A FACILE AND REGIOSPECIFIC TRITIATION OF SPHINGOSINE: SYNTHESIS OF (2S,3R,4E)-2-AMINO-4-OCTADECENE-1,3-DIOL-1-^{3}H

Tatsushi Toyokuni*, Mohammad Nisar**, Barbara Dean, and Sen-itiroh Hakomori
The Biomembrane Institute and University of Washington,
201 Elliott Ave West, Seattle, WA 98119

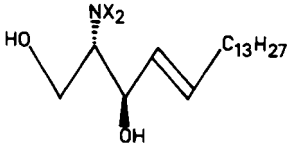
SUMMARY

Abstract: An easy technique for introduction of tritium into the 1-position of sphingosine was developed, employing regiospecific oxidation of the primary hydroxy group followed by reduction with NaB^3H_4. An improved preparation of N,N-dimethylsphingosine and its quaternization are also described.

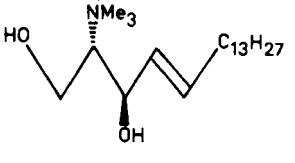
Key words: deuterium, tritium, sphingosine, N,N-dimethylsphingosine, N,N,N-trimethylsphingosine

INTRODUCTION

A breakdown product of cellular sphingolipids, (2S,3R,4E)-2-amino-4-octadecene-1,3-diol (sphingosine) (1), has been shown to be a modulator of protein kinase C (PK-C) (1) and epidermal growth factor (EGF) receptor kinase (2). Recently, it was reported that N,N-dimethylsphingosine (2) inhibits PK-C activity much more strongly than 1 and promotes EGF receptor kinase (3). Furthermore, it was shown that N,N,N-trimethylsphingosine (3) is a much more potent effector on PK-C and, in contrast to 2, inhibits EGF receptor kinase (4). These biological effects raise the possibility that 1, 2, and 3 may have a pharmacological use for the prevention of tumor growth and other pathological processes, since protein kinases are the pivotal enzymes in cell regulation and signal transduction (5). The



Sphingosine (1) X = H
N,N-Dimethylsphingosine (2) X = Me



N,N,N-Trimethylsphingosine (3)

---

* To whom correspondence should be addressed.

** On leave of absence, 1989-1991, from Department of Chemistry, University of Peshawar, Peshawar, Pakistan.

0362–4803/91/050567–08$05.00

Received 28 November, 1990
Revised 7 January, 1991

enzymatic transformation of 1 to 2 was recently demonstrated in the crude homogenate of mouse brain tissue, indicating the natural occurrence of N-methyltransferase responsible for conversion of 1 to 2 (6).

