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Characterization of Glucocerebrosides and the Active Metabolite 4,8-Sphingadienine from *Arisaema amurense* and *Pinellia ternata* by NMR and CD Spectroscopy and ESI-MS/CID-MS

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Supporting Information

ABSTRACT: Sphingolipid metabolites regulate cellular processes such as cell proliferation, differentiation, and apoptosis. In this study, glucocerebrosides (GluCer) from rhizomes of *Arisaema amurense* and *Pinellia ternata* were fully characterized using 1- and 2-dimensional nuclear magnetic spin resonance (NMR) and circular dichroism (CD) spectroscopy and tandem collision-induced dissociation mass spectrometry (ESI-MS/CID-MS). Three new acylated and seven known GluCer were elucidated with 4,8-sphingadienine (4,8-SD, d18:2) as backbone. 4,8-SD is a metabolite after enzymatical hydrolysis of GluCer in the gut lumen. In this study, 4,8-SD was hydrolyzed from GluCer and chromatographically purified on silica gel. In contrast to the GluCer, 4,8-SD showed cytotoxic effects in the WST-1 assay. GluCer with 4,8-SD as sphingoid backbone are present in plants consumed as food, such as spinach, soy, and eggplant.

KEYWORDS: *ESI-MS/CID-MS, glucocerebrosides, 4,8-sphingadienine, active metabolite*

INTRODUCTION

Sphingolipids (SLs) constitute a class of lipids present in both eukaryotic and prokaryotic cells. Their structure is composed of a sphingoid base that is *N*-acylated with a fatty acid and C-1 linked to a polar headgroup. Besides functioning as structural cell components, SLs and their metabolites are involved in the regulation of cellular processes such as cell proliferation, differentiation, and apoptosis.[1−3] Ceramide, sphingosine, and sphingosine-1-phosphate are mammalian SL metabolites known as biological active molecules.[1]

Cerebrosides are a class of sphingolipids and are structurally diverse with variations in chain length, the degree of saturation and/or hydroxylation and acetylation of the long-chain base.[3] In plants, the most abundant cerebrosides are glucocerebrosides (GluCer) which are primarily composed of a sphingoid base that is *cis* or *trans* 8-unsaturated and/or *trans* 4-unsaturated, 2-hydroxy fatty acids and glucose.[4,5] Dietary SLs are enzymatically lipolyzed in the intestines into ceramides and sphingoid bases after ingestion.[6−8,4,9]

Several SL breakdown products have been reported to inhibit proliferation and growth of cells of various cancer cell lines *in vitro* and *in vivo*.[10−12] Ahn and Schroeder reported ceramide and the sphingoid bases sphingosine and sphinganine to inhibit the proliferation and growth of human colon cancer cells by inducing apoptosis.[13,14] Ohta et al. reported an induction of apoptotic DNA fragmentation in HL-60 human myeloid leukemia cells after incubation with sphingosine and dimethyl-sphingosine.[15]

Analysis and structure elucidation of SLs has greatly been improved by the development in mass spectrometry of ionization techniques such as electrospray ionization and matrix-assisted laser desorption ionization as well as of fragmentation techniques such as collision-induced dissociation (CID).[16] These advances are well implemented in research of mammalian SLs. The mainstay for plants SLs however is to analyze structure details by methods using acid hydrolysis chromatographic separation and subsequent analysis by GC-MS.

Arisaema amurense Maxim. and *Pinellia ternata* (Thunb.) Breit (Araceae) are perennial plants native to East Asia of which the rhizomes are used as Chinese herbal medicine. The rhizomes are toxic and therefore traditionally processed by soaking or cooking in water with alum ($KAl(SO_4)_2{\cdot}12H_2O$). The herbs contain GluCer that were previously partly characterized.[17,18] The present findings give insight in the structure in relation to bioactivity of GluCer and their sphingoid backbone from Araceae species, which are also found in daily consumed plants. This is significant for understanding of the nutritional function of dietary GluCer. Thereby, the objective was to characterize GluCer from rhizomes of the two Araceae species using an advanced

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methodology. A combination of 1- and 2-dimensional (D) nuclear magnetic spin resonance (NMR) spectroscopy, electrospray-ionization spray mass spectrometry (ESI-MS), low energy tandem collision-induced dissociation mass spectrometry (ESI-MS/CID-MS), and circular dichroism (CD) spectroscopy was applied that provided full and accurate structure elucidation. Further, the cytotoxic effect of the isolated GluCer and their metabolite 4,8-sphingadienine (4,8-SD) was assessed in the WST-1 assay. This sphingoid base was previously shown to have effects on anti-inflammatory responses by *in vitro* ELISA experiments.[19]

MATERIALS AND METHODS

Materials. Dried, processed rhizomes of *Arisaema amurense* and *Pinellia ternata* were purchased from Plantasia (Oberndorf, Austria). The material was identified phytochemically based on comparison with literature[17,20] and with authentic material[21] as well as with dried, crude rhizomes of *P. ternata* obtained from Sichuan Neautus Traditional Chinese Medicine Co., Ltd. (Chengdu, China). Silica gel 60 of 0.063−0.200 mm was purchased from Merck, (Darmstadt, Germany) and RP C-18-OPN of 75 μm from Nacalai Tesque Inc. (Kyoto, Japan). All solvents used for extraction were of reagent grade, and those for chromatography and MS experiments were purchased from VWR (Vienna, Austria) and were of gradient grade. A cerebroside standard, containing soyacerebroside I and II, was purchased from Matreya LLC (Pleasant Gap, USA) (purity ≥98%).

