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Synthetic sulfogalactosylceramide (sulfatide) and its use for the mass spectrometric quantitative urinary determination in metachromatic leukodystrophies

Yanli Cui · Benoit Colsch · Carlos Alonso · Nicole Baumann · Jean-Claude Tabet · Jean-Maurice Mallet · Yongmin Zhang

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Abstract 3-*O*-Sulfogalactosylceramides (sulfatides) accumulate in the genetic disease metachromatic leukodystrophy which is due to a defect in the catabolic enzyme, arylsulfatase A. Clinical diagnosis is usually confirmed by *in vitro* enzymatic deficiency of arylsulfatase A activity. The diagnosis may be complicated because of arylsulfatase A pseudo-deficiencies and another cause of MLD, sphingolipid activator B deficiency. As large quantities of sulfatides can be found in the urine in this disease, sulfatiduria appears as an extremely useful test. As recently enzyme replacement is underway, the quantitative determination, using an internal standard, appears particularly useful as a follow-up. Thus a non-physiological sulfatide was synthesized for this purpose, *i.e.* 3-*O*-sulfo-β-D-C17 galactosylceramide (3-*O*-Sulfo-D-Galactosyl-β1′→1-*N*-Heptadecanoyl-D-*erythro*-Sphingosine). It has been prepared through condensation of an azidosphingosine derivative with a protected D-galactopyranosyltrichloroacetimidate. Reduction of the azide was followed by acylation of a C-17 fatty acid. The key step was achieved by selective sulfation of the desired hydroxyl group on the sugar residue of the galactosylceramide using the stannylene methodology to give a 3′-sulfated beta-galactosyl C-17 ceramide.

Keywords Sulfatide · Glycosphingolipid · Mass spectroscopy · Galactosyl ceramide

Y. Cui · J.-M. Mallet · Y. Zhang
Département de Chimie, Ecole Normale Supérieure,
UMR 8642: CNRS-ENS-UPMC,
24 rue Lhomond,
75231 Paris cedex 05, France

Y. Cui
Department of Chemistry, Zhejiang University,
Hangzhou 310027, China

Y. Zhang (✉)
ZJU-ENS Joint Laboratory of Medicinal Chemistry,
Zhejiang University,
Hangzhou 310058, China
e-mail: yongmin.zhang@ens.fr

B. Colsch · C. Alonso · N. Baumann · J.-C. Tabet
Université Pierre et Marie Curie,
UMR CNRS 7613, 4 Place Jussieu,
75252 Paris cedex 05, France

B. Colsch · N. Baumann
Université Pierre et Marie Curie, UMR INSERM 711,
Hôpital de la Salpêtrière, 47 Bd de l'Hôpital,
75651 Paris cedex 13, France

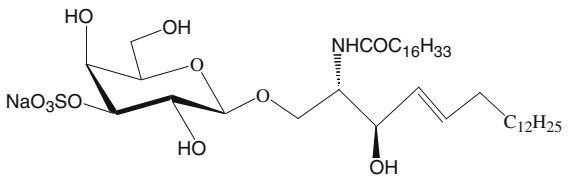
Introduction

Sulfatides (sulfogalactosylceramides) belong to the major acidic glycosphingolipids in mammalian tissues [1]. There are many isoforms in relation to the ceramide moiety, and in relation to fatty acid isoforms and to the nature of the sphingoid bases [2, 3]. It is abundant in the myelin sheath of both central and peripheral nervous system, but also present in neurons and glial cells [4], and other organs, and has been particularly studied in pancreas [5] and kidney [6]. Antisulfatide antibodies have been found in HIV patients with distal sensory neuropathies [7], in multiple sclerosis [8, 9] and in diabetic neuropathies [10]. These observations suggest that autoimmune responses directed against sulfatide contribute to the pathogenesis of autoimmune diseases.

Metachromatic leukodystrophy (MLD) is a genetic autosomal recessive disease caused by a deficiency in a catabolic enzyme arylsulfatase A (ASA) [11, 12]. Clinical diagnosis is usually confirmed by the *in vitro* analysis of arylsulfatase A, but may be complicated because of arylsulfatase A pseudo-deficiencies and another cause of MLD, sphingolipid activator B deficiency. As a large quantity of sulfatides can be detected in the urine in this disease [13–17], the search for sulfatiduria appears as an extremely useful test. As recently, enzyme replacement therapy is underway [18], the quantitative determination, using an internal standard would be particularly useful as a follow-up.

In order to analyze such glycosphingolipids, mass spectrometry was chosen for structural elucidation as well as for quantification. As these compounds exist in several isoforms with variable numbers of carbon atoms, their quantitative analysis requires either extensive purification or the use of a specific detection by using MS/MS scanning such as precursor ion spectra with a triple quadrupole mass spectrometer. In the negative ion mode, sulfatides yield under low energy CID conditions an abundant product ion at *m/z* 97 corresponding to the HSO_4^- anion. Therefore, the precursor ion scan of *m/z* 97 with a complex lipid mixture will lead only to the detection of the various sulfatide isoforms.

The use of an internal standard, a synthetic sulfatide which does not exist naturally, appears particularly useful for quantitative determinations and follow-up. This is why we choose to use a non-physiological sulfatide. Thus a non-physiological sulfatide was synthesized for this purpose, *i.e.* 3-*O*-sulfo-β-D-C17 galactosyl ceramide (3-*O*-Sulfo-D-Galactosyl β 1′→1-*N*-Heptadecanoyl-D-*erythro*-Sphingosine) **1**.



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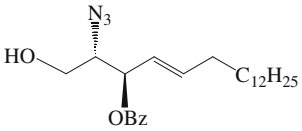
In this article we describe the synthesis of the C17 sulfatide, its structural characterization and its use for the quantitative determination of urinary sulfatides in metachromatic leukodystrophy.

