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Syriacin, a novel unusual sulfated ceramide glycoside from the freshwater sponge *Ephydatia syriaca* (Porifera, Demospongiae, Spongillidae)

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Abstract—Syriacin, a novel unusual sulfated ceramide glycoside with branched very-long-chain fatty acid, i.e., (all *Z*)-34*S*-methylhexatriaconta-5,9,12,15,18,21-hexaenoic acid, has been isolated from the freshwater sponge *Ephydatia syriaca*. Its structure was identified by means of extensive spectroscopic analysis (IR, UV, 2D NMR, MS, CD) and chemical degradation. Syriacin showed antifeeding activity against goldfish at natural concentration (~10 μg/ml).

1. Introduction

Sponges, an ancient and highly successful group of animals, common inhabitants of the benthos, have been living in the waters of the world for more than 600 million years, and can be found in all marine and many freshwater habitats.[1] In addition to many marine forms, the freshwater sponges belonging to the genus *Ephydatia* are typical representatives of Spongillidae and occur in different rivers and lakes around the world. A few natural compounds have been isolated from this genus.[2] *Ephydatia fluviatilis* contains almost exclusively Δ^5-sterols. In addition, *E. fluviatilis* contains sterols with the 24β configuration, which predominate over the 24α-epimers.[3] Phospholipids and fatty acids were also studied in *E. fluviatilis*.[4] Multibranched, polyunsaturated and very-long-chain fatty acids have been isolated from *Ephydatia syriaca*.[5,6]

This report is part of our investigation of marine and freshwater sponges[2,4,5] in the framework of a comprehensive program on the chemistry and biotoxicity of natural compounds. We isolated a novel unusual sulfated ceramide, named syriacin, with very-long-chain branched fatty acid having six double bonds.

2. Results and discussion

The extract of the freshwater sponge *E. syriaca*, which was collected in August 2003, in the Jordan River, was subjected to gel filtration chromatography on Sephadex LH-20. The fractions were further purified by gradient RP-HPLC to give glycoside (**1**, see Fig. 1), which was identified by its IR, MS, UV, CD, and ^{1}H and ^{13}C NMR spectroscopic data and by chemical degradation.

The IR spectrum of compound **1** has absorption bands at 3300–3400 (hydroxy groups), 1540, 1640, 3250 (amide),

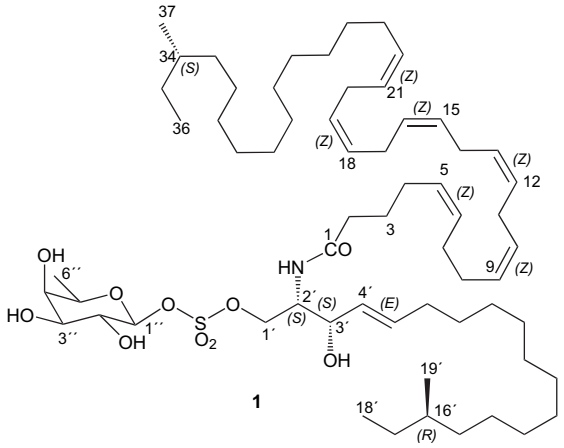

Figure 1. Structure of syriacin (**1**), a sulfated ceramide glycoside from the freshwater sponge *E. syriaca*.

Keywords: Ceramide; Glycoside; Sulfate; Freshwater sponge; *Ephydatia syriaca*; Antifeeding activity.
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1450, 2850, 2900 (the aliphatic chain), and 1070, 1230 (sulfate group) cm^{-1}.

The high-resolution mass spectrum of **1** shows a pseudomolecular ion peak $[M+Na]^+$ at *m/z* 1082.7666, which corresponds to the molecular formula $C_{62}H_{109}NO_{10}S$. The presence of a sulfate group was supported by the ion peaks at *m/z* 96 (SO_4) and 80 (SO_3) in FABMS.

The sulfate group was located at the C-1′ position from low-field shifts of the methylene signals of H-1′ (δ 4.28 and 4.18) and C-1′ (δ 74.2) (chemical shifts of typical ceramide are δ 62.6 and δ 3.94 and/or 3.68, respectively).[7]

The presence of the ester linked sulfo group was further confirmed enzymatically. β-D-Galactosidase from *E. coli* (EC 3.2.1.23) was used to hydrolyze compound **1** to afford fucose (**3**) and sulfoceramide (**1a**). The reaction mixture was lyophilized and then analyzed by FABMS; the corresponding ion at *m/z* 936.7090 $C_{56}H_{99}NO_6SNa$ $[M+Na]^+$ was identified. Although the enzyme used for the hydrolysis was not β-D-fucosidase (EC 3.2.1.38), β-D-galactosidase is known to be insensitive to substitution in position 6 of the substrate[8] and can be used for the purpose.