Extraction and GluCer Purification. Dried, processed rhizomes of *A. amurense* (1.9 kg) were pulverized and extracted by dichloromethane, and, subsequently, 1.4 kg of this material was extracted with methanol at 150 bar and 40 °C using an ASE 200 accelerated solvent extractor and a solvent controller (Dionex, Sunnyvale, CA, USA). Thereby every ca. 10 g of plant material in a capsule was extracted three times with 22 mL of solvent (66 mL/ca. 10 g). The combined dried extracts, dichloromethane extract (9.8 g) and methanol extract (10.7 g), were chromatographed over a silica column (length 1.5 m, ⌀ 2.5 cm) eluted by $CHCl_3$/MeOH/H_2O, 98/2/1 to 60/38/8.5, (v/v/v) to give eight fractions (D1−8) for the dichloromethane extract and eluted by $CHCl_3$/MeOH/H_2O, 70/22/3.5 to 60/40/10 (v/v/v) for the methanol extract (10.7 g) to give fifteen fractions (M1−15). Successive column chromatography of fractions D8 (3.6 g) and M1 (2.5 g) by RP C-18 CC (length 1 m, ⌀ 1.7 cm) (solvent methanol) and subsequent silica CC (length 1 m, ⌀ 1.7 cm) (solvent $CHCl_3$/MeOH/H_2O, 65/25/4, v/v/v) yielded five isomeric mixtures of GluCer. Out of 1.1 kg of pulverized dried, processed rhizomes of *P. ternata* a dichloromethane (3.1 g) and methanolic extract (7.9 g) were obtained. The two extracts were combined, dried, and subjected to silica CC (length 1 m, ⌀ 1.4 cm) (solvent $CHCl_3$/MeOH/H_2O, 65:25:4, v/v/v) to yield a fraction (389 mg) enriched in GluCer. Samples of the fractions enriched in GluCer and with pure GluCer were evaporated to dryness using a rotary evaporator and stored in the dark at −20 °C until analysis.

Optical Rotation, CD, UV, IR, and NMR Spectroscopy. Optical rotations were determined on a Perkin-Elmer 341 polarimeter of the GluCer dissolved in methanol. For CD and UV spectroscopy the GluCer and 4,8-SD were dissolved in methanol. CD spectra and UV spectra were obtained on a JASCO J-810 spectropolarimeter. For IR spectra, GluCer were ground in KBr powder and pressed in a thin-layered tablet form. The IR spectra were recorded on a Perkin-Elmer FT-IR spectrophotometer SPEKTRUM 1000. NMR spectra were recorded on a Bruker Avance 500 NMR spectrometer (UltraShield) using a 5 mm switchable probe (PA BBO 500SB BBF-H-D-05-Z, 1H, BB = 19F and 31P - 15N) with *z* axis gradients and automatic tuning and matching accessory (Bruker BioSpin). The resonance frequency for ^{1}H NMR was 500.13 MHz and for ^{13}C NMR 125.75 MHz. All measurements were performed for a solution in fully deuterated chloroform at 298 K. Standard 1D and gradient-enhanced (ge) 2D experiments, like double quantum filtered (DQF) COSY, NOESY, HSQC, and HMBC, were used as supplied by the manufacturer. Chemical shifts are referenced internally to the residual, nondeuterated solvent signal for chloroform ^{1}H (δ 7.26 ppm) or methanol ^{1}H (δ 3.31 ppm) and to the carbon signal of the solvent for chloroform ^{13}C (δ 77.00 ppm) or methanol ^{13}C (δ 49.00 ppm).

Hydrolysis of GluCer/Isolation and Characterization of 4,8-SD. A mixture of GluCer (40 mg) was heated in methanolic 1 M HCl at 70 °C for 7 h under reflux. The reaction mixture was neutralized and evaporated to dryness. The liberated sphingoid base was chromatographically purified on silica gel (solvent $CHCl_3$/MeOH/$NH_{3(aq)}$, 40/10/1, v/v/v). The pure compound (5.5 mg) was characterized by ^{1}H and ^{13}C NMR as an isomeric mixture of 4,8-sphingadienine (Δ^8 *E*/*Z* ∼ 1:1) and had a purity of at least 95% judging from NMR-spectra. Five mg of pure compound was further converted to the *N*-acetylated-*O*-trimethylsilylated derivative and subjected to GC-MS (EI mode; ionizing potential, 70 eV; carrier gas, He; gas flow 1.7 mL/min) using an 6890N Network GC (Agilent Technologies, Santa Clara CA) equipped with an DB-5 column (30 m × 0.25 mm i.d., 0.23 μm (Agilent Technologies) (temperature gradient 120 to 320 °C in 40 and 5 min hold on 320 °C), an 5973 inert Mass selective Detector, and a Combi PAL autosampler (CTC Analytics). One μL of sample (2 mg/mL) was injected of the prepared solutions with a split 1:10.