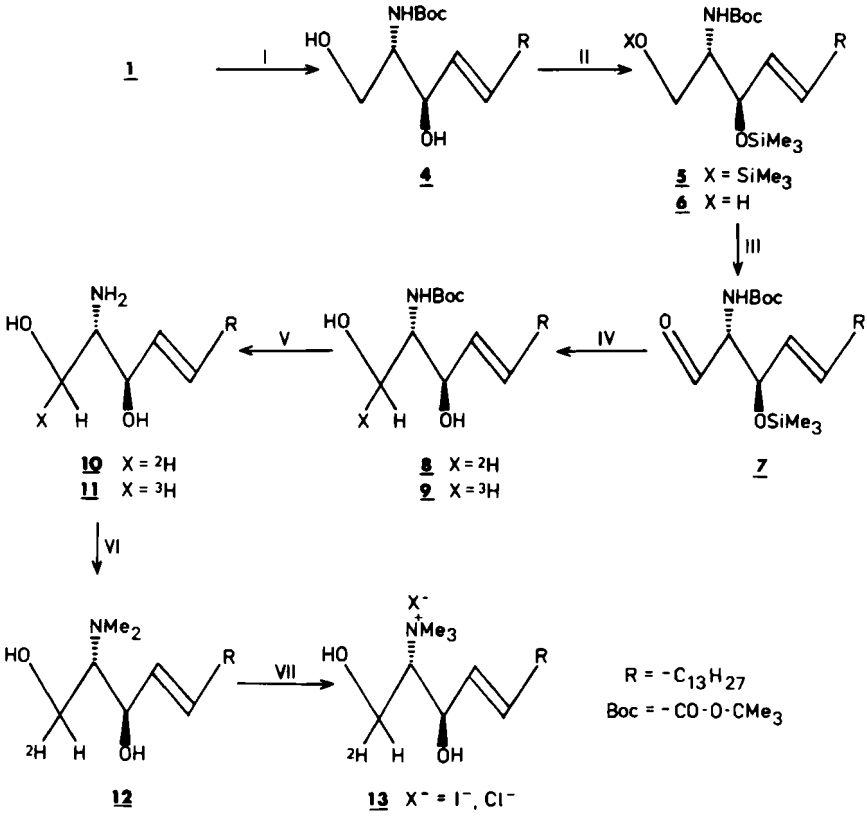
To gain further insight into their physiological functions and to study their pharmacological applications, the availability of simple procedures for introducing radioisotopes into these molecules is of importance. Four methods are available: a) a biosynthetic method starting from radioactive serine (7); b) labeling in the course of total synthesis (8); c) catalytic reduction in which tritium is added to the double bond yielding dihydrosphingosine-4,5-$^3H_2$ (9); and d) an oxidation-reduction method in which 1 is oxidized to the 3-keto derivative, which is then reduced back with NaB^3H_4, giving a mixture of 1 and its (3S)-isomer with tritium at the 3-position (10). Each of these methods, however, suffers disadvantages: a) a product with relatively low specific radioactivity (method a); b) multistage chemical syntheses (method b); and c) complete saturation (method c) and loss of stereochemistry at the 3-position (method d), both of which may result in physiological functions differing from those of the original molecule (3). We describe herein a convenient tritiation of 1 at the 1-position, featuring regiospecific oxidation of the primary hydroxy group, followed by reduction with NaB^3H_4. Thus, the method provides tritium labeled 1 (i.e., 11) with complete retention of the original sterochemistry.

RESULTS AND DISCUSSION

Only a few successful methods exist to oxidize the primary hydroxy group into the corresponding aldehyde while the secondary hydroxy group remains intact (11). In 1986 Schick et al. (12) reported a novel two-step method comprised of trimethylsilylation followed by oxidation with Collins reagent (13). This method was found to be successful for our purpose.

The readily available N-tert-butoxycarbonylsphingosine (4) (3b) was treated with a mixture of Me_3SiCl, $Me_3SiNHSiMe_3$, and dry pyridine under dry nitrogen (14) at room temperature for 1 h to give a 9:1 mixture of di-O- and mono-O-trimethylsilyl derivatives 5 and 6, which were separable by a flash column chromatography (15) on silica gel with 8:1 hexane-EtOAc as an eluent. In the ^1H NMR spectrum of 6, a triplet of doublets (J = 11.0 and 3.5 Hz) at δ 3.57 ppm and a broad doublet (J = 11.0 Hz) at δ 4.00 ppm due to the two H-1 protons changed to a doublet of doublets (J = 11.0 and 3.5 Hz) and a sharp doublet (J = 11.0 Hz), respectively, upon deuteration, being consistent with the assigned structure. Since compound 5 seemed to be slowly converted into 6 in contact with silica gel, the mixture of 5 and 6 was oxidized, without separation, with a Collins reagent, prepared from CrO_3 and dry pyridine (12a, 13), at room temperature for 1 h, yielding the desired aldehyde 7 as the major product. Purification of 7 was not performed due to its instability.

Scheme I



Reagents: I) Boc_2O; II) Me_3SiCl, $Me_3SiNHSiMe_3$, Pyr; III) Collins reagents; IV) NaB^2H_4/NaB^3H_4; V) CF_3COOH; VI) HCHO, $NaBH_3CN$, pH 4.8; VII) MeI.

