1-*O*-Alkyl-2-(ω-oxo)acyl-*sn*-glycerols from Shark Oil and Human Milk Fat Are Potential Precursors of PAF Mimics and GHB

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ABSTRACT: This study examines the feasibility that peroxidation and lipolysis of 1-*O*-alkyl-2,3-diacyl-*sn*-glycerols (DAGE) found in shark liver oil and human milk fat constitutes a potential source of dietary precursors of platelet activating factor (PAF) mimics and of gamma-hydroxybutyrate (GHB). Purified DAGE were converted into 1-*O*-alkyl-2-acyl-*sn*-glycerols by pancreatic lipase, without isomerization, and transformed into 1-*O*-alkyl-2-oxoacyl-*sn*-glycerols by mild autooxidation. The various core aldehydes without derivatization, as well as the corresponding dinitrophenylhydrazones, were characterized by chromatographic retention time and diagnostic ions by online electrospray mass spectrometry. Core aldehydes of oxidized shark liver oil yielded 23 molecular species of 1-*O*-alkyl-*sn*-glycerols with short-chain *sn*-2 oxoacyl groups, ranging from 4 to 13 carbons, some unsaturated. Autooxidation of human milk fat yielded 1-*O*-octadecyl-2-(9-oxo)nonanoyl-*sn*-glycerol, as the major core aldehyde. Because diradylglycerols with short fatty chains are absorbed in the intestine and react with cytidine diphosphate-choline in the enterocytes, it is concluded that formation of such PAF mimics as 1-*O*-alkyl-2-(ω-oxo)acyl-*sn*-glycerophosphocholine from unsaturated dietary DAGE is a realistic possibility. Likewise, a C_4 core alcohol produced by aldol-keto reduction of a C_4 core aldehyde constitutes a dietary precursor of the neuromodulator and recreational drug GHB, which has not been previously pointed out.

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Platelet-activating factor (PAF), 1-*O*-alkyl-2-acetyl-*sn*-glycerophosphocholine (GroPCho), is a biologically active phospholipid with diverse physiological and pathological effects in a variety of cells and tissues (1). Gamma-hydroxybutyric acid (GHB) is a simple four-carbon FA with an extraordinary range of physiological and pharmacological effects (2). PAF is known to be enzymatically synthesized by either the remodeling or the *de novo* pathways. PAF mimics, however, are generated by secondary peroxidation of unsaturated 1,2-diacyl-*sn*-GroPCho in cell membranes, which retain a short-chain residue esterified at the *sn*-2 position (3). This short-chain residue may contain either a ω-methyl, ω-aldehyde, ω-alcohol, or ω-carboxyl group for PAF-like activity. Investigations of the biological activities by multiple assays have shown that PAF-like lipids containing an *sn*-1 alkyl ether linkage are more effective than the corresponding *sn*-1 acyl derivatives, and that, in general, the shorter the *sn*-2 chain residue the more active the PAF mimic (4,5). Although it has been suggested (2) that GHB may also arise via lipid peroxidation, the exact mechanism has not been established.

In the present report, we demonstrate the feasibility of metabolic transformation of 1-*O*-alkyl-2,3-diacyl-*sn*-glycerols (DAGE) from shark liver oil and human milk into the corresponding core aldehydes, 1-*O*-alkyl-2-(ω-oxo)acyl-*sn*-glycerols, by mild autooxidation and lipolysis. We have previously shown that short-chain 1,2-diradyl-*sn*-glycerols are absorbed intact in the intestine (6,7) and that exogenous 1,2-diradyl-*sn*-glycerols are incorporated intact into the phosphatidylcholines (PtdCho) (8). We have shown elsewhere (9) that such PAF mimics prepared synthetically induce platelet aggregation and inhibit endothelium-dependent arterial relaxation. We postulate that the C_4 core aldehydes, either as glycerolipids or glycerophospholipids, are reduced to the corresponding C_4 core alcohols by endogenous aldol-keto reductases (10) before release into circulation as GHB. There has been no previous work on the core aldehydes arising from oxidation of alkyldiacylglycerols, although the nonvolatile oxidation products of triacylglycerols have been previously discussed (11–16).