Chemical synthesis

Synthesis of the β-galactosyl ceramide 9

The key building blocks in the synthesis of sulfated β-galactosyl ceramide **1** was (2S,3R,4E)-2-azido-3-*O*-benzoyl-4-octadecene-1,3-diol **2** and 2,3,4,6-tetra-*O*-acetyl-D-galactopyranosyl trichloroacetimidate **5**.



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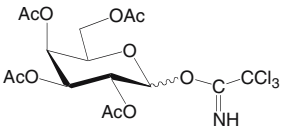

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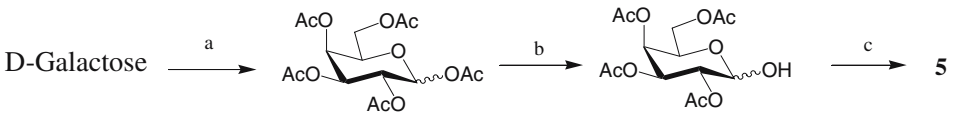
Interestingly, both **2** and **5** could be obtained from commercially available D-galactose. For the synthesis of **2**, a nine step procedure was used according to previous work [19, 20], whereas **5** was easily prepared by a three step procedure [21–23], as shown in Scheme 1. NMR spectra showed that the imidate was formed essentially in the α-form.

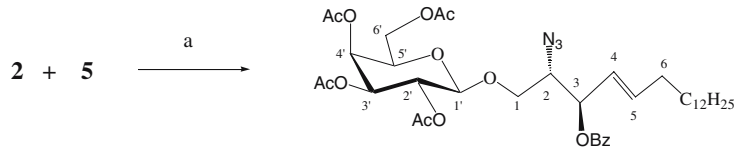
Condensation of trichloroacetimidate **5** with azidosphingosine derivative **2** was performed according to Schmidt's method [24]. When the reaction was promoted by BF_3 Et_2O, the desired product **6** was obtained in 56% yield. Using TMSOTf as promoter, **6** was obtained in a better yield (62%, Scheme 2). The β configuration of the newly introduced glycosidic linkage was confirmed from the ^{1}H NMR spectrum which showed the H-1′ (galactose unit) as a doublet at δ 4.52 ppm ($J_{1',2'}$=8.0 Hz). The NMR data are consistent with those reported in the literature [25, 26].

The azide group of compound **6** was reduced by triphenylphosphine [27] in a mixture of benzene and water at 50°C for 24 h to give an amino derivative **7**, which was not characterized at this stage and condensed directly with heptadecanoic acid in the presence of EDC [28] in dry CH_2Cl_2. The amide **8** was obtained in 72% yield (for two steps) as shown in Scheme 3. ^{1}H NMR spectrum of **8** showed a doublet at δ 5.82 ppm (J=9.2 Hz) corresponding to a proton of NH-CO group. The protecting groups of five hydroxy groups were subsequently removed under alkaline conditions (NaOMe) to provide **9** in 78% yield (Scheme 3). The ^{1}H NMR spectrum of **9** showed the existence of five free hydroxy groups: 2′-OH, 3-OH, 3′-OH,



3 **4**

Scheme 1 Reagents and conditions: **a** Ac_2O, NaOAc, 140°C, 1 h, 86%; **b** benzylamine, Et_2O, 0°C, 2.5 h, 70%; **c** Cl_3CCN, DBU, CH_2Cl_2, 0°C, 2 h, 92%



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Scheme 2 Reagents and conditions: **a** 1 equiv. of **2**, 1 equiv. of **5**, dry CH_2Cl_2, 4 Å MS, 0.5 h, rt, then 1.04 equiv. of TMSOTf, −40°C, 3 h, 62%

6′-OH, 4′-OH at δ 5.07 (d, *J*=2.8 Hz), 5.05 (d, *J*=5.5 Hz), 4.87 (d, *J*=4.8 Hz), 4.74 (t, *J*=5.5 Hz) and 4.53 (d, *J*= 4.4 Hz), respectively.

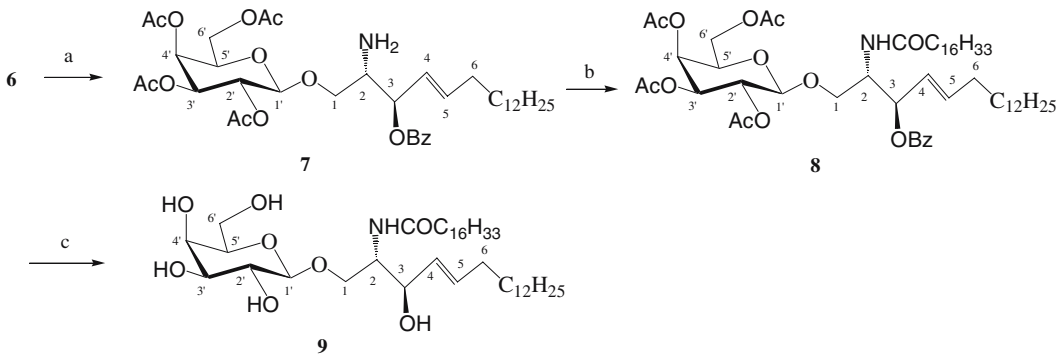
Synthesis of the mono- and disulfated β-galactosyl ceramides