Another enzyme used for splitting **1** was β-D-glucuronidase (EC 3.2.1.31) from the keyhole limpet, which exhibited a high sulfatase (EC 3.1.6.1) activity. FABMS analysis of the reaction mixture showed that the spectrum contains sulfofucose (**3a**) ion at *m/z* 267.0151 $C_6H_{12}O_8SNa$ $[M+Na]^+$. The two enzymatic reactions thus clearly show that the sulfate group forms a bridge between fucose and the ceramide.

The ^1H NMR spectrum revealed the presence of two secondary methyls at δ 0.82 and 0.83, respectively, six heteroatom-bearing methines, four of them in hexose, and an oxygenated methylene protons at δ 4.18 and 4.28, two trans olefinic protons at δ 5.57 and 5.69, 12 cis olefinic protons (δ 5.38–5.45), and a huge methylene envelope at δ 1.25–1.40 (Table 1). The ^1H NMR spectrum also showed, in the methyl region, two triplets at δ 0.85 and δ 0.86, respectively (ethyl termini). Interpretation of the ^1H–^1H COSY and HMQC spectra resulted in three partial structures, two of which were connected to long aliphatic chains.

The amide broad singlet at δ 6.51 allowed us to assign all the protons of the polar part of the sphingosine through the COSY spectrum. The lack of substitution of the fatty acid residue in α position was revealed by the presence of a characteristic triplet at δ ~2.2 of the α-protons of acyl in the ^1H NMR spectrum of **1**, and an intense correlation peak with the amide NH signal in the ROESY spectrum. In addition, both H_2-2′ and NH were shown to be coupled with the amidic carbon atom at δ 172.4 (C-1) in the HMBC spectrum. This correlation between H-2′ and C-1 not only confirmed the position of the nitrogen atom but also connected the two partial structures through an amide bond. Two olefinic protons at 5.57 and 5.69, respectively were found to be trans configuration, because the coupling constant was 15 Hz.

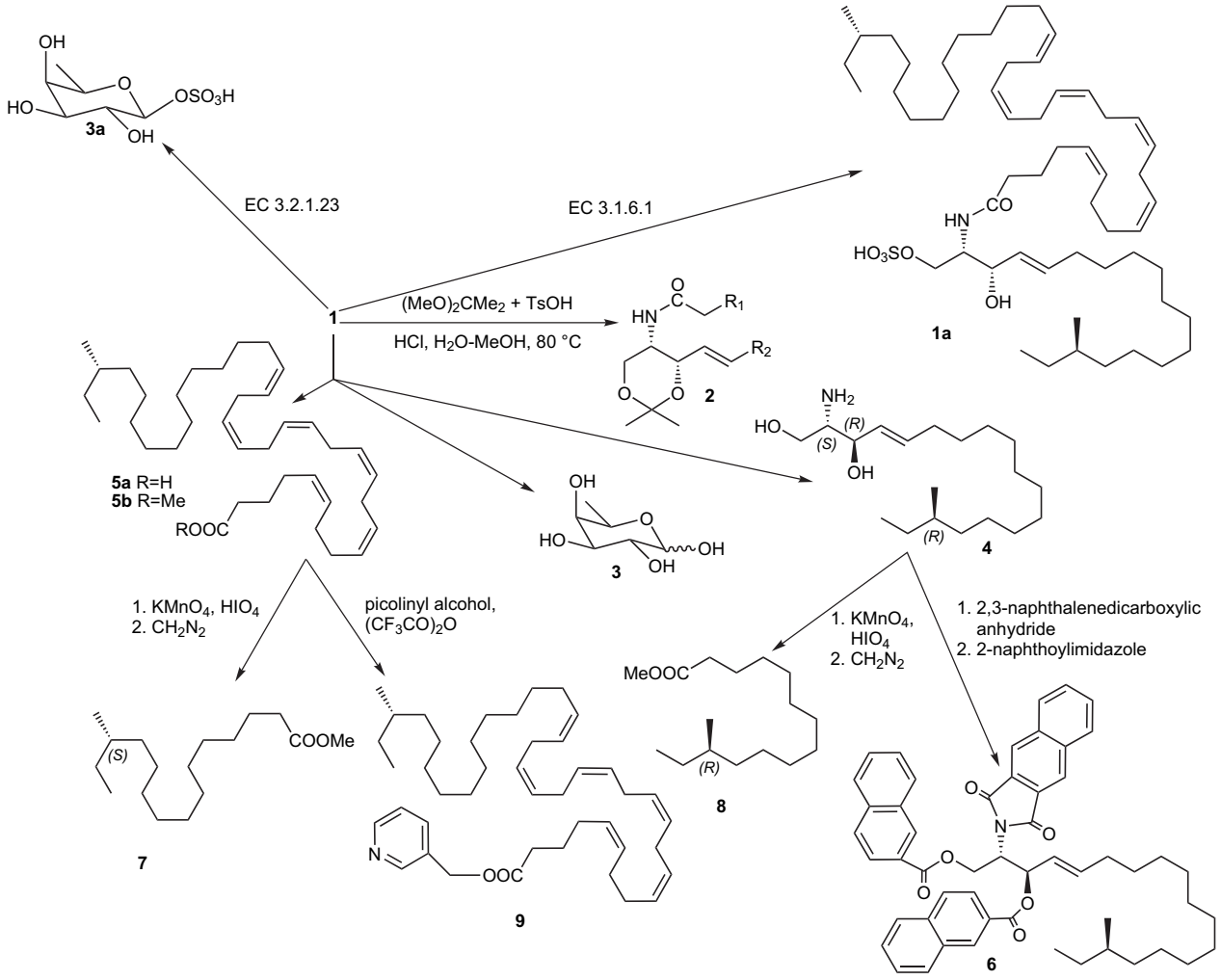
The ^{13}C NMR spectrum of **1** showed 14 olefinic carbons at δ 126–135, one anomeric carbon at δ 101.6, methylene carbons at δ 29.0–31.5, and two secondary methyls (δ 20.7 and 21.2, respectively; both quartets), further supporting the nature of **1**. Additionally, the carbon resonances at δ 74.2, 51.6, 78.3, 131.2, and 134.8 revealed the presence of a dihydroxyaminoene system in **1**.

