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Picomole Scale Stereochemical Analysis of Sphingosines and Dihydrosphingosines

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Abstract—We have developed a simple picomole (low nanogram) scale HPLC scheme which can separate all eight isomers of sphingosine and dihydrosphingosine thus leading to the identification of their relative and absolute configurations. The amino group of the sample is derivatized to its fluorescent *N*-naphthimide which is analyzed by normal and chiral phase HPLC, coupled with fluorescence peak detection. If necessary, the results of this HPLC method can be further corroborated by measurements of circular dichroic (CD) spectra of the *N*-naphthimido-derivatives and/or *N*,*O*-chromophoric derivatives. Copyright © 1996 Elsevier Science Ltd

Introduction

Sphingolipid-derived products are now recognized to play important roles in signal transduction and cell regulation.^{1,2} According to this paradigm, several sphingolipid metabolites such as sphingosine, ceramide, and sphingosine-1-phosphate, act as second messengers and bioactive molecules. Of these, sphingosine has been studied extensively with multiple biochemical and biological activities identified to date and with initial pre-clinical studies showing bioactivity. However, full analysis of biomedical and biochemical results are hampered by the lack of a general method for the stereochemical identification of all eight stereoisomers of sphingosines and dihydrosphingosines, namely, compounds 1A-4A and their dihydro compounds 1a-4a shown in Figure 1.^{3,4} The findings that the threoand erythro-isomers exhibit different activities5-9 emphasize the need of simple and highly sensitive nethods for their characterizations. We recently succeeded in developing a simple chemical/circular dichroic (CD) method for absolute configurational determinations of sphingosines.^{10a,11} According to this method, the 2-amino and the 1,3-diol groups are respectively converted into naphthimido derivatives and naphthoate esters 1B-4B¹² (Fig. 1) without protection-deprotection; each derivatized isomer exhibits CD spectra characteristic of its relative and absolute configurations thus providing the reference spectra.^{10a} A preliminary extension of our previous N,O-derivatization CD protocol to a dihydrosphingosine surprisingly showed that it differed from the parent sphingosine. Accordingly, the CD of the entire set of N,O-chromophoric derivatives of D- and L-erythro and D- and L-threo sphingosines and dihydrosphingosines (not prepared previously^{10a}) have now been measured. The CD of the *D*-erythro series 1B, 1b, and *L*-threo series **2B** and **2b** are summarized as authentic reference data at the end of this report in the two standard solvents, methylcyclohexane and acetonitrile: the enantiomeric **3B**, **3b**, **4B**, and **4b** series exhibited mirror image CD spectra as expected (not shown).

As a sequel to the previous CD study, this paper describes picomole scale stereochemical analysis in which the fluorescent naphthimido-derivatives of sphingosines (step 1 of previous protocol^{10a}) and dihydrosphingosines 1C-4C, 1c-4c are submitted to HPLC. The fluorescence-detected peaks are compared with those of authentic derivatives. The one-step derivatization can be performed in the lower picomole (nanogram) scale with a protocol employing capillary tubes for the reaction and a melting point apparatus as heater. Since the mononaphthimide CD curves in methylcyclohexane are surprisingly characteristic, although very weak (see below), the CD of separated samples should be measured for characterization purposes whenever possible; this will make it unnecessary to have authentic samples. If the amount is insuffifor CD measurements, cient some reference compounds are needed for comparison of the HPLC retention time. However, measurements of the intense characteristic CD spectra of di-O-naphthoyl-N-naphthimido (N,O-chromophoric) derivatives $1B-4B^{10a}$ and 1b-4b and comparisons with reference spectra establish the relative/absolute configurations in a facile and nonempirical manner.