Sulfated galactocerebrosides were synthesized by Marinier et al. [25]. In their preparation, the sulfation was performed with a series of galactosyl ceramide derivatives on which all the hydroxyl groups were appropriately protected except for the one to which the sulfation will take place. For example, a 2,6-di-*O*-benzoyl-4-*O*-acetyl-β-D-galactosyl ceramide (C-16 for fatty acid) was prepared and used to synthesize the 3′-sulfated galactosyl ceramide. It is evident that the multiple step protection–deprotection procedure makes the chemical synthesis more complicated. A concise method for preparing this class of compounds should be developed. We reported [29] a synthetic method to prepare the 3′-sulfated Lewis X trisaccharide using the stannylene methodology [30–33], the mono sulfation on Lewis X trisaccharide with four free hydroxyl groups at galactose residue occurred at position 3 of the galactose residue, resulting in a 3′-sulfated Lewis X trisaccharide derivative in 79% yield. The application of this method to the galactosyl ceramide **9** should provide the desired 3′-galactosyl ceramide. Thus, compound **9** was heated at reflux with one equivalent of dibutyltin oxide in a dry mixture of toluene and methanol (1:1) for 2 h. After removal of the solvent and evaporation under reduced pressure, the residue was reacted with two equivalent of trimethylamine-sulfur trioxide complex in dry DMF, followed by a cation exchange (Na^+) and purification on flash column chromatography, to give selectively the monosulfated derivative **1** (67%), which was easily separated from the disulfated derivative **10** (10%). This method was demonstrated efficient in the preparation of the 3′-sulfated β-galactosyl ceramide **1** by reaction of the intermediate 3′,4′-di-*O*-butylstannylene with the sulfur trioxide-trimethylamine complex (Scheme 4). Their structures were fully characterized by NMR spectroscopy, and further confirmed by mass spectrometry. Sulfation of 3′-OH (galactose unit) caused a downfield shift of signal of H-3′ of 0.63 and 0.65 ppm for **1** and **10** respectively, and sulfation of 6′-OH (galactose unit) caused a downfield shift of the signal of H-6′ of 0.27 ppm for **10**, compared to the non-sulfated compound **9**.

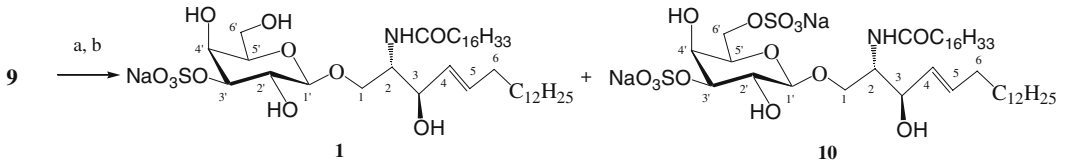
Characterization of synthetic sulfatide

General methods

Analytical grade chemicals were used for reactions and all solvents were dried over standard drying agents and freshly distilled prior to use. Optical rotations were measured at 20±2°C with a Perkin-Elmer Model 241 digital polarimeter, using a 10 cm, 1 ml cell. Fast Atom Bombardment (FAB) mass spectra were obtained with a JMS-700 spectrometer,



Scheme 3 Reagents and conditions: **a** 2.5 equiv. of triphenylphosphine, benzene, water, 50°C, 24 h, 90%; **b** 3.5 equiv. of heptadecanoic acid, 5.4 equiv. of EDC, dry CH_2Cl_2, 20 h, rt, 80%; **c** 0.05 M NaOMe, dry CH_2Cl_2/MeOH (1:1), rt, 24 h, 78%



Scheme 4 Reagents and conditions: **a** 1 equiv. of Bu_2SnO, toluene /MeOH (1:1), reflux, 2 h; **b** 2 equiv. of $Me_3N{\cdot}SO_3$ complex, DMF, rt, 40 h, Dowex resin (50X8-200, Na^+): **1** (67%), **10** (10%)

Electrospray Ionization (ESI) mass spectra were obtained with a LCT (Micromass UK) ESI-TOF spectrometer. ^1H NMR spectra were recorded with a Bruker DRX 400 spectrometer at ambient temperature. Assignments were aided by COSY experiments. ^{13}C NMR spectra were recorded at 100.6 MHz with a Bruker DRX 400 for solutions in $CDCl_3$ or DMS*O*-d6. Reactions were monitored by thin-layer chromatography (TLC) on a precoated plate of silica gel 60 F_{254} (layer thickness, 0.2 mm; E. Merk, Darmstadt, Germany) and detection by charring with sulfuric acid. Flash column chromatography was performed on silica gel 60 (230–400 mesh, Merck).

(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosy)-(1→1)-(2S,3R,4E)-2-azido-3-*O*-benzoyl-4-octadecene-l,3-diol **6**

A mixture of **2** (200 mg, 0.466 mmol) and **5** (229.6 mg, 0.466 mmol) in dry dichloromethane (20 ml) were stirred with powdered 4 Å molecular sieves (1 g) for 30 min. The temperature of the mixture was decreased to −40°C, trimethylsilyl trifluoromethanesulfonate (50 μl, 0.485 mmol) was then added dropwise. The mixture was stirred for another 3 h at −40°C. Triethylamine was then added, and this mixture was stirred until the pH became neutral. The molecular sieves were filtered through Celite and the solution was washed with water, saturated sodium bicarbonate, and brine, dried over anhydrous magnesium sulfate, filtered, and concentrated. The residue was purified by silica gel chromatography (dichloromethane/ethyl acetate 6:0.5) to afford the β-anomer **6** (110 mg, 62%). R_f=0.55 (cyclohexane/ethyl acetate 3:2). $[\alpha]_D$ −2.1 (*c* 0.67, $CHCl_3$). ref, $[\alpha]_D$ −2.6 (*c* 7, $CHCl_3$). HRMS (FAB^+): Calcd for $C_{39}H_{59}NO_{12}$ $[M+Na]^+$: 782.3840. Found 782.3823.

(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosy)-(1→1)-(2S,3R,4E)-2-amino-3-*O*-benzoyl-4-octadecene-l,3-diol **7**

6 (150 mg, 0.197 mmol) and thriphenylphosphine (129 mg, 0.494 mmol) was stirred in a mixture of benzene (12 ml) and water (0.45 ml) for 24 h at 50°C. After concentration, the residue was purified by silica gel chromatography (dichloromethane: methanol 15:1) to afford the title compound **7** (130.4 mg, 90%). R_f=0.68 (dichloromethane/methanol 15:1). HRMS (FAB^+): Calcd for $C_{39}H_{59}NO_{12}$ $[M+Na]^+$: 756.3935. Found 756.3954.