Table 1. ^1H and ^{13}C NMR data of syriacin (**1**) (measured in $CDCl_3$)

No.	^1H NMR	^{13}C NMR
1	—	172.4
2	2.18 (2H, t, *J*=6.3 Hz)	36.9
3	1.61 (2H, m)	25.6
4, 23	1.90 (4H, m)	27.2–27.9
5, 6, 9, 10, 12, 13, 15, 16, 18, 19, 21, 22	5.38–5.45 (12H, m)	126.0–135.0
7, 8	2.00–2.10 (4H, m)	31.2
11, 14, 17, 20	2.60–2.75 (8H, m)	25.0–26.0
24–32, 35	1.25–1.40 (20H, m)	29.0–31.5
33	1.24 (2H, m)	37.1
34	1.61 (1H, m)	35.4
36	0.85 (3H, t, *J*=7.1 Hz)	12.4
37	0.83 (3H, d, *J*=6.8 Hz)	21.2
1a′	4.18 (1H, dd, *J*=10.5, 5.2 Hz)	74.2
1b′	4.28 (1H, dd, *J*=10.5, 8.4 Hz)	
2′	4.01 (1H, ddd, *J*=8.6, 8.4, 5.2 Hz)	51.6
3′	4.14 (1H, dd, *J*=8.6, 7.2 Hz)	78.3
4′	5.57 (1H, dd, *J*=7.2, 15.0 Hz)	131.2
5′	5.69 (1H, dt, *J*=15.0, 6.9 Hz)	134.8
6′	1.96 (2H, m)	34.3
7′–15′, 17′	1.25–1.40 (20H, m)	29.0–31.5
16′	1.65 (1H, m)	35.4
18′	0.86 (3H, t, *J*=7.2 Hz)	11.6
19′	0.82 (3H, d, *J*=6.7 Hz)	20.7
NH	6.51 (1H, br s)	—
1″	5.28 (1H, d, *J*=7.5 Hz)	101.6
2″	4.26 (1H, dd, *J*=7.5, 8.5 Hz)	73.6
3″	4.39 (1H, dd, *J*=3.4, 8.5 Hz)	75.1
4″	4.15 (1H, dd, *J*=3.4, 1.4 Hz)	74.4
5″	4.42 (1H, dq, *J*=1.4, 6.7 Hz)	72.5
6″	1.52 (1H, d, *J*=6.7 Hz)	16.6

In the saccharide unit, the coupling system in the ^1H–^1H COSY spectrum of **1** started from the anomeric proton signal. This was established by tracing and joining the coupling points among C-1″–C-6″; this revealed contiguous coupling between H-1″ and H-2″, H-2″ and H-3″, H-3″ and H-4″, and H-5″ and 5″-Me. The coupling constants of the anomeric protons H-1″ ($J_{1''-2''}$=7.5 Hz) and H-2″ ($J_{2''-3''}$=8.5 Hz) of **1** suggest its axial orientation. The double-doublet signal of H-4″ suggested that H-4″ was equatorial. Selected NOE difference experiments were used to confirm the configuration of the monosaccharide residues in **1**, irradiation at H-5″ resulted in NOE enhancement at H-3″, confirming that both hydrogens were axial and that 5″-Me was equatorial. Finally, based on these spectral data, it was concluded that the monosaccharide unit of **1** was β-fucopyranose (6-deoxy-galactose). The presence of a β-fucopyranose moiety in **1** was confirmed by comparing the ^{13}C NMR chemical shifts of the monosaccharide unit with those of known monosaccharides.[9]

The relative stereochemistry of **1** was elucidated by chemical derivatization (Scheme 1). The sulfate group of **1** was easily removed by treatment with 2,2-dimethoxypropane under acidic conditions to furnish acetonide **2**. The *anti*-relationship of H-2′ and H-3′ in **2** was inferred from the coupling constant between H-2′ and H-3′ (*J*=8.6 Hz) as well as



Scheme 1. Reaction scheme of degradation compounds from syriacin (**1**).

from ROESY cross peaks between H-3′/H_3-21 and H-1b′/H_3-21 (Fig. 2).

