New C_{40}-Carotenoid Acyl Glycoside as Principal Carotenoid in *Salinibacter ruber*, an Extremely Halophilic Eubacterium

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The principal (>96% of total) carotenoid in the novel, extremely halophilic eubacterium *Salinibacter ruber*, here called salinixanthin (**1**), has been assigned the structure (all-*E*,2′*S*)-2′-hydroxy-1′-[6-*O*-(13-methyltetradecanoyl)-*β*-D-glycopyranosyloxy]-3′,4′-didehydro-1′,2′-dihydro-*β*,*ψ*-caroten-4-one by spectrometric (vis, EIMS, ^{1}HNMR, CD, GCMS) and chemical methods.

Saltern ponds are frequently colored due to the presence of *β*,*β*-carotene-rich *Dunaliella* cells or to C_{50}-carotenoids including bacterioruberin[1] produced by halophilic *Archaea*. Recently, it was recognized that also halophilic Bacteria may make up a significant fraction of the prokaryote community of saltern ponds,[2] where rod-shaped bacteria may represent up to 20% of the biomass in such environments. The latter bright red eubacterium has recently been isolated and named *Salinibacter ruber*. It requires at least 15% NaCl for growth and is distantly related to the *Cytophaga*−*Flavobacterium* group.[3,4]

We report here a detailed investigation on the carotenoids of *Salinibacter ruber*. Cells were lysed and extracted with acetone/methanol. Preliminary HPLC examinations revealed the presence of a major carotenoid (>96% of total), here called salinixanthin. After several precipitations of white contaminants from the crude extract, salinixanthin (**1**) has been isolated by TLC and preparative HPLC and characterized by vis, EIMS, ^{1}H−^{1}H COSY and TOCSY NMR (600 MHz), CD, and chemical reactions. The fatty acid residue has been analyzed by GC/MS as the methyl ester.

Visible spectra including λ_{max} and spectral fine structure (%III/II[5]) of **1**, its reduction ($NaBH_4$) product **2**, and allylic oxidation (*p*-chloranil) product **3** were compatible[6−8] with the monocyclic dodecaene chromophore shown in structure **1**, possessing a conjugated oxo group in the C-4-position and a *sec* allylic hydroxy group in the C-2′ position.

The polarity of **1** indicated a glycosidic structure. ^{1}H, ^{1}H−^{1}H COSY, and TOCSY NMR spectra defined structure **1**, including the *β*-D-glycoside and both ends of the ester moiety (Table 1).

Alkaline hydrolysis of **1** provided the more polar glycoside **4** and the free fatty acid. The esterifying acid was identified as the branched 13-methyltetradecanoic acid (C_{15}) by GC/MS of the corresponding methyl ester, compatible with the NMR evidence (Table 1).

The molecular formula of **1** ($C_{61}H_{92}O_9$) was confirmed via acetylated derivatives. Standard acetylation[9] of **1** provided a triacetate **5**, which was subsequently converted to the tetraacetate **6** upon prolonged treatment with acetic anhydride in pyridine. Also the free glycoside **4** provided only a tetraacetate **4b** upon standard acetylation.[9] The C-2′-hydroxy group was expected to be most resistant to acylation due to steric hindrance. This was confirmed by EIMS data with diagnostic cleavages for the tetraacetate **4b** (*m*/*z* 331, 564) and the triacetate **5** (*m*/*z* 225, 513, 564) (Figure 1).

The chirality at C-2′ remained to be determined. The Cotton effect of **1** (Figure 2) was weak and not very distinct. From our previous CD correlations of C-2′-ols, C-2′-*O*-glycosides, and C-2′-acetates with the monocyclic undecaene chromophore,[10] it is known that acetylation of C-2′-ols has a remarkable influence on the Cotton effect, leading to a stronger and more conservative type CD spectrum.[10,11] Whereas the triacetate **5** with a free hydroxy group at C-2′ had a weak and indistinct CD spectrum, the tetraacetate **6** showed a characteristic Cotton effect as for phleixanthophyll pentaacetate (**7**) (Figure 2).[10]

The 4-oxo group in the achiral ring of salinixanthin (**1**) is not expected to have any influence on the sign of the Cotton effect in comparison to **7**.[12] The CD spectrum of phleixanthophyll pentaacetate (**7**) has previously been correlated with that of plectaniaxanthin diester (**8**) and **9** with opposite C-2′ configuration. Subsequently, the 2′*R* chirality elucidated for plectaniaxanthin (**10**)[10] has been confirmed by total synthesis of the enantiomer.[13] The chirality of salinixanthin (**1**) could thus be assigned as 2′*S*.

