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Chemistry and Physics of Lipids 152 (2008) 78–85
CPL
CHEMISTRY AND PHYSICS OF LIPIDS
www.elsevier.com/locate/chemphyslip

An efficient and convenient synthesis of deuterium-labelled seminolipid isotopomers and their ESI-MS characterization

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Received 9 November 2007; received in revised form 6 February 2008; accepted 7 February 2008
Available online 14 February 2008

Abstract

Seminolipids **1a** and **1b** and galactosylalkylacylglycerols **2a** and **2b**, labelled with deuterium on the alkyl or acyl chain, respectively, were obtained isotopically and chemically pure through a straightforward synthesis from protected glycidyl galactoside **3** in an overall 22% yield. The identity and purity of compounds was ascertained by NMR spectroscopy and ESI mass spectrometry analysis. These labelled compounds are important as internal standards for quantification of these lipids by mass spectrometry, and they could also be used in metabolic studies in *in vitro* and even *in vivo* systems. Extension of the procedure could provide a route for the preparation of isotopomers of other compounds of the same general class.
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Keywords: Sperm–egg interaction; Seminolipid; Mammalian sulfoglycolipids; Deuterium isotopomers; ESI-MS

1. Introduction

The sulfated glycerogalactolipid seminolipid is the main glycolipid in mammalian spermatozoa and testis (up to 3% of the total lipids and 90% of total glycolipids in boar testis) (Ishizuka et al., 1973; Kornblatt et al., 1972; Ueno et al., 1977; Alvarez et al., 1990).

It was identified as a single molecular species with the general composition: 1-*O*-alkyl-2-*O*-acyl-3-*O*-β-D-(3′-sulfo)-galactopyranosyl-*sn*-glycerol. While most naturally occurring glycolipids exhibit a high degree of heterogeneity in their acyl and alkyl chain lengths, and in the degree of unsaturation and the presence or absence of hydroxylation, SGG from boar, rat (Kornblatt et al., 1972), human (Ueno et al., 1977) and bovine (Alvarez et al., 1990) spermatozoa is composed mainly of hexadecyl and hexadecanoyl alkyl and acyl chains, respectively.

Like sulfatide (cerebroside sulfate), the other main sulfoglycolipid in mammals, seminolipid **1** (SGG, Fig. 1) is biosynthesized by the sequential reactions of UDP-galactose:ceramide galactosyltransferase (CGT) which promotes galactosylation of alkylacylglycerol (Van der Bijl et al., 1996), and cerebroside sulfotransferase (CST) which is responsible for the sulfation of galactosylalkylacylglycerol **2** (GG, Fig. 1) (Honke et al., 1997; Zhang et al., 1990). Seminolipid is degraded to GG by the action of arylsulfatase A that catalyzes also the desulfation of sulfatide (Tanphaichitr et al., 2003).

Seminolipid is synthesized in primary spermatocytes and remains stable during spermatogenesis. It is essential for this process to occur normally (Fujimoto et al., 2000), although the precise role of SGG in spermatogenesis and other processes that

Abbreviations: ESI-MS, electrospray ionization mass spectrometry; MS/MS, tandem mass spectrometry; LC, liquid chromatography; Q1, first quadrupole; Q3, third quadrupole; THF, tetrahydrofuran; EDCI, (1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride; DMAP, 4-dimethylaminopyridine; EtOAc, ethyl acetate.

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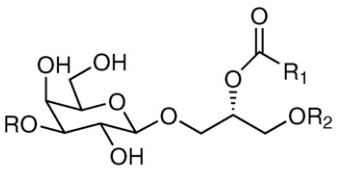
doi:10.1016/j.chemphyslip.2008.02.002



1 : R = SO_3Na, $R_1 = (CH_2)_{14}CH_3$, $R_2 = (CH_2)_{15}CH_3$
2 : R = H, $R_1 = (CH_2)_{14}CH_3$, $R_2 = (CH_2)_{15}CH_3$
1a : R = SO_3Na, $R_1 = (CH_2)_{14}CH_3$, $R_2 = (CH_2)_{15}CD_3$
2a : R = H, $R_1 = (CH_2)_{14}CH_3$, $R_2 = (CH_2)_{15}CD_3$
1b : R = SO_3Na, $R_1 = (CH_2)_{14}CD_3$, $R_2 = (CH_2)_{15}CH_3$
2b : R = H, $R_1 = (CH_2)_{14}CD_3$, $R_2 = (CH_2)_{15}CH_3$

Fig. 1. Structures of SGG, GG and their deuterium-labelled isotopomers.

take place during and following egg fertilization, is still not completely understood. SGG is exposed on the plasma membrane of spermatogenic cells and sperm, and like other glycolipids it contributes to the sperm cell membrane shape and stability (Attar et al., 2000; Bou Khalil et al., 2006), being also involved in recognition events (at least 20 proteins with affinity to SGG have been identified) and cell–cell adhesion. Specifically, SGG on the mammalian sperm head surface has direct affinity for the egg zona pellucida (ZP) and is an integral component of sperm lipid rafts, which are the ZP binding microdomains (White et al., 2000; Weerachatyanukul et al., 2001; Bou Khalil et al., 2006).