MALDI-TOF Analysis. An Ultraflex MALDI TOF (Bruker Daltonics) in the reflector mode was used. Surface-assisted laser desorption/ionization (SALDI) mass spectrometry using TiO_2 coated steel targets was performed as previously described.[22]

ESI-MS/CID-MS Analyses. An API 4000 QTrap mass spectrometer (Applied Biosystems) equipped with a Harvard Apparatus 11 Plus Syringe Pump (Harvard Apparatus) for direct flow infusion was used. The syringe pump was set at a flow rate of 0.010 mL/min and diameter 4.61 mm. The standard and the pure GluCer were diluted to 0.5 μg/mL in 1:1 methanol:tetrahydrofurane for MS experiments in positive mode and in methanol with 20% 5 mM aqueous ammoniumacetate. For ESI, the ion spray voltage was set at −4500 V in the negative mode and at 5500 V in the positive mode. Nitrogen served as a curtain gas (10 psi), the declustering potential, being the accelerating current from atmospheric pressure into high vacuum, was set at −55 V in the negative mode and at 76 V in the positive mode. GluCer were first identified in the Q1 positive and negative mode, and for further characterization the mass spectrometer was operated in the enhanced product ion mode (EPI), detecting the fragmentation of the $[M\text{-}H]^-$ or $[M\text{-}H]^+$ molecular ions (Q3) after collision (q2) with nitrogen as collision gas in high mode. For the EPI-experiments, the collision energy was set between 30 to 70 eV in the positive mode and −30 to −70 eV in the negative mode until optimal fragmentation was obtained. The ESI ionization source was a turbo tube.

Cell Culture. HL-60 (human promyeloid leukemia), HeLa-S3 (human cervix carcinoma), MCF-7 (human breast adenocarcinoma), RAW 264.7 (mouse leukemic monocyte macrophages), and HEK-293 (human embryonic kidney) cells were obtained from the American Type Culture Collection (ATCC). Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords as described before[23] and cultured in EGM medium plus provided supplements (Lonza, Braine-l'Alleud, Belgium). The other cells were maintained in RPMI 1640 (HL-60 and HeLa-S3) or DMEM (MCF-7, RAW 264.7 and HEK-293) medium supplemented with 10% heat inactivated fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin, at 37 °C in a humidified atmosphere of 5% CO_2. These media and supplements were purchased from Life Technologies (Carlsbad, CA, USA).

WST-1 Cell Proliferation/Viability Assay. Cells were seeded in 96-well plates (0.05×10^6 cells/well), grown for 24 h, and then incubated with test compounds or solvent (0.6% DMSO) for 24 h. Cell viability and metabolic activity was assessed by the WST-1 colorimetric method in triplicate, according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany). The absorbance was measured at 450 nm with a Tecan spectrophotometer GENios Pro (Tecan, Salzburg, Austria). IC_{50} values were calculated using GraphPad Prism software version 4.03. HL-60 cells were treated

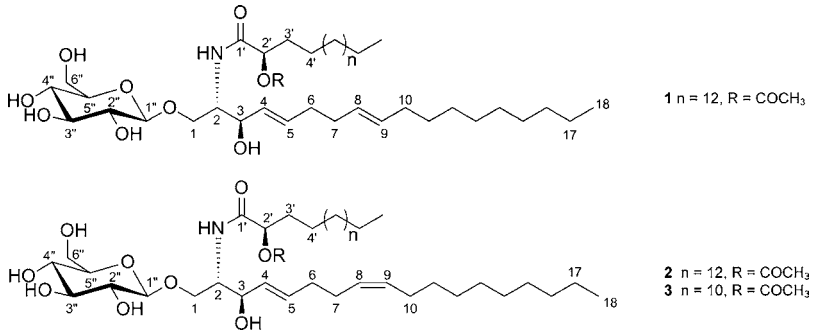

Figure 1. Structures of glucocerebrosides **1**–**3** isolated from processed rhizomes of *A. amurense* and *P. ternata*.

with etoposide (Tocris Bioscience, Bristol, UK) for 24 h as positive control (IC_{50} = 0.04 μM).

