Isolation, Structure Elucidation and Anti-microbial potential of a New Sphingolipid from *Salvadora oleoides*

Hafiza Naila Khalid, Ejaz Ahmed, Ahsan Sharif*, Sumra Amanat, Faiza Arshed, Muhammad Arshad Zahoor Hussain Farooqi
Institute of Chemistry, University of the Punjab, Quaid-e-Azam Campus, Lahore, 54590, Pakistan.
chahsansharif@yahoo.com*

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Summary: Salvadoramide (**1**) have been isolated from the ethyl acetate soluble fraction of the whole plant of *Salvadora oleoides*. The structure, (2R)-2-hydroxy-N-[(2S, 3S, 4R, 5E, 7E)-1, 3, 4-trihydroxy-5, 7-tetra decdiene-2-yl] tetradecanamide), was established by the use of sophisticated modern spectroscopic techniques (1D and 2D-NMR, EIMS, HREIMS, UV and IR) and chemical reactions. The compound was further evaluated for its antimicrobial activity.

Keywords*:* Salvadoramide, *Salvadora oleoides*, Sphingolipid, 1D and 2D NMR, Antimicrobial activity.

Introduction

Salvadoraceae comprises 3 genera and 11 species, widely distributed in subtropical regions of all over the world. *Salvadora* is one of the most important genus of Salvadoraceae consisting of perennial herbs, shrubs, and small tress. *Salvadora oleoides* is excessively used as nerve tonic, diuretic and cooling herb in Unani and Ayurvedic drug systems. This plant is characterized with wound healing and anti-inflammatory properties. Different parts of *Salvadora oleoides* has been used to cure various uterine and skin diseases [1-6]. Previously lignans, benzylamine, coumarin and amide derivatives have so far been reported form the *plant*. This arouses us to carry out further isolation studies on the natural products of this plant. We herein report the isolation and structure elucidation of a new sphingolipid (**1**) from the ethyl acetate soluble fraction of the *S. oleoides*. For its biological potential anti-microbial activity was performed, showed potent activity, using miconazol and amphotericin B as a positive control [7].

Experimental

General experimental procedures

Plant material

The whole plant of *Salvadora oleoides* (12 Kg) was collected from Margalla Hills, Islamabad, in May 2013 by Dr. Muhammad Sher from Department of Chemistry, AIOU, Islamabad, Pakistan. The plant was identified from Botany Department, Quaid e Azam University Islamabad, Pakistan.

Isolation

The shade dried plant material. (12 Kg) was crushed, ground, and. extracted with methanol. The methanolic extract was evaporated. under reduced pressure and the gummy material (0.5 Kg), was partitioned between. water, *n*-butanol, ethyl acetate chloroform, and *n*-hexane. soluble fractions. The ethyl acetate fraction was chromatographed. over silica gel in a column eluting with *n*-hexane-chloroform and chloroform-methanol mixtures in. increasing order of polarity to obtain 10 major fractions. labelled as A-J. The fraction C eluting with *n*-hexane-chloroform (6:4) was subjected to column chromatography. again and obtained several semi pure fractions labelled as C1-C5. The fraction C3 at 7.0:3.0 (*n*-hexane-chloroform) was again chromatographed over Preparative TLC using solvent system *n*-hexane-acetone (6:4) to afford **1** in a 22 mg.

Salvadoramide (**1**)

Colorless gummy solid; $[\alpha]_D^{25}$ +65.5 (c = 1.08, MeOH); UV (MeOH) λ_{max} (log ε_{max}) 233 (3.1); IR (KBr) ν_{max} cm^{-1} 3530-3342, 1650, 1640, 1555, 1410, 1340, 765. HREIMS, $[M^+]$ at *m/z* 483.4102 (calcd. for $C_{28}H_{53}NO_5$ 483.4164); EIMS *m/z* 465 (24), 447 (47), 429 (55), 317 (41), 259 (38), 224 (80), 209 (98), 169 (100), 137 (64), 43 (85). ^1H and ^{13}C NMR ($CDCl_3$, 500 MHz) see Table-1.

Antimicrobial Assay

Antibacterial and antifungal assays were performed by agar well diffusion method [8], and

*To whom all correspondence should be addressed.

agar tube dilution protocol [9], respectively. The results are illustrated in Table-2.

Results and Discussion

The methanolic extact of the whole plant of *Salvadora oleoides* was suspended in water and partitioned between *n*- hexane, ethyl acetate, and *n*-butanol soluble fractions. The ethyl acetate soluble fraction was evaporated to dryness to give brown gum and then submitted to VLC and repeated column chromatography over silica gel afforded **1**.

Table-1: ^{1}H-NMR (500 MHz) and ^{13}C NMR (125). Spectral Data of Salvadoramide (**1**).