Regiospecificity of this oxidation was confirmed by reduction of 7 with NaB^2H_4. Treatment of crude 7 with NaB^2H_4 in MeOH at room temperature for 30 min yielded deuterio derivative 8 as a colorless solid in 73% yield from 4. Acidolysis of 8 with CF_3COOH at 0° for 5 min produced sphingosine-1-^{2}H (10) quantitatively. It was clear from the ^{1}H-NMR spectra of 1 and 10 (Fig. 1) that 10

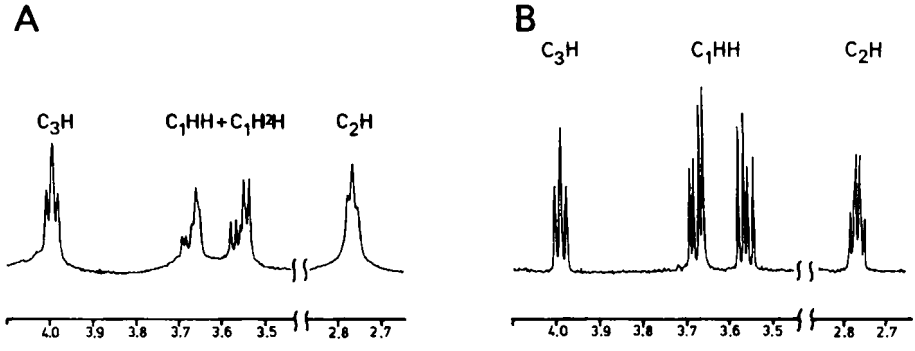


Fig. 1. ^{1}H NMR spectra (1:1 $CDCl_3$-CD_3OD) of **A**: sphingosine-1-^{2}H (10) and **B**: sphingosine (1).

was regiospecifically deuterated at C-1. The conversion of C_1HH to C_1H^2H collapsed the two pairs of doublets at δ 3.57 and 3.68 ppm (Fig. 1-B) to two doublets at δ 3.55 and 3.66 ppm, partially overlapping the pair of doublets due to the residual C_1HH (Fig. 1-A). The triplet of doublets at δ 2.77 ppm assignable to H-2 in **1** also collapsed to a broad triplet in **10**. Both $C_1\underline{H}^2H$ and $C_1{}^2H\underline{H}$ in **10** are 0.02 ppm more shielded than $C_1\underline{HH}$ in **1** due to the deuterium effect (16).

Sphingosine-1-^{3}H (**11**) was then synthesized by the same sequence of reactions as described above, except that **7** was treated with NaB^3H_4 (26 Ci/mmol) for 15 min and then with $NaBH_4$ for 15 min, leading to **9**: radiochemical purity 97%; specific activity 97 mCi/mmol.

Compound **10** was converted to N,N-dimethyl derivative **12** more efficiently than in the method previously reported (3b). Thus a mixture of **10** and 37% aq HCHO in a buffer solution (NaOAc-AcOH-H_2O, pH 4.8) was treated with $NaBH_3CN$ to give **12** in 80% yield.

Quaternization of **12** with iodomethane in dry $CHCl_3$ at room temperature in the dark overnight precipitated N,N,N-trimethyl derivative **13** ($Y^-=I^-$) in 86% yield, which was transformed to its chloride salt **13** ($Y^-=Cl^-$) by mixing with Dowex® 1X2 (Cl^-) resin in H_2O. Direct quaternization (17) of **1** was unsuccessful.

In conclusion, the present method allows the ready introduction of tritium to **1** (and thus **2** and **3**) with complete retention of their stereochemistry.

