

[19] Use of Circular Dichroism for Assigning Stereochemistry of Sphingosine and Other Long-Chain Bases

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Introduction

Sphingolipid signal transduction pathways are mediated by a wide variety of enzyme–substrate interactions. The key factor that ensures the fidelity of this pathway is the specificity between each enzyme and substrate. The specificity can be understood in terms of the molecular-level interactions, in which the asymmetric protein binding site accommodates its chiral substrate stereospecifically. In other words, stereochemistry of a substrate determines the binding affinity to its receptor enzyme—hence the subsequent course of the physiologic processes. In fact, each stereoisomer of sphingosine and dihydrosphingosine has been found to possess its own biologic properties,^{1–11} which underscores the significance of stereochemical assignment for both synthetic and natural sphingolipids.

This paper describes the application of an exciton chirality method to the stereochemical assignment of sphingosines and dihydrosphingosines,

- ¹ A. H. Merrill, S. Nimkar, D. Menaldino, Y. A. Hannun, C. Loomis, R. M. Bell, S. R. Tyagi, J. D. Lambeth, V. L. Stevens, R. Hunter, and D. C. Liotta, *Biochemistry* **28**, 3138 (1989).
- ² Y. Igarashi, S. Hakomori, T. Toyokuni, B. Dean, S. Fujita, M. Sugimoto, T. Ogawa, K. El-Ghaendy, and E. Racker, *Biochemistry* **28**, 6796 (1989).
- ³ T. J. Mullmann, M. I. Siegel, R. W. Egan, and M. M. Billah, *J. Biol. Chem.* **266**, 2013 (1991).
- ⁴ A. Bielawska, C. M. Linardic, and Y. A. Hannun, *J. Biol. Chem.* **267**, 18493 (1992).
- ⁵ R. Chao, W. Khan, and Y. A. Hannun, *J. Biol. Chem.* **267**, 23459 (1992).
- ⁶ M. Y. Pushkareva, W. A. Khan, A. V. Alessenko, N. Sahyoun, and Y. A. Hannun, *J. Biol. Chem.* **267**, 15246 (1992).
- ⁷ B. M. Buehrer and R. M. Bell, *J. Biol. Chem.* **267**, 3154 (1992).
- ⁸ A. Bielawska, H. M. Crane, D. Liotta, L. M. Obeid, and Y. A. Hannun, *J. Biol. Chem.* **268**, 26226 (1993).
- ⁹ A. Olivera, H. Zhang, R. O. Carlson, M. E. Mattie, R. R. Schmidt, and S. Spiegel, *J. Biol. Chem.* **269**, 17924 (1994).
- ¹⁰ R. A. Wolff, R. T. Dobrowsky, A. Bielawska, L. M. Obeid, and Y. A. Hannun, *J. Biol. Chem.* **269** (1994).
- ¹¹ N. Divecha and R. F. Irvine, *Cell* **80**, 269 (1995).

the structural core of sphingolipids.^{12,13} Exciton chirality is a very simple and highly sensitive analytical protocol utilizing circular dichroic (CD) spectroscopy. It requires only a low microgram quantity of the original sample material and allows stereochemical assignment of all possible configurational isomers of sphingosine and dihydrosphingosine. The method has already been employed for the stereochemical assignment of several sphingolipids from marine sources.^{14,15} Note that all of the chemical reactions and the purification methods are simple and straightforward, and the readers with limited chemical background should soon be able to obtain reproducible results after some practice.

Circular Dichroic Exciton Chirality Method

Circular dichroism is defined as the difference in absorption for the left and the right circularly polarized light ($\Delta\epsilon = \epsilon_L - \epsilon_R$, where ϵ_L and ϵ_R represent the absorptions of the left and the right helical rays, respectively). Because all chiral substances exhibit circular dichroism, CD spectroscopy has been used as a powerful tool for stereochemical analysis of many organic and inorganic compounds. Most of the commercial CD spectrometers are made for the detection of circular dichroism in the UV/VIS wavelength region. CD spectroscopy, therefore, is widely used for the conformational study of biopolymers, such as proteins and nucleic acids, both of which contain intrinsic UV absorbing chromophores. CD has also been applied to the stereochemical analysis of small organic molecules, one of the most successful applications being the exciton chirality method.^{16,17} This method is based on the through-space interaction between two or more chromophores, which may be preexisting within the substrate or introduced by derivatization of hydroxyl groups, amino groups, etc.; thus the original molecule of interest could be devoid of UV absorbing moieties. The CD of the substrate or derivatized substrate measures the through-space inter-

