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Neurosporaside, a Tetraglycosylated Sphingolipid from *Neurospora crassa*

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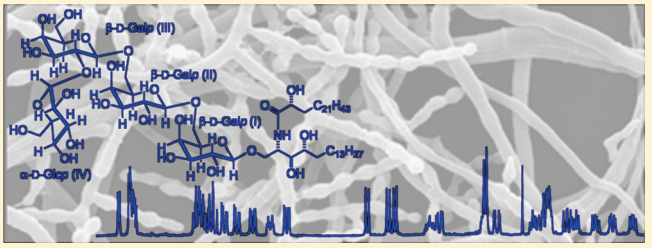
Supporting Information

ABSTRACT: The new tetraglycosylceramide neurosporaside (**1a**) has been isolated from the fungus *Neurospora crassa*. Neurosporaside is a tetraglycosylated glycosphingolipid characterized by a sugar chain unprecedented among natural glycoconjugates. The structure of neurosporaside was elucidated by extensive spectroscopic analysis and microscale degradation analysis, which allowed full structure elucidation using less than 1 mg of compound.



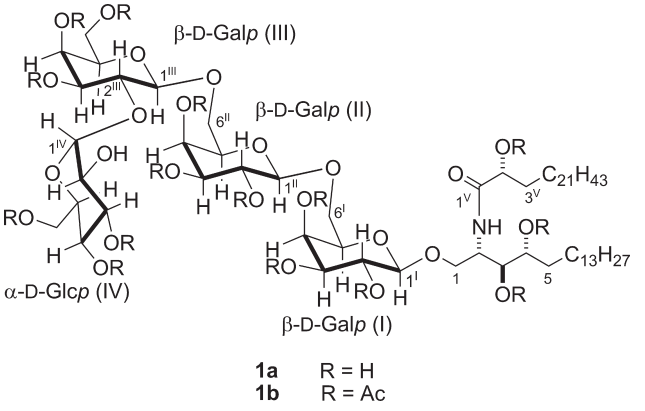
Fungi are known to produce a wide array of compounds, many of them unique to the members of this kingdom. Some of these compounds, their polymers, or their derivatives are an integral part of the barriers and gateways between the fungal cytoplasm and the environment. The significance of the integrity of the plasma membrane with regard to maintaining the fungal cell intact cannot be overestimated. In addition to serving as a barrier between the internal cellular components and the environment, the plasma membrane provides an architectural setting for proteins involved in cell wall biosynthesis (chitin and glucan synthases), transport, and signaling. Sphingolipids, sterols, and glycophospholipids are the prime lipid components of eukaryotic membranes and are particularly abundant in the plasma membrane.

Although all eukaryotic organisms produce sphingolipids, fungi use phytoceramide (a more hydroxylated sphingolipid than the mammalian ceramide counterpart) as the hydrophobic core for the synthesis of more complex sphingolipids,[1] namely, glycosphingolipids (GSLs). Although GSLs may have important roles in the cell, there are only few systematic studies of fungal GSLs. Since evidence[2] has been accumulating that GSLs may serve as key ligands for some endogenous lectins in animals, possible similar roles for fungal GSLs could be considered.

Two subclasses of GSLs appear to be widely expressed in the fungal kingdom: (i) the inositolphosphoceramides, in which the 1-hydroxyl group of ceramide is linked to *myo*-inositol and derivatives by a phosphodiester link, and (ii) the glycosylceramides, such as glucosylceramides (GlcCer) and galactosylceramides (GalCer), also called neutral GSLs.

Neurospora crassa's long history as an excellent genetic, biochemical, and cell biological model of fungal and higher eukaryotic biology, together with the recent availability of a fully sequenced and annotated genome,[3,4] makes it a convenient model ascomycete for studying almost any aspect of fungal growth and development. A previous report[5] on *N. crassa* showed that neutral GSLs were characterized as β-glucopyranosylceramides.