EXPERIMENTAL

General methods. - ^{1}H NMR spectra were recorded on a Bruker WM-500 spectrometer in $CDCl_3$, 1:1 $CDCl_3$-CD_3OD, or D_2O. Chemical shift standards were Me_4Si for $CDCl_3$ and $CDCl_3$-CD_3OD, and acetone (δ 2.09 ppm relative to TMS) for D_2O. TLC was performed on precoated Silica gel 60 F_{254} plates (Merck, Darmstadt; 0.25 mm) and visuallized by spraying with 0.5% orcinol in 10% aq H_2SO_4 or 0.2% ninhydrin in EtOH followed by heating. Preparative TLC was carried out on precoated Silica gel 60 plates (Merck, Darmstadt; 0.5 mm) and spots were detected by a H_2O spray. Silica gel used for flash column chromatography (15) was purchased from EM Science (Gibbstown, NJ; 230-400 mesh). High-resolution mass (HRMS) was obtained by M.E. Saylan of this Institute using JEOL JMS-HX 110 mass spectrometer under FAB mode. Radioactivity was measured on a Beckman LS 3801 Liquid Scintillation Counter.

Trimethylsilylation of N-tert-butoxycarbonyl-(2S,3R,4E)-2-amino-4-octadecene-1,3-diol (4). - Compound **4** (15 mg), prepared from (2S,3R,4E)-2-amino-4-octadecene-1,3-diol (sphingosine) (**1**)

(Sigma, St. Louis, MO) (3b), was treated with a mixture of Me_3SiCl (9.7 μL, 2.0 equiv), $Me_3SiNHSiMe_3$ (32.4 μL, 4.0 equiv), and dry pyridine (1 mL) under dry nitrogen at room temperature for 1 h. Addition of dry Et_2O (10 mL), followed by removal of a fine precipitate (NH_4Cl) using a syringe filter (0.2 μm) and concentration, yielded a colorless solid. This was further treated with dry Et_2O (10 mL) to remove residual NH_4Cl. The resulting colorless syrup (21 mg) was found to be a 9:1 mixture of 1,3-di-O-trimethylsilyl-N-tert-butoxycarbonyl-(2S,3R,4E)-2-amino-4-octadecene-1,3-diol **5** (R_f 0.8) and 3-O-trimethylsilyl-N-tert-butoxycarbonyl-(2S,3R,4E)-2-amino-4-octadecene-1,3-diol **6** (R_f 0.4) on TLC (3:1 hexane-EtOAc). Flash column chromatography (15) of the mixture on silica gel with 8:1 hexane-EtOAc as an eluent gave **5** and **6**. Compound **5**: colorless syrup; ^1H NMR ($CDCl_3$) δ 0.08 (s, 9) and 0.10 (s, 9) (2x$SiMe_3$), 0.88 (t, 3, J = 7.0 Hz, Me), 1.23-1.40 (m, 22, 11xCH_2), 1.43 (s, 9, CMe_3), 2.00 (q, 2, J = 7.0 Hz, 2xH-6), 3.54 (br s, 1, H-2), 3.58 (br d, 1, J = 10.5 Hz) and 3.76 (dd, 1, J = 11.0 and 4.2 Hz) (2xH-1), 4.13 (br s, 1, H-3), 4.70 (br s, 1, NH), 5.43 (dd, 1, J = 15.3 and 7.3 Hz, H-4), and 5.60 (dt, 1, J = 15.3 and 7.0 Hz, H-5); HRMS 544.4214 ($C_{29}H_{61}NO_4Si_2$+H, Δ-0.0005). Compound **6**: colorless syrup; ^1H NMR ($CDCl_3$) δ 0.11 (s, 9, $SiMe_3$), 0.88 (t,3, J = 7.0 Hz, Me), 1.23-1.40 (m, 22, 11xCH_2), 1.46 (s, 9, CMe_3), 2.03 (q, 2, J = 7.0 Hz, 2xH-6), 2.94 (br d, 1, J = 8.0 Hz, C_1-OH), 3.44 (br s, 1, H-2), 3.57 (td, 1, J = 11.0 and 3.5 Hz, changing to dd with J = 11.0 and 3.5 Hz on deutration) and 4.00 (br d 1, J = 11.0 Hz, changing to sharp d on deuteration) (2xH-1), 4.44 (s, 1, H-3), 5.32 (m, 1, NH), 5.45 (dd, 1, J = 15.0 and 5.9 Hz, H-4), and 5.70 (dt, 1, J = 15.0 and 7.0 Hz, H-5); HRMS 494.3628 ($C_{26}H_{53}NO_4Si$+Na, Δ-0.0013).

