methods

New methods for determining the enantiomeric purity of *erythro*-sphingosine

Shengrong Li, William K. Wilson,[1] and George J. Schroepfer, Jr.[2]

Departments of Biochemistry and Cell Biology and of Chemistry, Rice University, 6100 Main Street, Houston, TX 77005-1892

Abstract The enantiomeric purity of *erythro*-sphingosine samples can be determined simply, reliably, and accurately from ^{1}H or ^{19}F nuclear magnetic resonance spectra of the α-methoxy-α-(trifluoromethyl)phenylacetate (MTPA) derivative. As little as 0.1% of the minor enantiomer could be observed in a 1-mg sample, and detection limits of 1% and 5% were estimated for samples of 100 μg and 10 μg. The two *threo*-sphingosine enantiomers and four dihydrosphingosine stereoisomers were also differentiated by this technique, which served as an effective method for assessing the purity of sphingosine and dihydrosphingosine samples. Enantiomeric and diastereomeric purities could also be determined by normal-phase high performance liquid chromatographic analysis of the MTPA derivatives.—Li, S., W. K. Wilson, and G. J. Schroepfer, Jr. **New methods for determining the enantiomeric purity of *erythro*-sphingosine. *J. Lipid Res.* 1999.** 40: **764–772.**

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Sphingosine and other long-chain bases are the defining elements of sphingolipids, a complex and diverse class of lipids of increasing interest in biology and medicine. A large number of studies have demonstrated that the free sphingolipid bases and their 1-phosphate and *N*-acyl derivatives have important and varied biological activities (1–9). Investigations of the biological actions of these compounds require samples of defined structure and purity. d-*Erythro*-sphingosine (**1a**; **Fig. 1**) has traditionally been isolated from natural sources (10, 11), but total synthesis has become an important alternative (12–17 and references therein). A potential problem with total synthesis is the formation of stereoisomeric mixtures. Diastereomeric impurities can generally be detected by thin-layer chromatography (TLC), gas chromatography (GC), or nuclear magnetic resonance (NMR) spectroscopy (10) and removed by chromatographic methods. Enantiomeric impurities, by contrast, are difficult to remove and to detect.

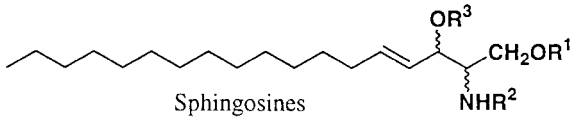
Optical rotation, the classical method for measuring the enantiomeric purity of sphingosine samples, suffers from low precision and numerous potential sources of error (18). These problems are exacerbated by the low specific rotation of sphingosine and its *N*-acetate and triacetate derivatives (10). Circular dichroism spectra, from which sphingosine and dihydrosphingosine isomers can be identified (19), would be subject to similar (albeit fewer) sources of error for determining enantiomeric purity. A simple and generally reliable nonoptical method is NMR analysis with chiral lanthanide shift reagents (20, 21), which have been used to measure the enantiomeric purity of an *N*,*O*-protected sphingosine (16). However, we did not observe *any* splitting of ^{1}H NMR signals at 500 MHz upon addition of $Pr(hfc)_3$ to racemic sphingosine (**1a** and **2a**) in $CDCl_3$. Another important method for measuring enantiomeric purity is HPLC on chiral phases (22). The *N*-benzoyl derivatives of the four dihydrosphingosine isomers **5a–8a** have been resolved by connecting two different chiral HPLC columns in tandem (23), and a CHIRALCEL OD column resolved enantiomeric pairs of the *N*-naphthimido derivatives of sphingosine and dihydrosphingosine (19). However, the promise of good separations is offset by the need to acquire chiral columns and the effort of validating the separation with authentic standards.

Chiral derivatizing agents are the basis of another popular method for measuring enantiomeric purity (21, 24). (*S*)-α-Methoxy-α-(trifluoromethyl)phenylacetate (MTPA) derivatives of chiral alcohols and amines are diastereomeric mixtures that can be analyzed by NMR or by GC or HPLC on achiral columns. NMR analysis of mono-MTPA derivatives has been used to determine the enantiomeric purity of model compounds (15) and synthetic intermediates (14) in total syntheses of sphingosine. For simplicity,

Abbreviations: COSY-DQF, ^{1}H-^{1}H correlation spectroscopy with double-quantum filtering; GC, gas chromatography; HMBC, heteronuclear multiple bond correlation; HPLC, high performance liquid chromatography; HSQC, heteronuclear single-quantum coherence; MTBE, methyl *tert*-butyl ether; MTPA, α-methoxy-α-(trifluoromethyl)phenylacetate; NMR, nuclear magnetic resonance (spectroscopy); $Pr(hfc)_3$, tris(3-(heptafluoropropylhydroxymethylene)-(+)-camphorato) praseodymium(III); TLC, thin-layer chromatography.

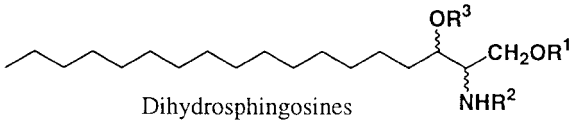
[1] To whom correspondence should be addressed.

[2] Senior author; deceased December 11, 1998.



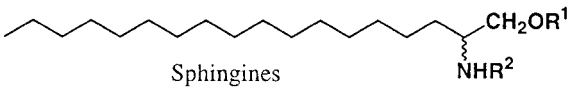
Sphingosines

D-*erythro*	**1a**	(2S, 3R)	$R^1 = R^2 = R^3 = H$
D-*erythro*	**1b**	(2S, 3R)	$R^1 = R^2 = R^3 = X$
L-*erythro*	**2a**	(2R, 3S)	$R^1 = R^2 = R^3 = H$
L-*erythro*	**2b**	(2R, 3S)	$R^1 = R^2 = R^3 = X$
L-*threo*	**3a**	(2S, 3S)	$R^1 = R^2 = R^3 = H$
L-*threo*	**3b**	(2S, 3S)	$R^1 = R^2 = R^3 = X$
D-*threo*	**4a**	(2R, 3R)	$R^1 = R^2 = R^3 = H$
D-*threo*	**4b**	(2R, 3R)	$R^1 = R^2 = R^3 = X$