In the course of our study to assess the sphingolipid profile and their relevance to the biology of *N. crassa*, we found a novel tetraglycosylated glycosphingolipid, neurosporaside (**1a**). In this paper, we report on the isolation and structure elucidation of neurosporaside, whose structure is unique in that the sugar moiety linked to the ceramide is formed by a chain of three β-galactopyranoside residues, with a glucopyranoside α-linked to the outer galactopyranoside.



RESULTS AND DISCUSSION

Lyophilized *N. crassa* mycelium obtained from liquid medium cultures was extracted with $CHCl_3$ and MeOH, and the combined

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extracts were partitioned between H_2O and *n*-BuOH. Using a standard procedure we developed to obtain a fraction composed mainly of glycolipids,[6] the organic phase was subjected to reversed-phase and normal-phase column chromatography. The glycolipid fraction was treated with acetic anhydride, and the peracetylated glycolipids were subjected to repeated HPLC on SiO_2 columns. Only two GSLs were found in the mixture: the major component was β-glucosylceramide (4.8 mg), known to be present in *N. crassa*, but significant amounts of a new peracetylated GSL (**1b**, 1.2 mg) were also isolated. While the sample appeared pure upon ^{1}H NMR analysis, mass spectral data showed that the sample was not chemically homogeneous, containing minor amounts of homologues with alkyl chains of different length. A final separation was therefore performed on reversed-phase HPLC, which gave pure neurosporaside peracetate **1b**, plus trace amounts of its homologues, which were not further characterized.

Structure Elucidation. Because the molecular weight of the peracetylated neurosporaside **1b** was higher than 2000, the upper *m/z* limit of our ESI mass spectrometer, only a doubly charged $[M + 2Na]^{2+}$ ion at *m/z* 1025 was visible in its ESI mass spectrum. Therefore, a small amount of **1b** was deacetylated with MeONa/MeOH to give compound **1a**. The mass spectrum of compound **1a** showed a clear pseudomolecular $[M + Na]^+$ ion peak at *m/z* 1354.8. A high-resolution measurement gave *m/z* 1354.8411, corresponding to the molecular formula $C_{66}H_{125}NO_{25}$ (calcd 1354.8433).

The planar structure of neurosporaside was elucidated by extensive 1D and 2D NMR analysis. In the ^{1}H NMR spectrum of the peracetyl derivative **1b** were present (i) an intense aliphatic chain signal at δ 1.25; (ii) several signals corresponding to oxymethine and oxymethylene groups between δ 5.25 and 3.45; (iii) an amide NH doublet at δ 6.70; and (iv) 16 acetyl methyl singlets between δ 2.22 and 1.96. These signals are indicative of a GSL structure. The ^{1}H NMR spectrum also showed a 6H triplet at δ 0.87 in the methyl region, indicating that both alkyl chains in the ceramide unit are unbranched.

Analysis of the 2D-NMR COSY enabled assignment of all the proton signals for the polar part of the sphinganine up to H_2-6 (see Table 1). The α-hydroxy substitution of the fatty acid residue was revealed by the absence in the ^{1}H NMR spectrum of **1b** of the characteristic fatty acid α-proton CH_2 signal and the presence of an acetoxymethine proton resonating at δ 5.13 (H-2^V), coupled with a methylene at δ 1.80 (H-3^V), which was, in turn, coupled with protons resonating in the broad alkyl chain signal at δ 1.25. In addition, a ROESY correlation of the signal at δ 5.13 with the amide NH doublet enabled connection of the NH to this α-hydroxy fatty acid unit. Therefore, the ceramide portion of the molecule is composed of a trihydroxylated, saturated sphinganine and an α-hydroxy fatty acid residue. The ^{1}H and ^{13}C NMR chemical shifts and the ^{1}H NMR multiplicities of the ceramide signals were all very close to those reported for the most common stereoisomer, i.e., that composed of a D-*ribo*-phytosphingosine and a 2*R*-hydroxy acid.[7] This configuration for the ceramide of neurosporaside was confirmed by microscale chemical degradation analysis, as reported below.