EXPERIMENTAL PROCEDURES

Materials. Crude deep-sea shark liver oil was a gift from Baldur Hjaltason, LYSI Ltd., Reykjavik, Iceland. The lyophilized

human milk sample was a gift from Dr. J. Cerbulis of the Eastern Regional Research Center, USDA, Philadelphia, PA. It was one of six milk samples obtained from nursing mothers in the Philadelphia area and used in a collaborative investigation of chloropropanediol diesters in human milk samples (17). The alkyldiacylglycerol composition of these samples ranged from 0.5 to 5 mol%. Linoleic acid, 4-dimethylaminopyridine, and *N*,*N*′-dicyclohexylcarbodiimide were obtained from Sigma-Aldrich (St. Louis, MO). 1-*O*-Octadecyl-*sn*-glycerol was obtained from Fluka (Ronkonkoma, NY). All solvents used were of analytical or HPLC grade.

Preparative TLC. Several preparative TLC systems were employed to purify the various transformation products. All TLC plates were prepared in the laboratory (200 × 200 × 0.25 mm) and activated for 2 h at 110°C before use. System A consisted of silica gel H, developed in hexane/diethyl ether (90:10, vol/vol). System B consisted of silica gel G containing 5% boric acid, developed in hexane/isopropyl ether/acetic acid (50:50:4, by vol). System C consisted of silica gel H, developed in hexane/diethyl ether/acetic acid (80:20:2, by vol). Lipids were visualized under UV light after spraying with 0.2% 2,7-dichlorofluorescein in ethanol (18), whereas the core aldehydes were visualized as purple areas after spraying with the Schiff base reagent (19). Migration of a component is given as the relative retention factor (R_f). Lipids and core aldehydes were recovered from the TLC plates by scraping off the gel, extracting it with chloroform/methanol (2:1, vol/vol), washing with water, drying over anhydrous sodium sulfate, evaporating under nitrogen, and dissolving in chloroform/methanol (2:1, vol/vol). The 2,7-dichlorofluorescein was removed with 1% ammonium hydroxide.

GLC. Injections were made at 100°C, and after 30 s the oven temperature was programmed at 20°C/min to either 130°C (FAME) or 180°C (diacetyl-derivatized 1-*O*-alkyl-*sn*-glycerols (GE)), and then to 240°C at 5°C/min (18). The GLC system consisted of a polar capillary column (SP 2380, 15 m × 0.32 mm i.d., Supelco, Mississauga, ON) installed in a Hewlett-Packard (Palo Alto, CA) Model 5880 gas chromatograph equipped with a flame ionization detector. Hydrogen was used as carrier gas at 3 psi. FAME and diacetyl-GE were identified on the basis of retention times (RT) compared with commercially available external reference compounds.

HPLC. Reversed-phase HPLC was performed with a Hewlett-Packard Model 1090 liquid chromatograph (Palo Alto, CA) using an HP ODS Hypersil C_{18} column (5 µm; 200 × 2.1 mm i.d.; Hewlett-Packard, Palo Alto, CA) and eluted isocratically with 100% Solvent A (methanol/water/30% ammonium hydroxide, 88:12:0.5, by vol) for 3 min, followed by a linear gradient to 100% Solvent B (methanol/hexane/30% ammonium hydroxide, 88:12:0.5, by vol) in 25 min, which was kept for another 6 min (20). Kim *et al.* (20) washed the HPLC column with 0.1 M ammonium acetate at 0.5 mL/min for 5 min at the end of each run and did not observe any ill effects on the performance of column or the quality of the mass spectra. When 2,4-dinitrophenylhydrazine (DNPH) derivatives were analyzed, the effluent was led through a UV detector (358 nm)

installed before the mass spectrometer. The flow was 0.4 mL/min.