The structure proposed earlier[6] for a minor carotenoid, 4-keto-phleixanthophyll, from *Mycobacterium phlei*, on the basis of chemical derivatizations and 100 MHz ^{1}H NMR, corresponds to the planar structure of the free glycoside of salinixanthin (**1**). 2′*S*-Chirality was later proposed by analogy with the major phleixanthophyll.[10] However, there is not a close phylogenic relationship between *M. phlei* and *S. ruber*. The *Cytophaga*−*Flavobacterium* group distantly related to *S. ruber* produces non-isoprenoid polyene pigments of the flexirubin type.[14] However, the 3-hydroxy carotenoid saproxanthin from *Saprospira grandis*,[15] the α-ketol fleixanthin, and the 4-oxo carotenoid deoxyfleixanthin from *Flexibacter* sp.,[16] as well as 2′-hydroxyflexixanthin and 3-deoxy-2′-hydroxyflexixanthin from gliding "*Taxeobacter*" spp.,[17] represent monocyclic undecaene carotenoids structurally related to the aglycone of salinixanthin (**1**). Indeed, the latter carotenoid is the 2′*R* enantiomer[13] of the aglycone of **1**.

The distribution of free carotenoid glycosides and of the less common carotenoid acyl glycosides have recently been reviewed.[18] Most of the carotenoid acyl glycosides are C_{40}-skeletal monocyclic carotenoids carrying a tertiary glycosyl

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Table 1. ^{1}H NMR (600 MHz) Data for Salinixanthin (**1**) and the Free Glucoside (**4**) in $CDCl_3/CD_3OD$, 1:1

		1			**4**	
	position	^{1}H δ (ppm)	multiplicity	J (Hz)	^{1}H δ (ppm)	multiplicity
aglycone	2	1.89	t	6.8	same	same
	3	2.51	t	6.8	chemical	multiplicities
	7	6.30	d	15.9	shifts as **1**	as **1**
	8	6.42	d	15.9		
	10	6.32	m[a]			
	11	6.70	m[a]			
	12	6.47	d	15.0		
	14	6.33	m[a]			
	15	6.69	m[a]			
	16/17	1.23	s			
	18	1.86	s			
	19	2.02	s			
	20	2.00	s			
	2′	4.13	d	7.3		
	3′	5.70	dd	7.3, 15.6		
	4′	6.40	d	15.6		
	6′	6.20	d	11.3		
	7′	6.63	dd	11.2, 14.9		
	8′	6.39	d	15.0		
	10′	6.25	d	11.3		
	11′	6.68	m[a]			
	12′	6.41	d	15.5		
	14′	6.31	m[a]			
	15′	6.70	m[a]			
	16′	1.23	s			
	17′	1.28	s			
	18′	1.93	s			
	19′	1.99	s			
	20′	2.01	s			
glycosyl	1″	4.55	d	7.8	4.56	d
	2″	3.26	dd	7.8, 9.1	3.23	dd
	3″	3.42	dd	9.1, 9.1	3.40	dd
	4″	3.28	dd	9.1, 9.1	3.25	dd
	5″	3.51	ddd	2.1, 7.7, 9.1	3.31	ddd
	6_a″	4.17	dd	7.7, −13.0	3.68	dd
	6_b″	4.44	dd	2.1, −13.0	3.85	dd
C_{15}-acyl	2‴	2.34	t			
	3‴	1.61	m[a]			
	4‴	1.29	m[a]			
	5−12‴	∼1.25	m[a]			
	13‴	1.51	th			
	14/15‴	0.87	d			