(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosy)-(1→1)-(2S,3R,4E)-2-heptadecanoylamino-3-*O*-benzoyl-4-octadecene-l,3-diol **8**

7 (150 mg, 0.204 mmol), heptadecanoic acid (193.1 mg, 0.71 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) (211.0 mg, 1.10 mmol) were stirred in dry dichloromethane (18 ml) under argon for 20 h at room temperature. After dilution by dichloromethane (60 ml), the mixture was washed with water, and brine, dried over anhydrous magnesium sulfate, filtered, and concentrated. The residue was purified by silica gel chromatography (dichloromethane/ethyl acetate, 5:0.4) to afford the title compound **8** (161.3 mg, 80%). R_f=0.44 (dichloromethane/ethyl acetate 5:0.4). $[\alpha]_D$ +2.5 (*c* 0.69, $CHCl_3$). ^1H NMR ($CDCl_3$, 400 MHz): δ 8.10–7.40 (m, 5H, C_6H_5), 5.91 (dt, 1H, $J_{4,5}$=14.8 Hz, $J_{5,6}$=6.8 Hz, H-5), 5.82 (d, 1H, *J*= 9.2 Hz, –NH–), 5.56 (dd, 1H, $J_{2,3}$=14.3 Hz, $J_{3,4}$=7.3 Hz, H-3), 5.51 (dd, 1H, $J_{3,4}$=7.3 Hz, $J_{4,5}$=14.8 Hz, H-4), 5.38 (dd, 1H, $J_{3',4'}$=3.0 Hz, $J_{4',5'}$<1 Hz, H-4′), 5.19 (dd, 1H, $J_{1',2'}$=7.8 Hz, $J_{2',3'}$=10.4 Hz, H-2′), 5.02 (dd, 1H, $J_{2',3'}$= 10.4 Hz, $J_{3',4'}$=3.4 Hz, H-3′), 4.55–4.48 (m, 1H, H-2), 4.46 (d, 1H, $J_{1',2'}$=7.8 Hz, H-1′), 4.08 (dd, 1H, $J_{1a,1b}$=9.9 Hz, $J_{1a,2}$=3.9 Hz, H-1a), 4.05 (dd, 1H, $J_{5',6'a}$=7.2 Hz, $J_{6'a,6'b}$= 11.2 Hz, H-6′a), 3.97 (dd, 1H, $J_{5',6'b}$=6.3 Hz, $J_{6'a,6'b}$= 11.2 Hz, H-6′b), 3.87 (ddd, 1H, $J_{4',5'}$=0.7 Hz, $J_{5',6'a}$= 7.2 Hz, $J_{5',6'b}$=6.3 Hz, H-5′), 3.70 (dd, 1H, $J_{1a,1b}$=9.9 Hz, $J_{1b,2}$=4.3 Hz, H-1b), 2.25–2.16 (m, 2H, $COCH_2$), 2.18, 2.06, 2.01, 1.97 (4s, 4×3H, 4×$COCH_3$), 2.09–2.02 (m, 2H, H–6), 1.67–1.60 (m, 2H, $COCH_2CH_2$), 1.33–1.25 (m, 48H, 24×CH_2), 0.90 (t, 6H, *J* 6.6 Hz, 2×CH_3). ^{13}C ($CDCl_3$, 100 MHz): δ 172.69, 170.20, 170.17, 170.01, 169.53, 165.13 (6×CO), 137.48 (C-5), 132.96 (CH, Ph), 130.18 (C, Ph), 129.58 (CH, Ph), 128.33 (CH, Ph), 124.62 (C-4), 100.83 (C-1′), 74.16 (C-3), 70.62 (C-3′, C-5′), 68.82 (C-2′), 67.20 (C-1), 66.78 (C-4′), 60.96 (C-6′), 50.54 (C-2), 36.81 ($COCH_2$), 32.28 (C-6), 31.86, 29.65–28.88, 22.63 (24×CH_2), 25.68 ($COCH_2CH_2$), 20.73, 20.61, 20.50 (4×$COCH_3$), 14.07 (2×CH_3). HRMS (FAB^+) Calcd for $C_{56}H_{91}NO_{13}$ $[M+Na]^+$: 1008.6388. Found 1008.6367.