Compound **5** seemed to be slowly converted into **6** in contact with silica gel. For the preparation of deuterium- and tritium-labeled sphingosine (**10** and **11**, respectively), the mixture of **5** and **6** was subjected to the next oxidation without separation.

N-tert-Butoxycarbonyl-(2S,3R,4E)-2-amino-4-octadecene-1,3-diol-1-^2H (8). The mixture of **5** and **6** (20.6 mg), obtained above, was dissolved in dry CH_2Cl_2 (0.5 mL). The solution was added dropwise to a pre-cooled solution of Collins reagent, prepared from CrO_3 (22.5 mg, 6.0 equiv to **4**) and dry pyridine (36.5 μL, 12 equiv to **4**) in dry CH_2Cl_2 (1 mL) (12a), with stirring in an ice-bath. After the addition, the ice was allowed to melt and the mixture was stirred vigorously for 1 h. The reaction mixture was then passed through a short column (ϕ 0.5 cm, ! 4 cm) of silica gel (70-230 mesh) and concentrated to a pale yellow syrup (18 mg) containing **7** as the major product at R_f 0.4 on TLC (8:1 hexane-EtOAc). Further purification of **7** was not performed due to its instability.

The crude **7** was dissolved in dry MeOH (1 mL) and treated with NaB^2H_4 (Aldrich, Milwaukee, WI;

98% atom ^2H) (2 mg) at room temperature for 30 min. The reaction mixture was acidified with Amberlite® IR 120 (H^+) resin and concentrated to dryness. Purification by preparative TLC (R_f 0.4, 1:1 hexane-EtOAc) yielded **8** (11 mg, 73% yield from **4**) as a colorless solid: HRMS 423.3318 ($C_{23}H_{44}{}^2HNO_4$+Na, Δ0.0009).

The ^1H NMR spectrum ($CDCl_3$) was identical with that of the authentic sample **4** (3b), except for the two H-1 protons which appeared as two multiplets centered at δ 3.70 and 3.92 ppm with the integral of ≈0.6 each.

(2S,3R,4E)-2-Amino-4-octadecene-1,3-diol-1-^2H (10). - Compound **8** (11 mg) was treated with CF_3COOH (0.5 mL) under dry nitrogen at 0° for 5 min. Concentration and treatment with Amberlite® IRA 400 (OH^-) resin in MeOH (1 mL) produced **10** (8.3 mg, quantitative) as a colorless solid: HRMS as $[M+H-H_2O]^+$ 283.2860 ($C_{18}H_{34}{}^2HNO$+H, Δ-0.0001).

The ^1H NMR spectrum (1:1 $CDCl_3$-CD_3OD) was identical with that of the authentic sample **1**, except for the two H-1 protons and H-2 proton as shown in Fig. 1.

(2S,3R,4E)-2-(Dimethylamino)-4-octadecene-1,3-diol-1-^2H (12). - To a solution of **10** (20 mg) in a buffer solution (1 mL, pH 4.8), prepared from NaOAc-$3H_2O$ (13.6 g), AcOH (6 mL), and H_2O (60 mL), was added 37% aq HCHO (0.2 mL) and the mixture was stirred at room temperature until it became homogeneous (for ≈10 min). The mixture was then treated sequentially at 5-min intervals with $NaBH_3CN$ (8 mg, 6 mg, and 4 mg) and MeOH (5 mL). After concentration, the residue was dissolved in $CHCl_3$ (1 mL) and washed successively with saturated aq $NaHCO_3$ (1 mL) and H_2O (1 mL). Concentration of the organic-layer and purification by preparative TLC (R_f 0.6, 4:1:0.1 $CHCl_3$-MeOH-conc NH_4OH) provided **12** (17.5 mg, 80%) as a colorless syrup: HRMS 329.3281 ($C_{20}H_{40}{}^2HNO_2$+H, Δ0.0001).