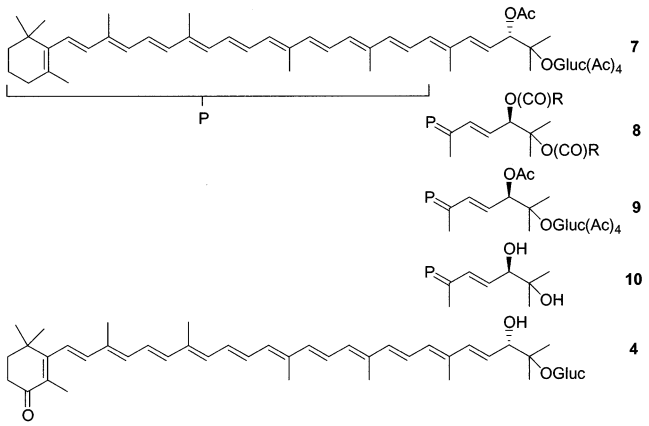
[a] The multiplicities of these signals could not be established due to overlapping with other signals.

group at C-1′, which is acylated in the C-6 position of the glucose moiety.

For *S. ruber* we have demonstrated here that the esterifying fatty acid is the branched 13-methyltetradecanoic acid, an acyl group that has also been shown to be a major constituent in the lipids of *S. ruber*.[19] MS data showed the molecular ion for the fatty acid at *m*/*z* 242 and for the acyl ion at *m*/*z* 225, compatible with a C15:0 fatty acid. NMR data indicated the presence of an iso fatty acid. By GC/MS co-chromatography of the methylated fatty acid derived from **1** with methyl 13-methyltetradecanoate, the structure of the fatty acid residue in **1** was confirmed. Interestingly, the same fatty acid was a major esterifying acid in the carotenoid acyl glycosides of *Mycococcus* spp. MY-18,[20] whereas common unbranched hexadecenoic and hexadecanoic acids were involved in the carotenoid acyl glycosides of *Chloroflexus aurantiacus* strains.[21]

It has been assumed that in prokaryotic organisms, which are lacking sterols, carotenoids with bipolar, linear, and rigid structures may be responsible for membrane stabilization.[22] A model where the fatty acid residue in the carotenoid acyl glycosides is curved back from the glucose moiety (cf. **1**, Scheme 1) into the lipid bilayer has been proposed.[23]

As to the function of carotenoids in extremely halophilic microorganisms, it has been demonstrated for *Archaea* that the red bacterioruberin-containing wild type of *Halobacterium salinarum* survived better than a colorless wild type in strong light.[24] For *S. ruber* exposed to strong light in saltern ponds, salinixanthin (**1**) might serve both these functions.



Experimental Section

General Experimental Procedures. UV/vis spectra were recorded on a Perkin-Elmer 552 spectrophotometer or directly of HPLC fractions during chromatography. CD spectra were

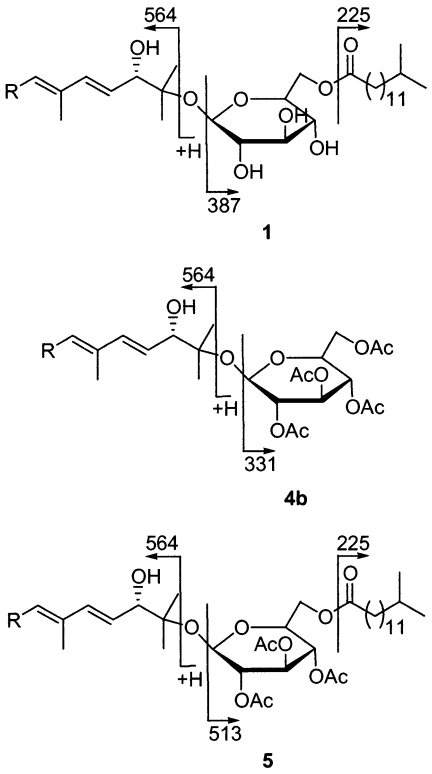


Figure 1. Diagnostic MS fragmentations of salinixanthin (**1**), its triacetate (**5**), and the tetraacetate (**4b**) of the free glucoside (**4**).

