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SYNTHESIS OF TRITIUM LABELLED PHOSPHONATE ANALOGUES OF SPHINGANINE-1-PHOSPHATE

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SUMMARY

Tritiated phosphonate analogues **9** and **10** are prepared as analogues of sphinganine-1-phosphate **4**. The key step in this synthesis is the catalytic tritiation of the triple bond in reduction of the protected diethyl-3-(S)-tert.-butoxycarbonylamino-4-hydroxy-5-tridecinyl-1-phosphonate by means of sodium boro[^{3}H]hydride as tritium source. These compounds are synthesized to study their metabolic stability and to evaluate their biological properties.

KEYWORDS: sphingosine-1-phosphate, sphinganine-1-phosphate, sphingolipids, signalling, lipid second messenger

INTRODUCTION

Sphingosine **1** and sphinganine **2** (fig. 1) are the long-chain bases most abundant in sphingolipids, e.g. ceramide, sphingomyelin, cerebrosides and gangliosides (1, 2). Sphinganine **2** itself is an intermediate in the biosynthesis of sphingolipids whereas sphingosine **1** is generated by degradation of ceramide (3). Interests in these intermediates of sphingolipid metabolism increased when sphingosine was found to inhibit strongly and specifically protein kinase C, a pivotal regulatory

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enzyme in cell growth (4). Although ceramide and sphingosine have been the subject of extensive studies, recently, attention has also been focused on sphingosine-1-phosphate **3** and sphinganine-1-phosphate **4**, the initial intermediates in the catabolism of the long-chain sphingoid bases. They are formed by the cytosolic enzyme sphingosine kinase (5, 6) which catalyses the ATP dependent phosphorylation at the 1-OH-position.

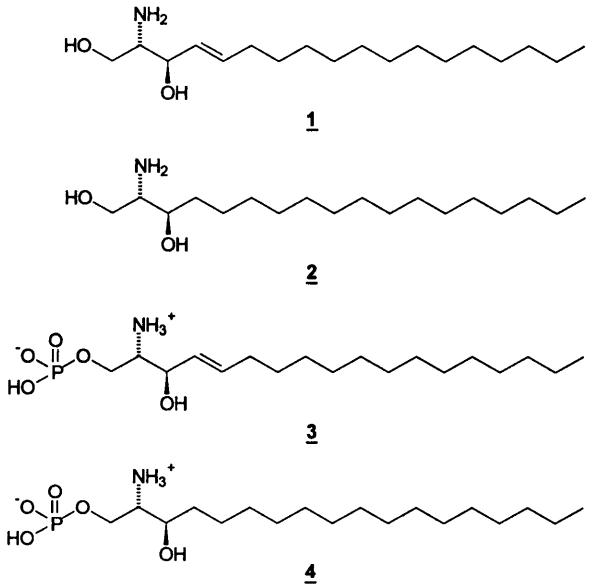


Figure 1

These sphingolipids are not only intermediary catabolites but also bioactive lipids with important functions including multitude of processes. Thus, activation of sphingosine kinase and enhanced formation of sphingosine-1-phosphate was shown to be induced by platelet-derived growth factor (7). Sphingosine-1-phosphate itself has been shown to induce DNA synthesis in Swiss 3T3 fibroblasts in a protein kinase C-independent manner (7). Furthermore, there is evidence that in various cellular systems sphingosine-1-phosphate can cause release of Ca^{2+} from internal stores independent of an inositol 1,4,5-trisphosphate-receptor mediated mechanism (8-10). In addition, sphingosine-1-phosphate decreases cellular cAMP levels and also causes a drastic decrease in isoproterenol- and forskolin-stimulated cAMP accumulation. These results suggest that

some of the sphingosine-1-phosphate-induced signaling pathways are mediated by G proteins that are substrates for pertussis toxin (11). On the other hand, it was demonstrated that sphingosine-1-phosphate activates a G_i protein-coupled plasma membrane receptor in a wide range of cellular systems, leading to increase in cytoplasmatic Ca^{2+} concentration, inhibition of adenylyl cyclase, and opening of G protein-regulated potassium channels (12).

The 1-phosphates **3** and **4** are degraded by the action of a pyridoxal phosphate dependent lyase to yield ethanolamine phosphate and a fatty aldehyde (13-15). In cultured skin fibroblasts sphinganine appeared to be another primary metabolic product of sphinganine-1-phosphate, indicating the action of a phosphatase (16). These findings propose that sphingosine-1-phosphate **3** and sphinganine-1-phosphate **4** may act as possible lipid second messengers controlling cell proliferation and intracellular Ca^{2+} release. The preparation of structural analogues with improved metabolic stability would be very useful for the study of the biological functions and effects of **3** and **4**. For these studies, and for the investigation of the physiological role of these intermediates in general, radioactively labelled analogues are required.