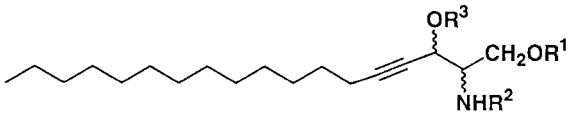
Dihydrosphingosines

D-*erythro*	**5a**	(2S, 3R)	$R^1 = R^2 = R^3 = H$
D-*erythro*	**5b**	(2S, 3R)	$R^1 = R^2 = R^3 = X$
L-*erythro*	**6a**	(2R, 3S)	$R^1 = R^2 = R^3 = H$
L-*erythro*	**6b**	(2R, 3S)	$R^1 = R^2 = R^3 = X$
L-*threo*	**7a**	(2S, 3S)	$R^1 = R^2 = R^3 = H$
L-*threo*	**7b**	(2S, 3S)	$R^1 = R^2 = R^3 = X$
D-*threo*	**8a**	(2R, 3R)	$R^1 = R^2 = R^3 = H$
D-*threo*	**8b**	(2R, 3R)	$R^1 = R^2 = R^3 = X$

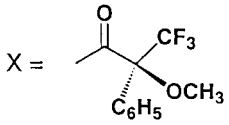


Sphingines

9a	(2S)	$R^1 = R^2 = H$
9b	(2S)	$R^1 = R^2 = X$
10a	(2R)	$R^1 = R^2 = H$
10b	(2R)	$R^1 = R^2 = X$



D-*erythro*	**11a**	(2S, 3R)	$R^1 = R^2 = R^3 = H$
D-*erythro*	**11b**	(2S, 3R)	$R^1 = R^2 = R^3 = X$
L-*erythro*	**12a**	(2R, 3S)	$R^1 = R^2 = R^3 = H$
L-*erythro*	**12b**	(2R, 3S)	$R^1 = R^2 = R^3 = X$
L-*threo*	**13a**	(2S, 3S)	$R^1 = R^2 = R^3 = H$
L-*threo*	**13b**	(2S, 3S)	$R^1 = R^2 = R^3 = X$
D-*threo*	**14a**	(2R, 3R)	$R^1 = R^2 = R^3 = H$
D-*threo*	**14b**	(2R, 3R)	$R^1 = R^2 = R^3 = X$



(S)-MTPA derivative

Fig. 1. Structures of sphingosine and dihydrosphingosine isomers, their (*S*)-MTPA derivatives, and related sphingolipid bases described herein.

we sought to apply this methodology by derivatizing sphingosine as the free base. Despite the potential complications of multiple MTPA groups, we easily converted sphingosine to its tris-MTPA derivative, and NMR analysis furnished accurate enantiomeric purities of *erythro*-sphingosine samples. Presented herein are the particulars of this simple and robust method and its extension to other sphingosine and dihydrosphingosine stereoisomers and to HPLC analyses.

EXPERIMENTAL PROCEDURES AND RESULTS

Materials and methods

d-*Erythro*-sphingosine (**1a**), l-*threo*-sphingosine (**3a**), and d-*erythro*-dihydrosphingosine (**5a**) were isolated from cow brain as described previously (10, 11) and showed high purity by TLC and ^{1}H NMR. l-*Erythro*-sphingosine (**2a**) was purchased from Sigma (St. Louis, MO) and showed ⩾99% purity by NMR as the MTPA derivative **2b**. dl-*Erythro*-sphingosine (**1a**, **2a**; analyzed by NMR as a 40:40:10:10:10:10:1:1 mixture of MTPA derivatives **1b**, **2b**, **3b**, **4b**, **11b**, **12b**, **13b**, and **14b**), dl-*threo*-sphingosine (**3a**, **4a**; analyzed by NMR as a 5:5:2:2 mixture of MTPA derivatives **3b**, **4b**, **13b**, and **14b**), dl-*erythro*-dihydrosphingosine (**5a**, **6a**; ~98% purity), and dl-*threo*-dihydrosphingosine (**7a**, **8a**; ~98% purity) were gifts from H. E. Carter, who received these materials in 1963 from Ciba Ltd. (Basel, Switzerland). This collection of dl mixtures, which included dl-*erythro*-2-amino-4-octadecyne-1,3-diol (**11a**, **12a**; analyzed by NMR as a 8:8:1:1 mixture of MTPA derivatives **11b**, **12b**, **13b**, and **14b**), was evidently synthesized by the methods of Grob and coworkers (25, 26). (2*S*)-Sphingine (**9a**, (2*S*)-2-aminooctadecan-1-ol) and dl-sphingine (**9a**, **10a**) were also gifts from H. E. Carter, who elucidated the structure of sphingine (27). The MTPA derivative of l-*threo*-dihydrosphingosine (**7b**) was prepared by hydrogenation of **3b** overnight over 10% palladium on carbon in tetrahydrofuran, followed by removal of the catalyst by filtration, evaporation to dryness, and purification of the residue on silica gel (30 × 5 mm column; elution with ethyl acetate–hexane 3:7 (5 ml)); NMR analysis showed a 1:3 mixture of **7b** and (2*S*)-sphingine (**9b**). Analogous hydrogenation of the MTPA derivative of d-*erythro*-sphingosine (**1b**) gave a 2:3 mixture of **5b** and **9b**. Similar hydrogenolysis during catalytic hydrogenation of d-*erythro*-sphingosine triacetate has been reported previously (27). (*S*)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride (98% ee) and tris(3-(heptafluoropropylhydroxymethylene)-(+)-camphorato) praseodymium(III) (Pr(hfc)$_3$) were purchased from Aldrich (Milwaukee, WI).

NMR spectra were measured on dilute $CDCl_3$ solutions with a Bruker AMX500 or AC250 spectrometer and referenced to internal tetramethylsilane at 0 ppm (^{1}H), $CDCl_3$ at 77.0 ppm (center line; ^{13}C), or $CFCl_3$ at 0 ppm (downfield line; ^{19}F). Spectra on the AMX500 were acquired on an inverse-detection probe at 25°C; spectra on the AC250 were obtained on the inner coil of a normal-geometry probe at ~22°C. Enantiomeric and diastereomeric purities were estimated by integration of spectra obtained with 0.5-Hz line broadening. Most 1D spectra were acquired for 5 min (128 scans) in regular 5-mm tubes, but 1–10 μg samples were acquired for 20–30 min in Shigemi tubes (also used for most 2D spectra). COSY-DQF (^{1}H-^{1}H correlation spectroscopy with double-quantum filtering), HSQC (heteronuclear single-quantum coherence), and HMBC

(heteronuclear multiple bond correlation) spectra were used to confirm structures and establish signal assignments; linear prediction was used sparingly to improve resolution in f_1. Chemical shifts measured to ±0.001 ppm precision were corrected for effects of strong coupling. No decomposition of the MTPA derivatives in $CDCl_3$ (freshly filtered through basic alumina) was observed, even upon storage for over 2 weeks (mainly at −20°C). Analytical TLC was performed using aluminum-backed silica gel 60 F_{254} plates (EM Science, Gibbstown, NJ). TLC plates were charred by spraying with 5% ammonium molybdate in 10% sulfuric acid followed by heating for 5 min at 80°C. HPLC was carried out on 5-μm Alltima C_{18} or silica columns (250 mm × 4.6 mm; 100 Å pore size) from Alltech Associates (Deerfield, IL) with UV detection at 210 nm. Normal-phase HPLC was done with an in-line solvent degasser.

