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Nanogram scale absolute configurational assignment of ceramides by circular dichroism

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Abstract

Ceramides play a central role in cell regulation and a variety of signal transduction pathways. A simple sensitive microscale circular dichroic (CD) method utilizing zinc porphyrins has been developed for assigning the absolute configurations of ceramides at the nanogram scale. The assignment is based on the CD spectra of the bis (zinc porphyrin) derivatives of ceramides with and without the presence of 1,3-diaminopropane. This method can also be applied to various sphingolipids after enzymatic cleavage to ceramides.

The ceramides, cleavage products of various sphingolipids, including gangliosides and cerebrosides, are involved in various signal transduction pathways.[1] Many extracellular stresses, such as tumor necrosis factor-α (TNF-α) and human immunodeficiency virus (HIV)[2] have been shown to activate sphingomyelinases that release ceramides which inhibit cell growth and induce apoptosis.[3] Since the ceramide bioactivity is governed by its configuration,[4] a nanogram scale method for its determination becomes critical.

The absolute configuration assignment of ceramide presents a challenging stereochemical problem with its acylated amine, 1,3-diol moieties, and the flexible acyclic structure. The stereochemistry of ceramides has been determined by correlating with sphingosines after hydrolysis of the amide group, but this can lead to epimerization and rearrangements due to the labile allylic hydroxyl group.[5] Thus, the lack of a sensitive method for determining the absolute configuration of ceramides has been an obstacle in understanding the relationship between the stereochemistry and their extremely diverse bioactivities. A microscale method for absolute configurational assignments of ceramides is reported in the following.

The CD exciton chirality method has been used extensively to determine the absolute stereochemistry of organic molecules in solution.[6] The presence of two or more chirally oriented chromophores in a molecule gives rise to characteristic bisignate CD curves, the sign of which is determined by the absolute skewness of the interacting chromophores.[6] We have reported an HPLC/CD method for the determination

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of absolute configuration of sphingosines and dihydrosphingosines at the picomole scale.[7] However, this method is not applicable to ceramides since it requires derivatization of the primary amino group with 2,3-naphthalenedicarboxylic anhydride. We have, therefore, used zinc tetraphenylporphyrins, the versatile and powerful chromophores, for CD studies of ceramides. In addition to their intense red-shifted absorption, e.g., ε=440 000 λ_{max}=419 nm, in CH_2Cl_2, for 5-(4′-carboxymethylphenyl)-10,15,20-triphenylporphyrin), porphyrins tend to undergo π–π stacking which have been utilized in absolute configurational determinations of diamines, amino acids and α-hydroxy acids.[8] Zinc porphyrins can bind with amines and compounds containing bis (zinc porphyrins) have been shown to form 1:1 macrocyclic complexes with diamines.[9] In the case of bis (zinc porphyrin) ceramide derivatives, a bidentate diamine such as 1,3-diaminopropane might possibly lead to intramolecular bridging of two zinc porphyrins to form 1:1 macrocyclic complexes, and thus provide a new type of steric differentiation. It was conceivable that derivatizations of ceramides with zinc tetraphenylporphyrins might allow direct configurational assignment without conversion to sphingosines.

The zinc porphyrin ceramide derivatives, the D-*erythro*-C18-bisZnTPP-ceramide (**1b**) and L-*threo*-C18-bisZnTPP-ceramide (**3b**),[10] are obtained in 70% yield by reacting ceramides[11] with 5-(4′-carboxyphenyl)-10,15,20-triphenylporphyrin (TPP-OH)[12,13] 1-(3-(dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDC) and dimethylaminopyridine (DMAP) in $CHCl_3$, followed by zinc acetate (Fig. 1). Although normally performed at the mg scale, a 50 ng scale derivatization is sufficient for CD. Thus, a solution of 1.2 mg TPP-OH, 1 mg EDC and 0.1 mg DMAP in anhydrous 30 µL $CHCl_3$ was added to a 100 µL capillary tube containing 50 ng D-*erythro*-C18-ceramide **1a**. The tube was sealed and heated at 85°C overnight in a capillary melting point apparatus.[7a] After TLC purification, the product was treated with 1 mg zinc acetate in 0.5 mL MeOH overnight at rt. HPLC purification yielded the D-*erythro*-derivative **1b** in 64% yield. HPLC[14] allows separation of *erythro*- and *threo*-isomers and a check for diastereomeric purity.