RESULTS AND DISCUSSION

Characterization of GluCer in *A. amurense* and *P. ternata*. MALDI-TOF analyses of fractions from the dichloromethane and methanol extracts led to the selection of GluCer-enriched fractions D8 and M1 from rhizomes of *A. amurense* for further purification using silica and RP-18 column chromatography. Thereby five isomeric mixtures of GluCer were separated, namely of compounds **1** and **2** (32 mg, Δ^8 *E/Z* 6:4), 2′-*O*-acetyl soyacerebroside I and compound **3** (7 mg, ratio Δ^8 *E/Z* 51:49); and of the following isomeric mixtures of GluCer previously reported by Jung et al.[17]: soyacerebroside I and II (71 mg, ratio Δ^8 *E/Z* 75:25); 1-*O*-β-D-glucopyranosyl-(2*S*,3*R*,4*E*,8*Z*)-2-[(2′(*R*)-hydroxyicosanoyl)-amido]-4,8-octadecadiene-1,3-diol and 1-*O*-β-D-glucopyranosyl-(2*S*,3*R*,4*E*,8*Z*)-2-[(2′(*R*)-hydroxyicosanoyl)amido]-4,8-octadecadiene-1,3-diol (16 mg, Δ^8 *E/Z* 44:56), and finally 1-*O*-β-D-glucopyranosyl-(2*S*,3*R*,4*E*,8*Z*)-2-[(2′-hydroxyoctadecanoyl)amido]-4,8-octadecadiene-1,3-diol and 1-*O*-β-D-glucopyranosyl-(2*S*,3*R*,4*E*,8*Z*)-2-[(2′-hydroxyoctadecanoyl)amido]-4,8-octadecadiene-1,3-diol (67 mg, Δ^8 *E/Z* 55:45). The new compounds **1**–**3** are the acetylated homologues of GluCer in rhizomes of *A. amurense* reported by Jung et al.; 2′-*O*-acetyl soyacerebroside I was previously reported in *P. ternata* (see Figure 1).[17,18,20] Further seven known GluCer were characterized as previously described.[17,18,20,24] The combination of ESI-MS/MS and NMR to characterize cerebrosides was previously used.[25,26] However, here the techniques were combined with CD spectroscopy by which full structure elucidation including stereochemistry could be obtained. All characterized GluCer contained 4,8-sphingadienine (4,8-SD) as sphingoid base, C-1 linked to glucose and *N*-acylated to a fatty acyl chain of differing length (C16 or C18), which was acetylated at C-2′.

Arisaenosides A (**1**) and B (**2**) were isolated as an isomeric mixture. ESIMS gave a *m/z* 784.5006 for $[M+H]^+$, corresponding to a molecular formula $C_{44}H_{81}NO_{10}$. The spectroscopic data of **1** and **2** (Table 1) exhibited the presence of an amide linkage, long chain(s), and a sugar, consistent with the glycocerebroside nature of **1** and **2**. In the ^{1}H spectra of **1** and **2** an anomeric proton appeared at δ 4.22 (1H, d, *J* = 7.6) and ^{13}C NMR signals resonated at δ 104.7 (C-1″), 75.1 (C-2″), 77.87 and 77.94 (C-3″,5″), 71.7 (C-4″) and 62.7 (C-6″) supporting the presence of a β-glucopyranose moiety. The glycosidic linkage was confirmed by HMBC correlations of H-1″ with C-1 and H-1 with C-1″ (Figure 2 and 3). ^{13}C NMR signals at δ 20.9 and ^{1}H NMR signals at δ 2.13 demonstrated the presence of an acetyl group. The position of the acetyl group at C-2′ of isomers **1** and **2** was confirmed by HMBC correlations between C-2′ and the acetyl group. ESI-MS/CID-MS with enhanced product ionization scan (EPI) of the negative $[M-H]^-$ precursor ion at *m/z* 782 yielded the products

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data of the Isomeric Mixture of Compounds 1 and 2 in CD_3OD

	1	2	1	2
position	δ_C		δ_H (*J* in Hz)	
Lipid Base Unit				
1a	69.7		4.16 (dd, 10.4, 5.1)	
1b	69.7		3.58 (dd, 10.1, 3.7)	
2	54.8		3.96 (m)	
3	72.7		4.11 (td,)	
4	131.4		5.48 (dd, 15.1, 7.6)	
5	134.3		5.73 (m)	
6	33.69		2.06 (m)	
7	33.7	27.9	2.06 (m)	2.07 (m)
8,9	132.0, 130.7	131.5, 130.0	5.42 (t, 4.8)	5.38 (t, 5.4)
10	33.3	28.3	1.97 (m)	1.97 (m)
11–17	23.8, 30.3, 30.4, 30.5, 30.6, 30.7, 30.8		1.19–1.44 (m)	
N-Acyl Unit				
1′	172.8			
2′	75.50, 75.55		4.91(dd, 12.9, 6.6)	
3′a	33.1		1.74 (m)	
3′b	33.1		1.74 (m)	
4′	26.2		1.19–1.44 (m)	
5′-17′	23.8, 30.3, 30.4, 30.5, 30.6, 30.7, 30.8			
2x CH_3	14.5		0.89 (t 6.6)	
Ac (CH_3)	20.9		2.13	
Ac (COO)	172.1			
Glucose Unit				
1″	104.7		4.22 (d, 7.6)	
2″	75.1		3.20 (dd, 8.5, 7.6)	
3″,5″	77.87, 77.94		3.35, 3.33, 3.26	
4″	71.7			
6″a	62.7		3.86 (dd, 12.0, 1.6)	
6″b	62.7		3.64 (dd, 12.0, 4.7)	

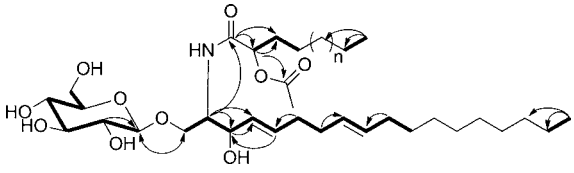

Figure 2. Key ^{1}H–^{1}H COSY (—, bold) and HMBC (→) correlations of **1** (*n* = 12).