Electrospray ionization MS (ESI-MS). Reversed-phase HPLC with online electrospray ionization MS (LC/ESI-MS) was performed by admitting the entire HPLC column effluent into a Hewlett-Packard Model 5988B quadrupole mass spectrometer (Palo Alto, CA) equipped with a nebulizer-assisted electrospray interface (Hewlett-Packard Model 59987A, Palo Alto, CA) as previously described (19). Nitrogen was used as both nebulizing (60 psi) and drying gas (60 psi, 270°C). Capillary voltage was set at 4 kV, the endplate voltage was 3.5 kV, and the cylinder voltage was 5 kV in the positive mode of ionization. In the negative mode, the values were −3.5 kV, −3 kV, and −3.5 kV, respectively. Both negative and positive ESI spectra were taken in the mass range 300–1100 amu. The capillary exit (CapEx) was set at 120 and −120 V in the positive and negative ion mode, respectively.
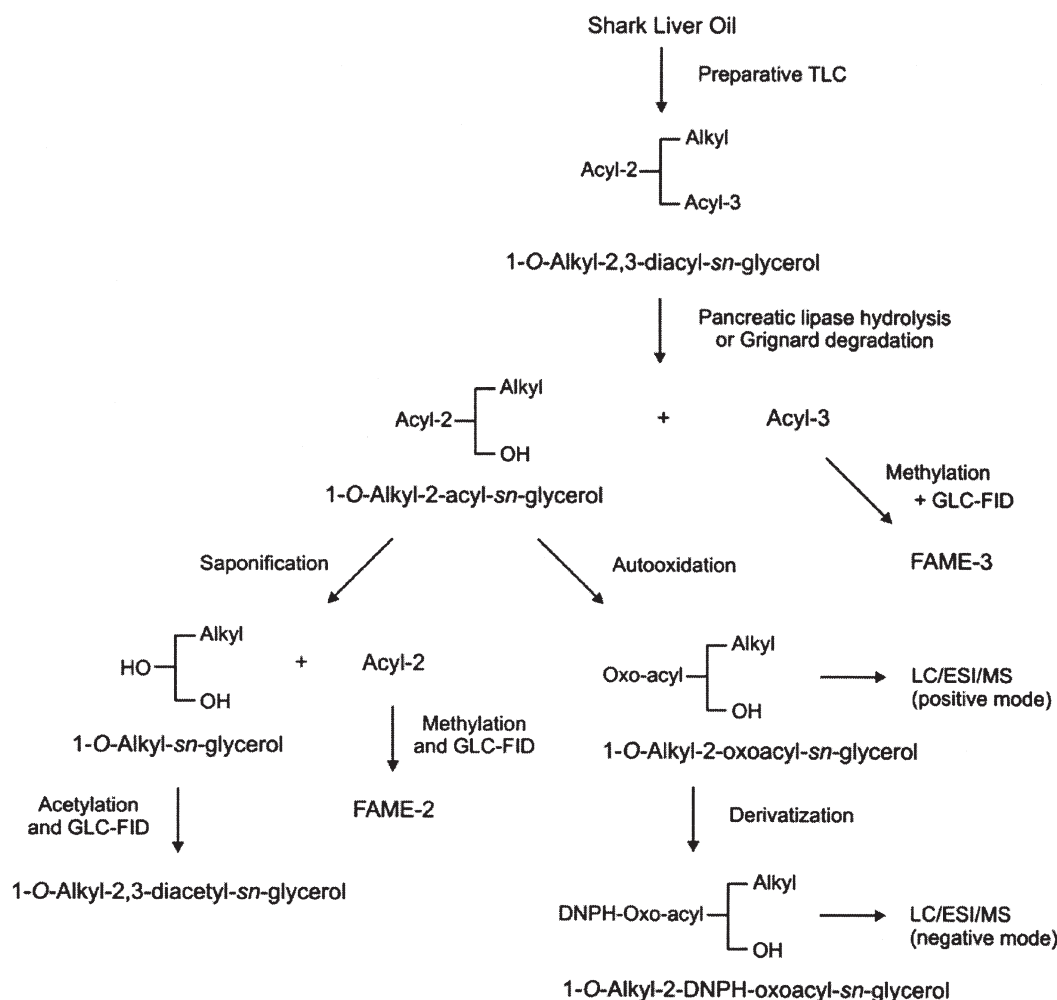
Preparation of DAGE from shark liver oil and human milk. A total lipid extract of freeze-dried human milk was prepared as previously described (21). DAGE was recovered from the human milk lipid extract and the shark liver oil by preparative double one-dimensional TLC (system A). The purified DAGE was subjected to regiospecific analysis to reveal the *sn*-1-*O*-alkyl-, *sn*-2 acyl-, and *sn*-3 acyl-chain composition and distribution. The complete procedure is outlined in Scheme 1.