Preparation of (*S*)-MTPA derivatives of sphingosine and dihydrosphingosine

(*S*)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride (30 μl, 160 μmol) was added to a solution of sphingosine or dihydrosphingosine (2 mg, 6.7 μmol) in pyridine (100 μl) followed by shaking for 5 min to mix the reagents. The mixture was left overnight at room temperature, quenched with water (2 ml), and extracted with dichloromethane (3 × 1.5 ml). The combined extracts were washed with water (1 ml) and brine (1 ml), dried over anhydrous sodium sulfate, and evaporated to a residue that was passed through silica gel (30 × 5 mm column; elution with ethyl acetate–hexane 3:7 (5 ml)). The eluate was evaporated to dryness in a stream of nitrogen and further dried in vacuo to a colorless oil. Less MTPA reagent was used for smaller reactions (10 μl for ≤100 μg sphingosine; 20 μl for 1 mg sphingosine). TLC on silica gel (ethyl acetate–hexane 3:7) showed a slight separation of some sphingosine and dihydrosphingosine isomers (R_f 0.46–0.52).

Analysis of D- and L-*erythro*-sphingosine by NMR

(*S*)-MTPA derivatives of d-*erythro*-sphingosine (**1b**), l-*erythro*-sphingosine (**2b**), and various mixtures thereof were analyzed by ^1H NMR at 250 and 500 MHz and by ^{19}F NMR at 235 MHz. ^1H and ^{19}F NMR chemical shifts and ^1H-^1H coupling constants are summarized in **Table 1**. Mixtures carefully prepared to have 100:30, 100:10, 100:5, and

TABLE 1. ^1H and ^{19}F NMR chemical shifts and ^1H-^1H coupling constants of (*S*)-MTPA derivatives of d-*erythro*-sphingosine and related sphingolipid bases[a–c]

	Sphingosine Isomers				Dihydrosphingosine Isomers			
	d-*erythro* **1b**	l-*erythro* **2b**	l-*threo* **3b**	d-*threo* **4b**	d-*erythro* **5b**	l-*erythro* **6b**	l-*threo* **7b**	d-*threo* **8b**
^1H chemical shifts (δ)								
H-1a	4.365	4.153	4.158	4.184	4.217	4.045	4.058	4.070
H-1b	4.387	4.342	4.300	4.261	4.366	4.420	4.161	4.134
H-2	4.529	4.474	4.462	4.475	4.485	4.468	4.498	4.489
H-3	5.451	5.42	5.483	5.514	5.161	5.196	5.096	5.197
H-4	5.225	5.41	5.164	5.262	1.48	1.69	1.47	1.67
H-5	5.776	5.960	5.736	5.808				
H-6	1.90	2.03	1.89	1.97				
N-H	6.96	6.96	6.96	7.13	6.94	7.14	6.85	7.11
1-OCH_3[d]	3.516	3.424	3.519	3.429	3.517	3.427	3.527	3.467
2-OCH_3	3.186	3.237	3.292	3.274	3.222	3.232	3.345	3.265
3-OCH_3[d]	3.423	3.517	3.464	3.473	3.420	3.545	3.489	3.476
^{19}F chemical shifts (δ)								
CF_3	−71.91	−71.97	−71.87	−72.03	−71.94	−72.05	−71.95	−71.88
CF_3	−69.48	−69.30	−69.42	−69.19	−69.23	−69.31	−69.35	−69.32
CF_3	−71.73	−71.60	−71.72	−71.90	−71.35	−71.16	−71.43	−71.65
^1H-^1H coupling constants (Hz)								
1a–1b	11.8	11.7	11.2	11.3	11.8	11.7	11.1	11.2
1a–2	4.7	4.6	5.3	5.1	5.5	6.0	6.2	5.9
1b–2	3.8	3.4	7.0	6.5	3.6	3.7	8.3	7.8
2–3	6.9	7.3	3.4	4.5	6.0	5.7	1.4	2.4
2-NH	9.2	9.3	9.3	9.0	9.2	9.3	9.6	9.3
3–4	7.8	~7†	8.2	8.0	7.3, 4.6	6.0, 6.0	7.7, 6.4	7.0, 7.0
4–5	15.4	15†	15.4	15.4				
5–6	6.9, 6.9	7†, 7†	6.8, 6.8	6.7, 6.7				

[a] ^1H NMR data obtained at 500 MHz in 5–15 mm $CDCl_3$ solution at 25°C and referenced to $Si(CH_3)_4$; ^{19}F NMR data obtained at 235 MHz at ~22°C and referenced to $CFCl_3$. Chemical shifts given to two (three) decimal places are accurate to ±0.01 (±0.001) ppm and were corrected for effects of strong coupling; samples containing excess water or ammonia showed larger variations, especially for the NH group. Essentially identical ^1H chemical shifts were obtained at 250 MHz at ~22°C after corrections for strong coupling. No stereochemical assignments are given for the C-1 protons (H-1a and H-1b).

[b] ^1H-^1H coupling constants marked by † are of low accuracy (±0.5 Hz). ^1H methoxy signals were distorted quartets ($^5J_{HF}$ 1.4 Hz for 2–MTPA and 1.2 Hz for 1– and 3–MTPA groups). ^{19}F signals appeared as broad singlets attributable to 5-bond couplings to CH_3 and aromatic protons.

[c] Sphingolipid base derivatives **1b–14b** also showed: δ 7.5–7.3 (m), 1.26 (br s), 0.880 (br t, 7 Hz). Olefinic compounds **1b–4b** showed allylic couplings of 1–1.5 Hz for H3–H5, H4–H6a, and H4–H6b.

[d] Assignments of 1– and 3–MTPA methoxy signals are uncertain and may be interchanged.

