SYNTHESIS OF TRITIUM LABELLED 2-VINYL DIHYDROSPHINGOSINE-1-PHOSPHATE

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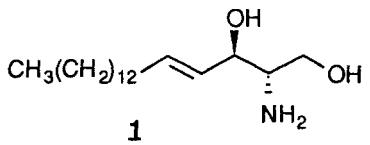
SUMMARY

The synthesis of [1,1,3-^{3}H]-2-vinyl dihydrosphingosine-1-phosphate is described. The key step in this synthesis is the reduction of methyl 2-trifluoro acetamido-2-vinyl nonadecanoate-3-one by means of ^{3}H-calcium diborohydride. This compound was synthesized to determine whether 2-vinyl dihydrosphingosine-1-phosphate inhibits the PLP-dependent sphingosine-1-phosphate lyase irreversibly *via* covalent modification.

KEYWORDS: sphingosine, sphingosine-1-phosphate lyase, [1,1,3-^{3}H]-2-vinyl dihydrosphingosine-1-phosphate, inhibitor, tritium labelling.



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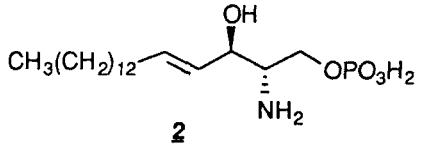

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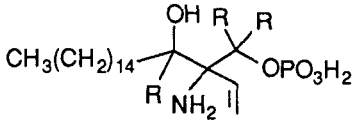

3 R = ^{1}H

4 R = ^{3}H

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CCC 0362–4803/95/030243–07

Received 28 June, 1994
Revised 10 September, 1994

INTRODUCTION

Sphingosine **1** and related long chain sphingoid bases are essential components of sphingolipids which occur in high abundance in biological membranes (1). Interests in these intermediates of sphingolipid metabolism increased when sphingosine was found to inhibit strongly and specifically protein kinase C, a pivotal regulatory enzyme in cell growth (2). Recently, metabolites of sphingosine have been shown to be produced in cells and to have potent effects on cell growth (3, 4, 5, 6). Thus, sphingosine-1-phosphate **2** which is produced from sphingosine by the action of a specific kinase (7, 8, 9), proved to be a very potent mitogen in Swiss 3T3 fibroblasts (5). Furthermore, there is evidence that **2** induces rapid and profound release of calcium from intracellular stores (3). Sphingosine-1-phosphate is degraded by the action of the PLP dependent sphingosine-1-phosphate lyase (aldolase) to yield ethanolamine-O-phosphate and hexadecenal (9, 10, 11, 12). The preparation of inhibitors would be very useful to provide information on the biological effects of blocking sphingosine catabolism *in vivo*. Preparation of radiolabelled irreversible inhibitors would aid in the isolation and purification of the lyase.

We have recently reported that 2-vinyl dihydrosphingosine-1-phosphate **3** is an inhibitor of sphingosine-1-phosphate lyase (13, 14). The inhibition with **3** was both time-dependent and irreversible, which suggested that compound **3** may be acting as a mechanism based inhibitor, i.e. a suicide substrate. To establish whether the 2-vinyl dihydrosphingosine-1-phosphate does in fact inhibit sphingosine-1-phosphate lyase *via* covalent modification, we have synthesized the tritium labelled [1,1,3-^{3}H]-2-vinyl dihydrosphingosine-1-phosphate **4**.

RESULTS AND DISCUSSION

The key step in the synthesis of tritium labelled 2-vinyl dihydrosphingosine-1-phosphate **4**, was the reduction of α-vinyl β-ketoester **6** by means of tritium labelled calcium diborohydride. The complete synthesis is outlined in Figure 1.