Hydrolysis with pancreatic lipase and Grignard degradation. Purified DAGE of shark liver oil and human milk were hydrolyzed by digestion with diethyl ether pre-extracted pancreatic lipase (22). The digestion was performed in the presence of gum arabic for 30 min, and the digestion products were extracted with diethyl ether. Alternatively, the purified DAGE were deacylated by Grignard degradation (23) in order to verify the results obtained from pancreatic lipase digestion. The degradation products were resolved and recovered by TLC (system B).

Preparation of FAME and diacetyl-GE. Purified fractions of DAGE, 2-MAGE, and 3-MAGE originating from shark liver oil were treated with 6% H_2SO_4 in methanol for 2 h at 80°C to produce FAME and GE. After the reaction, the lipids were extracted twice with chloroform. GE and FAME were resolved and recovered by preparative TLC (system C). Purified GE was derivatized to diacetyl-GE for 30 min at 80°C with acetic anhydride/pyridine (1:1, vol/vol; 75 µL). The profiles of FAME and diacetyl-GE were determined by GLC.

Autooxidation of 2-MAGE. Mild peroxidation was performed by flushing the purified 2-MAGE from either shark liver oil or human milk in a tube with oxygen, capping, and heating at 80°C for 3 h. The peroxidized 2-MAGE was analyzed by reversed-phase LC/ESI-MS.

Preparation of DNPH derivatives. Aldehyde preparations of oxidized 2-MAGE were derivatized by reaction with DNPH in the dark (0.5 mg in 1 mL 1 N HCl) for 2 h at room temperature and 1 h at 4°C (24). The DNPH derivatives were extracted with chloroform/methanol (2:1, vol/vol), dried over anhydrous sodium sulfate, evaporated under a stream of nitrogen, dissolved in chloroform/methanol (2:1, vol/vol), and analyzed by

SCHEME 1. Flow sheet for the regiospecific analysis of DAGE from shark liver oil and the procedure for isolation and characterization of core aldehydes from oxidized and digested DAGE.

reversed-phase LC/ESI-MS. Derivatization with DNPH, therefore, increased the detection limit for the core aldehydes by MS, and further provided additional ions for characterization of the alkyl ether core aldehydes, as well as an opportunity to monitor the components by UV detection at 358 nm.

Preparation of reference 1-O-octadecyl-2-(9-oxo)-nonanoyl-sn-glycerol. The esterfication of 1-*O*-octadecyl-*sn*-glycerol with linoleic acid was performed by the carbodiimide-mediated process (25). Linoleic acid (75 µmol), 1-*O*-octadecyl-*sn*-glycerol (100 µmol), and 4-dimethyl-aminopyridine (10 µmol) were dissolved in dry n-hexane. This solution was added to a suspension of *N,N'*-dicyclohexylcarbodiimide (100 µmol) in dry n-hexane and shaken vigorously for 17 h at room temperature. After filtration, solvent was evaporated under nitrogen, and the residue was purified by preparative TLC (system B). We have previously reported the LC/ESI-MS analysis of this and other related synthetic neutral ether lipids (26).

The synthesized and purified 1-*O*-octadecyl-2-octadeca-dienoyl-*sn*-glycerol was subjected to triphenylphosphine reductive ozonization as previously described (19). The resulting reference core aldehyde, 1-*O*-octadecyl-2-(9-oxo)nonanoyl-*sn*-

glycerol (R_f = 0.11), was purified by preparative TLC (system B) and analyzed by reversed-phase LC/ESI-MS, and its identity was established on basis of RT, averaged mass spectrum, and fragmentation pattern.