Severe diseases are associated with defective enzymes in glycolipid metabolism: in humans affected by metachromatic leukodistrophy the deficit of arylsulfatase A, and less frequently the deficit of the lipid transporter saposin B (also known as cerebroside sulfate activator protein), causes the accumulation of sulfatide and subsequent neurological damage (Norris et al., 2005), while in male mice lacking the CGT gene, the absence of GG and SGG may be responsible for apoptosis of germ cells, which subsequently leads to a spermatogenesis arrest at the primary spermatocyte level (Zhang et al., 2005). Since sperm SGG is involved in sperm–egg interaction, it is possible that decreased levels of sperm SGG are one of the causes of male infertility and/or subfertility. In this context, characterization, monitoring and quantitative determination of SGG and its metabolites (e.g., GG) in biological samples is important. Mass spectrometry, due to an unparalleled combination of sensitivity and specificity, is extensively applied to analyze and quantify lipids and glycolipids; in particular SGG and GG were analyzed by ESI-MS and their concentration in the total lipid extracts from mice testis was determined by LC-ESI-MS (Tadano-Aritomi et al., 2003). The accuracy of the quantitative determination of SGG and GG from biological extracts could be improved by the availability of suitable internal standards (Mills et al., 2005) with the same chemical structure as the analyte and the same fragmentation patterns. Such internal standards can be generated by incorporation of stable labels such as deuterium in the lipid chains of seminolipid.

With the aim of quantifying SGG and GG in biological samples, as well as studying their metabolism *in vivo*, we have synthesized two deuterium-labelled SGG isotopomers (Fig. 1): SGG **1a** with a [16,16,16-$^2H_3$]-ether chain and SGG **1b** with a [16,16,16-$^2H_3$]-fatty acyl chain. Since in our synthetic sequence (see Scheme 1) SGG **1a** and SGG **1b** are obtained by direct sulfation of the GG **2a** and GG **2b** precursors (Fig. 1), this route provides also a straightforward access to deuterium-labelled isotopomers of galactosylalkylacylglycerol.

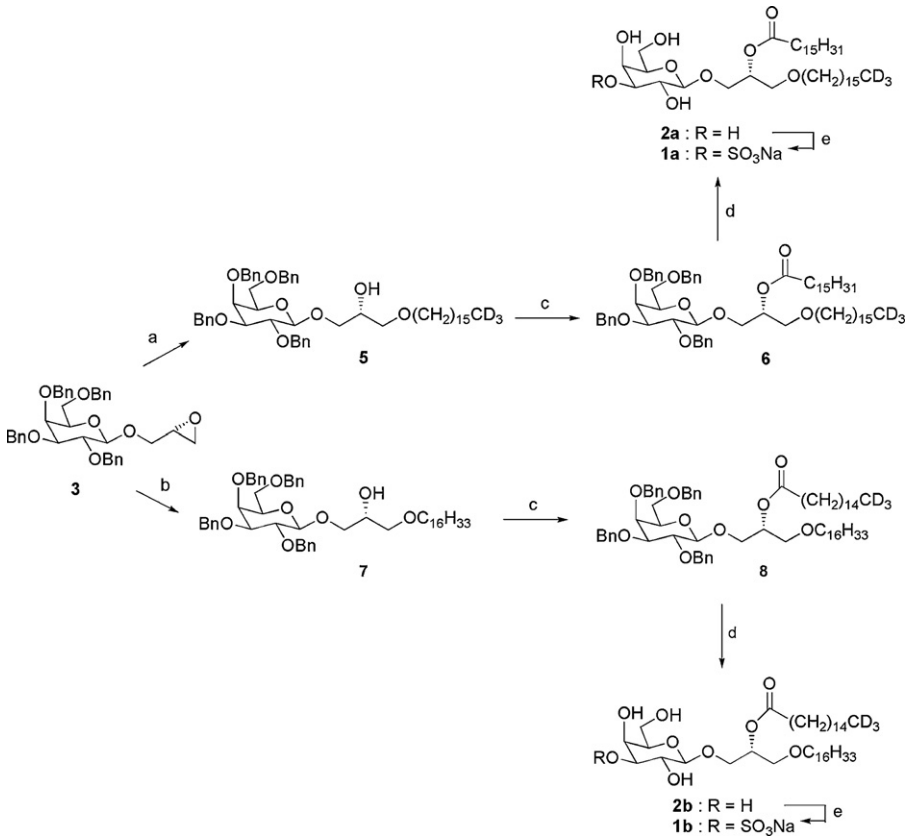
2. Experimental

2.1. General

Optical rotations were determined on a PerkinElmer 241 polarimeter in a 1 dm cell at 20 °C using chloroform solutions. All NMR spectra were recorded at 303 K with a Bruker FT-NMR Avance DRX500 spectrometer in $CDCl_3$ or $CDCl_3/CD_3OD$ solutions with TMS as internal standard; chemical shifts are reported as δ (ppm) relative to $CHCl_3$ fixed at 7.24 ppm for $CDCl_3$ and 7.60 ppm for $CDCl_3/CD_3OD$ solutions, for ^1H NMR spectra and relative to $CDCl_3$ fixed at 77.00 ppm for ^{13}C NMR spectra. All reactions were monitored by thin layer chromatography (TLC) on Silica Gel 60 F-254 plates (Merck) with detection by spraying with 50% H_2SO_4 solution or with phosphomolybdate-based reagent and heating to 110 °C. Flash column chromatography was performed on Silica Gel 60 (230–400 mesh, Merck). Dry solvents and liquid reagents were distilled prior to use: tetrahydrofuran (THF) was distilled from sodium; dichloromethane was distilled from calcium hydride; methanol was dried on 4 Å molecular sieves. All solvents and reagents were purchased from Aldrich; [16,16,16-$^2H_3$]-hexadecanoic acid (99.9 atom% D) was purchased from CDN isotopes. (*R*)-Glycidyl-2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranoside **3** and 1-*O*-hexadecyl-3-*O*-(2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)-*sn*-glycerol **7** were synthesized according to Lindberg et al. (2002); the physical data of the products agreed with those reported by these authors.