The sugar chain of neurosporaside, on the other hand, is unprecedented not only among natural GSLs but also in any known glycoconjugate, so its structure elucidation is described in detail here. The presence of four sugar units was revealed by four resonances in the characteristic anomeric region of the ^{13}C NMR spectrum at δ 95.4, 100.6, 100.8, and 102.6, which were associated through the HMQC spectrum to the relevant anomeric protons, resonating as doublets at δ 5.42 (J = 3.7 Hz, H-1^{IV}), 4.52 (J = 8.3 Hz, H-1^{II}), 4.44 (J = 7.9 Hz, H-1^{I}), and 4.42 (J = 7.7, Hz, H-1^{III}). These protons were used as starting points in the analysis of the COSY and *z*-TOCSY[8] spectrum (see Figure 1) for the sequential assignment of the proton signals in each monosaccharide unit, while the relevant carbon signals were assigned using the HSQC spectrum.

Table 1. ^{1}H and ^{13}C NMR Data ($CDCl_3$) for Neurosporaside Peracetate (1b)

position		δ_H (J in Hz)[b]	δ_C, mult[c]
1	a	3.88 (dd, 10.8, 3.6)	66.5 CH_2
	b	3.63 (dd, 10.8, 3.4)	
2		4.28 (dddd, 9.0, 8.4, 3.6, 3.4)	48.3 CH
2-N*H*		6.74 (d, 9.0)	
3		5.12 (dd, 3.1, 8.4)	71.6 CH
4		4.87 (ddd, 10.0, 3.1, 3.1)	73.2 CH
5	a	1.62 (m)	28.4 CH_2
	b	1.58 (m)	
1′		4.44 (d, 8.0)	100.8 CH
2′		5.09 (dd, 10.4, 8.0)	68.8 CH
3′		4.99 (dd, 10.4, 3.4)	71.0 CH
4′		5.37 (dd, 3.4, 1.0)	67.4 CH
5′		3.86[a]	72.1 CH
6′	a	3.76 (dd, 10.5, 6.4)	66.4 CH_2
	b	3.69 (dd, 10.5, 5.7)	
1″		4.52 (d, 8.0)	100.6 CH
2″		5.13 (dd, 10.4, 8.0)	68.6 CH
3″		5.04 (dd, 10.4, 3.4)	70.9 CH
4″		5.38[a]	67.1 CH
5″		3.92 (ddd, 7.1, 5.8, 1.1)	71.7 CH
6″	a	3.87[a]	66.7 CH_2
	b	3.55 (dd, 9.5, 5.8)	
1‴		4.42 (d, 7.7)	102.6 CH
2‴		3.80 (dd, 10.3, 7.7)	72.8 CH
3‴		4.93 (dd, 10.3, 3.4)	72.0 CH
4‴		5.38[a]	67.0 CH
5‴		3.86[a]	70.6 CH
6‴		4.09 (d, 6.7)	61.2 CH_2
1^{IV}		5.42 (d, 3.7)	95.4 CH
2^{IV}		4.81 (dd, 10.3, 3.7)	70.7 CH
3^{IV}		5.36 (dd, 10.2, 9.5)	69.5 CH
4^{IV}		5.08 (dd, 10.1, 9.5)	68.3 CH
5^{IV}		4.10 (ddd, 10.1, 4.2. 2.4)	67.6 CH
6^{IV}	a	4.26 (dd, 12.2, 4.3)	61.7 CH_2
	b	4.05 (dd, 12.2, 2.4)	
1^V			170.1 C
2^V		5.14 (dd, 7.0, 5.0)	74.0 CH
3^V		1.84 (m)	31.9 CH_2
Ac's	CH_3	2.219, 2.139, 2.136, 2.125, 2.093, 2.084, 2.070, 2.059, 2.053, 2.052, 2.050, 2.019, 2.009, 1.994, 1.970, 1.962	21.2−20.7 CH_3
	CO		171.3−169.5 C

[a] Overlapped by other signals. [b] Additional ^{1}H signals: δ 1.24 (broad band, alkyl chain protons), 0.87 (t, J = 7 Hz, *n*-chain Me groups). [c] Additional ^{13}C signals: δ 29.2 (CH_2), 14.3 (CH_3, ω).