RESULTS

Isolation of DAGE. Preparative TLC resolved the crude shark liver oil into six bands, which corresponded to monoacyl (monoradyl) glycerols (R_f = 0.01), free cholesterol (R_f = 0.13), triacylglycerol (R_f =0.37), DAGE (R_f = 0.55), cholesteryl esters (R_f = 0.93), and squalene (R_f = 0.97). The TLC bands were scraped off the plate and analyzed by high-temperature GLC, which indicated that the DAGE made up 55% of the shark liver oil. Reversed-phase LC/ESI-MS indicated that DAGE was composed of at least 50 species eluting between 10 and 37 min (chromatogram not shown). Similarly, the DAGE content of human milk was estimated to be approximately 1% of total fat and was made up of numerous species, of which only a few were abundant.

Regiospecific analysis of DAGE (26). The purified DAGE were subjected to a regiospecific analysis to reveal the *sn-1-O-*

alkyl, *sn*-2-acyl, and *sn*-3-acyl chain composition and distribution, which were determined by TLC (system B) and GLC, following pancreatic lipase hydrolysis and Grignard degradation.

Pancreatic lipase hydrolysis of the DAGE yielded 1-*O*-alkyl-2-acyl-*sn*-glycerols (2-MAGE, $R_f = 0.32$) as the major product (97%) and 1-*O*-alkyl-3-acyl-*sn*-glycerol (3-MAGE, $R_f = 0.41$) as the minor product (3%), along with GE ($R_f = 0.05$), free FA ($R_f = 0.66$), and original DAGE ($R_f = 0.90$).

The high recovery of 2-MAGE compared with 3-MAGE is consistent with the resistance of the *sn*-1 ether linkage to the action of most enzymes, and furthermore indicates a very low rate of isomerization and a low affinity of the pancreatic lipase for the *sn*-2-position of DAGE. The non-selective Grignard degradation yielded the 2-MAGE and 3-MAGE in equal amounts together with GE and the free FA as the tertiary alcohols (Grignard reaction products). The DAGE, 2-MAGE, and 3-MAGE fractions recovered from the pancreatic lipase digestion and Grignard degradation were treated with sulfuric acid/methanol to produce FAME and glyceryl ethers (GE), which were resolved by preparative TLC (system C). The purified GE were converted into the diacetyl GE by reaction with acetic anhydride and pyridine, and the FAME and the GE acetates were identified and quantified by GLC. Reversed-phase LC/ESI-MS analysis of 2-MAGE isolated from the shark liver oil following pancreatic lipolysis of DAGE showed a total of 49 species, of which 20 species were abundant, eluting between 10 and 26 min (chromatogram not shown).

Table 1 gives the regiospecific distribution of the fatty chains of shark liver oil DAGE as determined by GLC analysis of products of pancreatic lipolysis and Grignard degradation. The predominant *sn*-1-*O*-alkyl fatty chains were the monounsaturated alcohols (18:1n-9, 54.9%, and 16:1n-7, 12.2%) and saturated alcohols (16:0, 11.2%), with much smaller amounts of a diunsaturated alcohol (18:2n-6, 1%). Small amounts of odd-carbon saturated and monounsaturated fatty alcohols were also detected. Pancreatic lipase digestion and Grignard degradation gave similar FA profiles and selectivity for the *sn*-2- and *sn*-3-positions. The most abundant *sn*-2-FA were 16:0, 16:1n-7, 18:1n-9, 20:1n-9, 22:1n-11/13, 22:5n-3, and 22:6n-3, with 18:1n-9 accounting for more than 50% of the total. The most abundant *sn*-3-FA were 16:0, 16:1n-7, 18:0, 18:1n-9, 20:1n-9, 22:1n-11/13, and 22:4n-3. The FA 18:1n-9, 22:5n-3, and 22:6n-3 were preferentially associated with the *sn*-2-position, whereas 18:0, 20:1n-9, 22:1n-11/13, and 22:4n-3 were mostly in the *sn*-3-position.