2.2. Electrospray mass spectrometry

A triple quadrupole mass spectrometer (PerkinElmer Sciex API III$^+$) was tuned and calibrated as previously described (Glasgow et al., 1998). Samples (20 pmol/μL based on dry weight assuming 100% purity) were dissolved in methanol/chloroform (4/1, v/v, with or without 100 mM lithium chloride), and injected (10–20 μL/injection) in a stream of methanol/chloroform (4/1, v/v) entering an Ionspray™ ion source. Mass spectra (positive and negative ion mode) were obtained by scanning Q1 (*m/z* 200–1500, 0.3 Da step size, 1 ms dwell time, 4.56 s/scan, orifice 70 V). Fragment ion spectra (positive and negative ion mode) of Q1 pre-selected parent ions were produced by scanning Q3 (*m/z* 50–800, 0.3 Da step size, 2 ms dwell time, 5.13 s/scan, orifice 150–180 V) under collisionally activated conditions (collision gas: 10% nitrogen in argon), thickness instrumental setting (CGT) of 200, R_0–R_2 offset of 30 V). Averaging of all the scans accrued from each sample injection was achieved with the MacSpec™ computer program.



Scheme 1. (a) [16,16,16-$^2H_3$]-hexadecanol **4**, $BF_3{\cdot}OEt_2$, CH_2Cl_2, r.t., 60%; (b) see Lindberg et al. (2002); (c) [16,16,16-$^2H_3$]-hexadecanoic acid or hexadecanoic acid, EDCI, DMAP, CH_2Cl_2, r.t., 72 h, 75%; (d) H_2, Pd/C, EtOAc/MeOH 1:1, 24 h, 70%; (e) (i) Bu_2SnO, MeOH, reflux, 2 h, (ii) $Me_3N{\cdot}SO_3$, THF, r.t., 4 h, 70%.

2.3. Purification of pig testis SGG and GG

Total lipids were extracted from pig testes using the method of Bligh and Dyer (1959), as modified by Kates (1986). SGG was then purified from total testis lipids via Bio-sil A column chromatography, followed by preparative TLC (Kates, 1986; Tupper et al., 1994). To remove endogenous cations, the purified SGG was converted to the free acid form and then to the sodium salt form (SGG-Na^+) as previously described (Kates, 1986, Tupper et al., 1994). The purity of the final product was verified by mass spectrometry (see Section 3, Fig. 2) and analytical HPTLC using chloroform/methanol/water (65:25:4, v/v/v) as eluent and orcinol and Coomassie blue G-250 staining, which reveals glycolipids and all lipids bands, respectively. The purified SGG gave a single band with an R_f value of 0.329 with both stains. The yield was ~20 mg, accounting for approximately 1% of the total testis lipids. GG was further prepared from the purified SGG by mild acid hydrolysis (Kornblatt et al., 1972); it also appeared as a single band with an R_f of 0.659 and the identity was also confirmed by mass spectrometry (see Section 3, Fig. 2).

2.4. [16,16,16-$^2H_3$]-Hexadecanol (4)

To a stirred suspension of $LiAlH_4$ (0.33 g, 8.82 mmol) in THF (10 mL), a THF (10 mL) solution of [16,16,16-$^2H_3$]-hexadecanoic acid (0.50 g, 1.96 mmol) was added via cannula at 0 °C. The reaction was refluxed for 2 h, then quenched by dropwise addition of saturated Na_2SO_4. CH_2Cl_2 (40 mL) was added and the solution was filtered over celite cake, phases separated and the organic layer was dried over Na_2SO_4 and concentrated. Flash chromatography (*n*-hexane/EtOAc 8:2) of the product gave **4** (0.41 g, 85%) as a white solid. ^1H NMR ($CDCl_3$) δ 1.20–1.45 (m, 26H, $13CH_2$), 1.50–1.65 (m, 2H, CH_2), 3.70 (t, 3H, CH_2OH). ^{13}C NMR ($CDCl_3$) δ 22.4, 25.7, 29.4–29.7, 31.8, 32.8, 63.1. ESI-MS (positive-ion mode): *m/z* 268 $[M + Na]^+$.

2.5. 1-O-(16,16,16-$^2H_3$)-Hexadecyl-3-O-(2,3,4, 6-tetra-O-benzyl-β-D-galactopyranosyl)-sn-glycerol (5)

To a stirred solution of **3** (0.2 g, 0.34 mmol) and [16,16,16-$^2H_3$]-hexadecanol **4** (0.17 g, 0.68 mmol) and 4 Å molecular sieves in CH_2Cl_2 (8 mL) was added $BF_3{\cdot}Et_2O$ (0.1 mL, 10% in CH_2Cl_2). After 6 h more $BF_3{\cdot}Et_2O$ (0.1 mL) was added. After 48 h, the mixture was diluted with CH_2Cl_2 and washed with aqueous sat. $NaHCO_3$. The organic layer was filtered over celite cake, dried over Na_2SO_4 and concentrated. Flash chromatography (petroleum ether/EtOAc 8:2) gave **5** (0.18 g, 60%) as a white solid. $[\alpha]_D = -6.0$ (*c* 1). ^1H NMR ($CDCl_3$) δ 1.22–1.39 (m, 26H, $13CH_2$), 1.53–1.62 (m, 2H, CH_2), 3.18 (bs, 1H, OH), 3.40–3.65 (m, 8H, CH_2CH_2O, H-1a, H-1b, H-6′a, H-6′b, H-5′, H-3′), 3.79 (dd, $J_{3a,3b} = 11.0$ Hz, $J_{2,3a} = 6.7$ Hz, 1H, H-3a), 3.86 (dd, $J_{2,3} = 9.7$ Hz, $J_{1,2} = 7.7$ Hz, 1H, H-2′), 3.87–3.94 (m, 2H, H-4′, H-3b), 3.96–4.02 (m, 1H, H-2), 4.39 (d, $J_{1',2'} = 7.7$ Hz, 1H, H-1′), 4.42, 4.48, 4.64, 4.73, 4.77, 4.82, 4.91 and 4.96 (8d,

8H, CH_2Ph), 7.20–7.49 (m, 20H, Ph). ^{13}C NMR ($CDCl_3$) δ 22.4, 26.1, 29.3–29.7, 31.8, 68.7, 69.7, 71.6, 71.7, 73.1, 73.3, 73.4, 73.5, 73.5, 74.6, 75.3, 79.4, 82.2, 104.8, 127.6–128.4, 137.8–138.6. ESI-MS (positive-ion mode): found *m/z* 865.8, calculated 865.16 Da for $C_{53}H_{71}{}^2H_3O_8Na$ corresponding to $(M+Na)^+$.