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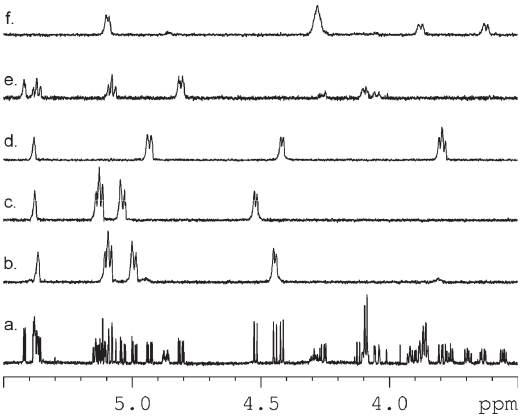


Figure 1. (a) Midfield region ^{1}H NMR spectrum of neurosporaside peracetate (**1b**); (b−f) Sections of the *z*-TOCSY spectrum of **1b**, showing the spin system of Gal-I (b), Gal-II (c), Gal-III (d), Glc-IV (e), and the sphingosine (f).

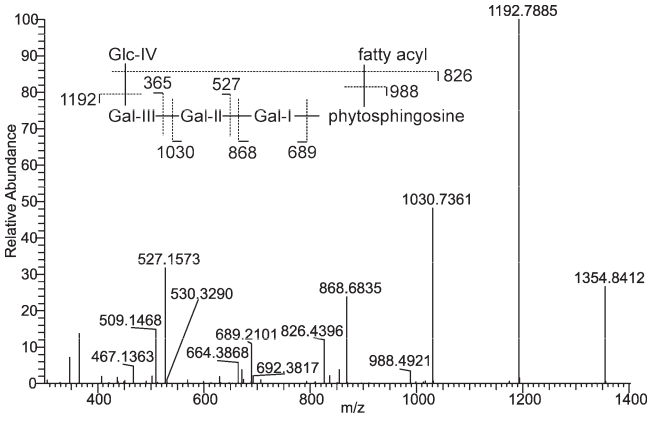


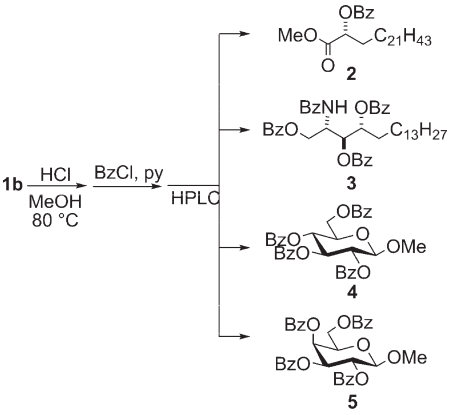
Figure 2. High-resolution MS/MS spectrum of neusporaside **1a**.

Unfortunately, the small coupling constant between H-4 and H-5 of the three galactose residues prevented us from assigning protons at positions 5 and 6 of this sugar using this method (this assignment is essential to determine the way that the sugars are linked to each other; see below). This problem can usually be overcome by looking at the cross-peak present in the ROESY spectrum between H-3 and H-5 of each sugar. This, indeed, allowed us to assign the protons at positions 5 and 6 of Gal-II, but not to distinguish those of Gal-I and Gal-III because the chemical shifts of H-5^{I} and H-5^{III} were coincident. A strong correlation peak between H$_2$-6^{III} and C-4^{III} in the HMBC spectrum was then the key to assigning the former protons and, as a consequence, also H-5^{III} and the protons at positions 5 and 6 of Gal-I.