recorded at room temperature on a Jobin-Yvon Mark IV dichrograph in ethanol using solutions with optical density 0.4−0.5 at 480 nm and recording the sample and background spectra twice each. ^{1}H NMR spectra were recorded on a Bruker Avance DPX 400 FT-NMR spectrometer and a Bruker Avance DRX 600 FT-NMR spectrometer with Xwin-NMR software version 2.6 in $CDCl_3$ or a $CDCl_3$/CD_3OD mixture with TMS as internal standard. All 2D spectra were recorded without spinning. HPLC was performed on a Hewlett-Packard Series HP1050 instrument equipped with a diode array detector with detection wavelengths set to 450 and 480 nm. A Brownlee Spheri-5 RP18 250 × 4.6 mm column was used with two different eluent systems: System 1: hexane/acetone/methanol/1 M ammonium acetate buffer (0:0:80:20 (0 min)−0:30:70:0 (30 min)−20:50:30:0 (50 min), 1.25 mL/min); System 2: acetone/methanol (0:100 (0−1 min)−15:85 (15 min), 1 mL/min). GC/MS spectra were recorded on a Fisons Trio 1000 with a BP-1 column. EIMS spectra were recorded on a Finnigan MAT 95 XL mass spectrometer. Silica/calcium carbonate (2:1) was used as stationary phase for preparative TLC.

Bacterial Material. *Salinibacter ruber* strain M31 (DSM 13855) was grown at 37 °C on a rotary shaker (180 rpm) in 2 L Erlenmeyer flasks containing 1 L of medium of the following composition (all concentrations in g/L): NaCl, 195; $MgSO_4 \cdot 7H_2O$, 25; $MgCl_2 \cdot 6H_2O$, 16.3; $CaCl_2 \cdot 2H_2O$, 1.25; KCl, 5.0; $NaHCO_3$, 0.25; NaBr, 0.625; yeast extract, 1.0; pH 7.0. Cells in the late-exponential growth phase were harvested by centrifugation (30 min, 5500*g*, 4 °C) and dried in a Savant Speedvac concentrator.

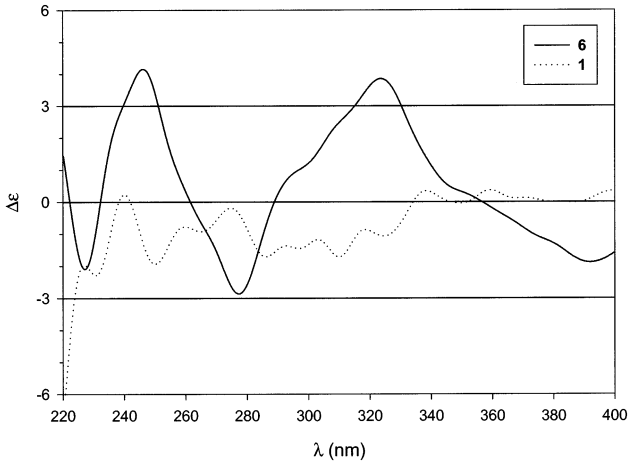


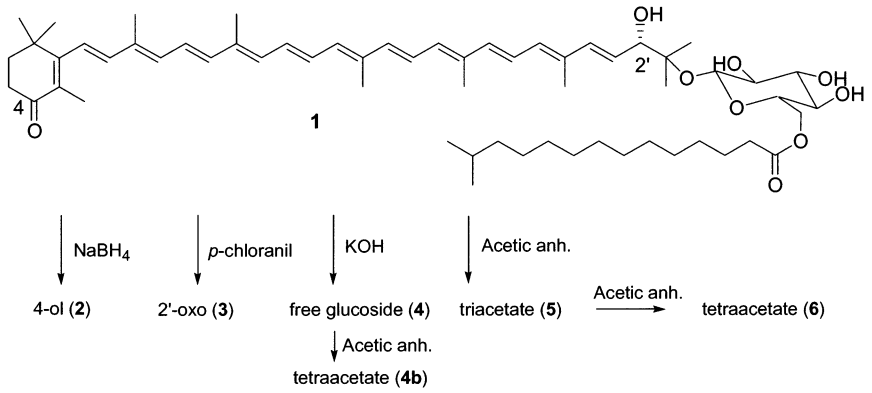
Figure 2. CD spectra in ethanol at room temperature of salinixanthin (**1**) and its tetraacetate (**6**).

Isolation of Carotenoids. General precautions for work with carotenoids were taken.[25] Lyophilized cells from *S. ruber* (9.4 g) were submitted to lysis with water (15 mL). The carotenoid was extracted with methanol/acetone (3:7, 3 × 150 mL) to give 2.5 mg of carotenoid ($E_{1\%,1cm} = 2500$). The solution was taken to dryness at reduced pressure and purified by repeated precipitation of white contaminants with cold acetone. The residue was subjected to CC (silica) with increasing percentage of acetone in hexane. The carotenoid was eluted with acetone/hexane (1:1). The solution was taken almost to dryness (reduced pressure, room temperature) and subjected to preparative TLC (acetone/hexane, 2:3) eluted with methanol.