Position No.	δ_H	δ_C
1	3.79 (dd, 3.7, 6.4)	61.5
2	5.06 (m)	52.9
3	4.58 (dd, 3.6, 7.0)	74.0
4	4.23 (dd, 5.2, 3.6)	72.1
5	5.98 (dd, 8.5, 5.5)	135.5
6	6.31 (dd, 14. 9, 9.9)	130.1
7	6.05 (dd, 9.9, 14.5)	128.0
8	5.67 (dt, 14.5, 6.8)	140.1
9	2.00 (dt, 13.2, 6.6)	35.0
10	1.33 (m)	33.1
11	1.30 (m)	32.0
12	1.33 (m)	32.1
13	1.33 (m)	22.5
14	0.88 (t, 7.0).	14.0
NH	nd	-
1′	-	175.7
2′	4.31 (dd, 4.6, 3.7)	73.6
3′	1.70-1.94 (q, 16.0, 11.5, 7.1, 3.6)	34.5
4′	1.32 (br m)	33.0
5′	1.28 (br m)	32.0
6′	1.33 (br m)	32.0
7′	1.33 (br m)	32.0
8′	1.33 (br m)	32.0
9′	1.33 (br m)	32.0
10′	1.33 (br m)	32.0
11′	1.33 (br m)	32.0
12′	1.33 (br m)	32.0
13′	1.30 (br m)	22.5
14′	0.88 (t, 7.0).	14.0

nd = not detectable

Table-2: Antibacterial and antifungal activity of compound **1**.

Microorganism	Zone of inhibition diameter (mm)	Standard drugs
Bacteria		**imipenem**
Gram-positive		
Bacillus subtilis	**25**	**33**
Shigella flexneri	**29**	**27**
Staphylococcus aureus	**20**	**33**
Gram-negative		
Escherichia coli	**30**	**30**
Pseudomonas aeruginosa	**20**	**24**
Salmonella typhi	**10**	**25**
Fungi		**Miconazole**
Trichophyton longifusus	**65**	**70**
Candida albicans	**73**	**110.8**
Microsporum canis	**66**	**98.4**
Fusarium solani	**48**	**73.25**
Candida glabrata	**58**	**110.8**

Values are inhibition. zones (mm) and an. average of triplicate; Conc. of sample. 4μg/ ml of DMSO. and St. drug, imipenem (10 μg/ disc) for antibacterial. activity; Size of well, 6 mm. (diameter); Concentration of Sample 200 μg/ ml of DMSO. for antifungal activity, Incubation temp. 27 (28 ± 1°C).; Incubation period 7 days.

Compound **1** was obtained as a colorless gummy solid. The ultraviolet spectrum showed absorption maxima at 233 nm, indicating the presence of conjugated double bond. The IR spectrum displayed bands of hydroxyl, amine (3530-3342 cm^{-1}) and olefinic (1640) cm-1 functionalities Furthermore the reading at 1650 and 1555 cm^{-1} suggested the nature of the compound **1** as secondary amide derivative [10]. The molecular formula was obtained by high resolution EIMS as $C_{28}H_{53}NO_5$, showed [M$^+$] peak at *m/z* 483.4102 (calcd 483.4164). Further peaks at *m/z* 465.3925, 447.3835, and 429.3660 were due to the loss of water molecule indicating the presence of three hydroxyl functionalities in the molecule. In the ^{1}H-NMR spectrum (Experimental section), the proton signals appeared at δ 6.31 (dd, J = 14. 9, 9.9 Hz), 6.05 (dd, J = 9.9, 14.5 Hz), 5.98 (dd, J = 8.5, 5.5 Hz), and 5.67 (dt, J = 14.5, 6.8 Hz) indicated the conjugated diene system. Terminal methyl groups appeared at δ 0.88 (t, J = 7.0 Hz, 6H). Two methylene groups were observed in the range of δ 1.70-1.94 (q, J = 16.0, 11.5, 7.1, 3.6 Hz) and 2.00 (dt, J = 13.2, 6.6 Hz) while the remaining methylene groups appeared at δ 1.27-1.33 as broad singlet (16 x CH_4). The oxymethine signals appeared at δ 4.58 (dd, 3.6, 7.0 Hz), 4.31 (dd, J = 4.6, 3.7 Hz) and 4.23 (dd, J = 5.2, 3.7 Hz) while the oxymethylene protons resonated at δ 3.79 (dd, J = 3.7, 6.4 Hz). On the basis of the coupling constant between the olefinic protons (J = 14.9 and 14.5 Hz) and the stereochemistry of the double bonds, *E*, was evident [11]. Inspection of the ^{13}C-NMR spectrum (BB and DEPT), Table 1, showed a prominent signal at δ 175.7 indicating an amide carbonyl carbon while the conjugated olefinic carbon atoms appeared at δ 140.1, 135.5, 130.1, and 128.0. The characteristic azomethine signal of a sphingolipid appeared at δ 52.9 while an oxymethine carbons resonated at δ 74.0, 73.6, and 72.1. Similarly the only oxymethylene singnal appeared at δ 61.5 while the methylene carbons of the aliphatic chains showed signals in the range of δ 35.0-22.5-along the two terminal methyl carbons at δ 14.0. The exact structure of the molecule was confirmed by 2D-NMR investigations in which ^{1}H-^{1}H COSY correlations of the azomethine proton (δ 5.06) showed interactions with oxymethylene protons (H-1 at δ 4.10 and 4.40) which further correlated with another oxymethine proton (H-4 at δ 4.23) indicating the position of two hydroxyl groups at C-3 and C-4, respectively. In HMBC correlations the position of third hydroxyl group was confirmed as C-2′ by observing J2 correlations of δ 4.31 with the amide carbonyl carbon (δ = 175.7), methylene carbon (δ = 33.0) and J3 correlations with methylene carbon at δ = 32.0. The position of the olefinic bonds were also confirmed by