Glycosphingolipids are often present in minute quantities in living organisms as inseparable mixtures of homologues with branched and unsaturated chains. Unfortunately, despite the progress of NMR and MS, chemical degradation is still an essential step in the study of these compounds. The degradation procedure for compound **1** that we used is quite complex and is summarized in Scheme 1. In order to determine the structures of the saccharide, sphingosine, and fatty acyl units of **1**, which would lead to a gross structure, compound **1** was subjected to acidic hydrolysis, and the reaction

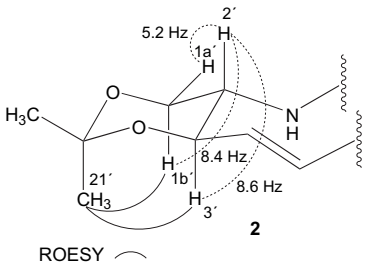


Figure 2. Coupling constants and ROESY correlations of acetonide (**2**).

products were partitioned between CH_2Cl_2 and H_2O/MeOH, giving an aqueous layer consisting of a saccharide (fraction 1) and an organic layer composed of a fatty acid and sphingosine (fraction 2).

The saccharide fraction (1) was used to determine the absolute configuration of the saccharide. The $[\alpha]_D^{21}$ of our fucopyranose (**3**) was +74.0; literature[10] reported $[\alpha]_D^{23}$ −76 for L or +75 for D form.

Fraction 2, containing the fatty acid (**5a**) and the long-chain aminodiol (**4**), was separated on silica gel column. The first eluted fraction 2a was analyzed as methyl ester (**5b**) by GC–MS and was shown to contain a branched polyunsaturated chain, which was further identified by MS, NMR, and chemical degradation (Scheme 1).

The presence of a double bond(s) in the molecule **9** (prepared from **5a**) causes a significant alteration in the mass spectrum, see Figure 3. The cationic site on the picolinyl nitrogen significantly reduces the double bond migrations and enabled us to locate the position of double bond(s). While this technique is universally applicable to all PUFA (polyunsaturated fatty acids), additional fragment ions present in the mass spectra of this picolinyl ester complicated the

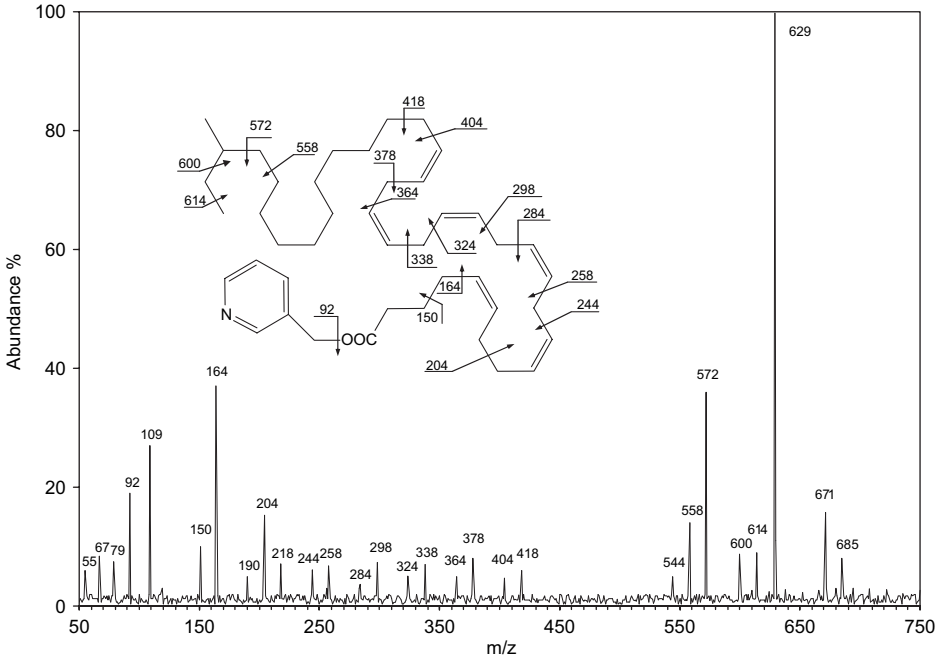


Figure 3. The APCI/MS of picolinyl ester of (all *Z*)-34*S*-methylhexatriaconta-5,9,12,15,18,21-hexaenoic acid.

clear interpretation of the exact position of branching. Oxidative splitting was therefore used to determine the branching position.