100:1 ratios of **1b** and **2b** showed ratios of 100:30.8, 100: 9.6, 100:4.9, and 100:1.1 by ^{1}H NMR and 100:31.2, 100:9.0, 100:4.4, 100:1.0 by ^{19}F NMR analysis of the MTPA derivatives. Methoxy (^{1}H) and trifluoromethyl (^{19}F) regions of the NMR spectra of the 100:30 and 100:1 mixtures together with spectra of the pure d- and l-*erythro*-sphingosine isomers are presented in **Fig. 2**. ^{13}C satellites in these spectra were distinguished from trace impurities by their intensities (0.55%) and coupling constants (290 Hz in ^{19}F spectra and 145 Hz for methoxy signals in ^{1}H spectra). Conversion of small quantities (1 mg, 100 μg, 10 μg, and 1 μg) of d-*erythro*-sphingosine to the MTPA derivative followed by ^{1}H NMR analysis at 500 MHz indicated detection limits for the minor isomer of 0.1%, 1%, 5%, and 20%, respectively.

The 250- and 500-MHz ^{1}H NMR spectra showed chemical shift nonequivalence (Δδ) of ~0.05 ppm for the upfield pair of methoxy signals, with peak widths at 2% height of 13 Hz (0.5 Hz line broadening), 10 Hz (no line broadening), or 7 Hz (mild Gaussian apodization). The trifluoromethyl signals of the 235-MHz ^{19}F NMR spectra showed Δδ values of ~0.2 ppm and corresponding peak widths of 23, 17, and 12 Hz. The ^{1}H methoxy and ^{19}F trifluoromethyl peaks were broadened by their mutual 1.3-Hz long-range ^{19}F–^{1}H coupling. Even at 250 MHz (or 235 MHz), the methoxy and trifluoromethyl peak separations were adequate for quantitative measurements. Certain ^{1}H NMR signals of the sphingolipid moiety (Table 1) showed good chemical shift nonequivalence (Δδ 0.2 ppm), but signal overlap and lower intensities made them less suitable for quantitative measurements.

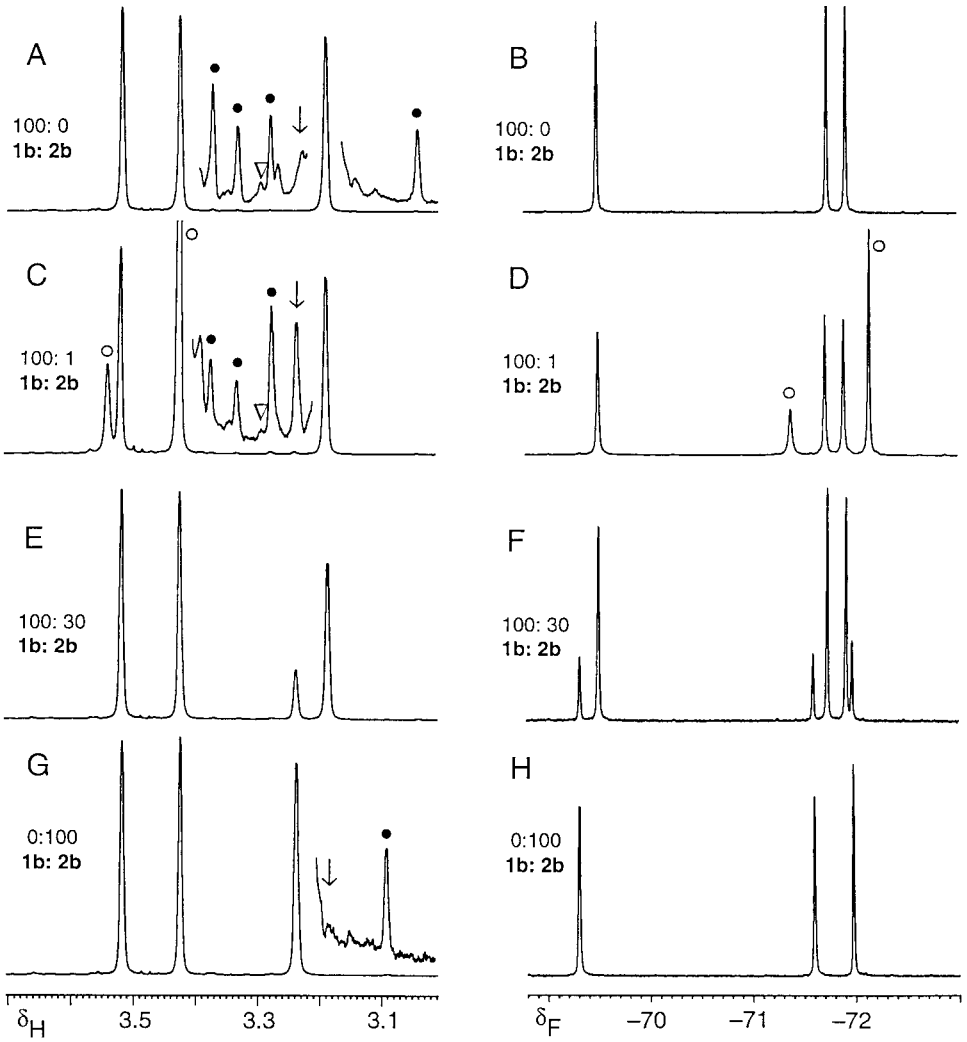


Fig. 2. ^{1}H and ^{19}F NMR spectra of (*S*)-MTPA derivatives of d-*erythro*-sphingosine (**1b**) (panels A and B), l-*erythro*-sphingosine (**2b**) (panels G and H), a 100:1 mixture of **1b** and **2b** (panels C and D), and a 100:30 mixture of **1b** and **2b** (panels E and F). ^{1}H and ^{19}F NMR spectra were acquired at 500 and 235 MHz, respectively, and processed with 0.5-Hz line broadening (2-Hz line broadening for panel D). Signals of trace enantiomeric impurities, l-*threo*-sphingosine derivatives, and ^{13}C satellites are marked by arrows, triangles, and closed circles (↓, ▽, and ●), respectively. The large signal at δ_H 3.42 in panel C represents a non-sphingolipid byproduct of derivatization coincident with one of the methoxy peaks of **1b** and has a correspondingly large ^{13}C satellite at δ_H 3.28. Other extraneous signals, marked by open circles (○), appear in panels C and D at δ_F −71.4, −72.2 and δ_H 3.53.

