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Short communication

An efficient, one-pot synthesis of various ceramide 1-phosphates from sphingosine 1-phosphate

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Abstract

An efficient, one-pot procedure for the synthesis of ceramide 1-phosphates with varying *N*-acyl substituents, to serve as tool compounds for analytical and biological investigations, was developed. Sphingosine 1-phosphate was silylated *in situ* to increase its solubility and to protect the 3-hydroxy functionality and then allowed to react with activated acid derivatives in the presence of diisopropylethylamine. Simultaneous cleavage of the silyl protecting groups and separation from reagents and by-products was achieved by medium pressure chromatography on reversed phase material. Thus, ceramide 1-phosphates with various fatty acid chains and with fluorescent and affinity labels attached to the sphingoid backbone were prepared in good yields.

Keywords: Biotin; Ceramide phosphate; Fluorescence; Sphingosine phosphate; Sphingolipids; Sphingolipidomics

1. Introduction

Ceramide 1-phosphate (C1P) is a sphingosine-based lipid generated from ceramide, which is the central molecule in the metabolism of sphingolipids, by ceramide kinase. The importance of C1P as signaling molecule has only recently begun to be recognized and investigated (Chalfant and Spiegel, 2005; Baumruker et al., 2005; Marcu and Chalfant, 2007; Graf et al., 2007). C1P is postulated to interact directly with cytosolic phospholipase A2 alpha resulting in activation and, hence, increased release of arachidonic acid and downstream inflammatory mediators (Pettus et al., 2004). Ceramide and C1P are naturally occurring as mixtures of analogues with a constant sphingosine backbone and *N*-acyl residues varying mainly in length ranging from C14 to C32 with optional α- or ω-hydroxy substitution. Therefore, C1P analogues with different, well defined *N*-acyl side chains (only few are commercially available) but also labeled C1P analogues are required as tool and reference compounds for further biological investigations such as metabolism and interaction studies as well as for measuring C1P concentrations in cells and tissues (sphingolipidomics).

Recently, we published two related methods to introduce a label into the sphingosine backbone of sphingolipids (Nussbaumer et al., 2005; Peters et al., 2007). The next goal was to develop an efficient method for the rapid preparation of C1P derivatives with various, defined labeled and unlabeled fatty acid residues for biological investigations. Most of the known synthesis methods for C1Ps require protection of the 3-hydroxy group. Beside enzymatic methods that are mainly used to prepare radiolabeled analogues and can deliver only very minute quantities, there is only one published procedure for phosphorylation of ceramide without the need of protection protocols (Byun et al., 1994). However, each corresponding ceramide is required in sufficient quantity as starting material and their low solubility is often a limiting factor. More recently, phosphorylation of *N*-Boc protected sphingosine and acylation of the deprotected phosphate diester followed by ester cleavage was reported for the synthesis of C1P (Szulc et al., 2000). We envisaged a fast and flexible alternative method starting from sphingosine 1-

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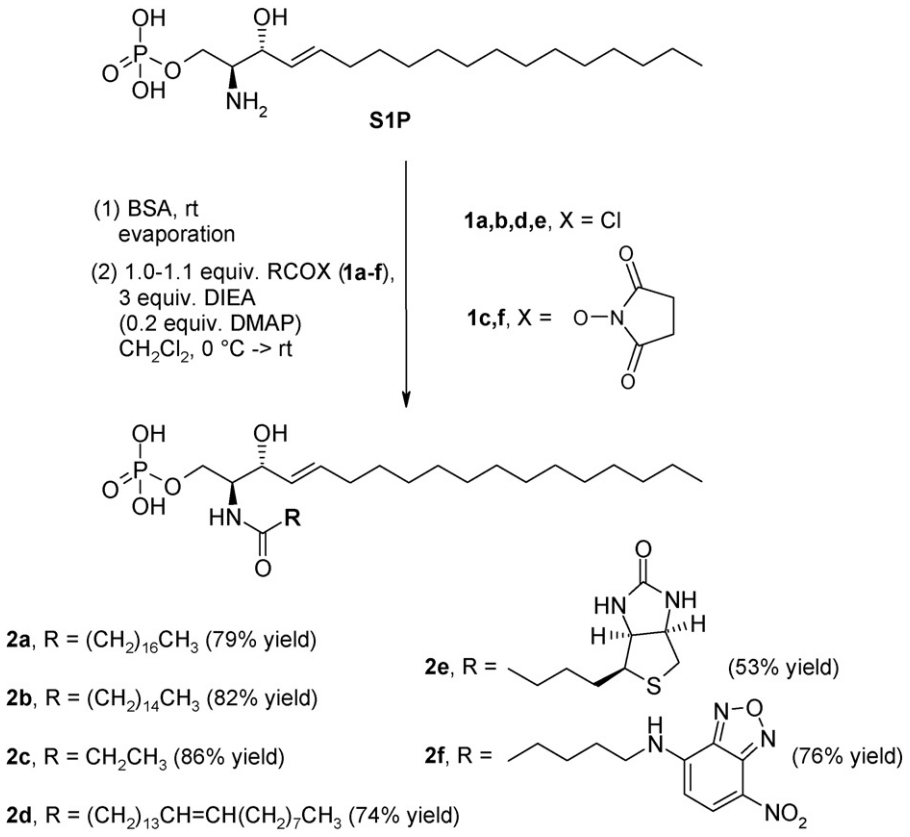