2.6. 1-O-(16,16,16-$^2H_3$)-Hexadecyl-2-O-palmitoyl-3-O-(2,3,4,6-tetra-O-benzyl-β-D-galactopyranosyl)-sn-glycerol (6)

Compound **5** (0.10 g, 0.12 mmol) was dissolved in CH_2Cl_2 (6 mL), then hexadecanoic acid (0.06 g, 0.24 mmol), EDCI (0.09 g, 0.48 mmol) and DMAP (0.04 g, 0.30 mmol) were added. The reaction mixture was stirred at room temperature for 96 h, then diluted with CH_2Cl_2 and washed with aqueous sat. $NaHCO_3$. The layers were separated and the organic layer was dried over Na_2SO_4 and concentrated. The crude product was purified by flash chromatography (*n*-hexane/EtOAc 9:1) affording **6** (0.096 g, 75%) as a foam. $[\alpha]_D = +1.6$ (*c* 1). ^1H NMR ($CDCl_3$) δ 0.91 (t, $J = 7.0$ Hz, 3H, CH_3), 1.20–1.45 (m, 50H, $25CH_2$), 1.53–1.62 (m, 2H, CH_2), 1.49–1.66 (m, 4H, $2CH_2$), 2.23–2.35 (m, 2H, CH_2), 3.37 (m, 1H, CH_2O), 3.44 (m, 1H, CH_2O), 3.50–3.56 (m, 2H, H-5′, H-3′), 3.57–3.67 (m, 4H, H-1a, H-1b, H-6′a, H-6′b), 3.72 (dd, $J_{3a,3b} = 10.5$ Hz, $J_{2,3a} = 4.5$ Hz, 1H, H-3a), 3.84 (dd, $J_{2',3'} = 9.7$ Hz, $J_{1',2'} = 7.5$ Hz, 1H, H-2′), 3.91 (d, $J_{3',4'} = 3.5$ Hz, 1H, H-4′), 4.03 (dd, $J_{3a,3b} = 10.5$ Hz, $J_{2,3b} = 4.7$ Hz, 1H, H-3b), 4.36 (d, $J_{1',2'} = 7.5$ Hz, 1H, H-1′), 4.43, 4.47, 4.64, 4.72, 4.76, 4.78, 4.94 and 4.96 (8d, 8H, CH_2Ph), 5.21 (m, 1H, H-2), 7.20–7.49 (m, 20H, Ph). ^{13}C NMR ($CDCl_3$) δ 14.1, 22.4, 22.7, 24.9, 26.1, 29.1–29.7, 31.8, 31.9, 34.4, 68.2, 68.7, 69.3, 71.3, 71.6, 73.1, 73.5, 73.5, 73.6, 74.6, 75.0, 79.3, 82.1, 104.3, 127.5–128.4, 137.9, 138.5, 138.6, 138.8, 173.3. ESI-MS (positive-ion mode): *m/z* 1103.0, calculated 1103.57 Da for $C_{69}H_{101}{}^2H_3O_9Na$ corresponding to $(M+Na)^+$.

2.7. 1-O-(16,16,16-$^2H_3$)-Hexadecyl-2-O-palmitoyl-3-O-(β-D-galactopyranosyl)-sn-glycerol (2a)

A mixture of **6** (0.06 g, 0.06 mmol) and 10% Pd on activated charcoal (0.05 g) in EtOAc–MeOH 1:2 (3 mL) was stirred under hydrogen atmosphere for 96 h, filtered over Celite and concentrated. Flash chromatography (CH_2Cl_2/MeOH 9:1) afforded the target compound **2a** (70%, 0.03 g) as a white solid. $[\alpha]_D = -3.2$ (*c* 0.75, $CHCl_3$/MeOH 1:1). ^1H NMR ($CDCl_3$/CD_3OD, 1:1) δ 0.87 (t, $J = 7.0$ Hz, 3H, CH_3), 1.20–1.45 (m, 50H, $25CH_2$), 1.49–1.70 (m, 4H, $2CH_2$), 2.34 (t, $J = 7.5$ Hz, 2H, CH_2), 3.40–3.50 (m, 4H, CH_2O, H-5′, H-3′), 3.52 (dd, $J_{2',3'} = 9.7$ Hz, $J_{1',2'} = 7.5$ Hz, 1H, H-2′), 3.57–3.67 (m, 2H, H-1a, H-1b), 3.70–3.76 (m, 2H, H-3a, H-6′a), 3.80 (dd, $J_{6a',6b'} = 11.5$ Hz, $J_{5',6b'} = 6.5$ Hz, H-6b), 3.86 (d, $J = 3.0$ Hz, 1H, H-4′), 3.95 (dd, $J_{3a,3b} = 11.0$ Hz, $J_{2,3b} = 5.5$ Hz, 1H, H-3b), 4.22 (d, $J_{1',2'} = 7.5$ Hz, 1H, H-1′), 5.20 (m, 1H, H-2). ^{13}C NMR ($CDCl_3$/CD_3OD, 1:1) δ 14.1, 22.7, 24.9, 26.1, 29.1–29.7, 31.2, 34.4, 61.7, 68.7, 69.1, 69.4, 71.3, 71.7, 73.5, 75.0, 104.3, 173.3. ESI-MS (positive-ion mode): found *m/z* 726.8, calculated 726.62 Da for $C_{41}{}^1H_{78}{}^2H_3O_9Li$ corresponding to $(M+Li)^+$. The physical data of **2a** is in agreement with those reported for the unlabelled compound (Lindberg et al., 2002).