The ceramide configurations can be assigned in the following manner from the CD shown in Fig. 1.

(i) Absolute configuration at C-3 (Fig. 1, I–IV). The D-ceramide derivatives **1b** and **4b** with 3*R* configuration yield negative CD couplets, e.g., D-*erythro*-ceramide **1b**: A=–219, while L-ceramide derivatives **2b** and **3b** with 3*S* configuration exhibit positive CD couplets, e.g., L-*threo*-ceramide **3b**: A=+110.

(ii) *Erythro/threo* stereochemistry at C-2/C-3. The CD amplitudes of *erythro* derivatives **1b/2b** (A=219) are two-fold those of *threo* derivatives **3b/4b** (A=110). The *erythro/threo* differentiation can be further supported by addition of 1,3-diaminopropane. With 20 equivalents of 1,3-diaminopropane, the signs of CD couplets of *erythro* derivatives **1b/2b** do not change but the amplitudes become much smaller, e.g., **1b**: A=-219→–40. In contrast, in the case of *threo* derivatives, the 1,3-diaminopropane addition induces sign inversion and doubling of amplitude, e.g., **3b**: A=+110→–193.

The correlation between the sign of the CD couplet and the C-3 stereochemistry can be accounted for by the stereoselective intramolecular π–π stacking of the two zinc porphyrins. The zinc porphyrin at C-1 would prefer stacking with the zinc porphyrin at C-3 from the side of the small group (*S*-group), i.e., the hydrogen, rather than from the large aliphatic chain (L-group) side for steric reasons. This leads to a counterclockwise twist between the transition dipole moments of the zinc porphyrins[8b,c] and a negative CD couplet (Fig. 2). Presence of the intramolecular π–π stacking between the two zinc porphyrins is proven by the split patterns of the ^{1}H NMR aromatic signals (Fig. 2), which is consistent with observations in other cases of stereoselective intramolecular porphyrin π–π stacking.[8b,c]

Addition of various acyclic α,ω-diamines, $H_2N(CH_2)_nNH_2$ (n=2–12), to bis (zinc porphyrin) ceramides **1b** and **3b** gave rise to changes in intensities and signs of the CD couplets, in addition to a ca. 10 nm red shift in Cotton effect extrema; of the various diamines tested, 1,3-diaminopropane gave the