Once the assignment of the sugar protons and carbons was completed, determination of the structure of the sugar chain was straightforward. The high-field chemical shift of H-5^{I} (δ 3.86), H-5^{II} (δ 3.92), H-5^{III} (δ 3.86), and H-5^{IV} (δ 4.10) showed that the relevant carbon atoms are involved in acetal (as opposed to ester) groups and, therefore, that all the sugars are in the pyranose form. Analysis of coupling constants revealed that sugars I, II, and II are β-galactopyranoses, because in each sugar all protons are axial, except for H-4. Likewise, sugar IV is an α-glucopyranose, because H-1^{IV} is the sole equatorial proton. Finally, the glycosylation sites and the sugar sequence were established by the HMBC cross-peaks of H-1^{I} with C-1, H-1^{II} with C-6^{I}, H-1^{III} with C-6^{II}, and H-2^{III} with C-1^{IV}, and confirmed by the ROESY cross-peak of H-1^{I} with H-1b, H-1^{II} with H-6^{I}b, H-1^{III} with H-6^{II}b, and H-1^{IV} with H-2^{III}.

The molecular formula deduced from the high-resolution mass spectrum indicated that the ceramide part of neurosporaside is composed of 42 carbon atoms, but did not provide information on how they are split between the sphingosine and the fatty acid. This information was provided by a high-resolution MS/MS experiment performed using the pseudomolecular ion of compound **1a** (*m/z* 1354.8411, $C_{66}H_{125}O_{25}NNa$) as the precursor ion. The spectrum (Figure 2) contained peaks at *m/z* 1192.7885 ($C_{60}H_{115}O_{20}NNa$, [M + Na − hexose]$^+$), 1030.7361 ($C_{54}H_{105}O_{15}NNa$, [M + Na − 2 hexoses]$^+$), 868.6835 ($C_{54}H_{105}O_{15}NNa$, [M + Na − 3 hexoses]$^+$), 689.2101 ($C_{24}H_{42}O_{21}Na$, [tetrahexose + Na]$^+$), 527.1573 ($C_{18}H_{32}O_{16}Na$, [trihexose + Na]$^+$), and 365.1050 ($C_{12}H_{22}O_{11}Na$, [dihexose + Na]$^+$), which confirmed the presence of an unbranched tetrasaccharide chain. More interestingly, the MS/MS spectrum also contained two peaks at *m/z* 988.4921 ($C_{42}H_{79}O_{23}NNa$, [M + Na − fatty acyl]$^+$) and 826.4396 ($C_{36}H_{69}O_{18}NNa$, [M + Na − hexose − fatty acyl]$^+$), which were indicative a C_{24} fatty acid and, therefore, of a C_{18} phytosphingosine.

Scheme 1. Microscale Chemical Degradation of Neurosporaside (1b)



Microscale degradation analysis (see Scheme 1) was performed to establish the absolute configuration of two sugars and of the stereogenic centers present in the ceramide. Compound **1b** (450 μg) was subjected to acidic methanolysis with 1 M HCl in 92% MeOH. The resulting mixture, composed of fatty acid methyl esters, free sphinganines, and methyl glycosides, was dried, benzoylated, and separated on normal-phase HPLC. The benzoylated fatty acid methyl ester **2**, the perbenzoylated phytosphingosine **3**, and the methyl β-glycosides**4** and **5** were obtained in pure form (the corresponding methyl α-glycosides coeluted in the conditions used). The absolute configuration of 2-benzoyloxytetracosanoate **2** was determined as *R* from its CD spectrum, matching that reported.[9] Sphingosine **3** was identified as (2*S*,3*S*,4*R*)-1,3,4-tri-*O*-benzoyl-2-benzamido-1,3,4-octadecatriol (D-*ribo*-phytosphingosine perbenzoate),[9] methyl glycoside **4** as methyl tetra-*O*-benzoyl-β-D-glucopyranoside,[10] and methyl glycoside **5** as methyl tetra-*O*-benzoyl-β-D-galactopyranoside[10] by comparison of the respective ^{1}H NMR and CD spectra with those reported. Therefore, neurosporaside (**1a**) is 1-*O*-[α-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl]-(2*S*,3*S*,4*R*)2-[2*R*-hydroxytetracosanoylamino]-1,3,4-octadecanetriol.