2.8. 1-O-(16,16,16-$^2H_3$)-Hexadecyl-2-O-palmitoyl-3-O-[3-O-(sodium oxysulfonyl)-β-D-galactopyranosyl]-sn-glycerol (1a)

Compound **2a** (0.02 g, 0.03 mmol), and Bu_2SnO (0.01 g, 0.045 mmol) were stirred in MeOH (2 mL) at reflux under Argon for 2 h. The solvent was removed under reduced pressure and the dibutylstannylene complex was treated with $Me_3N{\cdot}SO_3$ (0.008 g, 0.06 mmol) in THF (2 mL) for 2 h. The solvent was removed under reduced pressure, then the residue was dissolved in $CHCl_3$/MeOH 1:1 (2 mL), loaded onto a cation exchange resin column (Dowex 50 × 8 Na^+ form, 0.5 cm × 6 cm), eluted with $CHCl_3$/MeOH (1:1), concentrated under reduced pressure and subjected to flash chromatography ($CHCl_3$/MeOH 9:1) to give the target compound **1a** (70%, 0.017 g) as a foam. $[\alpha]_D = +3.3$ (*c* 0.4, $CHCl_3$/MeOH, 1:1). ^1H NMR ($CDCl_3$/CD_3OD, 1:1) δ 0.91 (t, $J = 7.0$ Hz, 3H, CH_3), 1.20–1.35 (m, 50H, $25CH_2$), 1.50–1.57 (m, 2H, CH_2), 1.58–1.66 (m, 2H, CH_2), 2.34 (m, 2H, CH_2), 3.39–3.52 (m, 2H, CH_2O), 3.55 (t, $J = 6.0$ Hz, 1H, H-5′), 3.58–3.67 (m, 2H, H-1a, H-1b), 3.70–3.84 (m, 4H, H-2′, H-6′a, H-6′b, H-3a), 3.95 (dd, $J_{3a,3b} = 11.0$ Hz, $J_{2,3a} = 5.5$ Hz, 1H, H-3b), 4.23 (dd, $J_{2,3} = 10.5$ Hz, $J_{3,4} = 3.0$ Hz, 1H, H-3′), 4.27 (d, $J_{3',4'} = 3.0$ Hz, 1H, H-4′), 4.33 (d, $J_{1',2'} = 7.5$ Hz, 1H, H-1′), 5.20 (m, 1H, H-2). ^{13}C NMR ($CDCl_3$/CD_3OD, 1:1) δ 13.6, 22.5, 24.8, 25.9, 28.9–29.6, 31.8, 34.2, 61.2, 67.1, 69.2, 69.3, 71.5, 71.6, 74.8, 80.6, 103.6, 174.1.

ESI-MS (negative-ion mode): found *m/z* 798.5, calculated 798.55 Da for $C_{41}{}^1H_{76}{}^2H_3O_{12}S$ corresponding to $(M-H)^-$.

2.9. 1-O-Hexadecyl-2-O-(16,16,16-$^2H_3$)-palmitoyl-3-O-(2,3,4,6-tetra-O-benzyl-β-D-galactopyranosyl)-sn-glycerol (8)

Compound **7** (0.10 g, 0.12 mmol) was dissolved in CH_2Cl_2 (6 mL), then [16,16,16-$^2H_3$]-hexadecanoic acid (0.06 g, 0.24 mmol), EDCI (0.09 g, 0.48 mmol) and DMAP (0.04 g, 0.30 mmol) were added. The reaction mixture was stirred at room temperature for 96 h, then diluted with CH_2Cl_2 and washed with aqueous sat. $NaHCO_3$. The layers were separated and the organic layer was dried over Na_2SO_4 and concentrated. The crude product was purified by flash chromatography (*n*-hexane/EtOAc 9:1) affording **8** (0.09 g, 70%) as a foam. $[\alpha]_D = +1.1$ (*c* 1). ^1H NMR ($CDCl_3$) δ 0.91 (t, $J = 7.0$ Hz, 3H, CH_3), 1.20–1.45 (m, 50H, $25CH_2$), 1.53–1.62 (m, 2H, CH_2), 1.49–1.66 (m, 4H, $2CH_2$), 2.23–2.35 (m, 2H, CH_2), 3.37 (m, 1H, CH_2O), 3.44 (m, 1H, CH_2O), 3.50–3.56 (m, 2H, H-5′, H-3′), 3.57–3.67 (m, 4H, H-1a, H-1b, H-6′a, H-6′b), 3.72 (dd, $J_{3a,3b} = 10.5$ Hz, $J_{2,3a} = 4.5$ Hz, 1H, H-3a), 3.84 (dd, $J_{2',3'} = 9.7$ Hz, $J_{1',2'} = 7.5$ Hz, 1H, H-2′), 3.91 (d, $J_{3',4'} = 3.5$ Hz, 1H, H-4′), 4.03 (dd, $J_{3a,3b} = 10.5$ Hz, $J_{2,3b} = 4.7$ Hz, 1H, H-3b), 4.36 (d, $J_{1',2'} = 7.5$ Hz, 1H, H-1′), 4.43, 4.47, 4.64, 4.72, 4.76, 4.78, 4.94 and 4.96 (8d, 8H, CH_2Ph), 5.21 (m, 1H, H-