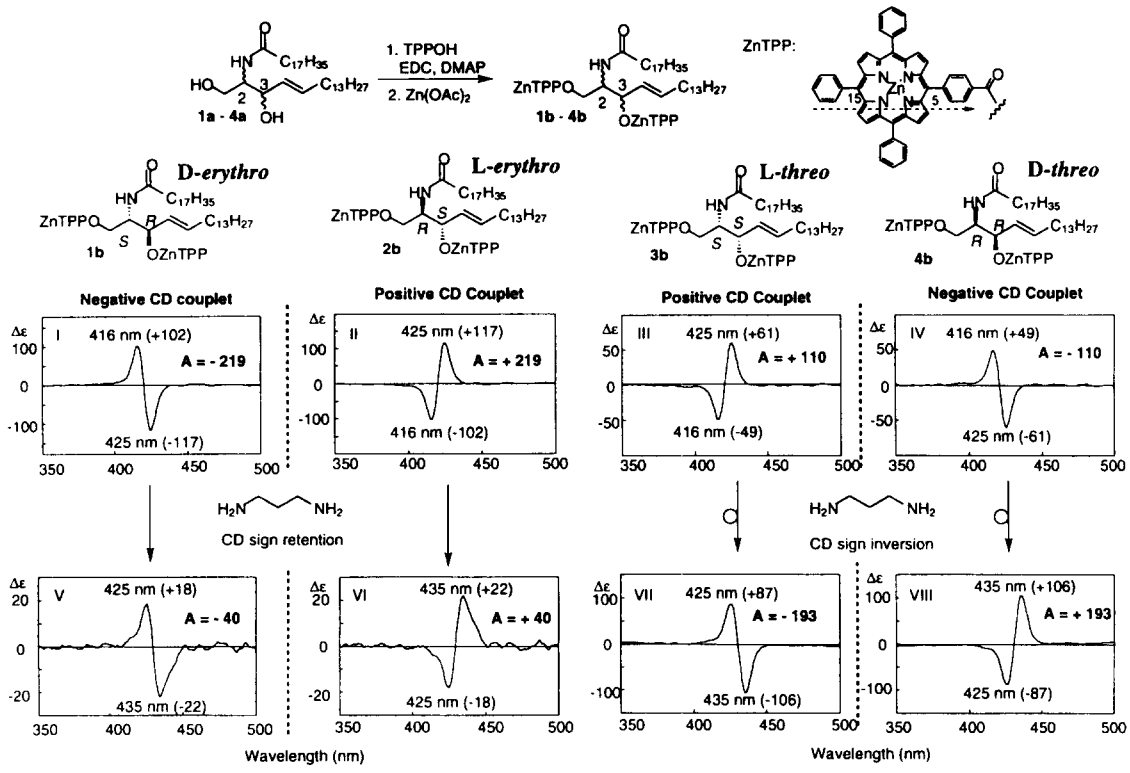

Figure 1. CD of D-*erythro*- and L-*threo*-ceramide derivatives **1b** and **3b**, in CH_2Cl_2, with/without 1,3-diaminopropane. CD of L-*erythro*- and D-*threo*-ceramides **2b** and **4b** (not measured) are presented as mirror images of those of **1b** and **3b**; the effective electric transition moment of ZnTPP is at the 5–15 direction shown by the dashed arrow

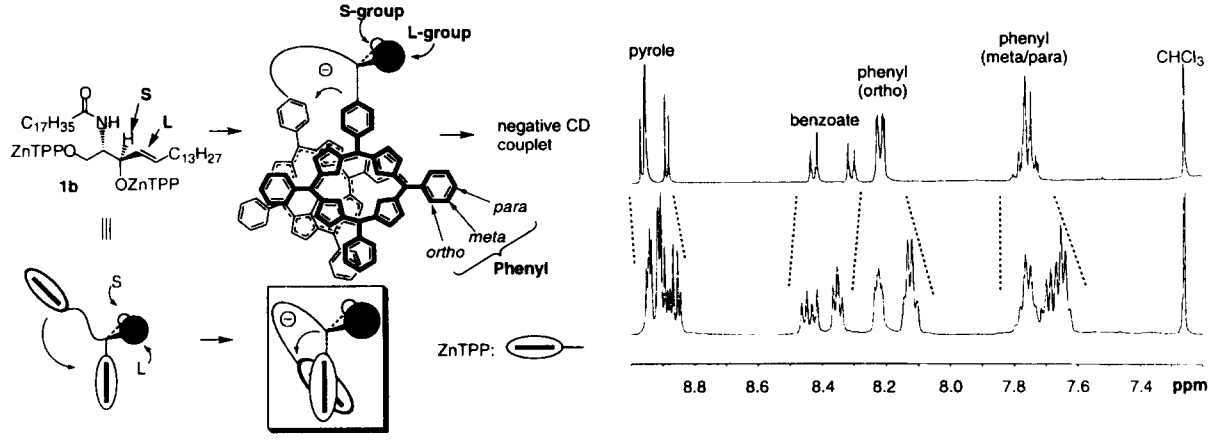

Figure 2. Intramolecular π–π stacking of two zinc porphyrins in D-*erythro*-ceramide derivative (**1b**) (the central nitrogens in the porphyrins are omitted for clarity). The aromatic signals of the ^{1}H NMR of **1b**, c=1×10^{-3} M in $CDCl_3$ (bottom spectrum), are split as opposed to those of 5-(4′-methylcarboxyphenyl)-10,15,20-triphenyl-zincporphyrin (top spectrum)

most pronounced effects (see Fig. 1). Through-space interaction between two intramolecularly bridged Zn-porphyrins instead of the original π–π stacking leads to the observed changes. Although the nature of these changes remains to be solved, the unexpected diamine-induced changes in the intensity and sign of exciton coupled CD turned out to be of great practical utility.