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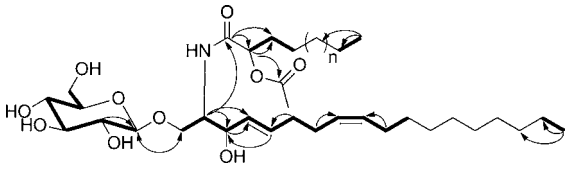

Figure 3. Key $^{1}H-^{1}H$ COSY (—, bold) and HMBC (→) correlations of **2** (n = 12) and **3** (n = 10).

ions at m/z 578 $[M-H-42-162]^-$ and m/z 560 $[M-H-60-162]^-$ due to the neutral loss of the OAc-group and a hexose unit from the precursor unit.

Compounds **1** and **2** could not be separated by chromatography. However, they could be distinguished from the coupling constants in the ^{1}H NMR spectra, indicating a mixture of *E*- and *Z*-geometry of the double bond at position Δ^8. The coupling constant ($J_{4,5}$ = 15.1 Hz) indicated an *E* geometry for Δ^4. The signals of C-7 (δ 33.7) and C-10 (δ 33.3) in the ^{13}C spectra for Δ^8 supported the presence of an (*E*)-8,9 double bond. The isomer **1** was therefore a cerebroside of the 4*E*,8*E*-sphingadienine-type. Based on signals of C-7 (δ 27.9) and C-10 (δ 28.3) a *Z* geometry was determined for Δ^8 of **2**, the isomer of **1**.[27] Therefore, **2** the isomer of **1** was designated as the 4*E*,8*Z*-sphingadienine-type cerebroside. From the signals in ^{1}H NMR spectroscopy the ratio of the isomers **1** and **2** (6:4) could be determined. The stereochemistry of **1** and **2** was verified by CD spectroscopy to be an analogue to that of the standard consisting of soyacerebroside I and II. Therefore, **1** and **2** were deduced to be in D-*erythro* stereochemistry (Figure 4).

The length of the fatty acyl chain and the sphingoid base of **1** and **2** could be determined by positive and negative MS/CID-MS in the EPI. The fragment ion of m/z 299 after CID of the negative $[M-H]^-$ precursor ion at m/z 782 in EPI represents the fatty acyl chain anion of **1** and **2** and was, in combination with NMR data, determined to be 2-acetoxy-octadecanoic acid. This fragment ion originated from the $[M-H]^-$ ion in three steps of fragmentation namely by neutral loss of the following: (1) the OAc-H_2O and (2) glucose-H_2O unit giving the $[M-H-42-162]^-$ ion at m/z 578 and (3) the 1-dehydroxy-1,4,8-sphingatrienine (MW 279 amu) as previously described.[26,28] Other detected fragments in negative MS/CID-MS mode from the $[M-H]^-$ ion were at m/z 740 ($[M-H-42]^-$), m/z 722 ($[M-H-60]^-$), and originated from the neutral loss of the OAc-group. The fragment ion at m/z 560 ($[M-H-60-162]^-$) originated from the $[M-H]^-$ ion by neutral loss of the OAc-group and the glucose unit. The fragment at m/z 262 ($[C_{18}H_{32}N]^+$) from the $[M+H]^+$ precursor ion at m/z 784 after CID, detected in positive mode, indicated 4,8-sphingadienine (d18:2Δ^4,Δ^8) as sphingoid base for **1** and **2**.[28] Based on described findings, the isomers arisaenoside A (**1**) and B (**2**) were characterized as 1-*O*-β-D-glucopyranosyl-(2*S*,3*R*,4*E*,8*E*)-2-[(2′(*R*)-acetoxyoctadecanoyl)amido]-4,8-octadecadiene-1,3-diol and 1-*O*-β-D-glucopyranosyl-(2*S*,3*R*,4*E*,8*Z*)-2-[(2′(*R*)-acetoxyoctadecanoyl)amido]-4,8-octadecadiene-1,3-diol, respectively.

Arisaenoside C (**3**), which can also be called 2′-*O*-acetyl soyacerebroside II, and the previously reported 2′-*O*-acetyl soyacerebroside I were isolated as an isomeric mixture.[18] ESIMS gave a m/z 756.5072 for $[M+H]^+$, suggesting a molecular formula of $C_{42}H_{77}NO_{10}$. Compound **3** was designated as the 4*E*,8*Z*-sphingadienine-type cerebroside. Compound **3** and 2′-*O*-acetyl soyacerebroside II were determined to be homologues of compounds **1** and **2**, with a C_{16} acyl chain rather than the C_{18} acyl chain of **1** and **2**.