LC/ESI-MS characterization of reference core aldehydes. The identities of all synthetic neutral ether lipids were established by combined TLC and LC/ESI-MS analysis. The 1-*O*-octadecyl-2-(9-oxo)nonanoyl-*sn*-glycerol was produced in high yield and purity by reductive ozonization of 1-*O*-octadecyl-2-(9-*cis*,12-*cis*)-octadecadienoyl-*sn*-glycerol. Figure 1A shows the total LC/ESI-MS positive ion current profile (CapEx +120 V) of synthetic 1-*O*-octadecyl-2-(9-oxo)nonanoyl-*sn*-glycerol (RT = 14.8 min). Figure 1B shows the full mass spectrum averaged over the entire peak with eight major ions being observed. The assignments for the observed ions, their corre-

TABLE 1
Regiospecific Distribution (mol%) of FA of Shark Liver Oil DAGE as Determined by GLC-FID After Pancreatic Lipase Hydrolysis or Grignard Degradation[a]

Fatty chain	*sn*-1 Alkyl	*sn*-2 Acyl		*sn*-3 Acyl	
		Lipase	Grignard	Lipase	Grignard
12:0	1.0	nd	nd	nd	nd
14:0	2.3	1.0	1.4	0.7	0.8
15:0	0.4	0.3	0.3	0.1	0.2
16:0	11.2	18.4	18.8	15.9	19.5
16:1n-7	12.2	4.7	4.9	3.2	3.2
16:2n-4	nd	0.7	0.7	0.9	1.0
17:0	0.6	nd	nd	nd	nd
17:1	2.2	nd	nd	nd	nd
18:0	2.9	1.2	1.7	4.6	3.7
18:1n-9	54.9	51.8	48.9	21.3	16.9
18:1n-7	4.6	nd	0.3	5.8	6.6
18:2n-6	1.0	0.8	0.8	0.7	0.2
18:3n-3	nd	0.3	0.4	0.4	0.3
18:4n-3	nd	0.2	0.2	0.2	0.1
19:0	0.6	nd	nd	nd	nd
20:1n-9	2.6	6.1	5.5	11.1	12.4
20:2n-6	nd	0.1	0.2	0.3	0.3
20:4n-6	nd	nd	0.1	nd	nd
20:5n-3	nd	0.1	0.3	0.5	0.2
22:1n-11/13	0.2	3.7	3.4	18.0	21.0
22:4n-3	nd	0.2	0.2	5.9	7.4
22:5n-3	nd	1.6	1.4	0.5	0.4
22:6n-3	nd	4.1	3.3	1.5	0.8
24:1n-3	nd	0.7	0.9	0.3	0.2