The α-vinyl β-keto ester **6** which was prepared as previously reported (13) was reduced and deprotected in one step to the aminodiol **7** by using ^{3}H-calcium diborohydride which was prepared *in situ* from calcium chloride and ^{3}H-sodium

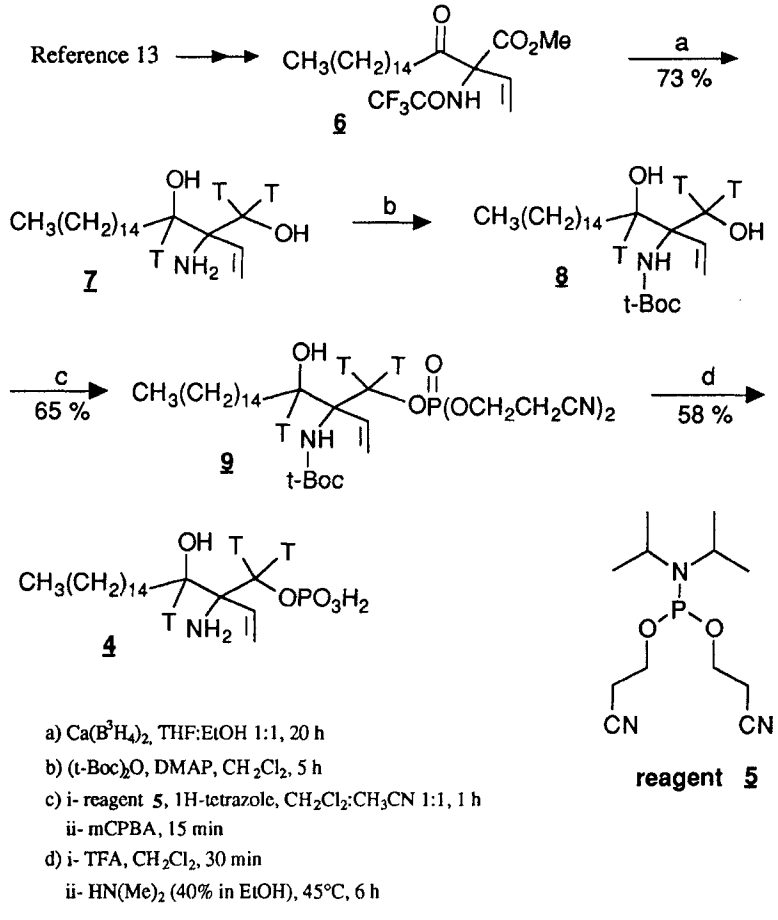

Figure 1. Synthesis of [1,1,3-^{3}H] 2-vinyl dihydrosphingosine-1-phosphate

borohydride (15). After removal of the reagent excess and labile tritium by an aqueous work-up, the crude material was purified by column chromatography to provide compound 7 with 90% chemical yield. The specific activity, 15 mCi/mmole, is only 14% of the theoritical maximum based upon the specific activity of the ^{3}H-sodium borohydride. This may be caused by the unfavorable isotope effect and/or hydrogen exchange into the reducing agent (16). The phosphate group was introduced using the monofunctional phosphitylation reagent 5 (17, 18, 19). For this purpose, the aminodiol 7 was N-protected with *tert*-butyloxycarbonyl (t-Boc) and condensed with 1 eq of 5 in the presence of 1H-tetrazole. The phosphite intermediate was not isolated but was oxidized *in situ* by means of m-CPBA to give the phosphotriester 9 in 70% yield. Conversion of 9 to 4, was achieved using the deprotection sequence shown in Figure 1. Treatment of

phosphotriester **9** with trifluoroacetic acid (TFA) resulted in complete removal of the Boc group. The free amine was not isolated prior to phosphate deprotection with a solution of dimethylamine (40 % in ethanol).

The purification of **4** by chromatography was precluded by the lack of solubility in most organic solvents. Instead, purification was achieved by dissolution of crude **4** in boiled acetic acid and precipitation by addition of water. After successive washings with water, acetone and diethylether, **4** was obtained in 58 % yield.

Assays (*in vitro*) are in progress to determine whether sphingosine-1-phosphate lyase is irreversibly inhibited by **3** *via* covalent modification.