The methyl ester of PUFA (**5b**) was not conjugated, as shown by the UV data (see Section 4). The double-bond stereochemistry was established by IR. All double bonds were *Z* because the IR spectrum of the PUFA exhibited absorption at 723 cm^{-1} and no absorption in the 960–980 cm^{-1} region.[11] This stereochemistry was further confirmed by ^{13}C NMR, since the allylic carbons (C-11, C-14, C-17, and C-20) resonated between 25 and 26 ppm (Table 1).[12] Consequently, the above data and deductions completed the sketchy features of the structure of the PUFA, with the exception of the absolute stereochemistry of the branching chain.

The absolute stereochemistry of the long-chain base was established using a method developed earlier.[13] The aminodiol (**4**) was converted by a two-step derivatization to 1,3-binaphthoate-2-*N*-naphthimido-sphingosine (**6**) and its circular dichroism spectrum was measured. The use of two different strongly absorbing chromophores leads to characteristic exciton split CD curves, which depend on the absolute skew of interacting chromophores and the pair wise additivity principle. In order to obtain diagnostic CD reference curves for all four sphingosine diastereomers, numerous bichromophoric combinations, including new chromophores, have been tested over the years,[13,14] we chose O-derivatization and N-derivatization with different chromophores.

In a non-polar solvent both the shapes and amplitudes of CD curves are distinctly different. The (2*S*,3*R*)-*erythro* derivative **6** exhibits a simple negative naphthimide/naphthoate exciton couplet with a negative Cotton effect at 254 μm ($\Delta\varepsilon$ −28) and a positive Cotton effect at 238 μm ($\Delta\varepsilon$ +65). The difference in $\Delta\varepsilon$ values may come from the presence of an extra methyl at C-16′ in **6**.

Both compounds (**4** and **5a**) were subjected to Lemieux oxidation with $KMnO_4/NaIO_4$ to convert sphingosine to a carboxylic acid with four less carbon atoms and the PUFA to a mixture of mono- and dicarboxylic acids. The obtained fatty acids were methylated with CH_2N_2 and analyzed by GC–MS; the results are reported below.

The methyl 13-methylpentadecanoate (**7**) was identified as the longest compound after splitting of PUFA (**5a**), while methyl 12-methyltetradecanoate (**8**) originated from sphingosine (**4**). The optical rotation values of both compounds were opposite; $[\alpha]_D^{23}$ for compound **7** was therefore +3.1; the literature value for free *S*-acid is $[\alpha]_D$ +3.3.[15] The $[\alpha]_D^{21}$ value of **8** was −4.7, which is in accordance with literature data ($[\alpha]_D$ −5.6 for *R* and +5.07 for *S*).[16] This value was a great surprise because this stereochemistry is unnatural (starting unit is probably derived from D-isoleucine). Thereby the absolute stereostructure of **1**, including both positions 34 and 16′, was finally determined as shown in Figure 1.

Based on our results, it was established that the isolated cerebroside occurred as a 2′*S*-(all *Z*)-34*S*-methylhexatriaconta-5,9,12,15,18,21-hexaenoylamino 3′*R*-hydroxy-16′*S*-methyl-octadece-4′*E*-enyl β-D-fucopyranosyl sulfate. The structure of the main product can be described by formula **1**.

Sponges, with their sessile lifestyle and soft unprotected body tissues, are in a strong need of chemical defenses. Accordingly, aquarium assays performed by previously described methods[17] showed that syriacin **1** at a concentration of 10 μg/mL, i.e., slightly below the natural concentration (17.2 μg/mL in our experiments), reduces feeding by goldfish to 13.4±0.6% of the syriacin-free control value (see

Section 4); this strong bioactivity suggests a role in the chemical defense of *E. syriaca*.

Ceramide 1-sulfates with structures similar to compound **1** but with different *N*-acyl moieties have been isolated from Bryozoa *Watersipora cucullata* as new potent inhibitors of human DNA topoisomerase I,[18] while sulfated ceramides isolated from the marine sponge *Discodemia calyx*[19] and from *Zoanthus* sp. (Coelenterata)[20] were found to be inhibitors of neuraminidase. *E. syriaca* syriacin contains a unique very-long-chain fatty acid with six double bonds—34-methylhexatriaconta-5,9,12,15,18,21-hexaenoic acid. Other very-long-chain fatty acids isolated from different freshwater sponges include, e.g., 5,9,23-triacontatrienoic acid (*Baicalospongia bacillifera*, *B. intermedia*, *Cortispongilla barroisi*, *E. syriaca*, *Lubomirskia baicalensis*, *Nudospongilla* sp.), 15,18,21,24-triacontatetraenoic and 15,18,21,24,27-triacontapentaenoic fatty acids (*B. bacillifera*, *B. intermedia* and *L. baicalensis*).[2]

The unusual branched starter units for the biosynthesis of branched chain fatty acids are obtained from the branched chain amino acids L-valine, L-leucine, and L-isoleucine via their corresponding catabolites.[21] Investigations of the biosynthesis of (2*S*)-methylbutanoate esters in apples[22] confirmed that (2*S*)-isoleucine was the precursor of (2*S*)-methylbutanoate. Isoleucine was also a precursor of anteiso-fatty acids in rat skin,[23] all four possible stereoisomers being selectively used for the biosynthesis of these acids. This study suggested the selective biosynthesis of the (*S*)-enantiomer of anteiso-fatty acid in rat skin from DL-isoleucine.

