$, 340 $[CH_3(CH_2)_{16}CH$=CHCH(OH)$CONH_2$ + H]$^+$, 281 (32), 211 (26).

Tanacetumide B (**2**): Colorless oil (3.5 mg): $[\alpha]_D^{23} - 6.67$ ($c = 0.03$, $CHCl_3$ + MeOH); IR ν_{max} (C_5D_5N): 3340, 2930, 2850, 1620, 1540 cm^{-1}, ^1H NMR ($CDCl_3$): $\delta = 0.80$ (3H, t, $J = 6.7$ Hz, H-21), 1.23 (br. s, CH_2 chain), 1.95 (2H, m, H-6), 3.65 (1H, dd, $J = 4.8$, 11.7 Hz, H-1a), 3.72 (1H, dd, $J = 4.1$, 11.7 Hz, H-1b), 4.00 (1H, m, H-2), 4.50 (1H, m, H-3), 5.27 (1H, dt, $J = 6.0$, 15.2 Hz, H-4), 5.35 (1H, dt, $J = 6.5$, 15.5 Hz, H-5), 7.42 (1H, d, $J = 8.6$ Hz, N*H*), ^{13}C NMR ($CDCl_3$): $\delta = 13.5$ (C-21), 22.2 (C-20), 24.8 (NHCO*C*H_3), 25.5 (C-19), 32.2 (C-6), 51.3 (C-2), 60.8 (C-1), 71.9 (C-3), 129.6 (C-4), 130.4 (C-5), 171.0 (NH*C*OCH_3), HREIMS: *m/z* 383.3383 (calcd. for $C_{23}H_{45}NO_3$, 383.3399); EIMS *m/z* (rel. int.): 383 $[M]^+$(1), 298 $[CH_3(CH_2)_{15}CH$=CHCH$(OH)_2]^+$, 281 $[CH_3(CH_2)_{15}CH$=CHCH(OH)]$^+$, 225 (34), 139 (22), 97 (34).

In vitro cholinesterase inhibition assay

Electric-eel acetylcholinesterase (EC 3.1.1.7), acetylthiocholine iodide 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB) and galanthamine were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade. Acetylcholinesterase inhibiting activity was measured by slightly modified the spectrophotometric method developed [15]. Acetylthiocholine iodide was used as substrate to assay acetylcholinesterase. The reaction mixture contained 140 μl of (100 mM) sodium phosphate buffer (pH 8.0), 10 μl of DTNB, 10 μl of test-compound solution and 20 μl of acetylcholinesterase was mixed and incubated for 15 min (25 °C). The reaction was then initiated by the addition of 10 μl acetylthiocholine. The hydrolysis of acetylthiocholine was monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine at a wavelength of 412 nm (15 min). Test compounds and the control was dissolved in EtOH. All the reactions were performed in triplicate in 96-well micro-plate in *SpectraMax 340* (Molecular Devices, USA). The percentage (%) inhibition was calculated as follows $(E - S)/E \times 100$, 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 substrate acetylthiocholine 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*, USA).

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