EXPERIMENTAL

Radioactivity was determined with a Beckman LS 7500 liquid scintillation counter. TLC plates, E. Merck silica gel 60 F_{254}, were scanned on a Berthold LB 2842 automatic TLC linear analyzer. Column chromatography was performed with Aldrich silica gel 230-400 mesh, 60 Å. NaB^3H_4 (specific activity 359.8 mCi/mmol) was purchased from New England Nuclear (Boston, Mass) and diluted with unlabelled $NaBH_4$ prior to use. All reagents used were obtained from Aldrich Chemical Company (Milwaukee, WI). NMR spectra were obtained on a varian VXR 300 MHz, chemical shifts are reported relative to TMS (δ = 0.0 ppm).

[1,1,3-^{3}H]-2-vinyl dihydrosphingosine **7**: To a solution of methyl 2-trifluoro acetamido-2-vinyl nonadecanoate-3-one **6** (41 mg, 0.09 mmol) in ethanol (2 mL) and cooled to -20°C, was added a suspension of $CaCl_2$ (10 mg, 0.09 mmol) in solution in THF (2 mL). The reaction mixture was treated with NaB^3H_4 (7 mg, 0.18 mmol, 25 mCi) added in small portions as a solution in THF:EtOH 1:1 (1 mL), and the temperature was allowed to reach room temperature. After 20 h, the solution was dried by a gentle stream of N_2 and the residue was dissolved in ethyl acetate (6 mL). The solution was washed with HCl (1N, 2 x 2 mL), saturated NaCl (2 x 1 mL), dried (Na_2SO_4) and concentrated yielding crude **7**. The crude product was chromatographed on a column of silica gel (1.2 cm x 5 cm) eluted with $CHCl_3$/MeOH (9:1) to provide [1,1,3-^{3}H]-2-vinyl dihydrosphingosine with 90 % yield. (1.2 mCi; Specific activity 15 mCi/mmol, 14% of theory). Product

identity was verified (R_f, ^1H NMR) by comparison with an authentic unlabelled sample. Radiochemical purity 97 % (by TLC: chloroform-methanol 9:1 $R_f = 0.2$). ^1H NMR ($CDCl_3/CD_3OD$ 7:3) δ 5.81 (m, 1H), 5.45 (m, 2H), 1.63 (m, 2H), 1.32 - 1.20 (m, 26H), 0.90 (t, J = 7 Hz, 3H).

N-tert-butyloxycarbonyl-[1,1,3-^{3}H]-2-vinyl dihydrosphingosine 8: To a stirred solution of **7** (27 mg, 0.082 mmol, 1.2 mCi) in dichloromethane (2 mL) was added di-*tert*-butyl dicarbonate (50 mg, 0.15 mmol). The solution was cooled to 0°C and 4-dimethyl aminopyridine (18 mg, 0.15 mmol) was added. The solution was stirred at room temperature for 5 h, diluted with dichloromethane (5 mL), washed with water (2 mL) and brine (5 mL). Drying over anhydrous sodium sulfate and evaporation provided N-t-Boc protected **7** as a single band by scanning the TLC plate (hexane-ethyl acetate 1:1 R_f = 0.42). This product was not further purified and synthesis was continued on the crude material.

N-tert-butyloxycarbonyl-[1,1,3-^{3}H]-2-vinyl dihydrosphingosine-1-[O,O`-di-(2-cyanoethyl)-phosphate] 9: To crude **8** in solution in acetonitrile:dichloromethane 1:1 (1 mL) was added reagent **5** (22 mg, 0.082 mmol) in solution in acetonitrile (0.5 mL) followed by 1H-tetrazole (10 mg, 0.15 mmol). The reaction mixture was stirred for 1 hour at room temperature. To the solution was added dropwise a solution of m-CPBA (16 mg, 0.09 mmol) in dichloromethane (0.5 mL). The solution was stirred at room temperature for 15 min and diluted with CH_2Cl_2 (5 mL), washed with $NaHCO_3$ (5 mL), water (3 mL). The solution was dried and evaporated to provide crude material which was purified by column chromatography eluted with (chloroform-methanol 9:1) to provide compound **9** with 61 % yield (0.7 mCi, specific activity 14 mCi/mmol, 58 % radiochemical yield). The radiochemical purity was found to be over 98 % by TLC in two solvent systems, (chloroform-methanol 9:1 $R_f = 0.36$; hexane-ethyl acetate 3:2 R_f = 0.15).