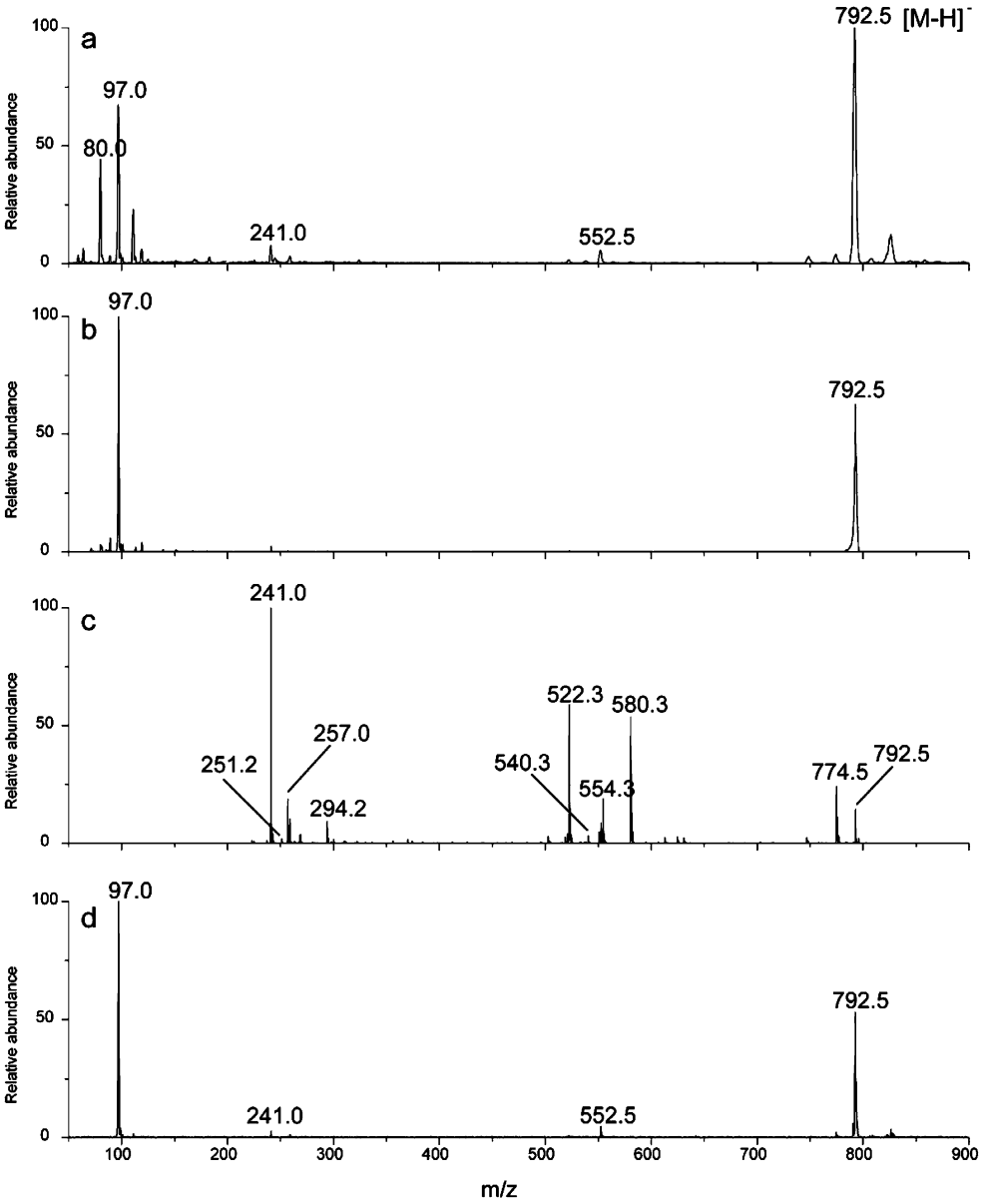
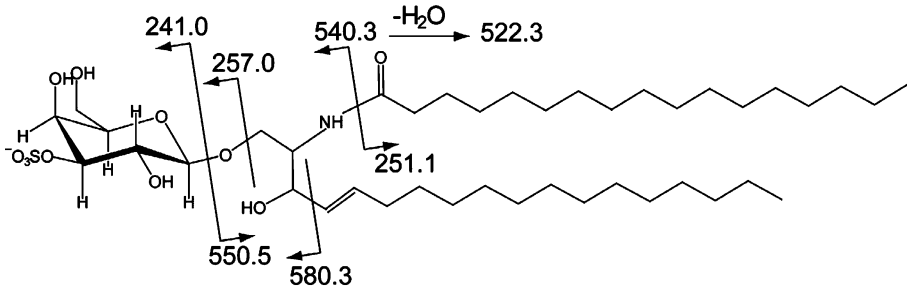


Fig. 1 **a** Negative ion ESI mass spectrum of synthetic sulfatide (*m/z* 792.5). **b**, **c** MS^2 experiments under CID conditions of singly-deprotonated ion $[M–H]^-$ recorded on the triple quadrupole and ion trap instruments respectively. **d** Precursor ion spectrum of [] (*m/z* 97) recorded using the ESI triple–quadrupole instrument showed the deprotonated synthetic sulfatide at *m/z* 792.5

(β-D-Galactopyranosy)-(1→1)-(2S,3R,4E)-2-heptadecanoylamino-4-octadecene-l,3-diol **9**

A solution of **8** (130 mg, 0.132 mmol) in 9 ml of 0.05 M NaOMe (dry dichloromethane and dry methanol, 1:1) was stirred under argon for 24 h at room temperature. The resin (Amberlite IR 120/H^+) was then added, and the mixture was stirred until the pH became neutral. The mixture was filtered, and the resin was washed with a mixture of dichloromethane and methanol (1:1). The filtrate was concentrated. The residue was purified by silica gel chromatography (dichloromethane/methanol 10:1) to provide the title compound **9** (73.4 mg, 78%) as a white solid. R_f=0.36 (dichloromethane/methanol 10:1). $[\alpha]_D$ −1.2 (*c* 1, $CHCl_3$: MeOH, 7:3). ^{1}H NMR (DMSO-d6, 400 MHz,): δ 7.68 (d, 1H, *J*=9.1 Hz, –NH–), 5.76 (dt, 1H, $J_{4,5}$=15.3 Hz, $J_{5,6}$=6.6 Hz, H-5), 5.57 (dd, 1H, $J_{4,5}$=15.3 Hz, $J_{3,4}$= 7.1 Hz, H-4), 5.07 (d, 1H, *J*=2.8 Hz, OH-2′), 5.05 (d, 1H, *J*=5.5 Hz, OH-3), 4.87 (d, 1H, *J*=4.8 Hz, OH-3′), 4.74 (t, 1H, *J*=5.5 Hz. OH-6′), 4.53 (d, 1H, *J*=4.4 Hz, OH-4′), 4.25 (d, 1H, $J_{1',2'}$=7.1 Hz, H-1′), 4.18 (dd, 1H, $J_{1a,1b}$=10.2 Hz, $J_{1a,2}$=4.7 Hz, H-1a), 4.11 (dd, 1H, $J_{2,3}$=13.4 Hz, $J_{3,4}$= 7.1 Hz, H-3), 4.03–3.96 (m, 1H, H-2), 3.87–3.84 (m, 1H, H-4′), 3.79–3.66 (m, 2H, H-6′a, H-6′b), 3.60 (dd, 1H, $J_{1a,1b}$ 10.2 Hz, $J_{1b,2}$ 3.7 Hz, H-1b), 3.55–3.46 (m, 3H, H-2′, H-3′, H-5′), 2.25 (t, 2H, *J* 7.4 Hz, $COCH_2$−), 2.19–2.10 (m, 2H, H-6a, H-6b), 1.72–1.60 (m, 2H, $COCH_2CH_2$), 1.56–1.38 (m, 48H, 24×CH_2), 1.07 (t, 6H, *J* 6.6 Hz, 2×CH_3). ^{13}C