¹² V. Dirsch, J. Frederico, N. Zhao, G. Cai, Y. Chen, S. Vunnam, J. Odingo, H. Pu, K. Nakanishi, N. Berova, D. Liotta, A. Bielawska, and Y. Hannun, *Tetrahedron Lett.* **36**, 4959 (1995).

¹³ A. Kawamura, N. Berova, V. Dirsch, A. Mangoni, K. Nakanishi, G. Schwartz, A. Bielawska, Y. Hannun, and I. Kitagawa, *Bioorg. Med. Chem.* **4**, 1035 (1996).

¹⁴ M. Ojika, G. Yoshino, and Y. Sakagami, *Tetrahedron Lett.* **38**, 4235 (1997).

¹⁵ V. Costantino, E. Fattorusso, A. Mangoni, M. Di Rosa, and A. Ianaro, *J. Am. Chem. Soc.* **119**, 12465 (1997).

¹⁶ N. Harada and K. Nakanishi, "Circular Dichroic Spectroscopy—Exciton Coupling in Organic Stereochemistry." University Science Books, Mill Valley, California, 1983.

¹⁷ N. Berova and K. Nakanishi, in "Circular Dichroism" (N. Berova, K. Nakanishi, and R. W. Woody, eds.), p. 337, Wiley-VCH, New York, 2000.

actions among the chromophores. Because the extent of interaction is proportional to the square of the chromophoric extinction coefficient, usage of strongly absorbing chromophores enables the analytical scale to be lowered to levels of just a few micrograms or less. In the case of conformationally rigid molecules, the exciton chirality method leads to a nonempirical determination of absolute configuration as shown in the following simple example of (1*S*,2*S*)-cyclohexanediol dibenzoate (**1**, Fig. 1).

The absolute sense of twist between the two hydroxyl groups is represented by the twist between the two benzoate electric transition moments since they are parallel to the two hydroxylic C–O bonds. The interaction splits the “excited states” of the chromophores into two energy levels, which is the so-called exciton coupling (Fig. 2). One of the two electric transitions, therefore, gives rise to a red-shifted absorption band (longer wavelength), while the other affords a blue-shifted band (shorter wavelength), and the two bands exhibit circular dichroism (or Cotton effects) of different signs to each other. When the absolute sense of twist between the two chromophores is clockwise (positive chirality), as is the case for **1** (Fig. 1), the red-shifted band absorbs the left circularly polarized light more than the right counterpart ($\Delta\epsilon > 0$), while the blue-shifted band absorbs