The ^1H NMR spectrum (1:1 $CDCl_3$-CD_3OD) was identical with that of the authentic sample **2** (3b), except for the two H-1 protons which appeared as a multiplet centered at δ 3.83 ppm with the integral of ≈1.1.

(2S,3R,4E)-2-(Trimethylammonio)-4-octadecene-1,3-diol-1-^2H (13) iodide (X^-=I^-)/chloride (X^-=Cl^-). - In order to establish the reaction condition, protio compound, (2S,3R,4E)-2-(trimethylammonio)-4-octadecene-1,3-diol (**3**) (iodide/chloride) was first synthesized.

To a solution of (2S,3R,4E)-2-(dimethylamino)-4-octadecene-1,3-diol (**2**) (3b) (30 mg) in dry $CHCl_3$ (1.5 mL) was added freshly distilled iodomethane (170 μL, excess) and the mixture was stirred at room

temperature in the dark overnight. Dilution with H_2O, followed by extraction with $CHCl_3$ (3 mLx4) and concentration, yielded **3** (iodide) (37 mg, 86%) as a yellow solid, which was converted to its chloride salt by mixing with Dowex® 1X2 (Cl^-) resin (0.5 g) in H_2O. Compound **3** (chloride): mp 224° decomp; ^1H NMR (D_2O) δ 0.88 (t, 3, J = 6.8 Hz, Me), 1.31 (br s, 22, 11xCH_2), 2.08 (q, 2, J = 6.8 Hz, 2xH-6), 3.29 (s, 9 N^+Me_3), 3.38 (br s, 1, H-3), 4.13 (br s. 2, 2xH-1), 5.57 (dd, 1, J = 3.1 and 3.4 Hz, H-4), and 5.90 (m, 1, H-5); HRMS 342.3371 ($C_{21}H_{44}NO_2$, Δ-0.0003).

Similarly compound **12** (10 mg) was reacted with iodomethane (55 μL) in $CHCl_3$ (0.5 mL) to give **13** ($X^-=I^-$). The iodide salt was converted to its chloride salt by treatment with Dowex® 1X2 (Cl^-) resin (0.1 g) in H_2O affording **13** ($X^-=Cl^-$) (9.7 mg, 84%) as a colorless solid: HRMS 343.3435 ($C_{21}H_{43}{}^2HNO_2$, Δ-0.0002).

The ^1H NMR spectrum (D_2O) was identical with that of **3**, prepared above, except that the two H-1 protons appeared as a multiplets centered at δ 4.12 ppm with the integral of ≈1.1.

N-tert-Butoxycarbonyl-(2S,3R,4E)-2-amino-4-octadecene-1,3-diol-1-^{3}H (9). - Compound **4** (15 mg) was transformed to **9** by the same sequence of reactions as described for the preparation of **8**, except that crude **7** was treated with NaB^3H_4 (Amersham, Arlington Heights, IL; 26 Ci/mmol) (10 mCi) for 15 min and then with $NaBH_4$ (2.0 mg) for 15 min. Purification by preparative TLC (1:1 hexane-EtOAc) gave **9** (10 mg, 67%; 2.51 mCi) as a colorless solid: radiochemical purity 97% (by TLC, 1:1 hexane-EtOAc); specific activity 100 mCi/mmol.

(2S,3R,4E)-2-Amino-4-octadecene-1,3-diol-1-^{3}H (11). - Compound **9** (10 mg, 2.51 mCi), prepared above, was treated with CF_3COOH (0.5 mL) and the reaction mixture was worked up as described in the preparation of **10**. Purification by preparative TLC (R_f 0.6, 4:1:0.1 $CHCl_3$-MeOH-NH_4OH) yielded **11** (7 mg, 93%; 2.28 mCi): radiochemical purity 97% (by TLC, 4:1:0.1 $CHCl_3$-MeOH-NH_4OH); specific activity 97 mCi/mmol.