The fragment ion of m/z 271 after CID of the negative $[M-H]^-$ precursor ion in EPI at m/z 754 represents the fatty acyl chain anion of **3** and was, in combination with NMR data,

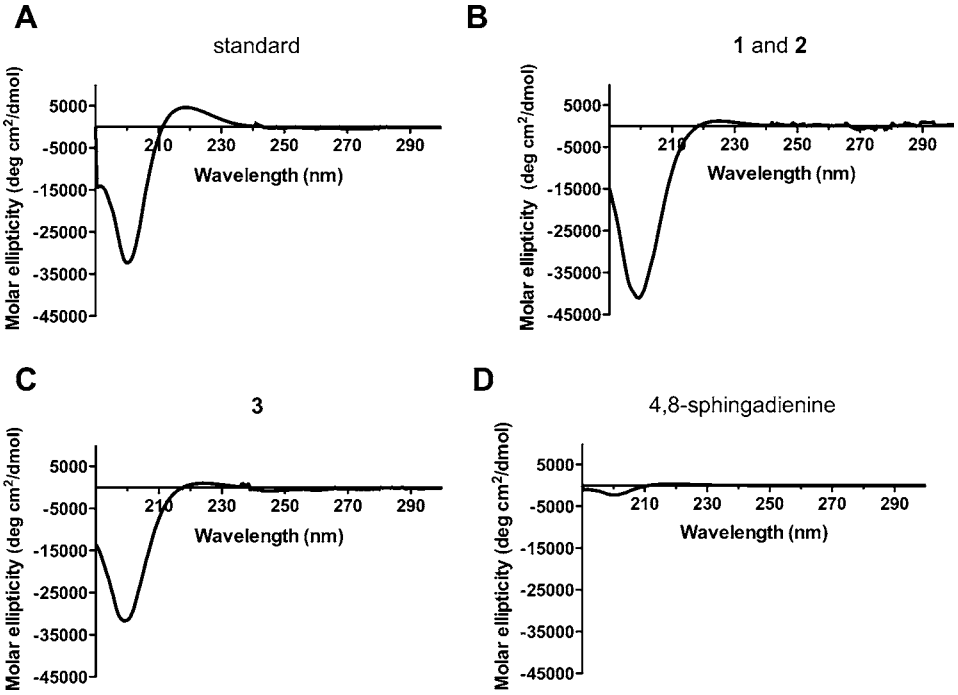

Figure 4. Molar ellipticity (deg cm^2/dmol) graphs derived from the CD spectra by correction for concentration of (A) the standard containing soyacerebroside I and II, (B) the isomeric mixture with arisaenoside A (**1**) and arisaenoside B (**2**); (C) the isomeric mixture of 2′-O-acetyl soyacerebroside I and arisaenoside C (**3**) and (D) 4,8-sphingadienine, all measured in solution in methanol.

determined to be 2-acetoxy-hexadecanoic acid. The fragment ion originated by a likewise fragmentation pathway as **1** and **2** and was consistent with literature values[28] and the standard glucocerebroside. Other detected fragments in negative MS/CID-MS mode from the $[M-H]^-$ ion at m/z 712 ($[M-H-42]^-$), m/z 694 ($[M-H-60]^-$) originated from the neutral loss of the OAc-group. The fragment ion at m/z 532 ($[M-H-60-162]^-$) originated from the $[M-H]^-$ ion by neutral loss of the OAc-group and the glucose unit.[26,28] From the signals in ^{1}H NMR spectroscopy the ratio of the isomers of 2′-*O*-acetyl soyacerebroside I and **3** (51:49) could be determined. Based on these findings, the isomer arisaenoside C (**3**) was characterized as 1-*O*-*β*-D-glucopyranosyl-(2*S*,3*R*,4*E*,8Z)-2-[(2′(*R*)-acetoxyhexadecanoyl)amido]-4,8-octadecadiene-1,3-diol.

4,8-SD as Active Metabolite in the WST-1 Assay. A mixture of GluCer was subjected to hydrolysis in 1 M methanolic HCl, which liberated the sphingoid base that was purified by silica column chromatography. The compound was identified as 4,8-SD by GC-MS analysis of its *N*-acetylated-*O*-trimethylsilylated derivative and by ^{1}H and ^{13}C NMR (Table 3). 4,8-SD is the sphingoid backbone of all the characterized GluCer in this study. From the signals in ^{1}H NMR spectroscopy the ratio of the *E*- and *Z*-isomers (ratio Δ^8 *E*/*Z* ~1:1) of 4,8-SD could be determined (see Figure 5).

The GluCer showed no cytotoxic effects when tested in HL-60 cells (data not shown). 4,8-SD was previously shown to induce apoptosis in Caco-2 human colon cancer cells by activation of caspases and decrease of intracellular *β*-catenin.[29] The metabolite was then evaluated for cytotoxic activity against cells of the HL-60, HeLa-S3, MCF-7, and RAW 264.7 cancer cell lines. 4,8-SD indicated a decrease in cell proliferation and viability with IC_{50} values of 42.8 *μ*M and 61.5 *μ*M respectively in HL-60 and RAW 264.7 cells. The cytotoxicity of the compound was also assessed in HUVEC and HEK-293 non-cancerous cells. The data indicated a general decrease in cell proliferation and viability with IC_{50} values of 36.4 *μ*M for HUVEC and 52.7 *μ*M for HEK-293 cells (Table 4).