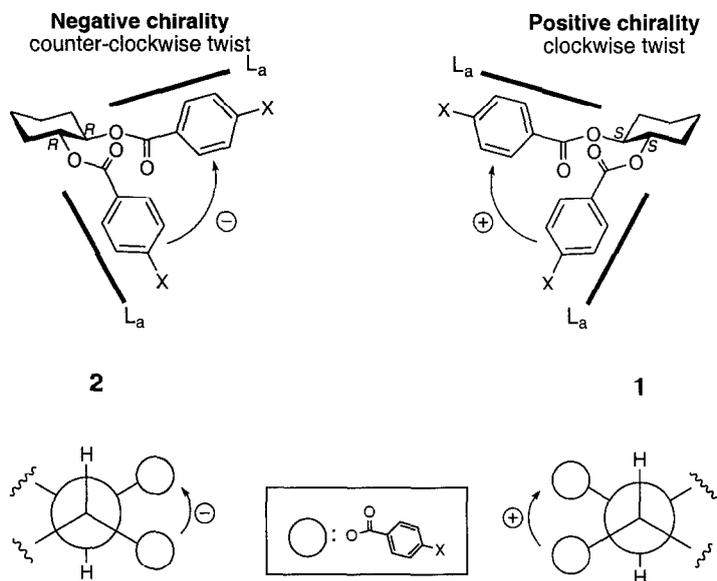
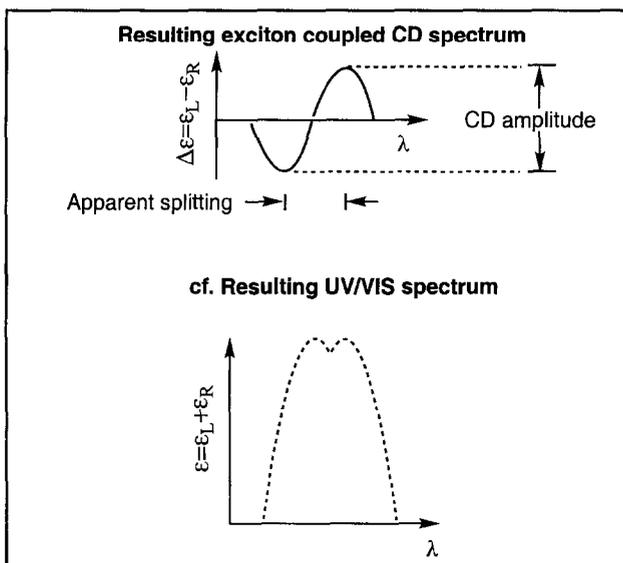
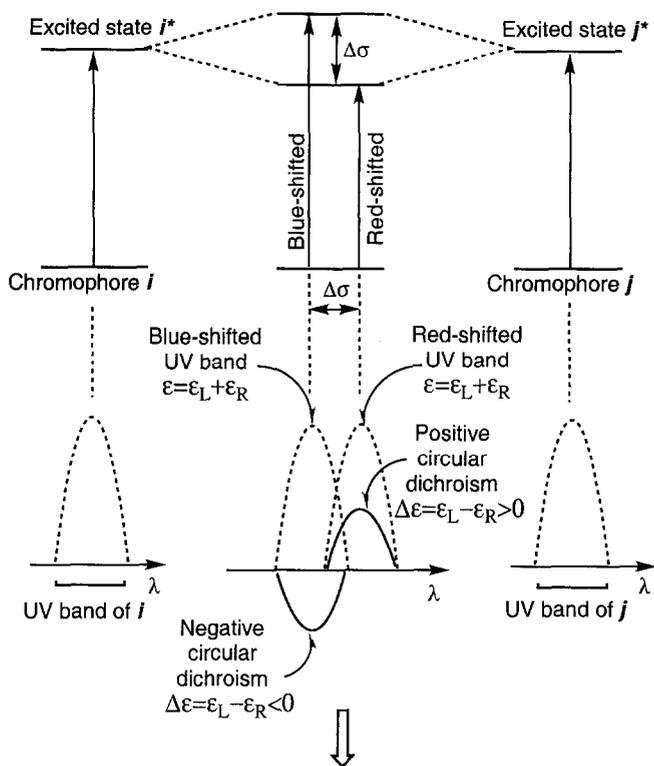


FIG. 1. The directions of electric transition moments of the *p*-substituted benzoate, the chromophoric L_a transitions, are represented by the thick lines. Positive chirality is defined by a clockwise twist between the transition moments as in **1**.

Exciton coupling



the right circularly polarized light more than the left one ($\Delta\epsilon < 0$). Therefore, the resulting CD curve, which is the summation of the two absorption bands with opposite signs, consists of a positive component at the longer wavelength side (first Cotton effect) and a negative component at the shorter wavelength side (second Cotton effect). Such a curve is defined as a "positive" exciton split CD or a positive CD couplet. On the other hand, a negative chirality, as in (1*R*,2*R*)-cyclohexanediol dibenzoate **2** (Fig. 1), leads to a negative CD couplet, i.e., negative first Cotton and positive second Cotton effects. Without exception, positive and negative chiralities afford positive and negative CD couplets, respectively. The absolute stereochemistry of conformationally rigid molecules, therefore, can be determined nonempirically by this method. The theoretical background of exciton chirality method¹⁶ and its applications to actual stereochemical problems of natural products¹⁷ have been described elsewhere.