Analysis of other sphingosine and dihydrosphingosine isomers by NMR

^{1}H and ^{19}F NMR spectra of (*S*)-MTPA derivatives of l-*threo*-sphingosine (**3b**), dl-*threo*-sphingosine (**3b**, **4b**), d-*erythro*-dihydrosphingosine (**5b**), dl-*erythro*-dihydrosphingosine (**5b**, **6b**), and dl-*threo*-dihydrosphingosine (**7b**, **8b**), a 1:3 mixture of l-*threo*-dihydrosphingosine (**7b**) and (2*S*)-sphingine (**9b**), a 2:3 mixture of d-*erythro*-dihydrosphingosine (**5b**) and (2*S*)-sphingine (**9b**), (2*S*)-sphingine (**9b**), and dl-sphingine (**9b**, **10b**) furnished chemical shifts and coupling constants for compounds **3b–8b** (Table 1) and **9b** and **10b** (see below). The methoxy and trifluoromethyl regions of dl mixtures of *threo*-sphingosine and *erythro*- and *threo*-dihydrosphingosine are shown in **Fig. 3**. In each case, at least one diastereomeric pair of methoxy or trifluoromethyl signals of the MTPA derivatives was resolved in an open spectral region. Of the 24 methoxy signals and 24 trifluoromethyl signals of the sphingosine and dihydrosphingosine isomers, the upfield set of 8 methoxy signals and the mid-field set of 8 trifluoromethyl signals showed the best peak dispersion. Isomer ratios could be measured from several ^{1}H NMR signal pairs (Table 1), but the ^{1}H methoxy and ^{19}F trifluoromethyl signals gave the best combination of high sensitivity and good peak dispersion (chemical shift nonequivalence of ~0.05 and ~0.2 ppm, respectively) despite the long-range ^{19}F–^{1}H coupling of 1.3 Hz that broadened the ^{1}H methoxy ($W_{0.5}$ 3 Hz) and ^{19}F trifluoromethyl ($W_{0.5}$ 4 Hz) peaks. Peak dispersion and sensitivity may be improved by increasing the NMR field strength or (more effectively) by use of decoupling to remove the long-range ^{19}F–^{1}H couplings.

Additional NMR signal assignments for compounds 1b–14b

^{1}H NMR signal assignments[3] were made from COSY-DQF spectra and by matching coupling constants from different resonances. Unequivocal assignments for each methoxy (^{1}H) and each trifluoromethyl (^{19}F) signal could not be established from 2D spectra, but chemical shift patterns among **1b–14b**, including comparisons with NMR data for MTPA derivatives of amines and alcohols (24) and sphingines **9b** and **10b** (see below), suggested that the upfield ^{1}H signals (δ 3.1–3.3) and downfield ^{19}F signals correspond to the 2–MTPA group and that signals near δ_F −72 belong to the 1–MTPA. Further comparisons suggested δ_H 3.52 and 3.43 for the 1–MTPA methoxy signals of the 2*S* and 2*R* isomers, respectively. ^{13}C NMR assignments for **1b** were determined from HSQC and HMBC spectra and chemical shift comparisons (10): δ_C 166.28 (1–C=O), 166.18 (2–C=O), 165.15 (3–C=O); 140.54 (C–5); 131.75, 131.68, 129.87, 129.73, 129.60, 128.68, 128.61, 128.47 (aromatic); 127.64, 127.28, 127.20 (aromatic, J_{CF} 1.4 Hz); 122.22 (C–4); 84.8, 84.6, 83.9 (1–MTPA, 3–MTPA, and 2–MTPA quaternary carbons, J_{CF} ca. 30 Hz); 74.88 (C–3), 64.03 (C–1), 55.46 (δ_H 3.516), 55.41 (δ_H 3.423), 54.73 (δ_H 3.186), 50.16 (C–2), 32.21, 31.91, 29.67, 29.66 (2C), 29.64, 29.55, 29.44, 29.34, 29.16, 28.55, 22.68, 14.10. NMR data for **9b**: δ_H 6.69 (d, 8.9 Hz, NH), 4.352 (dd, 11.3, 3.9 Hz, H–1), 4.393 (dd, 11.3, 3.8 Hz, H–1), 4.251 (m, H–2), 3.531 (q, 1.3 Hz, OCH_3), 3.285 (q, 1.3 Hz, OCH_3), 1.48 (m, H–3); δ_F −72.00, −69.30. NMR data for **10b**: δ_H 6.84 (d, 8.9 Hz, NH), 4.372 (dd, 11.3, 4.0 Hz, H–1), 4.359 (dd, 11.3, 3.6 Hz, H–1), 4.25 (m, H–2), 3.431 (q, 1.3 Hz, OCH_3), 3.295 (q, 1.3 Hz, OCH_3), 1.48 (m, H–3); δ_F −72.11, −69.42. NMR data for **11b**, **12b** (diastereomeric mixture): δ_H 7.17 (d, 9.0 Hz, NH), 5.615 (dt, 4.5, 2.0 Hz, H–3), 4.653 (dddd, 9.0, 6.3, 5.3, 4.4 Hz, H–2), 4.524 (dd, 11.6, 5.3 Hz, H–1), 4.405 (dd, 11.6, 6.3 Hz, H–1), 2.14 (m, H–6); 7.05 (d, ~8.7 Hz, NH), 5.644 (m, H–3), 4.62 (m, H–1, H–2), 4.199 (m, H–1), 2.22 (m, H–6); 3.552, 3.514, 3.421, 3.375, 3.238, 3.155 (all q, 1.3 Hz, OCH_3); δ_F −72.21, −72.15, −72.12, −71.89, −69.72, −69.43. NMR data for **13b**, **14b** (diastereomeric mixture) δ_H 7.04 (d, 9.0 Hz, NH), 5.624 (dt, 4.0, 2.1 Hz, H–3), 4.608 (dddd, ~9, 7.1, 5.8, 4.0 Hz, H–2), 4.393 (dd, 11.4, 5.9 Hz, H–1), 4.234 (dd, 11.4, 7.1 Hz, H–1), 2.09 (m, H–6); 7.18 (d, 9.0 Hz, NH), 5.598 (dt, 4.1, 2.1 Hz, H–3), 4.634 (dddd, 9.0, 6.9, 5.9, 4.0 Hz, H–2), 4.338 (dd, 11.4, 6.9 Hz, H–1), 4.241 (dd, 11.4, 6.1 Hz, H–1), 2.16 (m, H–6); 3.525, 3.514, 3.489, 3.429, 3.303, 3.203 (all q, 1.3 Hz, OCH_3); δ_F −72.18, −72.02, −72.00, −71.95, −69.67, −69.20.