Why isoleucine and alloisoleucine are incorporated into the syriacin molecule is not known. One of the possibilities is isomerization of isoleucine to form alloisoleucine, as described with coronamic acid, an ethylcyclopropyl amino acid derived from isoleucine.[24]

In our case a branched fatty acid was biosynthesized from leucine, which is in agreement with the (*S*)-configuration on carbon C-34. By contrast, sphingosine has (*R*)-configuration on carbon C-16′, which is in agreement with its biosynthesis from alloisoleucine. This configuration is unusual and, to our knowledge, no sphingosine with *R* anteiso-configuration has been found in the nature.

3. Conclusion

To the best of our knowledge, this is the first report of a sulfated ceramide containing an (*R*)-branched long-chain base and (*S*)-branched VLCPUFA (very-long-chain polyunsaturated fatty acids) from living organisms.

4. Experimental

4.1. General

General experimental procedures: UV–vis spectra were measured in MeOH within the range of 210–550 nm in a Cary 118 (Varian) apparatus. A Perkin–Elmer (Perkin–Elmer, Norwalk, CT, USA) model 1310 IR spectrophotometer was used for scanning IR spectroscopy as neat films. Circular dichroism (CD) spectroscopy was carried out under dry N_2 on a Jasco-500A spectropolarimeter at 24 °C. NMR spectra were recorded on a Bruker AMX 500 spectrometer (Bruker Analytik, Karlsruhe, Germany) at 500.1 MHz (^{1}H), 125.7 MHz (^{13}C). High- and also low-resolution MS were recorded using a VG 7070E-HF spectrometer (70 eV). HR-FABMS (positive ion mode) were obtained with a PEG-400 matrix. GC–MS of the fatty acid methyl esters were done using a Finnigan 1020 B (Finnigan MAT, San Jose, CA, USA) single-state quadrupole GC–MS instrument in the EI mode.

4.2. LC–MS/APCI

The LC–MS/APCI of picolinyl ester was realized as mentioned previously[25]—briefly: the HP 1090 series (HP 1090 series, Hewlett Packard, USA) was used with two columns (HIRPB-250AM 250×2.1 mm i.d., 5 mm phase particle). A quadrupole mass spectrometer system Navigator (Finnigan MAT, San Jose, CA, USA) had vaporizer temperature 400 °C, capillary heater temperature 220 °C, corona current 5 mA, sheath gas high-purity nitrogen, pressure ca. 380 kPa, and auxiliary gas (also nitrogen) flow rate 1500 ml/min. Ions with *m*/*z* 50–1500 were scanned with a scan time of 0.5 s, flow rate 0.37 ml/min. Picolinyl esters were separated using a gradient solvent program with acetonitrile/dichloromethane (90:10) and linear from 10 to 40 min acetonitrile/dichloromethane (70:30).

4.3. Chromatography of fatty acid methyl esters

GC–MS of FAME was done on a Finnigan 1020 B in EI mode. Splitless injection was 100 °C, and a fused silica capillary column (Supelcowax 10; 60 m×0.25 mm i.d., 0.25 mm film thickness; Supelco, Prague) was used. The temperature program was as follows: 100 °C for 1 min, subsequently increasing at 20 °C/min to 180 °C and at 2 °C/min to 280 °C, which was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm/s. All spectra were scanned within the range *m*/*z* 50–800. The mass spectra of methyl esters agreed with previously published data.[26,27]

4.4. Animal material

The freshwater sponge *E. syriaca* was collected in August 2003 in the spring of the Jordan River, Israel. The voucher specimens are deposited in the collection of the third author (V. M. Dembitsky). Fresh sponge was put into ethanol and stored at −10 °C under nitrogen.

4.5. Isolation

Sponge was extracted three times by butanol; the extracts were chromatographed on a Sephadex LH-20 column with chloroform/methanol 3:2 and then separated by RP-HPLC on a Discovery C18 column (Supelco) particle size 5 μm, length×i.d. (250 mm×21.2 mm) using a linear gradient from 20% H_2O and 80% acetonitrile to 1% H_2O and 99% acetonitrile over 25 min, with a flow rate of 9.9 ml/min and monitoring by a variable wavelength detector at 208 nm. The yield of compound **1** was 26.5 mg in the crude extract.

4.6. Syriacin (1)

Colorless powder (26.5 mg); $[\alpha]_D^{23}$ −18.3 (*c* 0.04, MeOH); UV λ_{max} (MeOH, nm) (log ε): 218 (3.07); IR (film, cm^{-1}) ν_{max} 3300–3400 (OH), 1540, 1640, 3250 (CONH), 1450, 2850, 2900 (CH_2), and 1070, 1230 (SO_4) cm^{-1}; HR-FABMS (*m/z*): 1082.7666 $[M+Na]^+$, calcd for $[C_{62}H_{109}NO_{10}S+Na]^+$ 1082.7668; NMR data, see Table 1.