ACKNOWLEDGEMENTS

We thank Drs. Y. Igarashi and Shaopei Cai for helpful discussions. This work was supported by funds from The Biomembrane Institute.

REFERENCES

1. a) Hannun Y.A., Loomis C.R., Merrill A.H., and Bell R.M. - J. Biol. Chem. 261: 12604 (1986). b) Hannun Y.A. and Bell R.M. - Science 243: 500 (1989).

2. a) Faucher M.F., Girones N., Hannun Y.A., Bell R.M., and Davis R.J. - J. Biol. Chem. 263, 5319 (1988). b) Northwood I.C. and Davis R.J. - J. Biol. Chem. 263: 7450 (1988).
3. a) Igarashi Y., Hakomori S., Toyokuni T., Dean B., Fujita S., Sugimoto M., Ogawa T., El-Ghendy K., and Racker E. - Biochemistry 28: 6796 (1989). b) Igarashi Y., Kitamura K., Toyokuni T., Dean B., Fenderson B., Ogawa T., and Hakomori S. - J. Biol. Chem. 265: 5385 (1990).
4. Endo K. and Igarashi Y. - unpublished.
5. a) Nishizuka Y. - Science 233: 305 (1986). b) Bell R.M. - Cell 45: 631 (1986).
6. Igarashi Y. and Hakomori S. - Biochem. Biophys. Res. Commun. 164: 1411 (1989).
7. Felding-Habermann B., Igarashi Y., Fenderson B.A., Park L.S., Radin N.S., Inokuchi J., Strassmann G., Handa K., and Hakomori S. - Biochemistry 29: 6314 (1990).
8. a) Gal A.E. - J. Label. Comp. 3: 112 (1967). b) Stoffel W. and Sticht G. - Hoppe-Seyler's Z. Physiol. Chem. 348, 1561 (1967).
9. Schwarzmann G. - Biochim. Biophys. Acta 529: 106 (1978).
10. a) Iwamori M., Moser H.W., and Kishimoto Y., J. Lipid Res. 16: 332 (1975). b) Ghidoni R., Sonnino S., Masserini M., Orlando P., and Tettamanti G. - J. Lipid Res. 22: 1286 (1981).
11. a) Tomioka H., Takai K., Oshima K., and Nozaki H. - Tetrahedron Lett. 22, 1605 (1981). b) Semmelhack M.F., Chou C.S., and Cortes D.A. - J. Am. Chem. Soc. 105: 4492 (1983). c) Nakano T., Terada T., Ishii Y., and Ogawa M. - Synthesis 774 (1986). d) Singh J., Kalsi P.S., Jawanda G.S., and Chhabra B.R. - Chem. Ind. (London) 21: 751-2 (1986). d) Anelli P.L., Banfi S., Montanari F., and Quici S. - J. Org. Chem. 54: 2970 (1989). e) Inokuchi T., Matsumoto S., Nishiyama T., and Torii S. - J. Org. Chem. 55, 462 (1990). f) Siedlecka R., Skarzewski J., and Miochowski J. - Tetrahedron Lett. 31: 2177 (1990).
12. a) Mahrwald R., Theil F., Schick H., Schwarx S., Palme H.-J., and Weber G. - J. Prakt. Chem. 328: 777 (1986). b) Kristen H., Vogel Ch., Wrubel F., Mahrawld R., Schick H. - J. Carbohydr. Chem. 7: 277 (1988).
13. Collins J.C., Hess W.W., and Frank F.J. - Tetrahedron Lett. 3363 (1968).
14. Sweeley C.C., Bentley R., Makita M., and Wells W.W. - J. Am. Chem. Soc. 85: 2479 (1963).
15. Still W.C., Kahn M., and Mitra A. - J. Org. Chem. 43: 2923 (1978).
16. a) Van G. and Tiers D. - J. Chem. Phys. 29: 963 (1958). b) Gutowsky H.S. - J. Chem. Phys. 31: 1683 (1959).
17. Sommer H.Z., Lipp H.Y., and Jackson L.L. - J. Org. Chem. 36: 824 (1971).