As an alternative to precipitation in cold acetone, the white contaminants could be removed by a different procedure. To the raw extract dissolved in benzene was added water to give two phases. Addition of methanol caused the precipitation of white contaminants in the hypophase. The hypophase was removed, and the procedure repeated with increasing amounts of hexane in the epiphase and stopped before the carotenoid turned hypophasic.

Salinixanthin ((all-*E*,2′*S*)-2′-hydroxy-1′-[6-*O*-(13-methyltetradecanoyl)-*β*-D-glucopyranosyloxy]-3′,4′-didehydro-1′,2′-dihydro-*β*,*ψ*-caroten-4-one) (1): UV−vis (acetone/MeOH, 7:3) λ_{max} 453 (sh), 482, 508 nm (%III/II^5 = 4); UV−vis (benzene) λ_{max} 462 (sh), 493, 524 nm (%III/II = 9); CD (EtOH), see Figure 2; ^{1}H NMR ($CDCl_3$−CD_3OD, 1:1, 600 MHz), see Table 1; EIMS (70 eV) *m*/*z* 968 [M$^+$] (0.05), 950 [M$^+$ − 18] (0.04), 862 [M$^+$ − 106 (xylene)] (0.05), 844 [M$^+$ − 106 − 18]

Scheme 1



(0.05), 744 [$M^+ - 224$] (0.16), 726 [$M^+ - 242$] (0.22), 582 [$M^+ - 386$] (1.1), 564 [$M^+ - 404$] (5.7), 548 [$M^+ - 404-16$] (4.4), 492 [$M^+ - 404 - 72$] (3.1), 472 [$M^+ - 404 - 92$] (1.1), 458 [$M^+ - 404 - 106$] (7.0), 442 [$M^+ - 404 - 106 - 16$] (6.0), 387 [$M^+ - 581$] (4.7), 242 [$M^+ - 726$] (18.2), 225 [$M^+ - 743$] (14.4), 43 (100).

(all-*E*,2′*S*)-2′-Hydroxy-1′-[6-*O*-(13-methyltetradecanoyl)-*β*-D-glucopyranosyloxy]-3′,4′-didehydro-1′,2′-dihydro-*β*,*ψ*-caroten-4-ol (2). $NaBH_4$ dissolved in ethanol (1.5 mL) was added to a solution of **1** (2 μg) in ethanol (1.5 mL). The reduction of **1** to **2**, monitored by UV−vis spectroscopy, was complete in 25 min. UV−vis (EtOH) λ_{max} 447, 475, 506 nm (%III/II = 46).

(all-*E*)-1′-[6-*O*-(13-Methyltetradecanoyl)-*β*-D-glucopyranosyloxy]-2′-oxo-3′,4′-didehydro-1′,2′-dihydro-*β*,*ψ*-caroten-4-one (3). *p*-Chloranil (1.5 mg) was added to a solution of **1** in benzene (2.5 mL). No reaction was observed after 4 h. *p*-Chloranil (1 mg) and traces of iodine[26] were further added. The reaction mixture was taken to dryness after 28 h. Two zones were eluted with methanol from preparative TLC (acetone/hexane, 1:1), R_f = 0.71 and 0.50, corresponding to **3** and **1**, respectively. UV−vis (EtOH) λ_{max} 501 nm.

(all-*E*,2′*S*)-2′-Hydroxy-1′-*β*-D-glucopyranosyloxy-3′,4′-didehydro-1′,2′-dihydro-*β*,*ψ*-caroten-4-one (4). To a solution of **1** in diethyl ether (15 mL) was added KOH in methanol (10%, 15 mL). The reaction was quenched with saturated aqueous NaCl after 2 h. The organic phase was washed with water three times and taken to dryness at reduced pressure, redissolved in $CHCl_3$, and subjected to preparative TLC (20% hexane in acetone) and preparative HPLC (System 1). UV−vis (HPLC System 1, 21.8 min) λ_{max} 481, 508 nm (%III/II < 4); ^{1}H NMR, see Table 1.