T_1 relaxation measurements

The following T_1 relaxation times were measured on nondegassed samples by the inversion–recovery method: **1b**, **2b**, 0.6 s (H–1), 0.8 s (NH), 0.9 s (H–2), 1.1 s (H–3, 2–MTPA methoxy), 1.2 s (H–4, H–5), 1.3 s (1– and 3–MTPA methoxy); **3b**, **4b**, 0.6 s (H–1), 0.9 s (NH), 1.0 s (H–2), 1.1 s (H–3, 2–MTPA methoxy), 1.2 s (H–4, H–5), 1.4 s (1– and 3–MTPA methoxy); **5b**, **6b**, 0.6 s (H–1), 0.9 s (H–2, H–3, NH), 1.0 s (2–MTPA methoxy), 1.3 s (1– and 3–MTPA methoxy); **7b**, **8b**, 0.5 s (H–1), 0.7 s (H–2, H–3, NH), 0.9 s (2–MTPA methoxy), 1.2 s (1– and 3–MTPA methoxy). A similar ^{19}F NMR experiment for **1b**, **2b** showed T_1 values of 0.6 s (slightly less for the 2–MTPA trifluoromethyl). In ^{1}H and ^{19}F experiments, all pairs of signals corresponding to different diastereomers differed in T_1 by <0.1 s. Consequently, relatively short pulse repetition times (e.g., 2–3 s) will not favor one stereoisomer over another. This conclusion was consistent with the observation that methoxy peak pairs in NMR spectra of MTPA derivatives of racemic mixtures had virtually equal intensities. Because of slightly shorter T_1 values for 2–MTPA signals relative to 1– and 3–MTPA signals, comparisons should be made between these different sets of MTPA signals only if the NMR data were acquired with a sufficient relaxation delay.

Analysis of sphingosine and dihydrosphingosine isomers by HPLC

Various mixtures of sphingosine and dihydrosphingosine isomers **1b–8b** were analyzed by HPLC on a

[3] The validity of the method for measuring enantiomeric and diastereomeric purities is not dependent on NMR signal assignments. Assignments were important in elucidating the composition of diastereomeric mixtures and may be helpful in assigning the stereochemistry of other sphingosine derivatives.

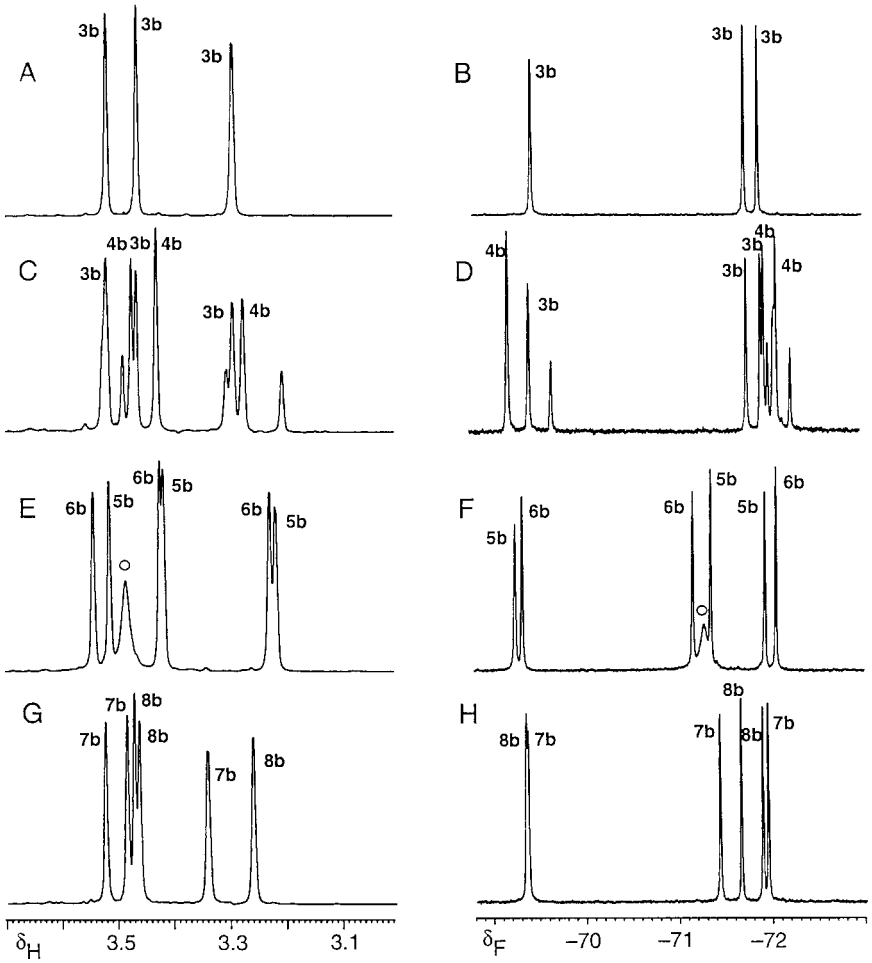


Fig. 3. ^{1}H and ^{19}F NMR spectra of (*S*)-MTPA derivatives of l-*threo*-sphingosine (**3b**) (panels A and B), dl-*threo*-sphingosine (**3b**, **4b**) (panels C and D), dl-*erythro*-dihydrosphingosine (**5b**, **6b**) (panels E and F), and dl-*threo*-dihydrosphingosine (**7b**, **8b**) (panels G and H). ^{1}H and ^{19}F NMR spectra were acquired at 500 and 235 MHz, respectively, and processed with 0.5-Hz line broadening. Minor signals in panels C and D represent **13b** and **14b**. Broad signals marked by open circles (○) in panels E and F (δ_H 3.49; δ_F −71.3) are attributable to a non-sphingolipid MTPA byproduct.

reversed-phase (C_{18}) column with acetonitrile and methanol–water and on a normal-phase (silica) column with methyl *tert*-butyl ether (MTBE)–hexane. Retention times are given in **Table 2**, and representative separations on the normal-phase system are shown in **Fig. 4**. The HPLC separations gave ca. 13,000 theoretical plates (acetonitrile), 9,000 plates (methanol–water), and 10,000 plates (normal phase). A resolution factor R_S of 1.5 (2% valley between equal-sized symmetrical peaks) at t_R 15 min would require a peak separation of 0.8 min (acetonitrile), 0.95 min (methanol–water), and 0.9 min (normal phase); R_S of 1.0 (27% valley between peaks) would require peak separa-

TABLE 2. HPLC retention times of (*S*)-MTPA derivatives of d-*erythro*-sphingosine and related sphingolipid bases[a,b]

	Sphingosine Isomers				Dihydrosphingosine Isomers			
	d-*erythro* **1b**	l-*erythro* **2b**	l-*threo* **3b**	d-*threo* **4b**	d-*erythro* **5b**	l-*erythro* **6b**	l-*threo* **7b**	d-*threo* **8b**
Reversed-phase HPLC, acetonitrile (t_R, min)	15.1	15.4	14.8	15.5	17.5	17.5	18.7	17.6
Reversed-phase HPLC, methanol–water 96:4 (t_R, min)	17.3	16.9	17.5	16.8	20.4	19.3	21.5	20.7
Normal-phase HPLC, MTBE–hexane 1:9 (t_R, min)	12.1	15.4	12.3	8.7	11.0	13.3	11.3	7.7

[a] HPLC data were obtained at 1 ml/min on 5-μm Alltima C_{18} or silica columns (250 mm × 4.6 mm). Retention times of non-retained peaks were 2.5–2.6 min.