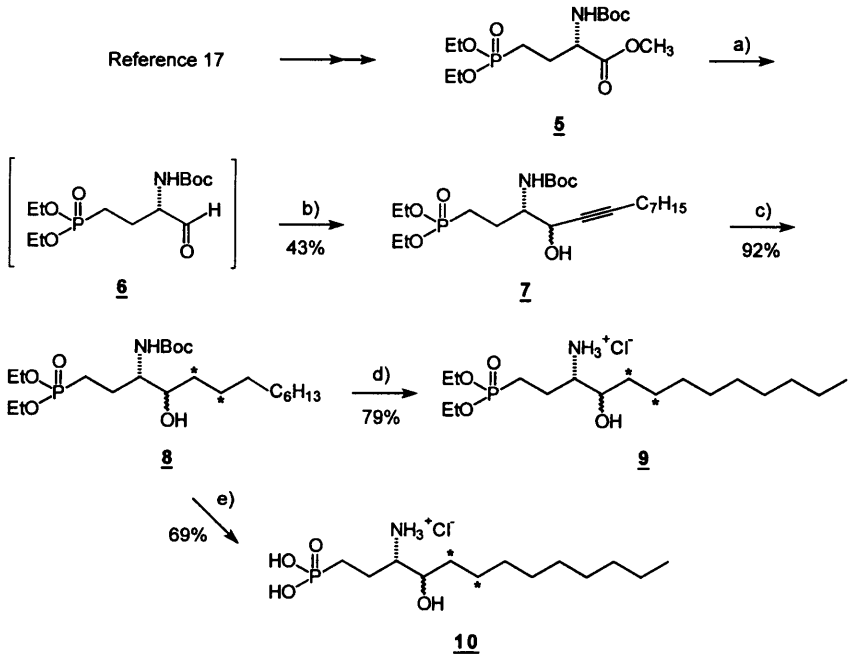
We have recently reported the synthesis of structural analogues of sphingosine-1-phosphate **3** and sphinganine-1-phosphate **4** modified in the headgroup (17, 18). First experiments have shown that these analogues can release Ca^{2+} from internal sources in the same manner as sphingosine-1-phosphate (19). To establish the metabolic stability and the metabolism of these analogues we synthesized the tritium labelled sphinganine-1-phosphonates **9** and **10**.

RESULTS AND DISCUSSION

The key step in the synthesis of the tritium labelled sphinganine-1-phosphonates **9** and **10** was the reduction of the triple bond **7** by using tritium gas and palladium as catalyst according to the procedure of Schwarzmann (20). In this method, the catalyst and the tritium gas are produced *in situ* through the reaction of palladium-II-acetate with sodium boro[^{3}H]hydride. The complete

synthesis is outlined in Figure 2. Other methods used in tritiation of sphingolipids are based on the Wilzbach tritium gas irridiation technique (21, 22) and the catalytic addition of tritium gas to double bonds (23 - 26) all of which require either sophisticiated apparatus and/or tedious work up procedures. Besides catalytic hydrogenation of the CC-double bond with tritium, radioactively labelled sphingoids are obtained by selective oxidation of the allylic hydroxy group and subsequent reduction of the keto group with sodium boro[^{3}H]hydride (27 - 29).

Treatment of 7, which was prepared as reported previously (18), with sodium boro[^{3}H]hydride and palladium-II-acetate led to the crude product of protected sphinganine-1-phosphonate 8.



a) diisobutylammoniumhydride, toluene; b) 1-nonine, n-BuLi, THF;
c) NaB[^{3}H$_4$], Pd(OAc)$_2$, THF, RT; d) MeOH/HCl; e) 4 N HCl
* : indicates position of label

Figure 2. Synthesis of the tritium labelled sphinganine-1-phosphonates

The specific activity, 3.3 Ci/mmol, was 82.5% of the theoretical maximum based upon the information given on the sodium boro[^{3}H]hydride. After removal of excess reagent and the labile tritium by an aqueous work-up, the crude material was purified by column chromatography to provide compound **8** in 92% chemical yield.

Deprotection of the phosphonate diethylester **8** with a saturated methanolic solution of hydrogen chloride afforded the tritiated hydrochlorides **9** in high chemical yield and a specific acitivity (3.3 Ci/mmol). Treatment of the protected phosphonate diethylester **8** with 4 N HCl solution resulted in complete removal of the Boc group and the diethylester groups. The purification of **10** by chromatography was not possible due to the lack of solubility in most organic solvents. Therefore, the crude product was dissolved in boiling 4 N hydrogen chloride solution and **10** was precipitated, by addition of water, as white amorphous powder. After successive washings with water and diethyl ether, **10** was obtained in 69% chemical yield and with a specific acitivity of 3.3 Ci/mmol).