With respect to conformationally flexible molecules, such as sphingosines, however, the analysis becomes more complicated. Because a chromophoric derivative of a flexible molecule gives a CD curve that is arising from a mixture of many conformers,¹⁸ it is not possible to unequivocally assign the stereochemistry by the sign of the CD couplet. However, for compounds with significant biologic importance, it is worthwhile to prepare a set of reference spectra for all possible stereoisomers, and use this library for the stereochemical assignment of the unknown. This alternative approach is still highly sensitive and does not require any authentic sample as reference. In addition to sphingosines and dihydrosphingosines,^{12,13} which are discussed below, CD reference curves for other classes of acyclic compounds, such as bacteriohopanoids,¹⁹ brassinosteroids,²⁰ and heptopyranosides/heptofuranosides,²¹ have also been prepared for such purposes.

¹⁸ N. Zhao, P. Zhou, N. Berova, and K. Nakanishi, *Chirality* **7**, 636 (1995).

¹⁹ N. Zhao, N. Berova, K. Nakanishi, M. Rohmer, P. Mougnot, and U. J. Jurgens, *Tetrahedron* **52**, 2777 (1996).

²⁰ A. Kawamura, N. Berova, K. Nakanishi, B. Voigt, and G. Adam, *Tetrahedron* **53**, 11961 (1997).

²¹ I. Akritopoulou-Zanze, K. Nakanishi, H. Stepowska, H. Grzeszczyk, B. Zamojski, and N. Berova, *Chirality* **9**, 699 (1997).

FIG. 2. Exciton coupling of two identical chromophores with positive chirality, as in **1** of Fig. 1. The red-shifted and blue-shifted bands show positive and negative circular dichroism, respectively. Summation of the two bands with opposite signs gives rise to the exciton coupled CD spectrum shown, which is defined as a positive CD couplet.

Application of Exciton Chirality Method to Sphingosine and Dihydrosphingosine

Sphingosine and dihydrosphingosine contain two stereogenic centers at the sites of the 2-amino and 3-hydroxyl groups, thus giving rise to a total of eight isomers: *D-erythro* (2*S*,3*R*), *L-threo* (2*S*,3*S*), *L-erythro* (2*R*,3*S*), and *D-threo* (2*R*,3*R*) of sphingosines and dihydrosphingosines (Fig. 3). All eight isomers can be differentiated by CD spectroscopy after appropriate chromophoric derivatization, i.e., 1,3-bisnaphthoate-2-*N*-naphthimido derivative, which can be prepared by a simple two-step reaction sequence (Fig. 4). Reference CD spectra for all isomers have already been prepared (Fig. 5).¹³ Therefore, the configuration of any sphingosine sample, natural or synthetic, can be checked by chemical derivatization, followed by CD