Scheme 5 C17 synthetic sulfatide fragmentations

(DMSO-d6, 100 MHz): δ 171.79 (CO), 131.42 (C-4), 131.28 (C-5), 104.44 (C-1′), 75.17, 73.07, 70.64 (C-2′, C-3′, C-5′), 70.69 (C-3), 68.90 (C-1), 68.03 (C-4′), 60.36 (C-6′), 52.99 (C-2), 35.56 ($CO\mathit{C}H_2$), 31.23 (C-6), 31.69, 29.14–28.64, 22.02 ($24\times CH_2$), 25.33 ($COCH_2\mathit{C}H_2$), 13.83 ($2\times CH_3$). HRMS (FAB^+) Calcd for $C_{41}H_{79}NO_8$ $[M+Na]^+$: 736.5703. Found 736.5720.

[3-*O*-(Sodium sulfonato)-β-D-galactopyranosy]-(1→1)-(2S,3R,4E)-2-heptadecanoylamino-4-octadecene-l,3-diol **1**
[3,6-di-*O*-(sodium sulfonato)-β-D-galactopyranosy]-(1→1)-(2S,3R,4E)-2-heptadecanoylamino-4-octadecene-l,3-diol **10**

A mixture of **9** (30 mg, 0.04 mmol) and dibutyltin oxide (10 mg, 0.04 mmol) in dry toluene and methanol (2 ml, 1:1) was refluxed for 2 h (the solution became clear). Then the solvent was distilled at 50°C through rotary evaporation to give a yellowish syrup, which was evaporated to dryness under diminished pressure (2 h). The residue was dissolved in DMF (2 ml), $SO_3{\cdot}NMe_3$ (11.1 mg, 0.08 mmol) was introduced, the mixture was stirred under argon for 40 h at room temperature. Methanol (1 ml) was added. After stirring for 10 min, the reaction mixture was concentrated. The residue was then treated with resin (Dowex 50X8-200, Na^+ form) using a solution (dichloromethane/methanol, 1:1) and stirred for 15 min. The mixture was filtered, the resin was washed with dichloromethane/methanol (1:1), and the filtrate was concentrated. The residue was purified by silica gel chromatography with dichloromethane/methanol (6:1) to give the title compound **1** (23 mg, 67.1%). Eluting with dichloromethane/methanol (4:1) then provided the title compound **10** (4 mg, 10.4%). A small amount of the starting material **9** was also recovered from the chromatography (3 mg, 10%).

Compound **1** R_f 0.4 (dichloromethane/methanol, 5:1). $[\alpha]_D$=+1.2 (*c* 1.0, $CHCl_3$: MeOH, 7:3). ^{1}H NMR (DMSO-d6, 400 MHz): δ 7.70 (d, 1H, *J* 9.0 Hz, –NH–), 5.74 (dt, 1H, $J_{4,5}$=15.3 Hz, $J_{5,6}$=6.7 Hz, H-5), 5.57 (dd, 1H, $J_{4,5}$= 15.3 Hz, $J_{3,4}$=7.1 Hz, H-4), 5.30 (d, 1H, *J*=2.5 Hz, OH-2′), 5.09 (d, 1H, *J*=5.5 Hz, OH-3), 4.82 (t, 1H, *J*=5.6 Hz, OH-6′), 4.66 (d, 1H, *J*=4.5 Hz, OH-4′), 4.37 (d, 1H, *J*=7.1 Hz, H-1′), 4.19–4.12 (m, 3H, H-3′, H-4′, H-1a), 4.13–4.07 (m, 1H, H-3), 4.01–3.94 (m, 1H, H-2), 3.76–3.62 (m, 5H, H-1b, H-2′, H-5′, H-6′a, H-6′b), 2.23 (t, 2H, *J* 7.4 Hz, $COCH_2$), 2.17–2.11 (m, 2H, H-6a, H-6b), 1.70-1.60 (m, 2H, $COCH_2C\mathit{H}_2$), 1.53–1.38 (m, 48H, $24\times CH_2$), 1.07 (t, 6H, *J*=6.6 Hz, $2\times CH_3$). ^{13}C (DMSO-d6, 100 MHz): δ 171.77 (CO), 131.29 (C-4, C-5), 104.29 (C-1′), 79.00, 74.89, 70.95, 69.23, 66.20 (C-2′, C-3′, C-4′, C-5′, C-3), 68.81 (C-1), 60.07 (C-6′), 53.18 (C-2), 35.62 ($CO\mathit{C}H_2$), 31.79, 31.32, 31.30 (C-6, $2\times CH_2$), 29.16–28.72, 22.11 ($22\times CH_2$), 25.37 ($COCH_2\mathit{C}H_2$), 13.96 ($2\times CH_3$). HRMS (ESI, MeOH–CH_2Cl_2): Calcd for $C_{41}H_{78}NO_{11}S$ $[M-H]^-$: 792.5296. Found 792.5293.