[b] Reversed-phase half-height line widths varied from 0.3 min (t_R 15 min) to 0.4 min (t_R 18 min) for acetonitrile and from 0.4 min (t_R 17 min) to 0.5 min (t_R 21 min) for methanol–water 96:4. Normal-phase line widths varied from 0.2 min (t_R 8 min) to 0.3 min (t_R 13 min).

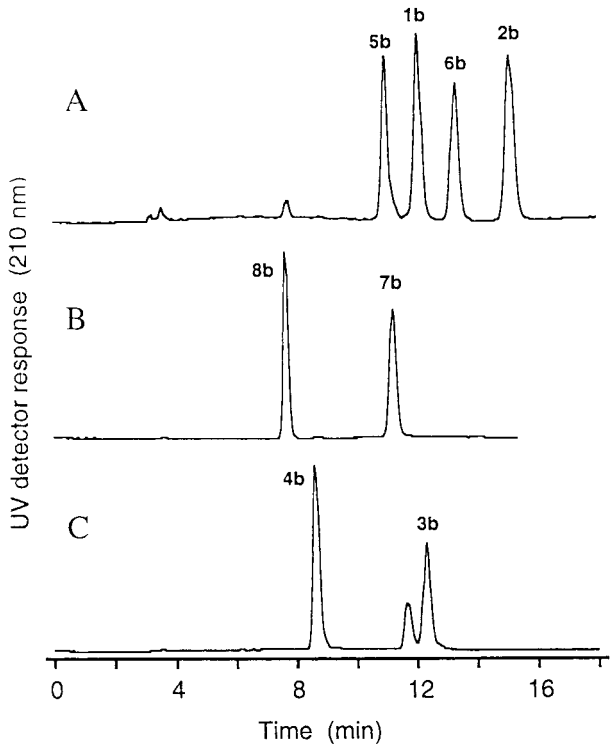


Fig. 4. Normal-phase HPLC separations of (*S*)-MTPA derivatives of sphingosine and dihydrosphingosine isomers: A, mixture of d-*erythro*-sphingosine (**1b**), l-*erythro*-sphingosine (**2b**), and dl-*erythro*-dihydrosphingosine derivatives (**5b**, **6b**); B, dl-*threo*-dihydrosphingosine derivatives (**7b**, **8b**); C, dl-*threo*-sphingosine derivatives (**3b**, **4b**). In panel C, the peak at t_R 11.8 and the additional intensity of the peak at t_R 8.7 min are attributable to minor components (**13b**, **14b**) in the dl-*threo*-sphingosine sample. Conditions: 5-μm Alltima silica column (250 mm × 4.6 mm); MTBE-hexane 1:9 at 1 ml/min; UV detection at 210 nm.

tions of 0.5–0.6 min. By these criteria, peaks corresponding to the separations of most interest (e.g., **1b** and **2b**; **1b** and **3b**; **5b** and **6b**) would be poorly resolved for quantitative work in either reversed-phase system. The normal-phase system would be capable of providing credible quantitation of mixtures of *erythro*-sphingosine and *erythro*-dihydrosphingosine enantiomers but not of mixtures of d-*erythro* and l-*threo* diastereomers.

MTPA byproducts and other components that might interfere with analyses

Some samples of MTPA derivatives of sphingolipid bases showed additional ^{1}H and ^{19}F NMR signals attributable to MTPA byproducts (see Figs. 2C, 2D, 3E, and 3F). These signals fell outside the 2–MTPA region (δ_H 3.1–3.4; δ_F ca. −69) used for measuring the enantiomeric purity of *erythro*-sphingosine, but their ^{13}C satellites could be distracting (Fig. 2C). The extraneous signals did not match resonances of methyl MTPA (δ_H 3.91, 3.56; δ_F −72.3) or the carboxylic acid (δ_H 3.57; δ_F −71.5), although the latter may show considerable variation in chemical shift. In one case (Fig. 2C, 2D), one set of contaminants (δ_H 3.53; δ_F −71.4) was removed by extraction from dichloromethane into aqueous $NaHCO_3$ and another set was not (δ_H 3.42; δ_F −72.2). These byproducts, which could interfere with some purity analyses, might be avoided by a TLC purification similar to that described for preparing sphingosine derivatives on a nanogram scale (19). Another potential source of interference is the presence of homologs, such as C_{20}-sphingosine.[4] These homologs, easily detected by GC methods, would probably not be distinguished from C_{18}-sphingosine in either the NMR or normal-phase HPLC analyses but might cause confusion in reversed-phase analyses described herein and those on chiral columns.

DISCUSSION

We have shown that the enantiomeric purity of sphingosine can be measured simply and accurately by ^{1}H or ^{19}F NMR analysis of the MTPA derivative. The tris-MTPA derivative was easy to prepare and furnished excellent reporter groups for NMR analysis. Methoxy signals corresponding to each enantiomer were cleanly resolved in both 250- and 500-MHz ^{1}H NMR spectra of a racemic mixture, and trifluoromethyl signals in ^{19}F NMR spectra at 235 MHz showed even better chemical shift nonequivalence (Fig. 2). Adequate ^{1}H and ^{19}F peak separations on a 250-MHz spectrometer indicated that the enantiomeric purity of sphingosine can be measured on virtually any current NMR instrument. The high accuracy and sensitivity of the present method was demonstrated by analysis of several *erythro*-sphingosine mixtures of known enantiomeric composition. At 500 MHz with an inverse-detection probe, 0.1% of the minor enantiomer could be detected with 1-mg samples. With a 5% detection limit for the minor enantiomer, analyses required as little as 10 μg of sphingosine.

Similar separations were observed for the enantiomers of *threo*-sphingosine, *erythro*-dihydrosphingosine, and *threo*-dihydrosphingosine. As with the *erythro*-sphingosine MTPA derivatives, the strong intensity and good separation of the ^{1}H methoxy and ^{19}F trifluoromethyl signals led to accurate and sensitive measurement of enantiomeric purities. The signal dispersion was sufficiently large in both ^{1}H and ^{19}F NMR spectra of sphingosine derivative **1b** that any stereoisomeric contaminants and any dihydrosphingosine stereoisomers could be detected by simple inspection of the methoxy or trifluoromethyl region (Figs. 2 and 3). An obvious application is the analysis of commercial sphingosine samples, some of which we and others (19) have found to contain significant amounts of stereoisomeric and dihydrosphingosine impurities.