Biochemical studies and experiments are in progress to determine the metabolic stability and the biological properties of **9** and **10**.

EXPERIMENTAL

Solvents were purified in the usual way and/or were argon-saturated prior to use. Tetrahydrofuran is freed of peroxides by passing over basic alumina, followed by saturation with dry argon. Water sensitive reactions were carried out in flame-dried glassware under argon. ^{1}H-NMR spectra were performed on a Bruker AM-400 instrument using tetramethylsilane as internal standard. Elemental analyses were performed with a CHN-O-Rapid analyser (Hereaus, Osterode, Germany) at the Institut für Organische Chemie und Biochemie, Bonn, Abteilung Mikroanalyse. Melting points are uncorrected. R_f values refer to TLC performed on silica gel (Merck, 60 F_{254}) with mobile phases noted. Preparative purification of products was by flash chromatography using silica gel

(Merck, 63-200 μM) or using reversed-phase silica gel (Merck, LiChroprep RP-18, 40-63 μM) under normal pressure. All reagents were of analytical reagent grade or better. Sodium boro[^{3}H]hydride was supplied by Amersham (specific activity: 269 GBq/mmol, 205 mCi/mg). The radioactivity was measured in a Packard liquid scintillation counter (1900CA). Following purification by TLC of the tritiated sphinganine-1-phosphonates, radioactivity was determined and qualified by the use of a Fuji BAS 1000 Bio Imaging analyser (Raytest, Pforzheim, Germany).

Diethyl-3-(S)-tert-butoxycarbonylamino-4-hydroxy-5-tridecinyl-1-phosphonate 7: Methyl ester **5** (365 mg, 1.03 mmol) was dissolved in dry toluene (3.3 ml) and cooled to -78°C. To this cooled solution 1.5 M diisobutylaluminium hydride in toluene (1.2 ml, 1.81 mmol) was added. The rate of addition was adjusted to keep the bath temperature below -70°C. This procedure took approximately 1 h to be completed. After stirring for additional 2 h at -78°C, TLC analysis (dichloro-methane/methanol 30:1, R_f = 0.22) showed the formation of the desired aldehyde **6**. The reaction was quenched by slowly adding 210 μl methanol. Again the temperature of the bath was kept below -70°C. The resulting white suspension was slowly poured into 5 ml of ice-cold 1 N hydrochloric acid and stirred for additional 15 min. After extraction of the aqueous layer with ethyl acetate (3 x 6 ml), the combined organic layers were washed with brine (1 x 5 ml), dried over $MgSO_4$ and concentrated under reduced pressure to give 270 mg of crude product **6** (aldehyde) as a colourless oil. To a solution of 1-nonine (357 μl, 2.17 mmol) in dry THF (6.15 ml) was added a 1.6 M solution of n-BuLi (1.23 ml, 1.96 mmol) under argon at -23°C. The resulting suspension of the lithium alkide was stirred at this temperature for 30 min and then cooled to -78°C. For the further reaction, a solution of the crude aldehyde **6** (353 mg, 0.87 mmol), prepared as described before, in dry THF (4 ml) was added dropwise with a syringe. Stirring of the colourless solution was continued for additional 3 h at -78°C. The reaction was quenched by addition of 16 ml of saturated aqueous ammonium chloride and the mixture was allowed to warm to room temperature. The resulting solution was extracted with ethyl acetate (3 x 5 ml) and the combined organic extracts

were washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure to give a colourless oil. Purification of the residue by flash chromatography on silica gel (dichloromethane/methanol 30:1 R_f = 0.28) afforded 181 mg (43%) of 7 as a 2 : 1 mixture of diastereomers. The diastereomeric ratio was estimated by the integration of 4-OH signal.

^{1}H NMR (400 MHz, $CDCl_3$): d = 0.83 (t, J = 6.1 Hz, 3 H, CH_3); 1.17-1.54 (m, 19 H, $C(CH_3)_3$, Alkyl-CH_2); 1.32 (t, J = 7 Hz, 6 H, $P(OCH_2CH_3)_2$); 1.62-2.03 (m, 4 H, PCH_2CH_2); 2.14 (m, 2 H, $CH_2C{\equiv}C$); 2.81 and 2.93, (d, br, J = 8 Hz, 1 H, diastereomeric OH, integration ratio 2 : 1), 3.71 (m, 1 H, CHNH); 4.04 (dq, $^3J_{HH}$ = 7 Hz, $^3J_{HP}$ = 4 Hz, 4 H, $P(OCH_2CH_3)_2$); 4.36 (m, 1 H, CHOH); 4.81 (br, d, J = 9 Hz, 1 H, NH).