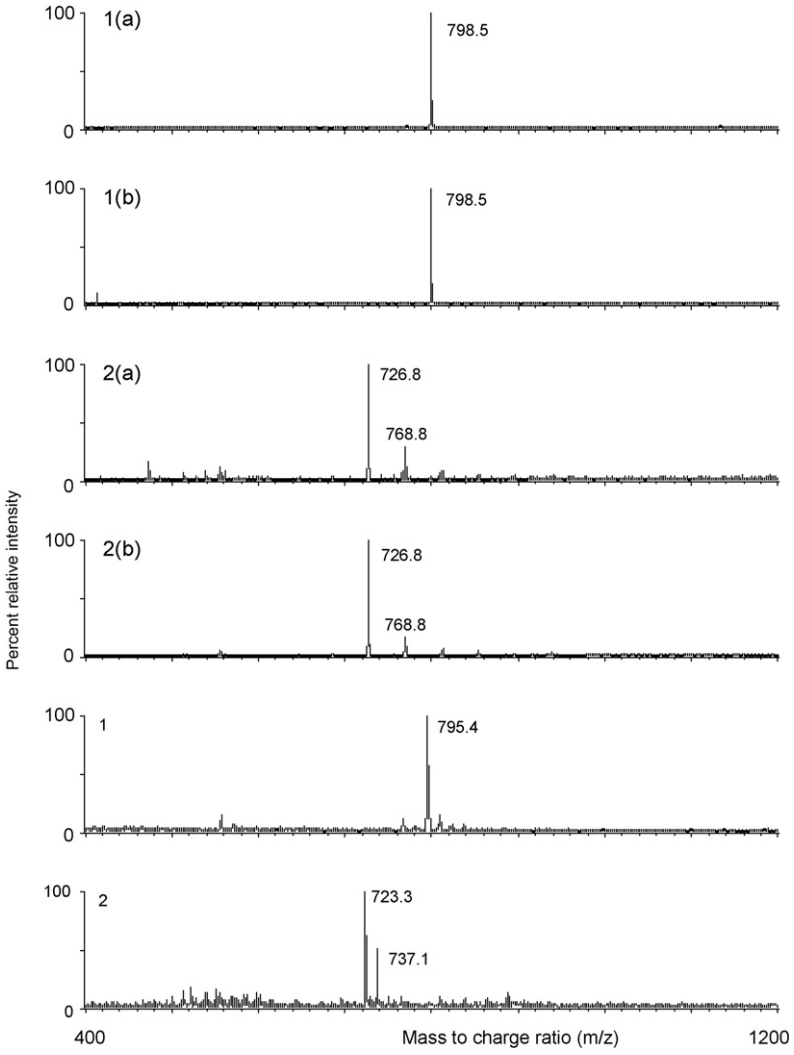


Fig. 2. ESI mass spectra of SGG **1a** (panel 1a), SGG **1b** (panel 1b), and natural (unlabelled) SGG **1** purified from pig testis (panel 1), GG **2a** (panel 2a), GG **2b** (panel 2b), and natural (unlabelled) GG **2** purified from pig testis (panel 2). Spectra **1a**, **1b** and **1** were obtained from solutions in methanol/chloroform (4/1, v/v), spectra **2a**, **2b** and **2** were obtained from solutions in methanol/chloroform (4/1, v/v) containing 100 mM lithium chloride, as described in Section 2.

2), 7.20–7.49 (m, 20H, Ph). ^{13}C NMR ($CDCl_3$) δ 14.1, 22.4, 22.7, 25.0, 26.1, 29.1–29.7 (23C), 31.9, 34.4, 68.3, 68.7, 69.4, 71.3, 71.6, 73.1, 73.5 (2C), 73.6, 74.8, 75.0, 79.3, 82.1, 104.3, 127.3–128.4 (20C), 137.9, 138.5, 138.6, 138.8, 173.3. ESI-MS (positive-ion mode): *m/z* 1103.0, calculated 1103.57 Da for $C_{69}H_{101}{}^2H_3O_9Na$ corresponding to $(M+Na)^+$.

*2.10. 1-O-Hexadecyl-2-O-(16,16,16-$^2H_3$)-palmitoyl-3-O-(β-D-galactopyranosyl)-sn-glycerol **(2b)***

Compound **2b** was prepared from 0.08 g of **8** as described for **2a**. Purification of the crude material by flash chromatography (CH_2Cl_2/MeOH 9:1) afforded **2b** as a white solid. (0.04 g, 75%). $[\alpha]_D = -2.5$ (*c* 0.5, $CHCl_3$/MeOH 1:1).^{1}H NMR ($CDCl_3$/CD_3OD, 1:1) δ 0.87 (t, $J = 7.0$ Hz, 3H, CH_3), 1.20–1.45 (m, 50H, $25CH_2$), 1.53–1.62 (m, 2H, CH_2), 1.49–1.66 (m, 4H, $2CH_2$), 2.34 (t, $J = 7.5$ Hz, 2H, CH_2), 3.40–3.50 (m, 4H, CH_2O, H-5′, H-3′), 3.52 (dd, $J_{2',3'} = 9.7$ Hz, $J_{1',2'} = 7.5$ Hz, 1H, H-2′), 3.57–3.67 (m, 2H, H-1a, H-1b), 3.70–3.76 (m, 2H, H-3a, H-6′a), 3.80 (dd, $J_{6a',6b'} = 11.5$ Hz, $J_{5',6b'} = 6.5$ Hz, H-6b), 3.86 (d, $J = 3.0$ Hz, 1H, H-4′), 3.95 (dd, $J_{3a,3b} = 11.0$ Hz, $J_{2,3b} = 5.5$ Hz, 1H, H-3b), 4.22 (d, $J_{1',2'} = 7.5$ Hz, 1H, H-1′), 5.20 (m, 1H, H-2). ^{13}C NMR ($CDCl_3$/CD_3OD, 1:1) δ 14.1, 22.7, 24.9, 26.1, 29.1–29.7, 31.2, 34.4, 61.7, 68.7, 69.1, 69.4, 71.4, 71.8, 73.5, 75.0, 104.3, 174.0. ESI-MS (positive-ion mode): found *m/z* 726.8, calculated 726.62 Da for $C_{41}{}^1H_{78}{}^2H_3O_9Li$ corresponding to $(M+Li)^+$. The physical data of **2b** is in agreement with those reported for the unlabelled compound (Lindberg et al., 2002).