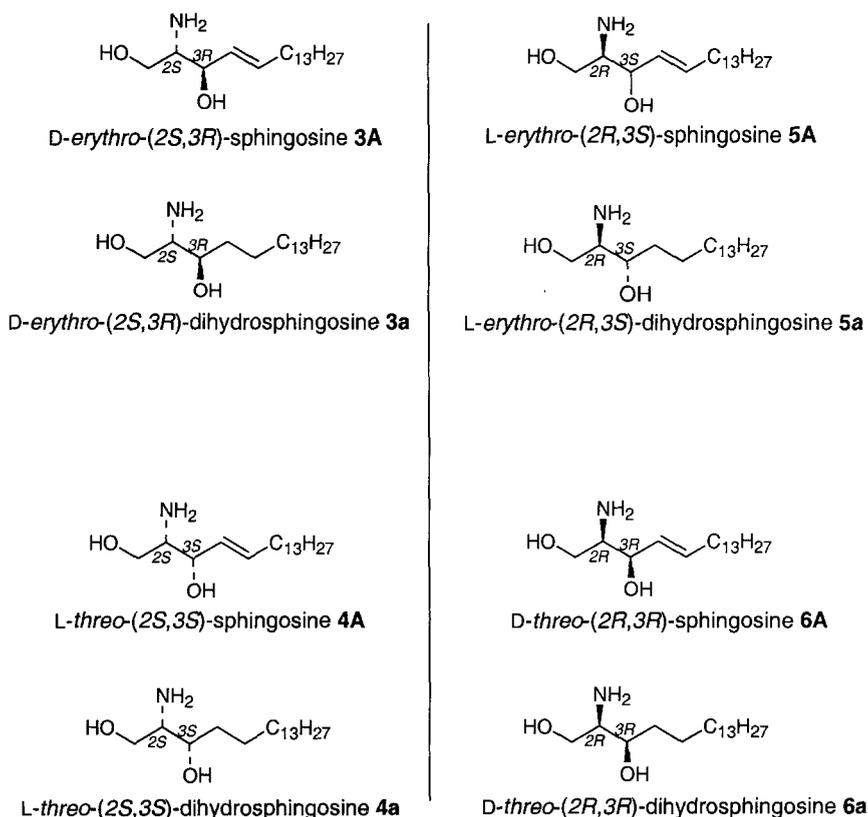


FIG. 3. Structures of sphingosines and dihydrosphingosines.

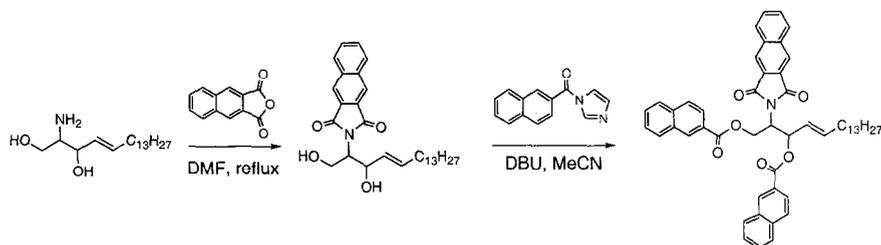


FIG. 4. Two-step chromophoric derivatization.

measurements. Note that the CD spectra of sphingosines and dihydrosphingosines are not the same, reflecting the difference in conformation of the unsaturated and saturated series (Fig. 5). The CD spectra of *L-erythro* and *D-threo* isomers are the mirror images of *D-erythro* and *L-threo* isomers, respectively.¹³ Because conformational distribution of the derivative depends on the solvent, each isomer was measured in two different solvents, acetonitrile (polar) and methylcyclohexane (nonpolar). The CD measurements in two different conditions ensure the stereochemical identity of unknown species in this analysis.

Selection of chromophores was the key to the successful differentiation of these structurally close isomers (Fig. 4).¹² Monochromophoric derivatization, such as perbenzoylation or pernaphthoylation, cannot clearly differentiate the isomers since all stereochemical information is condensed within a narrow UV range of the single chromophore. Differentiation becomes easier if the derivative contains several different chromophores so that stereochemical information is spread out over a wider absorption range. The naphthimide group was selected for the derivatization of the primary amino group at C-2 because of its intense UV band (ϵ 64,000, λ_{\max} 258 nm, acetonitrile) and fluorescence, both positive attributes for microscale manipulation. On the other hand, the 1,3-hydroxyl groups were derivatized to 2-naphthoates,²² also an intensely absorbing chromophore (ϵ 58,000, λ_{\max} 234 nm, acetonitrile) with an absorption maximum well separated from that of the naphthimide. The combination of the two different chromophores and the resulting complex chromophoric interactions made it possible to obtain characteristic CD curves covering the range of 200–320 nm for each isomer.

Two-Step Chromophoric Derivatization

Before the derivatization of sphingosine sample, one reagent, 2,3-naphthalenedicarboxylic acid anhydride, has to be prepared from the corre-

²² N. Ikemoto, L.-C. Lo, and K. Nakanishi, *Angew. Chem. Int. Ed. Engl.* **31**, 890 (1992).