Results and Discussion

Despite the presence of only two chiral centers, the assignments of absolute and relative configurations of sphingosines have been hampered because of their amphiphilic nature, presence of the allylic double bond, and acyclic structure where ¹H NMR coupling constants do not give definitive structural information. In our previous CD studies, numerous chromophoric combinations were checked in order to distinguish the subtle stereochemical differences prior to development of the simple two step *N*-naphthimidation/*O*-naphthoylation sequence which could be performed with a few micrograms (Scheme 1).^{10a,12,13}

Picomole scale HPLC analysis based on selective N-derivatization

Although the micro-scale non-empirical CD led to unambiguous assignments of sphingosines without reference compounds,^{10a} a protocol using HPLC¹⁴ and fluorescence detection¹⁵ seemed to be an attractive alternative for several reasons. First, only one derivatization is needed. Namely, direct naphthimidation without hydroxyl group protection yields the fluorescent *N*-naphthimido derivatives, which could be subjected to both normal and chiral phase chromatography. Second, the high sensitivity of fluorescence detection would lower the analysis to the nanogram (picomole) level. On the other hand, possible obstacles expected were as follows. Although the substrates may be analyzed by HPLC, at the onset it was not clear whether the HPLC resolution would be sufficient to differentiate all eight stereoisomers of sphingosines and their dihydro derivatives. Moreover, there was the problem of reducing the derivatization scale to the picomole level which would be required for the analysis of real biomedical metabolites.

The optically pure synthetic sphingosines (D-*erythro* 1A, L-*threo* 2A, L-*erythro* 3A, and D-*threo* 4A)^{16,17} and dihydrosphingosines (D-2H-*erythro* 1a, L-2H-*threo* 2a, L-2H-*erythro* 3a, D-2H-*threo* 4a)^{16,17} were derivatized to the N-naphthimido-derivatives (C and c series; See Fig. 1) according to the method previously described.^{10a,13} Several authentic mixtures were prepared from these optically pure samples and tested by both normal and chiral phase HPLC with various solvent systems. A



A and a series: $R_1 / R_2 = H$



Figure 1. Sphingosines, dihydrosphingosines, and their derivatives. D-erythro-(2S,3R)-sphingosine (1A), D-erythro-(2S,3R)-dihydrosphingosine (1a), L-threo-(2S,3S)-sphingosine (2A), L-threo-(2S,3S)-sphingosine (2A), L-erythro-(2R,3S)-sphingosine (3A), L-erythro-(2R,3S)-dihydrosphingosine (3A), D-threo-(2R,3R)-sphingosine (3A), D-threo-(2R,3R



Scheme 1. Two step derivatization for CD analysis of sphingosines based on exciton chirality method.^{10,11} (a) Naphthalenedicarboxylic acid anhydride, acetonitrile¹³ reflux; (b) 2-naphthoylimidazole, DBU, acetonitrile, rt.

major problem turned out to be the separation of mixtures of threo-isomers, such as the mixture of 1-threo 2C and 1-2H-threo 2c on normal phase and the mixture of *L*-threo **2C** and *D*-threo **4C** on chiral phase. With accumulation of chromatographic data, it became clear that chloroform improves the differentiation of these mixtures on both normal and chiral phases, while high contents of alchohols such as methanol and 2-propanol, hinder the separation. These findings led to the development of a sequence of normal and chiral phase HPLC protocols which could differentiate all eight isomers. Figure 2 shows the result of the separation of all eight isomers. Namely, the mixture is first submitted is normal phase HPLC upon which four peaks corresponding (\pm) -threo-(2C, to 4C), (\pm) -2H-threo-(2c, 4c), (\pm) -2H-erythro-(1c, 3c), and (\pm) -erythro-(1C, 3C) mixtures appear as base-line separated peaks with the threo series preceding the erythro compounds (Fig. 2, I). Further passage of the individual peaks through the chiral column,

CHIRALCEL OD, separates the two enantiomers (Fig. 2, II).

The protocol for scaling down is described in the following. An excess of 2,3-naphthalenedicarboxylic acid anhydride in pyridine was added to the sample, sphingosine or dihydrosphingosine, placed at the bottom of a melting point capillary tube, and the tube was put in a melting point apparatus set at 110-114 °C, just below the boiling point of pyridine (Fig. 3, I). It should be noted that for the naphthimidation to proceed in high yield, the sample should be heated in anhydrous pyridine with a freshly sublimed anhydride. The capillary tube was taken out from the apparatus after the solvent had evaporated from the open end of the capillary tube, and the residue was then partially purified by analytical scale thin layer chromatography (TLC) to remove unreacted anhydride and polar byproducts, such as 2,3-naphthalenedicarboxylic acid (Fig. 3, II). The yield according to this picomole scale