In summary, a nanogram scale protocol to determine the absolute configurations of ceramides has been developed. The length of the fatty acid on the amino group does not influence the CD as shown by the fact that the CD of bis (zinc porphyrin) C12 ceramides (data not shown) are identical to those of bis (zinc porphyrin) C18 ceramides. The present method can be used to re-examine absolute configurations of ceramides and re-evaluate their bioactivities. This is particularly the case where stereochemical discrepancies have been noted between endogenous ceramides and the corresponding sphingosines.[15] The method should also be applicable when determining the absolute configurations of ceramides obtained upon enzymatic degradation of glycosphingolipids, sphingomyelines, and gangliosides.[1c] Application of the protocol to ceramides isolated from various cell lines and tissues is underway.

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10. Compound **1b**: TLC (2% MeOH/CH_2Cl_2) R_f: 0.6; UV–vis (CH_2Cl_2): 548 (27 000), 419 (914 000); ^{1}H NMR (400 MHz, $CDCl_3$:CD_3OD 1:1): δ 0.65–1.78 (several m, 58 H), 2.18 (m, 1H), 2.33 (m, 1H), 4.78 (dd, *J*=7.3, 11.4, 1H), 4.84 (dd, *J*=4.4, 11.4 Hz, 1H), 4.97 (m, 1H), 5.18 (m, 1H), 5.80 (dd, *J*=7.0, 15.4 Hz, 1H), 5.94 (t, *J*=7.0 Hz, 1H), 6.15 (m, 1H), 7.57–7.75 (several m, 18H), 8.05–8.20 (several m, 12H), 8.34 (t, *J*=7.6 Hz, 4H), 8.47 (t, *J*=7.6 Hz, 4H), 8.71–8.85 (m,

16H). FABMS (*m/z*): 1974 ($M+H^+$). Compound **3b**: TLC (2% MeOH/CH_2Cl_2) R_f: 0.5; UV–vis (CH_2Cl_2): 548 (27 000), 419 (914 000); ^1H NMR (400 MHz, $CDCl_3$:CD_3OD 1:1): δ 0.65–1.78 (several m, 58 H), 2.19 (m, 1H), 2.33 (t, *J*=7.4 Hz, 1H), 4.70 (m, 2H), 4.95 (m, 1H), 5.79 (dd, *J*=7.6, 15.3 Hz, 1H), 5.98 (dd, *J*=7.6, 7.5 Hz, 1H), 6.17 (m, 1H), 7.57–7.75 (several m, 18H), 8.05–8.20 (several m, 12H), 8.34 (t, *J*=7.6 Hz, 4H), 8.47 (t, *J*=7.6 Hz, 4H), 8.71–8.85 (m, 16H). FABMS (*m/z*): 1974 ($M+H^+$).

11. The ceramides **1a** and **3a** were prepared in around 95% yield from the corresponding commercially available sphingosines (Matreya Inc.) treated with stearoyl chloride and triethylamine in CH_2Cl_2.
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13. The TPP-OH will be commercially available from TCI America.
14. Retention times: 20 min for the D-*erythro*-C18-bisZnTPP-ceramide (**1b**) and 23 min for the L-*threo*-C18-bisZnTPP-ceramide (**3b**) under the following conditions: column: YMC-Pack Sil, 150×4.6 mm I.D.; solvent system: 0–5 min 0.5% MeOH/CH_2Cl_2, 5–25 min MeOH/CH_2Cl_2, 25–30 min MeOH/CH_2Cl_2; UV–det: 418 nm; Flow: 1 mL/min.
15. Hannun, Y. A. and Bielawska, A. E., personal communication.