4.7. Acetonide (2)

A mixture of **1** (1.8 mg), 2,2-dimethoxypropane (0.1 ml), and *p*-TsOH (catalytic amounts) in CH_2Cl_2 (0.5 ml) was stirred at room temperature for 3 h. After addition of triethylamine (10 µl) and H_2O (2 ml), the reaction mixture was extracted with ether (3×2 ml). The ether extract was evaporated and chromatographed on a silica gel column with $CHCl_3$/MeOH (100:1) to yield (1.3 mg, i.e., 87%) of acetonide **2**. ^1H NMR ($CDCl_3$) δ 1.66 (2H, m, H-3), 1.99 (2H, m, H-6′), 3.68 (1H, dd, *J*=5.2, 13.6 Hz, H-1a′), 3.77 (1H, dd, *J*=8.4, 13.6 Hz, H-1b′), 3.79 (1H, ddd, *J*=8.4, 8.6, 5.2 Hz, H-2′), 2.24 (2H, m, H-2), 4.32 (1H, dd, *J*=8.6, 6.9 Hz, H-3′), 5.34 (1H, dd, *J*=15.0, 6.9 Hz, H-4′), 5.71 (1H, dt, *J*=15.0, 6.9 Hz, H-5′); HR-FABMS (*m/z*): 896.7833 $[M+Na]^+$, calcd for $[C_{59}H_{103}NO_3+Na]^+$ 896.7836.

4.8. Enzymatic cleavage of 1

Tris buffer solution (3 ml, 200 mM), pH 6.3 and **1** (1 mg) in deionized water (2 ml) were mixed and further equilibrated at 37 °C. After 10 min, 100 units of sulfatase solution (β-D-glucuronidase (EC 3.2.1.31) from the keyhole limpet) was added and mixed for 5 min. The solution was lyophilized and analyzed by mass spectrometry.

Citrate buffer (3 ml, 400 mM), pH 3.5 and **1** (1 mg) in deionized water (2 ml) were mixed and further equilibrated at 25 °C. After 10 min, 100 units of sulfatase solution (β-D-galactoside (EC 3.2.1.23) from the *E. coli*) was added and mixed for 15 min. The solution was lyophilized and analyzed by mass spectrometry.

4.9. Hydrolysis of 1

Compound **1** (24.0 mg) was dissolved in 1 M HCl (1 ml) in 50% MeOH and the solution was kept for about 12 h at 80 °C in a sealed tube. The reaction mixture was dried under nitrogen, and partitioned between $CHCl_3$ and H_2O/MeOH (8:2). The aqueous layer was concentrated to give a saccharide (fraction 1). The organic layer was concentrated and dissolved in a small quantity of $CHCl_3$ and the solution was passed through a silica gel column. Elution with 1% pyridine in $CHCl_3$ (15 ml) gave a mixture of acids (fraction 2a), and subsequent elution with 1% pyridine in MeOH afforded a sphingosine (fraction 2b).

4.9.1. Fraction 1. The water from fraction 1 was evaporated to dryness and an α,β-anomeric mixture (2.1 mg, 56%) of D-fucopyranose $[\alpha]_D^{21}$ +74.0 (*c* 0.15, MeOH) was obtained as a colorless syrup.

4.9.2. Fraction 2a. The component (11.0 mg) after hydrolysis of compound **1** was identified by APCI/MS as picolinyl ester (**9**) and also as methyl ester (**5b**) by ^1H, ^{13}C NMR, MS, and chemical degradation. Methyl ester (**5b**) was prepared by treatment of **5a** (9.5 mg) with diazomethane—yield 9.5 mg.

The free fatty acid (1.5 mg) was dissolved in diethyl ether (1 ml) and converted into the mixed anhydride derivatives by reaction with trifluoroacetic anhydride (0.1 ml) at 30 °C for 8 h. The excess reagent was evaporated, 10% solution of nicotinyl alcohol in tetrahydrofuran (0.25 ml) was added and the mixture was left at 50 °C for 2 h. Diethyl ether (1 ml) and hexane (0.2 ml) were added and the mixture was washed with water (0.2 ml), 1 M HCl (0.2 ml, three times), and water (0.2 ml, three times) and dried. The solvents were evaporated under reduced pressure and picolinyl ester was further analyzed by LC–MS/APCI.