Figure 2. HPLC chromatograms of a mixture of all eight isomers of sphingosines and dihydrosphingosines. (I) Normal phase HPLC (YMC-Pack SIL, S-3 μ m, 120 A, 150 × 4.6 mm; eluent: hexane:chloroform:ethyl acetate (35:45:20); flow rate: 1 mL/min; fluorescence detection: $\lambda_{ex} = 260$ nm, $\lambda_{em} = 370$ nm). (II) Chiral phase HPLC (CHIRALCEL OD, 250 × 4.6 mm; eluent for i and ii (*threo* isomers): hexane:chloroform:2-propanol (63:35:2); eluent for iii and iv (*erythro* isomers): hexane:2-propanol, 78:22; flow rate: 1 mL/min; Fluorescence detection: $\lambda_{ex} = 260$ nm, $\lambda_{em} = 370$ nm.

protocol is around 40%, which was checked by running two to three microgram scale reactions with *D-erythro*sphingosine **1A** and measuring the UV absorbance of the naphthimido product **1C**, 260 nm (ϵ 61,000, methylcyclohexane); the naphthimidation yield, however, is around 80% when the reaction is performed with regular flasks (instead of melting point tubes) using >50 µg of sample (see Experimental). The minimal derivatization scale carried out so far is 7.4 ng (24.6 pmol): further optimization could possibly reduce the scale down by at least one more digit.

Commercial sphingosines were derivatized and analyzed as described above. One such example is presented in Figure 4. Normal phase analysis of a sphingosine isolated from bovine brain sphingomyelin showed only one peak corresponding to erythro-sphingosine (Fig. 4, I), and subsequent chiral phase analysis confirmed that this isomer is D-erythro-sphingosine 1C (Fig. 4, II). However, analyses of some commercial samples isolated from natural sources showed they mixtures of sphingosines isomeric were and dihydrosphingosines.

CD spectra of N monoderivatives

The CD of mono-derivatized sphingosines (1C and 2C) and dihydrosphingosines (1c and 2c) were measured to check the characteristic pattern originating from the asymmetric environment around the naphthimidogroup. It turned out that although the observed Cotton effects arising from the ¹B_b, ¹L_a, and ¹L_b transitions of the naphthimido chromophore were rather weak, each



Figure 3. Sample preparation protocol for picomole scale HPLC analysis. (I) (a) sample (sphingosine or dihydrosphingosine) solution in chloroform:methanol (1:1); (b) evaporation, pump; (c) 2,3-naphthalenedicarboxylic acid anhydride in pyridine. Reaction: 110-114 °C, 10 h. (II) TLC pre-purification: hexane:ethyl acetate (1:1), developed twice. The spots of the naphthimido-derivatives were invisible when picomole quantities were derivatized. The area enclosed by the dotted frame was scraped off and extracted with ethyl acetate.

isomer exhibited surprisingly unique CD curves (Fig. 5). Therefore, provided a few microgram quantity of sample is available, the CD measurements of HPLC-isolated compounds furnish further evidence of the compound identity.

Nanomole scale analysis based on exciton coupled CD of *N*,*O*-chromophoric derivatives

The set of four dihydrosphingosines, not available earlier, were submitted to the N.O-derivatization protocol.^{10a} Synthetic dihydrosphingosines 1a and 2a^{16,17} were first derivatized to the N-naphthimide by 2,3-naphthalenedicarboxylic refluxing with acid anhydride in anhydrous pyridine.¹³ The N-naphthimido dihydrosphingosines 1c and 2c were then converted to final products 1b and 2b by naphthoylation.¹² As reported previously,^{10a} the UV and CD spectra of each isomer were taken in two solvents, the nonpolar methylcyclohexane and polar acetonitrile, in order to enhance the differences in the characteristic shapes of the CD curves. Table 1 lists the ɛ-values of *D-erythro*sphingosine 1B, L-threo-sphingosine 2B, D-2H-erythro 1b, and L-2H-threo 2b. The CD spectra of D-erythrodihydrosphingosine 1b in the two solvents were similar to those of *D*-erythro-sphingosine 1B, although differences were seen in the ratios of the two Cotton effects (Fig. 6, I and II).^{10b} In contrast, the CD spectra of *L-threo*-dihydrosphingosine **2b** were found to be substantially different from the corresponding unsaturated sphingosine 2B (Fig. 6, III and IV). The characteristic CD curves depicted in Figure 6 completes the CD data base for stereochemical analysis of sphingosines and dihydrosphingosines, and serve as a reference for the analysis of unknown samples.