the HMBC correlations in which the proton at δ 5.98 showed C-3 (δ 74.0) and C-7 (δ 128.0). The ^{1}H-^{1}H COSY correlations of δ 5.98 (H-5) showed correlations with δ 4.23 (H-4) and δ 6.31 (H-6). Which in turn again showed correlations with olefinic proton at δ 6.05 (H-7) showing its position at C-5 and C-7. The position of the double bond and length of the fatty acid chain were further confirmed by fragment analysis and methanolysis. Methanolysis was achieved with HCl in MeOH, yielded a 2-hydroxytetradecanoic acid methyl ester, which was further characterized by mass spectrometry showing [M^{+}] peak at *m/z* 258 and its optical rotation [α]D^{25}=+5.52 indicated the *S*-configuration [12]. These data confirmed that both chains of compound **1** were of equal length with the double bonds located in the sphingosine base. The diagnostic ion peaks in EIMS spectrum at *m/z*=209 and *m/z* 259 were due to the cleavage of the amide bond, confirmed the length of the fatty acid consisting of 14 carbons [Fig 1]. The absolute stereo-chemistry of the sphingosine base was assumed to be 2S, 3S, 4R, because the ^{1}H and ^{13}C NMR signals assigned to C-1, C-2, C-3, C-4, and H-2 were in good agreement with those sphingolipids composed of (2S, 3S, 4R)-phytosphingosine and (2R)-2-hydroxy fatty acids [13-15]. This assumed stereochemistry was further supported by the NOESY spectrum. In NOESY spectrum the azomethine proton at δ 5.06 (H-2) showed correlations with H_{β}-1 at δ 4.10 and H-2′ at δ 4.31. Similarly, H-3 at the δ=4.58 showed correlation with H_{α}-1 at δ 4.40. In natural sphingolipids from higher plants, the stereo-chemistries at C-2, C-3, and C-4 are already defined as 2S, 3S and 4R, and many of them have been reported for double bonds at C-5 and C-7 [16-17]. On the basis of these evidences, the structure of salvadoramide (**1**) have been assigned as (2R)-2-hydroxy-N-[(2S, 3S, 4R, 5E, 7E)-1,3,4-trihydroxy-5,7-tetra decdiene-2-yl] tetradecamide [Fig 1].

The isolated compound (**1**) was tested for their antimicrobial activity (Table-2). It showed significant antimicrobial activity against *Salmonella typhi*, *Bacillus subtilis*, *Staphylococcus aureus*, *Shigella flexneri*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Trichophyton longifusus*, *Fusarium solani*, *Microsporum canis*, and *Candida glabrata*.

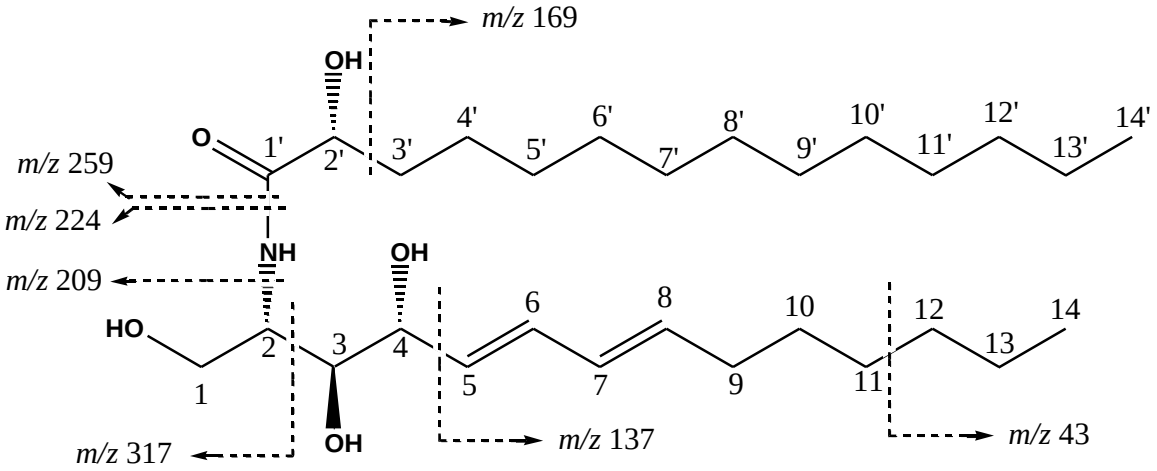

Fig. 1: Structure and Mass fragmentation of Salvadoramide (**1**).

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