[1,1,3-^{3}H]-2-vinyl dihydrosphingosine-1-phosphate 4: To a stirred solution of **9** (31 mg, 0.05 mmol, 0.7 mCi) in dichloromethane (1 mL) was added trifluoroacetic acid (1 mL). After 30 min of stirring at room temperature, the solution was evaporated under reduced

pressure, dissolved in methanol and evaporated, and this sequence was repeated twice. The crude product obtained was dissolved in dimethylamine (40 % in ethanol, 10 mL). The solution was stirred at 45°C for 6 h and evaporated to dryness. The crude product was dissolved in boiling acetic acid (1 mL), the solution was cooled to 0°C and water (1 mL) was dropwise added. The solution was kept at 0°C for 10 min and centrifuged. The supernatant was removed, this step was repeated again. The final pellet was washed with water (1 mL), acetone (2 mL), diethylether (2 mL) and dried under vacuum to afford compound **4** with 58 % as a white solid (12 mg, 0.39 mCi, specific activity 13.8 mCi/mmol, 56 % radiochemical yield). The radiochemical purity of **4** was found to be over 96 % by analysis of the product in three different solvent systems. (n-butanol-acetic acid-water 3:1:1 R_f = 0.51; Chloroform-methanol-water 60:35:8 R_f = 0.25; chloroform-methanol-water-acetic acid 30:30:2:5 R_f = 0.34). Product identity (R_f) was firmly established by comparison with authentic unlabelled material.

ACKNOWLEDGMENTS

We thank Dr. Roscoe O. Brady for his support and his interest in this work.

REFERENCES

1. Hakomori S. Am.-Rev. Biochem.50: 733 (1981).
2. Hannun Y. A. and Bell R. M.-Science. 235: 670 (1987).
3. Ghosh T. K., Bian J. and Gill D. L.-Science. 248:1653 (1990).
4. Buehrer B. M. and Bell R. M.-J. Biol. Chem. 267: 3154 (1992).
5. Zhang H., Desai N. N., Olivera A., Seki T. and Spiegel S.-J. Cell. Biol. 114: 155 (1991).
6. Sadahira Y., Ruan F. Hakomori S. and Igarashi Y.-Proc. Natl. Acad. Sci. USA. 89: 9686 (1992).
7. Stoffel W., Assman G. and Binczek E.-Hoppe-Seyler`s Z. Physiol. Chem. 351: 635 (1970).
8. Stoffel W., Sticht G. and Le Kim D.-Hoppe-Seyler`s Z. Physiol. Chem. 349: 1745 (1968).
9. Keenan R. W. and Maxam A.-Biochem. Biophys. Acta. 176: 348 (1969).

10. Keenan R. W. and Haegelin B.-Biochem. Biophys. Res. Comm. 37: 888 (1969).
11. Louie D. D., Kisic A. and Schroepfer G. J. Jr.-J. Biol. Chem. 251: 4557 (1976).
12. Van Veldhoven P. P. and Mannaerts G. P.-Ad. Lip. Res. 26: 69 (1993).
13. Boumendjel A. and Miller S. P. F.-Tetrahedron Lett. 35: 817 (1994).
14. Boumendjel A. and Miller S. P. F.-J. Med. Chem., manuscript in preparation.
15. Luly J. R., Dellaria J. F., Plattner J. J., Soserquist J. L. and Yi N.-J. Org. Chem. 52: 1487 (1987).
16. Kanda Y.-J. Labelled Cpds & Radiopharm. 31, 1 (1991).
17. Bannwarth W. and Trzeciak. A.-Helv. Chim. Acta. 70: 175 (1987).
18. Kratzer B, and Schmidt R. R.-Tetrahedron Lett. 34: 1761 (1993).
19. Boumendjel A. and Miller S. P. F.-J. Lip. Res., accepted (1994).