Compound **10**: R_f 0.52 (ethyl acetate:isopropanol:H_2O, 4:2:0.5). $[\alpha]_D$=+2.6 (*c* 0.23, $CHCl_3$: MeOH, 7:3). ^{1}H NMR (DMSO-d6, 400 MHz): δ 7.73 (d, 1H, *J*=8.9 Hz, –NH–), 5.74 (dt, 1H, $J_{4,5}$=15.4 Hz, $J_{5,6}$=6.5 Hz, H-5), 5.56 (dd, 1H, $J_{4,5}$=15.4 Hz, $J_{3,4}$=7.0 Hz, H-4), 5.31 (d, 1H, *J*=2.5 Hz

Table 1 Quantitative analysis of sulfatides in urine of MLD patients and control individuals

Patients	Age at sampling (years)	Urinary sulfatide concentration (nmol/l)	ASA activity (nmol min^{-1} mg $proteins^{-1}$)
1	22	1,280	3.2
2	33	862	10
3	26	723	1.8
4	34	2,066	8.7
5	37	274	5.8
Controls (*n*=5)	24–23	Not detected	55–80

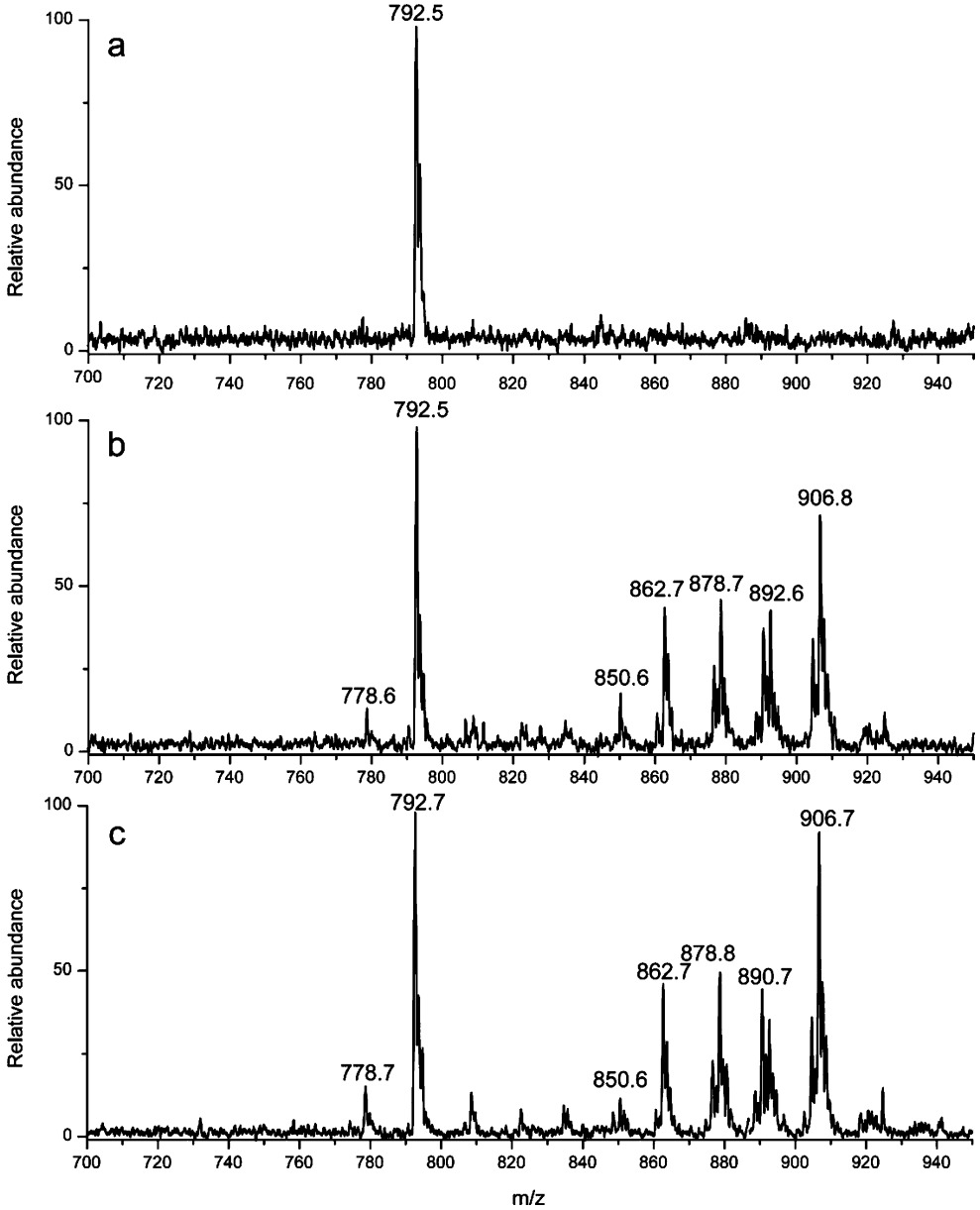


Fig. 2 Precursor ion scans (negative mode) of *m*/*z* 97 in urine of control (**a**), motor form of adult metachromatic leucodystrophy (**b**) and psycho-cognitive form of adult metachromatic leucodystrophy (**c**). The ESI triple quadrupole instrument showing the same sulfatide profiles between the two MLD forms. The precursor ion scan of *m*/*z* 97 in control displayed no detectable sulfatide signals