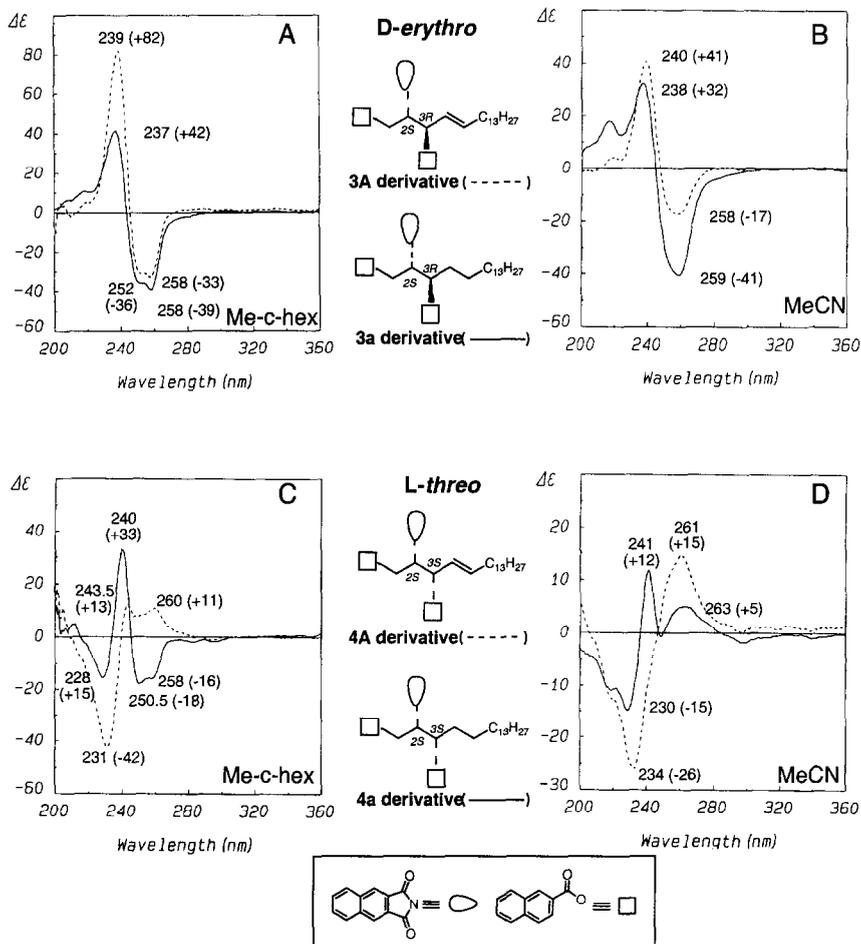


FIG. 5. CD spectra of 1,3-bisnaphthoate-2-N-naphthimide derivatives. (A) *D-erythro*-sphingosine (dotted line) and *D-erythro*-dihydrosphingosine (solid line) derivatives in methylcyclohexane. (B) *D-erythro*-sphingosine (dotted line) and *D-erythro*-dihydrosphingosine (solid line) derivatives in acetonitrile. (C) *L-threo*-sphingosine (dotted line) and *L-threo*-dihydrosphingosine (solid line) derivatives in methylcyclohexane. (D) *L-threo*-sphingosine (dotted line) and *L-threo*-dihydrosphingosine (solid line) derivatives in acetonitrile.

sponding diacid,²³ which is commercially available; all the other chemicals used in the derivatization can be obtained from commercial sources and used without further purification. 2,3-Naphthalenedicarboxylic acid (500 mg, 2.3 mmol) is placed in a dried 10-ml round-bottomed flask. Acetic

²³ B. H. Nicolet, J. A. Bender, F. C. Whitmore, and W. F. Singleton, in "Organic Synthesis" 2nd ed. (H. Gilman and A. H. Blatt, eds.), Coll. Vol. 1, p. 410. John Wiley & Sons, New York, 1941.

anhydride (2 ml, 21 mmol) is added, and the mixture is refluxed for 3 hr under inert gas (argon, nitrogen, etc.). The dark brown solution is cooled to room temperature. The crude product, which should be solidified by then, is collected by filtration with a Büchner funnel and washed with acetic acid and ether. The material is then dried at 100° overnight. The brownish anhydride is then purified by sublimation and stored in a desiccator.

The two-step derivatization is carried out as follows (Fig. 4). The first derivatization step, which uses the reagent prepared above, converts the 2-amino group into the naphthimide as follows. Pyridine was used as the solvent for this step in the previous report,¹³ but a subsequent study found that dimethylformamide (DMF) gives more reproducible results, possibly by preventing by-product formation. The sample sphingosine (50 μg , 0.16 μmol) and 2,3-naphthalenedicarboxylic acid anhydride (36 μg , 0.18 μmol , 1.1 equivalent) are placed in a dried 10-ml round-bottomed flask. The mixture is dissolved in anhydrous DMF (200 μl) and refluxed under inert gas for 12 hr.