*2.11. 1-O-Hexadecyl-2-O-(16,16,16-$^2H_3$)-palmitoyl-3-O-[3-O-(sodium oxysulfonyl)-β-D-galactopyranosyl]-sn-glycerol **(1b)***

Compound **1b** was prepared from **2b** (0.03 g, 0.04 mmol) as described for **2a**; purification of the crude material by flash chromatography (CH_2Cl_2/MeOH, 9:1) gave the target compound **1b** (70%, 0.024 g) as a foam. $[\alpha]_D = +2.5$ (*c* 1, $CHCl_3$/MeOH,

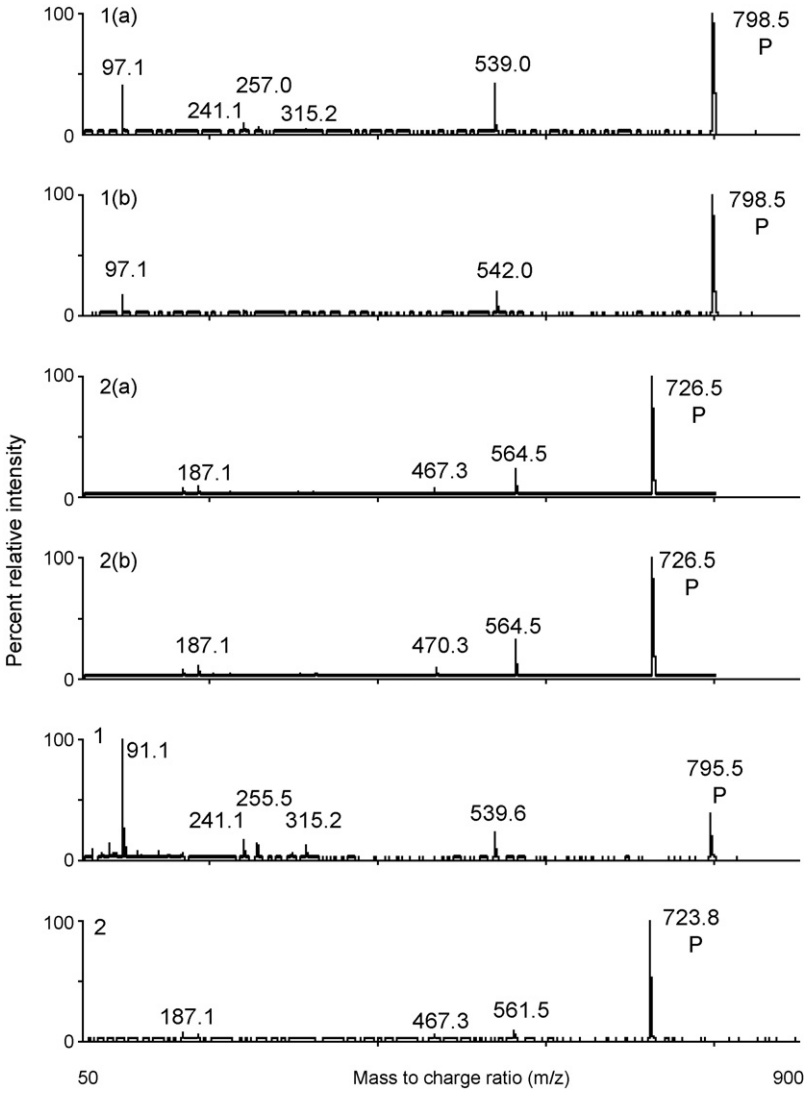


Fig. 3. ESI tandem mass spectra produced as described in Section 2 under collisionally activated dissociation conditions of designated parent ions (P) from SGG **1a** (panel 1a), SGG **1b** (panel 1b), natural (unlabelled) SGG **1** purified from pig testis (panel 1), GG **2a** (panel 2a), GG **2b** (panel 2b), and natural (unlabelled) GG **2** purified from pig testis (panel 2). Spectra **1a**, **1b** and **1** were obtained from solutions in methanol/chloroform (4/1, v/v), spectra **2a**, **2b** and **2** were obtained from solutions in methanol/chloroform (4/1, v/v) containing 100 mM lithium chloride.

1:1). ^{1}H NMR ($CDCl_3/CD_3OD$, 1:1) δ 0.91 (t, $J = 7.0$ Hz, 3H, CH_3), 1.20–1.35 (m, 50H, $25CH_2$), 1.50–1.57 (m, 2H, CH_2), 1.58–1.66 (m, 2H, CH_2), 2.34 (m, 2H, CH_2), 3.39–3.51 (m, 2H, CH_2O), 3.55 (t, $J = 6.0$ Hz, 1H, H-5′), 3.58–3.67 (m, 2H, H-1a, H-1b), 3.70–3.84 (m, 4H, H-2′, H-6′a, H-6′b, H-3a), 3.95 (dd, $J_{3a,3b} = 11.0$ Hz, $J_{2,3a} = 5.5$ Hz, 1H, H-3b), 4.23 (dd, $J_{2,3} = 10.5$ Hz, $J_{3,4} = 3.0$ Hz, 1H, H-3′), 4.26 (d, $J_{3',4'} = 3.0$ Hz, 1H, H-4′), 4.33 (d, $J_{1',2'} = 7.5$ Hz, 1H, H-1′), 5.20 (m, 1H, H-2). ^{13}C NMR ($CDCl_3/CD_3OD$, 1:1) δ 13.6, 22.4, 24.8, 25.9, 28.9–29.6, 31.8, 34.2, 61.2, 67.1, 69.2, 69.3, 71.5, 71.6, 74.8, 80.6, 103.6, 174.0. ESI-MS (negative-ion mode): found *m/z* 798.5, calculated 798.55 Da for $C_{41}{}^{1}H_{76}{}^{2}H_3O_{12}S$ corresponding to $(M - H)^-$.