Conclusion

We present two independent methods for the absolute and relative configurational determinations of sphingosines and dihydrosphingosines (Fig. 7). If the available sample amount is >1 microgram, the N,O-derivatization protocol^{10a} followed by CD measurements (Fig. 6) will establish the stereochemistry of the sphingosines and dihydrosphingosines listed in Figure 1 without reference samples. The highly sensitive HPLC method described above can be performed with picomole (nanogram or less) amounts of sample; if the amount of HPLC-isolated N-derivative(s) permits CD measurements, reference to Figure 5 will establish the identity of sample(s). However, as may be the case in the analysis of samples of actual metabolic origin where the amount is extremely limited, the HPLC of N-derivative(s) will require parallel runs with several authentic samples. Since most isomers of sphingosines and dihydrosphingosines are now commercially available as synthetic samples,¹⁸ references should be prepared from some of the available isomers.¹⁹ These two highly sensitive and diagnostic analytical methods for stereochemical assignments should contribute in studies aimed at clarifying the roles and/or mechanisms



Figure 4. HPLC analysis of natural sphingosine (commercial). (I) Normal phase HPLC (YMC-Pack SIL, S-3 μ m, 120 A, 150 × 4.6 mm; eluent: hexane:chloroform:ethyl acetate (35:45:20); flow rate: 1 mL/min; fluorescence detection: $\lambda_{ex} = 260$ nm, $\lambda_{em} = 370$ nm). (II) Chiral phase HPLC (CHIRALCEL OD, 250 × 4.6 mm; eluent: hexane:2-propanol (78:22); flow rate: 1 mL/min; fluorescence detection: $\lambda_{ex} = 260$ nm, $\lambda_{em} = 370$ nm).

of the sphingosines and dihydrosphingosines on a molecular structural basis. The above mentioned

analyses were performed with sphingosines and dihydrosphingosines with $\rm C_{18}\mbox{-}fatty$ acid moieties. In



Figure 5. CD spectra of N-naphthimido-derivatives. (I) D-erythro 1C. (II) D-2H-erythro 1c. (III) L-threo 2C. (IV) L-2H-threo 2c.

general unknown cases, in which sphingosine bases could be a mixture of homologues, the HPLC peaks should be submitted to MS measurement.

Experimental

Materials

Optically pure sphingosines and dihydrosphingosines were synthetically obtained.^{16,17} Natural sphingosine was purchased from Sigma, Serdary Research Labora-

Table 1. E-Values of compounds 1B, 2B, 1b and 2b

ε-Value in ethylcyclohexane	ε-Value in acetonitrile
2,000 (238 nm),	126,500 (237 nm),
9,000 (258 nm)	130,000 (280 nm)
0,000 (238 nm),	130,000 (238 nm),
4,500 (259 nm),	58,500 (260 nm)
5,000 (238 nm),	127,500 (238 nm),
0,500 (258 nm)	73,000 (260 nm)
3,000 (237 nm),	135,500 (237 nm),
	e-Value in ethylcyclohexane 22,000 (238 nm), 79,000 (258 nm) 40,000 (238 nm), 44,500 (259 nm), 55,000 (238 nm), 30,500 (258 nm) 13,000 (237 nm),

tories Inc., Matreya Inc., and Biomol. 2,3-Naphthalenedicarboxylic acid, acetic acid anhydride, 2-naphthoic acid, 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU), and 1,1'-carbonyldiimidazole were purchased from Aldrich. HPLC grade solvents were obtained through Fisher Scientific. All solvents and reagents were used directly without further purification unless otherwise specified. CD spectra were measured by JASCO J-720 spectropolarimeter. Parameters for CD measurement were as follows, Bandwidth 1.0 nm, Slit width Auto, Sensitivity 10 mdeg, Response 4 s, Start wavelength 400 nm, End wavelength 200 nm, Scan speed 100 nm/min, Step resolution 0.5 nm, Accumulation 4. Fluorescence spectra were taken by a Perkin-Elmer Luminescence Spectrometer LS-50B. Parameters for fluorescence measurement were as follows: excitation, 260 nm; emission, 340 to 500 nm; acan speed, 240 nm/min; excitation slit, 3.5 nm; emission slit, 3.5 nm. HPLC analysis was carried out with a Perkin-Elmer Model Series 4 Liquid Chromatograph terminal coupled with a Hewlett-Packard 1046 Programmable Fluorescence Detector. The normal phase HPLC column was YMC-Pack SIL (YMC, Inc., 150 × 4.6 mm i.d., S-3 μm, 120 Å). The chiral phase HPLC column was CHIRALCEL OD (Chiral Technologies, Inc., 250 × 4.6 mm i.d.). TLC plate used for both analysis and