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Neurosporaside is a tetraglycosylated sphingolipid with a sugar chain unprecedented among natural glycoconjugates. Neurosporaside is present in *N. crassa* in lesser, but significant amounts (about 25%) compared to the only other GSL detected, β-glucosylceramide.

Fungi exhibit great variability in lipid composition. In addition to the functional roles, lipid patterns have been considered a potential and useful tool for taxonomic classifications. Until now, GSLs from fungi have been described only to a limited extent. Results obtained so far point to a genus-/species-specific relationship, and this information can be used as a part of a more complex taxonomic approach. Screening for the GSL composition has now been made easier and much more accurate by the huge improvement of instrument-based analytical techniques. In this work, a combination of NMR and MS studies made possible the isolation and stereostructural identification of a novel tetraglycosylated sphingolipid from a small amount of biological material.

Previous studies on the GLS composition of *N. crassa* reported only β-glucopyranosylceramide to be present, except for a paper published in 1974,[11] in which a glycolipid with a general $(Gal)_3$-GluCer structure was reported from *N. crassa*. The compound was poorly characterized, and neither the sugar sequence nor the glycosylation positions were determined, so that it is not possible to define which of the many possible compounds with this general structure was actually found. In addition, no details were given on the absolute stereochemistry of the sugars nor on the stereochemistry of the glycosidic linkages. These are of course extremely relevant issues, because the interaction of GSLs with the receptors (or other cellular components) that may determine their biological activity is strongly dependent on the overall shape of the carbohydrate chain (as we have demonstrated in the past with other GSLs),[12] for which glycosylation positions and anomeric configurations play as important a role as the number and the nature of the sugar monomers do.

Identification of the structural components of the fungal membrane is a prerequisite for the eventual understanding of fungal growth and development, especially in cases where structural abnormalities linked to the plasma membrane and/or its adjacent cell wall prevail.[13−16] In addition, the biosynthesis of GSLs that are present in fungi, but absent in humans, may offer unique targets for antifungal drugs.[17] The identification of neuroporaside is only the first step of a more general study focused on the comprehension of lipid structural biology in fungi.

EXPERIMENTAL SECTION

General Experimental Procedures. High-resolution ESIMS and ESIMS/MS spectra were acquired on a Thermo LTQ Orbitrap mass spectrometer. ESIMS experiments were performed on an Applied Biosystems API 2000 triple-quadrupole mass spectrometer. The spectra were recorded by infusion into the ESI source using MeOH as the solvent. Optical rotations were measured at 589 nm on a Jasco P-2000 polarimeter using a 10 cm microcell. ^{1}H and ^{13}C NMR spectra were determined on a Varian UnityInova spectrometer equipped with a cryogenic probe at 700 and 175 MHz, respectively; chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δ_H 7.26, δ_C 77.0). For an accurate measurement of the coupling constants, the one-dimensional ^{1}H NMR spectra were transformed at 64K points (digital resolution: 0.09 Hz). Homonuclear ^{1}H connectivities were determined by COSY and *z*-TOCSY[8] experiments. Through-space ^{1}H connectivities were evidenced using a ROESY experiment with a mixing time of 450 ms. The HSQC spectra were optimized for $^1J_{CH}$ = 142 Hz, and the HMBC experiments for $^{2,3}J_{CH}$ = 8.3 Hz. High-performance liquid chromatography (HPLC) was performed using a Varian Prostar 210 apparatus equipped with a Varian 350 refractive index detector or a Varian 325 UV detector.

Biological Material. Wild-type *Neurospora crassa* strain 74-OR23-1 (FGSC 987)[18] was cultured in 250 mL flasks containing 100 mL of Vogel's minimal medium with 1.5% (w/v) sucrose as previously described.[19] The biomass obtained from 5 flasks of 22 h old cultures (5 × 100 mL) was harvested by filtration, frozen in liquid nitrogen, and lyophilized.