GluCer and 4,8-SD and Their Nutritional Role. The GluCer that are composed of 4,8-SD as the sphingoid backbone are besides in the two studied Araceae species present in daily consumed plants such as spinach, soybean, and eggplant.[4,30] The daily intake of SLs in foods including dairy, meat, and egg products was estimated at 300−400 mg in the United States.[17] In Japan in food 50 mg SLs were estimated to be from plant sources.[30,31]

The absorption, uptake, and metabolism of SLs was previously reported.[7,32,33] We observed that 4,8-SD was in contrast to the poorly soluble complex GluCer well soluble. As suggested from our observations 4,8-SD is well absorbed by intestinal epithelial cells.[7,31,34] Sugawara et al. reported that digestibility of maize GluCer is similar to that of mammalian cerebrosides. However, the metabolic fate of sphingoid bases of plant origin within enterocytes differs from that of sphingosine (d18:1). In contrast to sphingosine, the plant-derived sphingoid bases (d18:2) were transported out of the cell by P-glycoprotein after absorption in human Caco-2 cells.[7,35] Fyrst et al. reported that sphingadienines (d18:2) in general have the advantage over other SLs of being slowly metabolized and of having a long half-life in intestinal epithelial cells.[10] The bioavailability of 4,8-SD in the mucosal cells of the intestines after ingestion of complex SLs is unknown. The present findings reinforce that metabolism of GluCer in the gut lumen

Table 2. ^{1}H and ^{13}C NMR Spectroscopic Data of the Isomeric Mixture of 2′-*O*-Acetyl Soyacerebroside I and Compound 3 in CD_3OD

	2′-*O*-acetyl soyacerebroside I	3	2′-*O*-acetyl soyacerebroside I	3
position	δ_C		δ_H (*J* in Hz)	
Lipid Base Unit				
1a	69.7		4.11 (dd, 12.8, 4.1)	
1b	69.7		3.71 (td, 10.5, 3.8)	
2	54.8		3.63 (m)	
3	72.6		4.11 (td, 15.6, 4.1)	
4	131.5		5.48 (dd, 15.6, 7.6)	
5	134.3		5.73 (m)	
6	33.7		2.09 (m)	
7	33.6	27.8	2.07 (m)	2.07 (m)
8,9	132.0, 130.7	131.5, 130.0	5.42 (t, 4.8)	5.37 (t, 5.7)
10	33.3	28.3	1.97 (m)	1.97 (m)
11−17	23.8, 30.3, 30.4, 30.5, 30.6, 30.7, 30.8		1.20−1.45 (m)	
N-Acyl Unit				
1′	172.8			
2′	75.50, 75.54		4.93 (m)	
3′a	33.1		1.75 (m)	
3′b	33.1		1.54 (m)	
4′	26.2		1.20−1.45 (m)	
5′-15′	23.8−30.8			
2x CH_3	14.5		0.89 (t, 6.6)	
Ac (CH_3)	20.9		2.13 (s)	
Ac (COO)	172.1			
Glucose Unit				
1″	104.7		4.22 (d, 7.9)	
2″	75.51		3.18 (dd, 9.1, 7.9)	
3″,5″	77.94, 77.88		3.37, 3.27, 3.26 (m)	
4″	71.7			
6″a	62.7		3.86 (dd, 10.7, 1.6)	
6″b	62.7		3.68 (dd, 11.7, 4.7)	

Table 3. ^{1}H and ^{13}C NMR Spectroscopic Data of the Isomers 4*E*,8*E*-Sphingadienine (4*E*,8*E*-SD) and 4*E*,8*Z*-Sphingadienine (4*E*,8*Z*-SD) in $CDCl_3$

		4*E*,8*E*-SD	4*E*,8*Z*-SD	4*E*,8*E*-SD	4*E*,8*Z*-SD
position		δ_C		δ_H	
1	CH_2	59.67		3.79	
2	CH	56.56		3.26	
3	CH	71.35		4.43	
4	CH	127.65[a]	127.38[a]	5.47	
5	CH	135.13[a]	133.99[a]	5.81	
6	CH_2	32.47		2.10	
7	CH_2	32.65		1.95	2.11
8	CH	130.76		5.36	5.31
9	CH	131.20		5.39	
10	CH_2	31.91		2.04	2.01
11−15	CH_2	29.71; 29.69; 29.63; 29.37; 29.30		1.37−1.20	
16	CH_2	31.91		1.25	
17	CH_2	22.68		1.29	
18	CH_3	14.11		0.88	

[a]Assignment (*E*/*Z*) interchangeable.

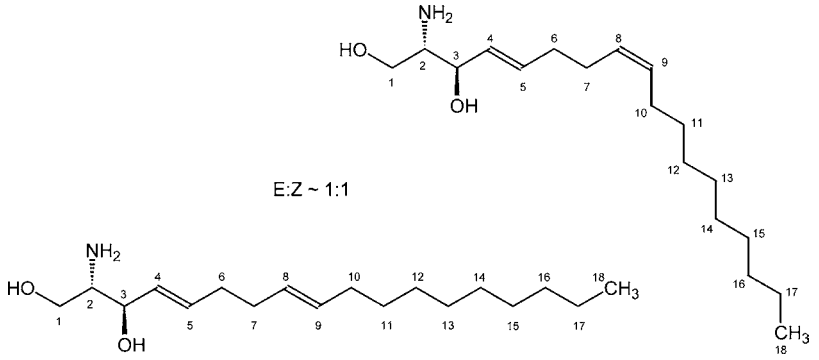

Figure 5. Structures of isomers of 4,8-sphingadienine or 2-amino-1,3-dihydroxy-4,8-octadecadiene (d18:2) from glucocerebrosides from rhizomes of *A. amurense*. and *P. ternata*.