Figure 6. Circular dichroic (CD) spectra of NO-chromophoric derivatives. (I) 1a (dotted line), 2H-1a (solid line) in methylcyclohexane; (II) 1a (dotted line), 2H-1a (solid line) in acetonitrile; (III) 2a (dotted line), 2H-2a (solid line) in methylcyclohexane; (IV) 2a (dotted line), 2H-2a (solid line) in acetonitrile.



I. Nanomole scale determination of relative/absolute configurations

Figure 7. Protocols for stereochemical analysis of sphingosines and dihydrosphingosines. (I) Nanomole scale CD method. Following the two step derivatization, CD measurement non-empirically determines the absolute configuration. (II) Picomole scale HPLC protocol. Stereochemistry of the *N*-naphthimide is identified by a sequence of normal and chiral phase HPLC.

preparation was silica gel 60 F-254, 0.25 mm, E. Merck. NMR spectra were recorded on Varian VXR 400 instrument and performed in CDCl₃ or in benzene- d_6 . Chemical shifts (δ) are reported in ppm downfield from internal TMS and coupling constants (J) in Hz. Low-resolution and high-resolution FAB mass spectra were measured on a JEOL JMS-DX303 HF mass spectrometer using a glycerol matrix and Xe ionizing gas. CI mass spectra were measured on a NERMAG R10-10 spectrometer with NH₃ as ionizing gas. The melting point apparatus used for the picomole scale derivatization was Thomas Hoover Capillary Melting Point Apparatus.

2,3-Naphthalenedicarboxylic acid anhydride. 2,3-Naphthalenedicarboxylic acid anhydride was prepared from the corresponding diacid by refluxing with 2 equiv of acetic anhydride for 12 h.²⁰ The obtained anhydride was washed with acetic acid and ether, and dried at 100 °C overnight. The brownish crude anhydride was further purified by sublimation (ca. 160 °C) before use. Purity was checked by TLC (hexane:ethyl acetate, 1:1). CIMS m/z 198 [M]⁺.

Picomole scale derivatization for HPLC analysis of sphingosine mixture

The naphthimidation step, the first step described above, was scaled down to picomole (nanogram) level with the following simple method. A sample solution (typically, ca. 50 ng, 167 pmol, in 10 μ L of chloroform: methanol, 1:1) was transferred to the melting point capillary tube (75 × 1.5–1.8 mm i.d.: the tube was originally 90 mm long, and the top part, 15 mm, was truncated), and the solvent was removed by evaporation followed by pumping overnight. The sample was added an excess of 2,3-naphthalenedicarboxylic acid anhydride¹³ (1 µg in 5 µL of anhydrous pyridine), and put into the melting point apparatus set at 110–114 °C. The solvent, pyridine, was refluxed and gradually evapd from the open top of capillary. The reaction tube was removed from the melting point apparatus when the tube was dried out: it usually took ca. 10 h, though it could be varied by the size of capillary and the apparatus. The crude reaction mixture contained polar byproduct(s), such as 2,3-naphthalenedicarboxylic acid, which could be easily removed by TLC (40×10 mm, hexane:ethyl acetate, 1:1, developed twice) before HPLC analysis (See Fig. 3, II).