Extraction and Isolation. Lyophilized *N. crassa* (1.2 g) was extracted with a mixture of MeOH and $CHCl_3$ (1:1, 3 × 500 mL) and then with $CHCl_3$ (3 × 500 mL); the combined extracts were partitioned between H_2O and *n*-BuOH. The organic layer was concentrated in vacuo and afforded 614 mg of an oil, which was chromatographed on a column packed with RP-18 silica gel. A fraction (88 mg) eluted with MeOH/$CHCl_3$ (1:1) was further chromatographed on a SiO_2 column, giving a fraction [11 mg, eluent EtOAc/MeOH (7:3)] mainly composed of glycolipids. This fraction was peracetylated by treatment with Ac_2O in pyridine for 12 h. The acetylated glycolipids were subjected to HPLC separation on an SiO_2 column [Phenomenex Luna SiO_2, 5 μ, 250 × 4.60 mm; eluent *n*-hexane/*i*-PrOH (8:2); flow 1 mL min^{-1}], affording a fraction composed of β-glucosylceramide peracetate (4.8 mg), identified by comparison of its ^{1}H NMR spectrum with that of an authentic sample, and a fraction (1.2 mg) containing neurosporaside and its minor homologues. Final separation on an RP-18 column [Phenomenex Luna C18; 5 μ, 250 × 4.60 mm; eluent *n*-hexane/*i*-PrOH (8:2); flow 1 mL min^{-1}] yielded pure neurosporaside peracetate (**1b**, 0.9 mg). A second 500 mL culture of *N. crassa* was extracted and separated using the same procedure; β-glucosylceramide peracetate and neurosporaside peracetate were obtained in approximatively the same amounts (4.2 and 1.0 mg, respectively).

*Neurosporaside peracetate (**1b**):* colorless oil, $[\alpha]^{25}_D$ +6.8 ($CHCl_3$, *c* 0.05); ^{1}H and ^{13}C NMR, Table 1; ESIMS (positive ion mode, MeOH) *m*/*z* 1025 ($[M + 2Na]^{2+}$).

Deacetylation of 1b. Compound **1b** (50 μg) was dissolved in 95 μL of MeOH, and 5 μL of a 0.4 M solution of MeONa in MeOH was added. The reaction was allowed to proceed for 18 h at 25 °C in a sealed vial; then the reaction mixture was dried under nitrogen, and the residue partitioned between water and *n*-BuOH. After removal of the solvent, the organic layer was analyzed without any further purification by ESIMS (positive ion mode, MeOH), *m*/*z* 1354.8 ($[M+Na]^+$), and high-resolution ESIMS (positive ion mode, MeOH), *m*/*z* 1354.8411 ($C_{66}H_{125}NO_{25}$ gives 1354.8433).

Degradation Analysis of 1b. Neurosporaside **1b** (450 μg) was subjected to acidic methanolysis with 1 M HCl in 92% MeOH (500 μL) for 16 h at 80 °C. The resulting mixture was dried under nitrogen and then benzoylated for 16 h at 80 °C with benzoyl chloride (10 μL) in pyridine (200 μL) at 25 °C for 16 h. The reaction was quenched with MeOH and then dried under nitrogen. Methyl benzoate was mostly removed by keeping the residue under vacuum with a freeze-drier for 60 h. The mixture was purified on normal-phase HPLC [column Phenomenex Luna SiO_2; 5 μ, analytical 250 × 4.60 mm; eluent *n*-hexane/*i*PrOH (99:1); flow 1 mL min^{-1}]. Four peaks present in the chromatogram were identified as (*R*)-2-benzoyloxytetracosanoate (**2**, t_R = 3.7 min), D-*ribo*-phytosphingosine perbenzoate (**3**, t_R = 8.3 min), methyl tetra-*O*-benzoyl-β-D-glucopyranoside (**4**, t_R = 10.6 min), and methyl tetra-*O*-benzoyl-β-D-galactopyranoside (**5**, t_R = 11.6 min), by comparison of their retention times, ^{1}H NMR spectra, and CD spectra with those reported.[9,10]

ASSOCIATED CONTENT

Supporting Information. ^{1}H, COSY, ROESY, TOCSY, HSQC, and HMBC NMR spectra of compound **1b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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