After cooling to room temperature, the sample is dried under vacuum pumping, and purified by TLC (5 \times 20 cm, E. Merck silica gel 60 F-254, 250 μm). [Note: Wash the TLC plate before use with ethyl acetate since the purified product is extracted to ethyl acetate. The washing can be done by developing a new TLC plate (20 \times 20 cm) with ethyl acetate, which is then dried and cut into the appropriate size.] When hexane–ethyl acetate (1:1) is used as the mobile phase, R_f values of the *erythro* isomers are around 0.26, while that of the *threo* isomers are around 0.38. The product spot on the developed TLC plate, which is seen under the UV lamp, is scraped and extracted to ethyl acetate. The yield can be checked with the UV absorption (*N*-naphthimide, ϵ 64,000, λ_{max} 258 nm, acetonitrile). At least 50% yield (44 μg of the product) should be attainable after some practice.

The purified product, *N*-naphthimide, is then submitted to the second step, in which the 2-naphthoate group is attached to the 1,3-hydroxyl groups.²² *N*-Naphthimide (44 μg , 0.08 μmol) is placed in a dried 10-ml round-bottomed flask, dissolved in anhydrous acetonitrile (200 μl), 1-(2-naphthoyl)imidazole (200 μg , excess) is added, and a catalytic amount of DBU (1 drop of 10% DBU solution in anhydrous acetonitrile) is introduced. The mixture is stirred at room temperature for 1 hr. The solvent is evaporated and the final product is purified by TLC (5 \times 20 cm, E. Merck silica gel 60 F-254, 250 μm , prewashed with ethyl acetate). Hexane–ethyl acetate (3:1) gives R_f values of ca. 0.36 and 0.39 for the *erythro* and *threo* isomers, respectively. The product spot is scraped and extracted with ethyl acetate. The yield of this step should easily exceed 70% after some practice. The purified 1,3-bisnaphthoate-2-*N*-naphthimido derivative is then submitted to the CD measurements in acetonitrile (polar) and methylcyclohexane (nonpolar).

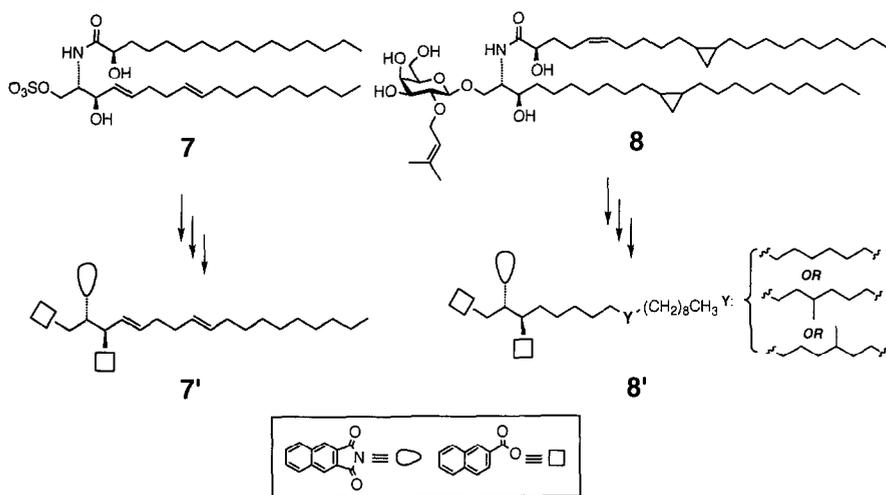


FIG. 6. Applications to unknown sphingolipids; a ceramide 1-sulfate **7** and a glycosphingolipid **8**. The sphingosine moieties of **7** and **8** were derivatized to the 1,3-bisnaphthoate-2-*N*-naphthimides, **7'** and **8'**, which gave CD curves close to those of *D*-erythro-sphingosine and *D*-erythro-dihydrosphingosine, respectively.