OH-2′), 5.11 (d, 1H, *J*=5.4 Hz, OH-3), 4.80 (d, 1H, *J*= 5.0 Hz, OH-4′), 4.38 (d, 1H, *J*=7.7 Hz, H-1′), 4.19–4.15 (m, 2H, H-1a, H-3′), 4.13–4.09 (m, 1H, H-4′), 4.08–3.93 (m, 4H, H-2, H-3, H-6′a, H-6′b), 3.83–3.79 (m, 1H, H-5′), 3.69–3.63 (m, 2H, H-1b, H-2′), 2.23 (t, 2H, *J*=5.4 Hz, $COCH_2$), 2.17–2.11 (m, 2H, H-6a, H-6b), 1.71–1.59 (m, 2H, $COCH_2CH_2$), 1.53–1.38 (m, 48H, 24×CH_2), 1.07 (t, 6H, *J*=6.8 Hz, 2×CH_3). ^{13}C (DMSO-d6, 100 MHz): δ 171.81 (CO), 131.25, 131.23 (C-4, C-5), 104.15 (C-1′), 78.61 (C-3′), 72.73 (C-5′), 71.12 (C-3), 69.13 (C-2′), 69.07 (C-1), 66.65 (C-4′), 64.96 (C-6′), 53.17 (C-2), 35.63 ($COCH_2$), 31.78 (C-6), 31.30, 31.29, 29.10–28.70, 22.09 (24×CH_2), 25.36 ($COCH_2CH_2$), 13.93 (2×CH_3). HRMS (ESI: MeOH–CH_2Cl_2): Calcd for $C_{41}H_{77}NO_{14}S_2Na$ $[M–H]^-$: 894.4683. Found 894.4688.

Mass spectrometric characterization of synthetic sulfatide by negative ion electrospray

The synthetic sulfatide (Exact Mass: 793.53738 u) was analyzed using an ESI ion-trap mass spectrometer (PUBLI) (Esquire 3000; Bruker, Bremen, Germany) operated using the negative ion mode with a scan rate of 13,000 Th/s using a *m*/*z* range of 3,000 Th (ion ejection to β_z=2/3). Sequential MS^n experiments were performed by collision-induced dissociation (CID) under resonant excitation conditions. The automated ion charge control was set to 10,000 to avoid space charge effect. The low mass cut-off used during the CID experiments was set automatically by the instrument software (28% of the precursor ion mass to charge ratio). Additional experiments were performed using an ESI

triple-quadrupole instrument (Micromass Quattro I, Manchester, UK) operated in negative ion mode. The precursor ion scan mode was used (PUBLI). Argon was used as collision gas at pressure of 2.5×10^{-4} mbar. The cone voltage, and collision energy were 100 and 150 V, respectively).

Mass spectrometry

The Fig. 1a presents the ESI mass spectrum of the synthetic C17 sulfatide displaying the depronated molecule $[M–H]^-$ at *m/z* 792.5 and also the *m/z* 97 ion and *m/z* 80 ion corresponding to HSO_4^- and $SO_3^{\cdot-}$ respectively. In order to confirm the structure of the sulfatide, an MS/MS experiment was carried out on this precursor ion using the triple quadrupole instrument (Fig. 1b) but under these conditions only the *m/z* 97 ion is produced. This experiment was repeated using the ion trap instrument (Fig. 1c). In this case, several product ions are displayed but the *m/z* 97 ion is not detected as it is below the low mass cut off. The attribution of the different product ions is presented in Scheme 5. The observed product ions are consistent with the C17 sulfatide. In particular, the galactose-sulfate moiety is, confirmed by the presence of the *m/z* 241 ion (Fig. 1c) that corresponds to its dehydrated product. In the same way the *m/z* 520 ion corresponds to the lysosulfatide produced through the fatty acid moiety loss. Mechanisms for the decomposition of deprotonated sulfatides under low collision conditions have been proposed by Turk et al. [2].

Detection of sulfatiduria

Patients

Patients in whom sulfatiduria was determined, were characteristic patients, juvenile or adult, with metachromatic leukodystrophy. They had either motor or major behavioural and cognitive deficiencies. Their molecular defects have been characterized [12, 15]. ASA activity was tested in both patients and controls as previously published [15].

Lipid extract

Urinary samples were extracted as previously published [15, 17]. Briefly, 12.5 nmol of synthetic sulfatide was added in 10 ml of urines. Urines were acidified with two drops of concentrated acetic acid and were left overnight in the cold. After 10 min of centrifugation at 2,500 rpm, the pellet was collected and extracted by 5 ml chloroform/methanol 2/1 (v/v), using sonication. After sonication, the extract was washed with 1 ml 0.88% KCl. The lipid extract was evaporated to dryness and redissolved in 500 µl of methanol in view of mass spectrometry analysis. All samples had been previously tested on thin-layer chromatography using alpha-naphthol as reagent to detect sulfatides [15].

The obtained quantitative results are summarized in Table 1. Sulfatides were detected in MLD patients with concentrations ranging from 274 to 2,066 nmol/l of urine. No sulfatides were detected on the five urine control samples. Urinary sulfatides concentrations were consistent with the enzymatic results *i.e.* high sulfatides concentrations are obtained for low ASA activity (Fig. 2).

Sulfatiduria was studied in several MLD adolescent or adult patients using this standard as well as another standard C12-sulfatide (data not shown). Results were identical. The mass profile of sulfatides in urine shows essentially the presence of the following fatty acid C22:1 (*m/z* 862.7), C22:0 (OH) (*m/z* 878,8), C24:0 (*m/z* 890,7), C23:0 (OH) (*m/z* 892.6), C24:0 (OH) (*m/z* 906.7)

Interestingly, whatever the clinical form, cognitive or motor (Rauschka) the results were identical. The results were identical to those observed by Whitfield et al. [16] except to the fact that these authors did not use a non-physiological standard. By this method, no sulfatiduria was detected in controls ($n=5$) in agreement with Lugowska et al. [13].

In conclusion, the quantitative analysis of sulfatides appears particularly useful in view of future enzyme replacement therapy. The use of an internal, non-physiological standard, allows the quantitative follow-up. Furthermore, this method is independent of different factors which have rendered difficult the comparison of results, such as urinary volume, quantity of total urinary lipids. Thus this quantitative method should allow easy comparison of results between laboratories. This particularly sensitive method may allow possibly the detection of sulfatides in other diseases as has been previously suspected [34].

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