The component **5b** was further identified by UV, IR, ^1H, ^{13}C NMR, MS, and chemical degradation: UV λ_{max} (MeOH, nm) (log ε) 209 (3.36); IR (neat) λ_{max} 3500 (OH), 3010 (=CH), 2950, 2930, 2870, 1710 (C=O), 1460, 1435, 1410, 1380, 1370, 1240, 940, 720 (HC=CH, *Z*) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.99 (3H, t, *J*=6.8 Hz, H-36), 1.09 (3H, d, *J*=6.9 Hz, H-37), 1.24 (2H, m, H-33), 1.26–1.39 (18H, m, H-25, -32, -35), 1.61 (1H, m, H-34), 1.90 (4H, m, H-4, -23), 2.00–2.10 (4H, m, H-7, -8), 2.18 (2H, m, H-2), 1.61 (2H, m, H-3), 2.32 (2H, m, H-24), 2.60–2.75 (8H, m, H-11, -14, -17, 20), 3.62 (3H, s, OCH_3), 5.38–5.45 (12H, m, H-5, -6, -9, -10, -12, -13, -15, -16, -18, -19, -21, -22); ^{13}C NMR ($CDCl_3$) δ 12.4 (C-36), 21.2 (C-37), 25.0–26.0 (C-11, -14, -17, -20), 25.6 (C-3), 27.2–27.9 (C-4, -23), 29.0–31.0 (C-25, -32, -35), 31.2 (C-7, -8), 31.4 (C-24), 35.4 (C-34), 37.1 (C-33), 36.9 (C-2), 53.2 (OCH_3), 128.0–129.5 (C-5, -6, -9, -10, -12, -13, -15, -16, -18, -19, -21, -22), 173.0 (C-1); HREIMS (*m/z*): 552.4903 $[M]^+$, calcd for $[C_{38}H_{64}O_2]^+$ 552.4906.

To a solution of **5b** (4.0 mg) in 1 ml of *t*-BuOH were added 0.04 M solution of K_2CO_3 (0.2 ml), an aqueous solution of 0.023 M $KMnO_4$ (0.4 ml), and 0.09 M $NaIO_4$ (0.4 ml). The reaction was allowed to proceed at 37 °C for 18 h. After acidification with 2.5 M H_2SO_4, the solution was decolorized with a saturated solution of Na_2SO_3 and extracted twice with 4 ml of ether. The combined extracts were dried and the resulting carboxylic acid was methylated with CH_2N_2. The methyl ester (**7**) was analyzed by GC–MS: methyl (*S*)-13-methylpentadecanoate (**7**), yield 1.2 mg (60%) $[\alpha]_D^{24}$ +3.1 (*c* 0.09, $CHCl_3$), literature data[16] $[\alpha]_D^{21}$ +3.3; HREIMS (*m/z*): 270.2556 $[M]^+$, calcd for $[C_{17}H_{34}O_2]^+$ 270.2559.

4.9.3. Fraction 2b. A first portion of fraction 2b (3.1 mg) of the sphingosine (**4**) from compound **1** was subjected to oxidative cleavage with $KMnO_4$/$NaIO_4$ as described above. GC–MS analysis of the resulting methyl ester gave the methyl (*R*)-12-methyltetradecanoate (**8**); $[\alpha]_D^{24}$ −4.7 (*c* 0.08, $CHCl_3$) literature[18] $[\alpha]_D^{22}$ −5.6 (*c* 2.06, $CHCl_3$); HREIMS (*m/z*) 256.2401 $[M]^+$, calcd for $[C_{16}H_{32}O_2]^+$ 256.2402.

A second portion (3.0 mg) of the sphingosine (**4**) and freshly sublimed 2,3-naphthalenedicarboxylic acid anhydride (3 mg) were dissolved in anhydrous pyridine (1 ml) and refluxed

under stirring for 12 h. The reaction mixture was purified by preparative TLC (hexane/EtOAc 1:1, strongly fluorescent band at R_f ~0.60). The obtained *N*-naphthimido derivative of sphinganines was dissolved in anhydrous acetonitrile (0.2 ml), and 2-naphthoylimidazole (10 mg) and a catalytic amount of 1,8-diazabicyclo-[5.4.0]undec-7-ene were added. The reaction mixture was stirred under argon for 2 h, then dried and purified by preparative TLC (hexane/EtOAc 3:1, strongly fluorescent band at R_f ~0.65) to obtain the pure compound **6** (yield 2.7 mg, 35%); CD (MeCN, nm) λ_{max} 238.5 ($\Delta\varepsilon$ +33.3), 260.0 (−42.3); HR-FABMS (*m*/*z*) 824.3924 $[M+Na]^+$, calcd for $[C_{53}H_{55}NO_6Na]^+$ 824.3927.

4.10. Antifeeding activity assay

Purified syriacin **1** was dissolved in a minimal volume of methanol and mixed with alginate-based food matrix[17] (100 μl) until all organic and water-soluble components were distributed uniformly throughout the paste. The alginate food matrix was then dispensed by a 0.1 ml syringe into a $CaCl_2$ solution (0.25 M), forming a strand that was allowed to harden for 2 min. The hardened strand was rinsed with filtered water and cut into 3 mm pellets with a scalpel. Control pellets were prepared identically but without the addition of natural or synthetic compounds. Feeding assays were performed with goldfish (*Carassius auratus*).

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References and notes

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