Most ^{1}H NMR chemical shifts of the individual MTPA isomers are given in Table 1 to ±0.001 ppm precision

[4]Samples of sphingolipid bases **1a**, **3a**, and **5a** analyzed herein were obtained by hydrolysis of a mixture of cerebrosides and sphingomyelin from cow brain (11). Unlike gangliosides, cerebrosides and sphingomyelin contain low levels of C_{20} bases, and GC–MS analysis of **1a** showed negligible levels (<0.5%) of the C_{20} homolog.

(±0.01 ppm for ^{19}F data), which allows reliable identification of individual isomers and any stereoisomeric contaminants. Unlike chromatographic methods, NMR does not require calibration with authentic standards. Chemical shifts matching our data in Table 1 to ±0.001 ppm (or ca. ±0.01 ppm for ^{19}F) can be obtained by acquiring NMR spectra on a reasonably clean sample in dilute $CDCl_3$ solution (≤10 mm) at approximately 25°C (or ~22°C for ^{19}F) and using tetramethylsilane or $CFCl_3$ as a calibration reference. The ^1H NMR chemical shifts of the MTPA derivatives of sphingosine isomers showed much better sample-to-sample reproducibility than those of the free base, which may vary by 0.2 ppm (10). Although triacetate derivatives also give reproducible NMR data and can be used to distinguish *threo* and *erythro* isomers (10), the MTPA derivatives provided markedly better NMR analyses because of higher sensitivity, the ability to distinguish enantiomers, and better chemical shift nonequivalence between stereoisomers.

The MTPA derivatives of sphingosine and dihydrosphingosine isomers were also analyzed by HPLC (Table 2). Reversed-phase HPLC with either acetonitrile or methanol–water separated some isomers, but little or no resolution was observed for several isomeric pairs, including d- and l-*erythro*-sphingosine (**1b** and **2b**). Normal-phase HPLC on silica gel gave much better separations, and all of the sphingosine and dihydrosphingosine stereoisomers were well resolved except those of the d-*erythro* and l-*threo* pairs. Relative to NMR, HPLC suffered from lower peak dispersion, the need for careful calibration with authentic standards, and much greater potential for gross errors because quantitation is dependent on measurement of a single peak for each component. However, HPLC provides better sensitivity and the capability for high throughput of samples at low cost.

As with other methods of measuring enantiomeric purity, errors can arise in the present technique by discrimination against one of the enantiomers during derivatization, workup, NMR or HPLC analysis, and data reduction. To avoid partial kinetic resolution of enantiomers, all reactions were carried out for 24 h in the presence of a large excess of reagent with the aim of complete formation the tris-MTPA derivatives. No evidence of bis-MTPA derivatives was observed in NMR spectra. Racemization would seem highly improbable in MTPA derivatizations of sphingosine, and epimerization at the allylic 3-hydroxyl was not observed under the mild reaction conditions described herein. The MTPA derivatives were isolated by filtration of the crude reaction mixture through silica gel with a strong solvent to avoid any possible separation of diastereomers. This necessarily crude procedure sometimes left the analyte contaminated with MTPA byproducts. Interference from the resulting extraneous NMR signals and their ^{13}C satellites was negligible in milligram-scale analyses but adversely affected the sensitivity and reliability in microgram-scale work. Another potential source of error is low enantiomeric purity of the MTPA reagent. The (*S*)-MTPA chloride used herein appeared to be of high enantiomeric purity and no interfering NMR signals from (*R*)-MTPA species were observed.[5] Measurements of T_1 relaxation times in ^1H and ^{19}F NMR spectra of representative mixtures showed that long relaxation delays to avoid discrimination among diastereomeric species are generally unnecessary. We calculated enantiomeric ratios from integration of NMR peak areas, but ratios calculated from peak heights were only slightly less accurate despite differences in peak widths owing to differences in long-range ^{19}F–^1H couplings constants. Peak areas were also used for HPLC analyses. Perhaps the best evidence that enantiomer discrimination does not occur at any stage of analysis is the agreement of observed NMR measurements with known enantiomeric compositions of samples prepared by mixing various amounts of the individual enantiomers.

In summary, we have presented new methods for determining the stereoisomeric purity of any isomer of sphingosine and dihydrosphingosine. The MTPA derivatives are easily prepared and can be analyzed by NMR or HPLC. Both ^1H and ^{19}F NMR give results of high reliability and require no authentic standards and only a few minutes of time on instrumentation that is commonly available in research settings. The NMR methods work best with samples of ≥100 μg and should be useful for determining the purity of sphingosine from total synthesis. HPLC analysis, despite the considerable effort required for setup, calibration, and validation and its susceptibility to major errors from adventitious impurities, is the only practical alternative for measuring enantiomeric and diastereomeric purities of small biological samples, which may contain only nanogram levels of sphingosine. The NMR and HPLC methods described herein represent major improvements over previous methodology for determining the stereoisomeric purity of sphingosine and dihydrosphingosine. These new methods should be valuable for assuring the purity and authenticity of sphingosine and dihydrosphingosine samples used in biomedical research.

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[5] The two chiral centers of sphingosine and the three MTPA chiral centers can produce 16 distinct diastereomers. Designating the chirality of the three MTPA groups as 1*S**,2*S**,3*S** and supposing a 1% level of the (*R*)-MTPA isomer, we can predict that the 2*S*, 3*R*, 1*R**, 2*R**, 3*R** isomer (d-*erythro*-sphingosine derivatized entirely with the (*R*)-MTPA contaminant) would have an intensity of 0.0001% of that of the normal 2*S*, 3*R*, 1*S**, 2*S**, 3*S** isomer. Similarly, the (*R*)-MTPA contaminant would not contribute significantly to NMR signal intensities of other sphingosine isomers. In this regard, the tris-MTPA derivative of sphingosine is superior to a mono-MTPA derivative for analysis of enantiomeric purity. However, for each tris-MTPA sphingosine stereoisomer, three artifactual diastereomers of 1% intensity would be produced, i.e., 1*S**,2*S**,3*R**; 1*S**,2*R**,3*S**; and 1*R**,2*S**,3*S**, and their enantiomers would be produced at 0.01% intensity. In the analysis of a single sphingosine isomer, 9 artifactual MTPA NMR signals from 3 artifactual diastereomers would appear at a level of 1% and 9 others at a 0.01% level. If all four sphingosine stereoisomers are present in equal amounts, 36 artifactual NMR signals would be produced at a 1% level. This potential problem is best avoided by using MTPA reagent of high enantiomeric purity.

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