Analysis: $C_{22}H_{42}NO_6P$ (447.274) calcd. (%): C 59.00, H 9.46, N 3.13

found (%): C 58.60, H 9.33, N 3.26;

FAB MS: ($C_{22}H_{43}NO_6P$, MW 448.282) at *m/z* 448.

[^{3}H]-Diethyl-3-(S)-tert.-butoxycarbonylamino-4-hydroxy-5-tridecyl-1-phosphonate 8: 5.59 mg (12.5 µmol) of compound 7 , dissolved in 300 µl of dry peroxide-free THF, were after addition of acetic acid (25 µl) frozen in a screw-capped vial under argon atmosphere in liquid nitrogen. This freezing procedure was repeated after the addition of sodium boro[^{3}H]hydride (0.48 mg, 12.5 µmol, 100 mCi) dissolved in 300 µl 1M sodium hydroxide. Finally, after flushing with argon, 125 µl (6.25 µmol) of 50 mM palladium-II-acetate in THF were layered over the frozen section and the reaction mixture was frozen again under an argon atmosphere in liquid nitrogen. The vial was then promptly capped, care being taken that any air above the solution had been replaced by argon. After warming to room temperature, the hydrogenation reaction was allowed to proceed, with vigorous agitating (Vortex), for at least 48 h at 25°C. To complete the hydrogenation reaction, solid, unlabelled borohydride (1.2 mg, 30 µmol) was added to the reaction mixture with further addition of palladium-II-acetate in THF (6.25 µmol in 125 µl), following the procedure as outlined above. This mixture was agitated for another 24 h at room temperature. For work-up, the reaction mixture

was poured into 2 ml water and 2 ml methanol. The solution was applied to a reversed-phase silica gel column (LiChoprep RP-18) to adsorb the tritiated **8**. Exchangeable tritium was removed by washing the reversed-phase silicia gel with 100 ml water. The tritiated product **8** was then eluted with 50 ml methanol and 75 ml methanol/chloroform (1:1). The solvents were evaporated to dryness in a nitrogen-stream and the residue was dissolved in ethyl acetate. The crude labelled diastereomers **8** obtained thus were purified over a small column containing 2 ml of silica gel (Merck 40-63 µm) with ethyl acetate as eluent to provide **8** with 92% yield (5.19 mg, 1402.67 MBq, 37.91 mCi, specific activity 122.19 Bq/mmol, 3300 Ci/mol. The radiochemical purity was determined by TLC in two systems (chloroform/methanol 30:1 R_f = 0.24, ethyl acetate R_f = 0.13) and found to be over 98%. The labelled product **8** was TLC identical to an authentic unlabelled sample.

[^{3}H]-Diethyl-3-(S)-amino-4-hydroxy-5-tridecyl-1-phosphonate hydrochloride 9: The diastereomeric amino alcohols **8** (1.5 mg, 3.32 µmol, 663.04 MBq, 17.92 mCi) were dissolved in 3 ml methanol, saturated with hydrogen chloride and stirred for 12 h at ambient temperature. The reaction mixture was extracted once with diethyl ether (2 ml) which was discarded. The solvent was evaporated to dryness in a nitrogen-stream to yield 1.02 mg (79%) of **9** (8.65 mCi, 320.05 MBq, specific acitivity 122.19 Bq/mmol, 3300 Ci/mol) as a yellow oil. The radiochemical purity was determined by TLC (chloroform/methanol/$CaCl_2$ 0.22% w/v 60:35:8 R_f = 0.48) and found to be over 97%. The labelled product **9** was TLC identical to an authentic unlabelled sample.

[^{3}H]-3-(S)-amino-4-hydroxy-5-tridecyl-1-phosphonic acid hydrochloride 10: The N-t-Boc protected amino alcohols **8** (2 mg, 4.43 µmol, 884.3 MBq, 23.9 mCi) were dissolved in 3 ml 4 N HCl and refluxed under argon for 10 h. The solvent was evaporated to dryness in a nitrogen-stream, suspended in methanol and evaporated, and this sequence was repeated twice. The crude product was dissolved in boiling 4 N HCl (1 ml) and **10** was precipitated with water (10 ml) as a

white amorphous compound. The solution was kept at 0°C for 15 min and centrifuged. The supernatant was removed, and this step was repeated again. The final pellet was washed several times with water (2 ml) and diethyl ether (2 ml) and dried under reduced pressure (40°C, 12 Torr, 12 h) to afford compound **10** with 69% yield as a white amorphous solid (1.02 mg, 331.52 MBq, 8.96 mCi, specific activity 122.19 Bq/mmol, 3300 Ci/mol). The radiochemical purity of **10** 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 = 0.56; chloroform/methanol/$CaCl_2$ 0.22% w/v R_f = 0.23; chloroform/ methanol/water/acetic acid 30:30:2:5 R_f = 0.31). Product identity (R_f) was firmly established by comparison with authentic unlabelled material.

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