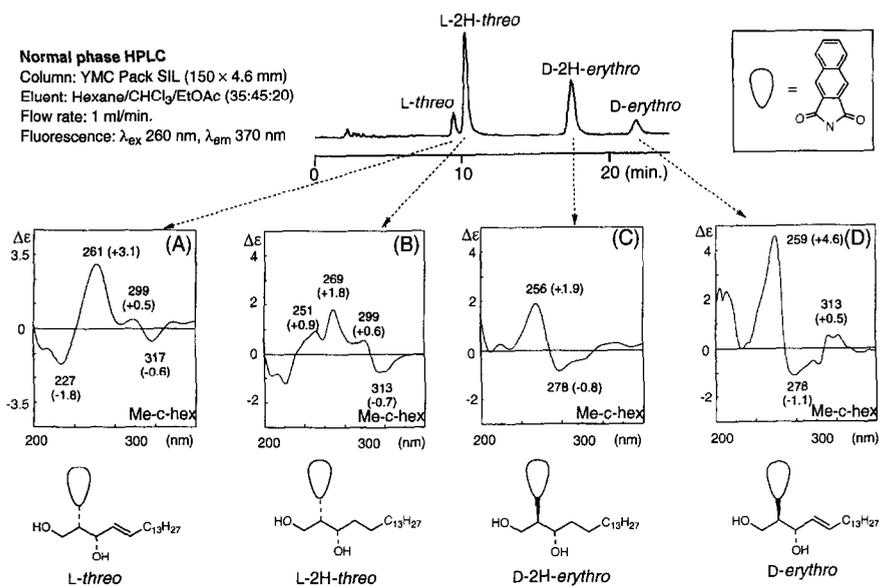


FIG. 7. Normal-phase HPLC separation of *N*-naphthimide derivatives, and their CD spectra in methylcyclohexane. (A) *L*-threo-Sphingosine derivative; (B) *L*-threo-dihydrosphingosine derivative; (C) *D*-erythro-dihydrosphingosine derivative; and (D) *D*-erythro-sphingosine derivative.

This CD method has been employed for the configurational assignment of several sphingolipids from marine sources (Fig. 6).^{14,15} The sphingosine moiety from a ceramide 1-sulfate **7** was converted into the 1,3-bisnaphthoate-2-*N*-naphthimido derivative **7'**, which gave a negative CD curve closely resembling that of the *D*-*erythro*-sphingosine derivative.¹⁴ Another sphingosine from a glycosphingolipid **8** was also derivatized to **8'**, which afforded a negative CD close to *D*-*erythro*-dihydrosphingosine derivative.¹⁵ It seems that the double bond at C-4 affects the conformational profiles around the chromophore attachment sites, which is reflected to the CD shapes of the sphingosine derivative and **7'**. On the other hand, the double bond at C-8 in **7'** and cyclopropyl group at C-11 in **8'** do not appear to affect the CD outcome.

Attempts to Lower the Scale of Stereochemical Analysis

In addition to the CD method, a high-performance liquid chromatography (HPLC) protocol has been developed for the stereochemical analysis of sphingosine and dihydrosphingosine.¹³ The sample material is derivatized to its *N*-naphthimide in this method, and analyzed by the sequential normal and chiral phase HPLC, which can differentiate all stereoisomers. The advantages of this HPLC protocol are that the method requires only one-step derivatization and the fluorescence detection of *N*-naphthimido derivative makes it possible to lower the analysis scale to the low nanogram level: Because each *N*-naphthimido derivative shows a weak but distinct CD curve, stereochemical assignment could also be done by CD spectroscopy provided the purified derivative is enough for the detection of the weak CD arising from the chiral environment surrounding the naphthimido group (Fig. 7). The drawbacks of the sequential HPLC protocol are that the method requires authentic derivatives for HPLC calibration; and the nanogram-level derivatization and purification requires experience handling small quantities of these materials.

There is an approach to improve the sensitivity of the exciton chirality method, in which fluorescence of chromophoric derivative is measured to obtain the CD curve, so-called fluorescence detected CD (FDCD).²⁴ Although FDCD has not been applied to sphingolipid analysis, it would theoretically lower the CD detection level to the nanogram scale. The limiting factor here, however, is not the sensitivity of FDCD, but the two-step derivatization, which is not easily scaled down to the nanogram level.

²⁴ J.-G. Dong, A. Wada, T. Takakuwa, K. Nakanishi, and N. Berova, *J. Am. Chem. Soc.* **119**, 12024 (1997).