[a]Average of two determinations. nd, not detected.
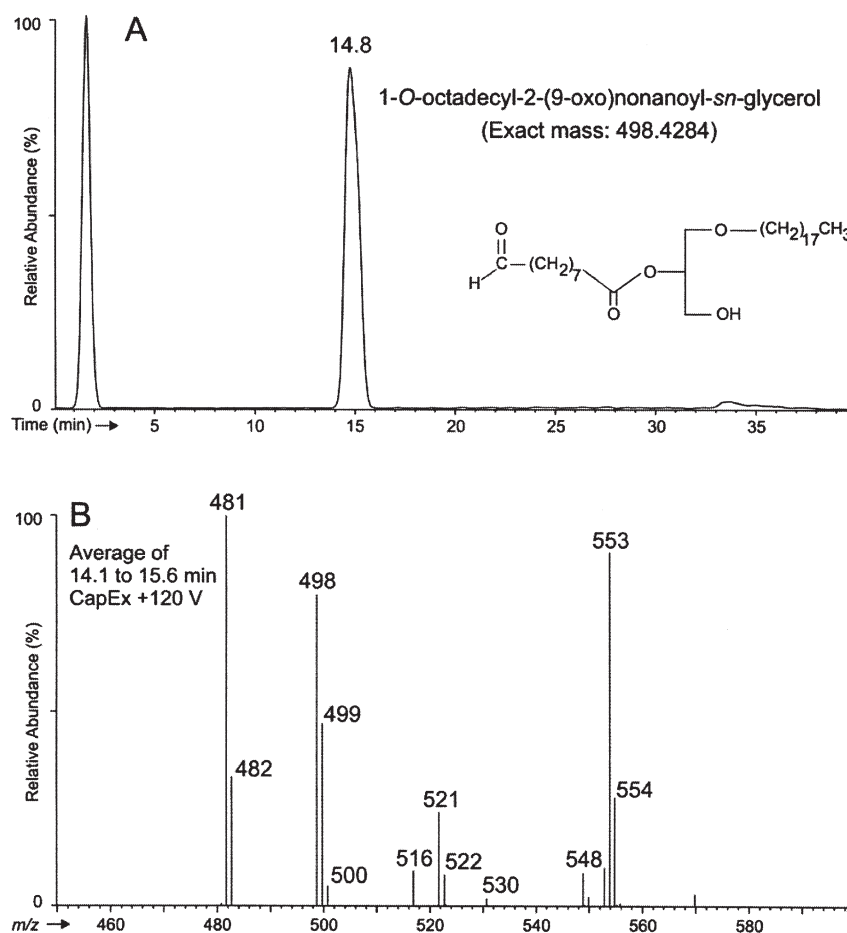
FIG. 1. Reversed-phase LC/ESI-MS analysis of synthetic 1-*O*-octadecyl-2-(9-oxo)nonanoyl-*sn*-glycerol. (A) Total positive ion current profile. (B) Full mass spectrum averaged over the entire peak, zoomed to *m/z* 450–600, in (A). All of the ions detected in the spectrum were assigned to the original reference compound. Note the high abundance for the *m/z* 499 ion relative to *m/z* 498, suggesting that the *m/z* 499 ion is not entirely composed of the ^{13}C isotopic ion of *m/z* 498. The LC/ESI-MS analysis showed that the optimal diagnostic ion of the 1-*O*-alkyl-2-(ω-oxo)acyl-*sn*-glycerols is the [M − 17]$^+$ ion, which in (B) corresponds to *m/z* 481. Additional details are given in Table 2, Scheme 2, and the text.

sponding ^{13}C isotopic components, and their relative abundances are presented in Table 2. The proposed molecular structures for these assigned ions are shown in Scheme 2. Sodium and ammonium adducts are common features of ESI, and it is also known that aldehydes in methanol solutions, as encountered in the mobile phase, are converted to the corresponding neutral hemi-acetal form (a methanol adduct).

The presence of an ion at *m/z* 498.70 corresponding to the [M]$^+$ ion was unexpected and only observed in the samples with the underivatized monoalkylglycerols containing a free aldehyde ester group. We confirmed this observation with the corresponding 3-isomer reference compound, 1-*O*-octadecyl-3-(9-oxo)nonanoyl-*sn*-glycerol (data not shown). It would be expected that the observed ion would have *m/z* 499.75 corresponding to the protonated molecular ion [M + H]$^+$. As indicated in Table 2 and Scheme 2, we propose that the [M]$^+$ ion at *m/z* 498.70 arises from a dehydrated ammonium adduct, [M + NH$_4$ − H$_2$O]$^+$, and not directly from the ionization as a radical cation, [M]$^{+\cdot}$. Another unexpected observation was the relatively high abundance (59%) of the *m/z* 499.75 ion compared with the *m/z* 498.70 ion, which is much higher than the calculated contribution of the ^{13}C isotope (32%) for this compound. The other ions have the ^{13}C contribution showing the expected ~32% relative abundance (Table 2). This suggests that the peak at *m/z* 499.75 is actually composed of two different species, [(M + ^{13}C$_1$) + NH$_4$ − H$_2$O]$^+$ and the protonated molecular ion, [M + H]$^+$. As indicated in Table 2 and Scheme 2, we propose that the [M + H]$^+$ ion can also arise indirectly from the neutral loss of ammonia from the molecular ammonium adduct, [M + NH$_4$ − NH$_3$]$^+$.

Due to the lengthy and complicated ion assignment and the multiple possible pathways of ion formation, we have decided to describe the ions as mass difference from the exact mass of the 1-*O*-alkyl-2-(ω-oxo)-*sn*-glycerol, that is, [M ± X]$^+$.

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