Stereochemical analysis of sphingosine by HPLC

A standard sample was prepared from the eight possible isomers (D-erythro 1C, L-threo 2C, L-erythro 3C, and D-threo 4C) and dihydrosphingosines (D-2Herythro 1c, L-2H-threo 2c, L-2H-erythro 3c, and D-2H-threo 4c), which had been prepared from optic-ally pure synthetic samples.^{16,17} The mixture was analyzed on silica gel HPLC column (YMC-Pack SIL; eluent: hexane:chloroform:ethyl acetate, 35:45:20; flow rate: 1 mL/min; fluorescence detection: $\lambda_{ex} = 260$ nm, $\lambda_{em} = 370$ nm: See Fig. 2, I). The four peaks, (\pm) -2H-threo-. corresponding (\pm) -threo-, to (\pm) -2H-erythro-, and (\pm) -erythro-sphingosine (eluted in this order), were collected and further analyzed on chiral phase HPLC column (CHIRALCEL OD.; eluent: (1) hexane:chloroform:2-propanol, 63:35:2; for the analyses of (\pm) -threo- and (\pm) -2H-threo mixtures. (2) hexane:2-propanol, 78:22; for (\pm) -erythro- and (\pm) -2H-erythro mixtures); flow rate: 1mL/min; fluorescence detection: $\lambda_{ex} = 260 \text{ nm}$, $\lambda_{em} = 370 \text{ nm}$: See Fig. 2, **II**). The retention time of each isomer on both normal and chiral phases was used as the standard reference for the composition analysis of natural sphingosine. Natural sphingosine was derivatized with the nanogram scale method described above and analyzed with the same conditions.

Sub-micromole scale derivatization of dihydrosphingosines for CD measurement

A solution of the mixture of dihydrosphingosine (50 µg, 0.16 µmol) and 2,3-naphthalenedicarboxylic acid anhydride¹³ (36 µg, 0.18 µmol, 1.1 equiv) in anhydrous pyridine (0.1 mL) was placed in a 10 mL round bottomed flask and refluxed under argon for 12 h. The whole mixture was then evapd, and N-naphthimide was purified by prep. TLC [hexane:ethyl acetate, 1:1, 70] μ g, 80% yield, R_f (1c) = 0.26, R_f (2c) = 0.38]. The prep. TLC plate was developed twice with methanol (washing) before use. The naphthimide (70 µg, 0.13 µmol) was then dissolved in anhydrous acetonitrile (0.1 mL) and stirred at room temperature for 1 h with 2-naphthoylimidazole¹² and a catalytic amount of DBU. The solution was evapd, and the final product was purified by prep. TLC (hexane:ethyl acetate, 3:1, 80 μ g, 80% yield, R_f (1b) = 0.36, R_f (2b) = 0.39).

Large scale preparation (2–3 mg scale) for NMR measurement was also carried out with the method described above. ¹H NMR and HRMS data of each compound are as follows.

1b: ¹H NMR (400 MHz, benzene- d_6): δ 0.91 (t, 3H, J=7.0 Hz), 1.10–1.30 (m, 24H), 1.50–1.64 (m, 2H), 1.90–2.06 (m, 2H), 5.23 (dd, 1H, J=11.4, 4.0 Hz), 5.46 (dd, 1H, J=11.4, 8.8 Hz), 5.56 (ddd, 1H, J=8.8, 7.7, 4.0 Hz), 6.63 (ddd, 1H, J=7.7, 7.7, 4.0 Hz), 6.98–7.18 (m, 5H), 7.21 (m, 1H), 7.28 (dd, 2H, J=6.1, 3.3 Hz), 7.36 (d, 2H, J=8.8 Hz), 7.41 (d, 1H, J=8.1 Hz), 7.51 (d, 1H, J=8.1 Hz), 7.57 (d, 1H, J=8.8 Hz), 7.62 (d, 1H, J=8.1 Hz), 7.94 (s, 2H), 8.16 (dd, 1H, J=8.7, 1.6 Hz), 8.37 (dd, 1H, J=8.6, 1.7 Hz), 8.68 (s, 1H), 8.95 (s, 1H). FABHRMS *m/z*: calcd for C₅₂H₅₅O₆N [M]⁺ 789.4029, found 789.4017.