Table 4. Cytotoxic Effect (IC_{50} Value (μM)) of 4,8-Sphingadienine and Etoposide As Positive Control against HL-60, HeLa-S3, MCF-7, RAW 264.7, HUVEC, and HEK-293 Cells *in Vitro* after 24 h As Was Assessed in the WST-1 Assay

	IC_{50} (μM)					
compound	HL-60	HeLa-S3	MCF-7	RAW 264.7	HUVEC	HEK-293
4,8-sphingadienine	42.8	>100	>100	61.5	36.4	52.7
etoposide	0.04	-	-	-	-	>5

by enzymes is required to yield bioactive metabolite 4,8-SD.[36] The double bonds of the sphingoid backbone are considered to be important for the biological activity.[12]

GluCer can be fully and rapidly characterized by a combination of NMR and CD spectroscopy and ESI-MS/CID-MS. ESI-MS/CID-MS analysis revealed the GluCer to differ in length of the carbon chains of the fragments, namely the sphingoid base and fatty acyl chain. 1- and 2-D NMR spectra provide information on the position of fatty acids, glucose and hydroxyl- and acetyl groups and the degree of saturation. Isomers of a GluCer and that of the obtained 4,8-SD after hydrolysis differed in *E/Z* geometry at the double bond at position Δ^8 and were distinguished exclusively by ^{1}H NMR. The stereochemistry was verified by CD spectroscopy by comparison with the standard GluCer. The ESI-MS/CID-MS approach is simpler and more sensitive than previous methods which are prone to introduction of artifacts and loss of structural information.[37] 4,8-SD was revealed as the active metabolite. Further work may serve to determine the bioavailability and nutritional role of the 4,8-SD and other sphingoid bases from sphingolipids in plants in our daily diet.

Structural Details 1−3. ***Arisaenoside A (1) and Arisaenoside B (2)*** *(Isomeric Mixture).* White, amorphous powder; $[\alpha]^{20}_D$ +6.0° (*c* 0.2, MeOH); UV λ_{max} (MeOH) (log ε) 196 (3.49) nm; IR (KBr) 3342, 2918, 2850, 1734, 1724, 1662, 1646, 1541, 1528, 1466, 1457, 1378, 1252, 1084, 1040, 963, 710, 620 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; (+)MALDI-TOF *m/z* 806 $[M+Na]^+$, 822 $[M+K]^+$; (+)ESI-MS/MS *m/z* 554, 484, 430, 262; (-)ESI-MS/MS *m/z* 740, 722, 578, 560, 299; ESIMS *m/z* 784.5006 $[M+H]^+$ (calcd for $C_{44}H_{82}NO_{10}$, 784.5939).

Arisaenoside C (3) *(Isomeric Mixture with 2′-O-Acetyl Soyacerebroside I).* White, amorphous powder; $[\alpha]^{20}_D$ +5.8° (*c* 0.1, MeOH); UV λ_{max} (MeOH) (log ε) 196 (3.61) nm; IR (Kbr) 3398, 2929, 2852, 1733, 1654, 1541, 1465, 1457, 1382, 1244, 1080, 1040, 711, 620 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2; (+)MALDI-TOF *m/z* 778 $[M+Na]^+$, 794 $[M+K]^+$; (+)ESI-MS/MS: *m/z* 526, 510, 456, 262; (-)ESI-MS/MS *m/z* 712, 694, 532, 271; ESIMS *m/z* 756.5072 $[M+H]^+$ (calcd for $C_{42}H_{78}NO_{10}$, 756.5626).

4,8-Sphingadienine. Brownish yellow, amorphous solid; ^{1}H and ^{13}C NMR data, see Table 3. EIMS of *N*-acetylated-*O*-trimethylsilylated derivative *m/z* 73, 174, 309, 334, 378, 468.[38]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H and ^{13}C NMR spectra of compounds **1**−**3** and 4,8-sphingadienine; (tandem) MS spectra of compounds **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

ASE, accelerated solvent extractor; CC, column chromatography; CD, circular dichromism; CID, collision-induced dissociation; EI, electron ionization; EPI, enhanced product ionization; ESI-MS/CID-MS, electrospray ionization collision induced dissociation mass spectrometry; GC-MS, gas chromatography mass spectrometry; GluCer, glucocerebrosides; SL, sphingolipid; MALDI-TOF, matrix assisted laser desorption/ionization-time-of-flight; NMR, nuclear magnetic resonance; SALDI, surface-assisted laser desorption/ionization; RP, reversed phase; 4,8-SD, 4,8-sphingadienine

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