3. Results and discussion

Seminolipid **1** (carrying hexadecyl and hexadecanoyl chains) was first synthesized by Gigg (Gigg, 1978) and its properties confirmed the structure of the natural material. This elaborate route exploits 3-*O*-allyl-L-glycerol as starting material and requires tedious manipulation of protecting groups both on the glycerol acceptor and the galactosyl donor.

The glycidyl galactoside **3**, derived from *S*-glycidol, was recently described (Lindberg et al., 2002) as a precursor in the synthesis of galactoglycerolipid tumor antigens. Compound **3** was the key intermediate to obtain the deuterated SGG **1a,b** and GG **2a,b**.

For the preparation of d_3-GG **2a**, labelled on the ether chain, opening of the epoxide in **3** with [16,16,16-$^{2}H_3$]-hexadecanol **4** (obtained by $LiAlH_4$ reduction of [16,16,16-$^{2}H_3$]-hexadecanoic acid) gave the galactosylglycerol derivative **5** labelled on the hexadecyl chain (route a, Scheme 1). Then, acylation with unlabelled hexadecanoic acid following standard procedures afforded protected **6** in 75% yield after purification. Hydrogenolytic debenzylation of [16,16,16-$^{2}H_3$]-ether **6**, followed by flash chromatography, yielded GG **2a** in 70%. Selective

sulfation (Guilbert et al., 1994) finally gave the target SGG **1a** in 70% yield. Selective functionalization at position 3 of galactose was effectively accomplished through reaction of dibutylstannilene acetal with $SO_3{\cdot}Me_3N$, as indicated by the downfield shift of the H-3′ signal (Franchini et al., 2007) in the ^{1}H NMR spectrum of SGG **1a**.

Vice versa, by opening of the epoxide in **3** with unlabelled hexadecanol (route b, Scheme 1) we obtained the known alcohol **7** (Lindberg et al., 2002), that was acylated with commercially available [16,16,16-$^2H_3$]-hexadecanoic acid leading to the protected derivative **8** carrying the [16,16,16-$^2H_3$]- label on the fatty acyl chain. Removal of the benzyl groups afforded GG **2b** in 70% yield after purification. Finally sulfation of GG **2b**, conducted in the same conditions as for **2a** gave the target SGG **1b** in 70% yield; regioselectivity was once again confirmed by the downfield shift of the signal of H-3′ in the ^{1}H NMR spectrum of SGG **1b**.

Mass spectrometry analysis was conducted on the labelled isotopomers **1a, 2a** and **1b, 2b** to verify the possibility of using them as internal standards for *in vivo* or *in vitro* metabolic studies. A mass spectrometry assay based on ESI coupled to tandem mass spectrometry has been developed to monitor sulfatide depletion and cerebroside formation, taking advantage of the strong signals these glycolipids yield both in negative (for sulfatides) and positive (for cerebroside in the presence of lithium) ion mode (Hsu and Turk, 2001; Norris et al., 2005). An extension of this method is now being developed to monitor SGG (negative ion mode) and GG (positive ion mode as the lithiated adduct) concentrations (Faull, K.F., Norris, A. J., Yaghoubian, A., Panza, L., Tanphaichitr, N., Ronchetti, F. et al., unpublished data). Negative ion ESI-MS of SGG **1a** and **1b** (Fig. 2, panel 1a and 1b) showed as expected intense ions at *m/z* 798 corresponding to the $[M-H]^-$ anion; while the positive ion spectra of GG **2a** and **2b** in the presence of lithium (Fig. 2, panel 2a and 2b) gave intense ions at *m/z* 726 corresponding to the lithiated adducts $[M+Li]^+$; for a comparison ESI spectra of natural (unlabelled) SGG (Fig. 2, panel 1) and GG (Fig. 2, panel 2) from pig testis are reported.

The MS/MS spectrum (Fig. 3, panel 1a) generated from the $[M-H]^-$ ion at *m/z* 798 (corresponding to SGG **1a**, deuterated on the alkyl chain), gave rise to an intense product ion at *m/z* 542 due to the loss of the fatty acyl chain [M–$C_{16}O_2H_{31}$]; while in the MS/MS spectrum of SGG **1b** (Fig. 3, panel 1b), in which the label was present in the acyl moiety, an intense product ion was observed at *m/z* 539 generated from the parent ion at *m/z* 798, due to the loss of the [16,16,16-$^2H_3$]-hexadecanoyl chain. MS/MS spectra of the desulfated compounds, GG **2a** and GG **2b** (Fig. 3, panel 2a and 2b), show a similar fragmentation pattern and allow confirmation of these assignments. These results are also in agreement with those previously obtained by ESI-MS analysis performed on seminolipid from the mouse testis (Tadano-Aritomi et al., 2003), and allow us to unambiguously validate that the product ion at *m/z* 539 derives from elimination of the acyl residue from the parent molecular ion of seminolipid **1** and not by β-cleavage of the ether chain.

In conclusion we have provided a straightforward synthesis of four glycolipid isotopomers along with their NMR and mass spectrometric analysis; extension of this procedure could provide a route for the preparation of isotopomers of other compounds of the same general class. The use of these compounds, synthesized with high isotopic and chemical purity, for metabolism studies will be the subject of future research and hopefully publications.

Acknowledgments

This work was supported by the University of Milan. Kessiri Kongmanas holds a scholarship from the Development and Promotion of Science and Technology Talented Project, Thailand.

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