2b: ¹H NMR (400 MHz, benzene- d_6): δ 0.91 (t, 3H, J = 7.0 Hz), 1.20–1.40 (m, 24H), 1.57 (m, 2H), 1.84 (m, 2H), 5.07 (dd, 1H, J = 11.0, 5.1 Hz), 5.40 (dd, 1H, J = 11.0, 9.1 Hz), 5.50 (ddd, 1H, J = 9.1, 7.2, 5.1 Hz), 6.11 (ddd, 1H, J = 7.2, 7.2, 5.4 Hz), 6.96 (m, 2H), 7.06 (m, 2H), 7.11 (m, 2H), 7.18 (m, 2H), 7.38 (m, 2H), 7.43 (br d, 1H, J = 8.8 Hz), 7.56 (br d, 1H, J = 8.0 Hz), 7.89 (s, 1H), 8.18 (dd, 1H, J = 8.6, 1.7 Hz), 8.32 (dd, 1H, J = 8.6, 1.6 Hz), 8.72 (s, 1H), 8.90 (s, 1H). FABHRMS *m/z*: calcd for C₅₂H₅₅O₆N [M]⁺ 789.4029, found 789.4046.

1C: ¹H NMR (400 MHz, CDCl₃): δ 0.89 (t, 3H, J=7.5 Hz), 0.95–1.35 (m, 22H), 1.90 (m, 2H), 3.03 (d, 1H, J=4.0 Hz), 3.38 (dd, 1H, J=9.5, 3.6 Hz), 4.15 (ddd, 1H, J=12.4, 3.6, 3.4 Hz), 4.26 (m, 1H), 4.40 (m, 1H),

4.76 (ddd, 1H, J=7.4, 7.4, 4.0 Hz), 5.53 (dd, 1H, J=15.3, 7.6 Hz), 5.69 (ddd, 1H, J=15.3, 7.0, 6.6 Hz), 7.72 (dd, 2H, J=3.3, 2.9 Hz), 8.07 (dd, 2H, J=3.3, 2.8 Hz), 8.35 (s, 2H). FABHRMS *m*/*z*: calcd for C₃₀H₄₂NO₄ [M+H]⁺, 480.3114, found 480.3120.

1c: ¹H NMR (400 MHz, CDCl₃) δ 0.87 (t, 3H, J = 7.0 Hz), 1.15–1.70 (m, 28H), 2.05 (s, 0.5H), 2.17 (s, 0.5H), 3.40 (m, 0.5H), 3.54 (d, 0.5H, J = 4.8 Hz), 4.02–4.20 (m, 2H), 4.21–4.36 (m, 2H), 7.73 (dd, 2H, J = 3.3, 2.9 Hz), 8.08 (dd, 2H, J = 3.3, 2.8 Hz), 8.36 (s, 2H). FABHRMS *m*/*z*: calcd for C₃₀H₄₄NO₄ [M+H]⁺, 482.3271, found 482.3254.

2C: ¹H NMR (400 MHz, CDCl₃): δ 0.88 (m, 3H), 0.95–1.40 (m, 24H), 1.95 (m, 2H), 2.85 (br s, 1H), 3.56 (br s, 1H), 4.08 (m, 2H), 4.50 (dd, 1H, *J*=11.1, 6.2 Hz), 4.71 (br s, 1H), 5.45 (dd, 1H, *J*=15.4, 5.4 Hz), 5.81 (ddd, 1H, *J*=15.4, 7.3, 6.7 Hz), 7.70 (m, 2H), 8.04 (m, 2H), 8.30 (s, 2H). FABHRMS *m/z*: calcd for C₃₀H₄₂NO₄ [M+H]⁺, 480.3114, found 480.3124.

2c: ¹H NMR (400 MHz, CDCl₃): δ 0.87 (t, 3H, J=7.0 Hz), 1.20–1.60 (m, 28H), 2.67 (m, 1H), 3.61 (d, 1H, J=10 Hz), 4.01–4.17 (m, 3H), 4.49 (m, 1H), 7.73 (dd, 2H, J=3.3, 2.9 Hz), 8.08 (dd, 2H, J=3.3, 2.8 Hz), 8.36 (s, 2H). FABHRMS *m*/*z*: calcd for C₃₀H₄₄NO₄ [M+H]⁺, 482.3271, found 482.3278.

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19. It should be noted that the fluorescence intensity of N-naphthimido-sphingosines (1C and 2C) is approximately half that of the dihydro series (1c and 2c) in the normal phase HPLC solvent system (data not shown); this should be taken into account in cases where fluorescence intensities are used in estimating the composition ratio.

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