(all-*E*,2′*S*)-2′-Hydroxy-1′-[2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyloxy]-3′,4′-didehydro-1′,2′-dihydro-*β*,*ψ*-caroten-4-one (4b). Acetic anhydride (0.1 mL) was added to a solution of **4** in pyridine (1 mL). Diethyl ether was added and the reaction quenched by addition of saturated aqueous NaCl after 4 h. The organic phase was washed with water five times to remove traces of pyridine. UV−vis (HPLC System 1, 32.3 min) λ_{max} 481, 507 nm (%III/II < 4); EIMS (70 eV) *m*/*z* 912 [M^+] (0.19), 806 [$M^+ - 106$ (xylene)] (0.43), 564 [$M^+ - 348$] (1.9), 458 [$M^+ - 348 - 106$] (4.1), 331 [$M^+ - 581$] (5.5), 43 [Acetyl$^+$] (100).

(all-*E*,2′*S*)-2′-Hydroxy-1′-[2,3,4-tri-*O*-acetyl-6-*O*-(13-methyltetradecanoyl)-*β*-D-glucopyranosyloxy]-3′,4′-didehydro-1′,2′-dihydro-*β*,*ψ*-caroten-4-one (5). The acetylation of **1** followed the same procedure as the acetylation of **4** to **4b**. UV−vis (HPLC System 2, 11.7 min) λ_{max} 480, 510 nm (%III/II < 4); EIMS (70 eV) *m*/*z* 1094 [M^+] (0.04), 1034 [$M^+ - 60$] (0.02), 1002 [$M^+ - 92$ (toluene)] (0.02), 988 [$M^+ - 106$ (xylene)] (0.12), 942 [$M^+ - 60 - 92$] (0.04), 928 [$M^+ - 60 - 106$] (0.07), 564 [$M^+ - 530$] (3.9), 548 [$M^+ - 530 - 16$] (3.5), 513 [$M^+ - 581$] (4.0), 472 [$M^+ - 530 - 92$] (0.8), 458 [$M^+ - 530 - 106$] (6.6), 442 [$M^+ - 530 - 106 - 16$] (3.6), 225 [Acyl$^+$] (50), 43 [Acetyl$^+$] (100).

(all-*E*,2′*S*)-2′-Acetoxy-1′-[2,3,4-tri-*O*-acetyl-6-*O*-(13-methyltetradecanoyl)-*β*-D-glucopyranosyloxy]-3′,4′-didehydro-1′,2′-dihydro-*β*,*ψ*-caroten-4-one (6). The acetylation of **5** to **6** followed the same procedure as the acetylation of **1** to **5**, except that 0.5 mL of acetic anhydride was employed and the reaction was run for 19 h. The product was purified by preparative HPLC (System 2). UV−vis (HPLC System 2, 12.2 min) λ_{max} 481, 510 nm (%III/II < 4); CD (EtOH) 222 ($\Delta\epsilon$ 0) 228 (−2.1) 232 (0) 247 (4.1) 262 (0) 278 (−2.3) 289 (0) 324 (3.8) 358 (0) nm, see Figure 2; EIMS (70 eV) *m*/*z* 1136 [M^+] (0.05), 1076 [$M^+ - 60$] (0.02), 1044 [$M^+ - 92$] (0.01), 1030 [$M^+ - 106$] (0.06), 564 [$M^+ - 572$] (0.08), 548 [$M^+ - 572 - 16$] (0.92), 513 [$M^+ - 623$] (0.97), 242 [$M^+ - 894$] (3.3), 225 [$M^+ - 911$] (16), 43 (100).

Identification of the Acyl Residue in Salinixanthin (1). The water phase from the hydrolysis of salinixanthin was acidified by addition of dilute sulfuric acid to pH 4. The fatty acid was extracted into diethyl ether, the extract taken to dryness, and the residue redissolved in methanol (5 mL). Boron trifluoride diethyl etherate (1 mL) was added. The reaction was quenched by addition of water after refluxing for 5 min, and the fatty acid ester extracted into isopropyl acetate. The methyl ester was analyzed by GC/MS and co-chromatographed with a mixture of acids of authentic *n*-alkanes and methyl 13-methyltetradecanoate.

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