Scheme 1. Synthesis of ceramide 1-phosphates **2a–f** from S1P.

phosphate (S1P) which should provide C1Ps **2** with defined *N*-acyl substituents in one step (Scheme 1). S1P has become commercially available in 100 mg batches at a reasonable price.

2. Experimental procedures

2.1. Materials

Stearoyl chloride (**1a**), palmitoyl chloride (**1b**), nervonic acid and biotin were purchased from Sigma–Aldrich, *N*-succinimidyl-proprionate (**1c**) from Wako Pure Chemical Industries, Ltd., NBD-aminohexanoic acid *N*-hydroxyl succinimidyl ester (**1f**) from Marker Gene Technologies, Inc., and D-erythro-sphingosine-1-phosphate (S1P) from Toronto Research Chemicals, Inc. All reagents were used without further purification.

Nervonoyl chloride (**1d**) and biotin chloride (**1e**) were prepared from the corresponding acids by treatment with oxalyl chloride for 5 h at room temperature followed by evaporation to dryness in high vacuum and used immediately.

The purity of products **2** was checked by ^{1}H NMR, and the structures were further confirmed by ^{13}C NMR, ^{31}P NMR and HRMS. NMR spectra were recorded on a 400 MHz Bruker Avance spectrometer and chemical shifts are reported in δ units (ppm) relative to tetramethylsilane and phosphoric acid, respectively, as internal standard. Chromatography was performed using a Biotage SP4 equipment. HRMS spectra were recorded on a Bruker Daltonics 9.4T APEX-III FT-MS.

2.2. Synthesis

General procedure: synthesis of *N*-[(1*S*,2*R*,3*E*)-2-hydroxy-1-[(phosphonooxy)methyl]-3-heptadecen-1-yl]-octadecanamide (C18-ceramide 1-phosphate, **2a**).

Sphingosine 1-phosphate (10.3 mg, 0.027 mmol) was stirred with *N*,*O*-bis(trimethylsilyl)acetamide (BSA, 400 μl) at room temperature overnight. After evaporation under high vacuum, the residue was dissolved in dry dichloromethane (DCM, 400 μl) and treated at 0 °C with diisopropylethylamine (15 μl, 0.08 mmol) and stearoyl chloride (400 μl from a stock solution of 82 mg stearoyl chloride in 4 ml of dry DCM, corresponding to 0.027 mmol). The mixture was stirred for 2 h, concentrated under vacuum to about 30% of volume and then soaked onto a reversed phase chromatography samplet (Biotage^{TM}). The samplet was dried, loaded onto an RP-18 reversed phase column, and the product was isolated by starting the chromatographic purification with H_2O:MeOH:NH_4OH = 50:50:1, followed by an increasing gradient of MeOH to achieve a final solvent mixture of MeOH:NH_4OH = 100:1. The fractions were analyzed by TLC (silica gel, *n*BuOH:MeOH:H_2O:NH_4OH = 8:1:1:1) and MS. Concentration, dissolution in a small amount of MeOH/dichloromethane and precipitation with acetone gave 13.8 mg (79%) of **2a** as a colourless powder.

Analogously, the following compounds were prepared, with the only exception that 0.2 equivalents of 4-dimethylaminopyridine (DMAP) was added when *N*-hydroxysuccinimide esters **1c**,**f** (1.1 equivalents) were used.

N-[(1*S*,2*R*,3*E*)-2-hydroxy-1-[(phosphonooxy)methyl]-3-heptadecenyl]-hexadecanamide (C16-ceramide 1-phosphate, **2b**), 82% yield.

N-[(1*S*,2*R*,3*E*)-2-hydroxy-1-[(phosphonooxy)methyl]-3-heptadecenyl]-propanamide (C3-ceramide 1-phosphate, **2c**), 86% yield:

^{1}H NMR (CD_3OD): δ 5.72 (dt, $J = 15.2 + 6.7$ Hz, 1H; H-4), 5.48 (dd, $J = 15.2 + 7.6$ Hz, 1H; H-3), 4.10–4.24 (m, 2H), 3.90–3.95 (m, 2H), 2.22 (qua, $J = 7.6$ Hz, 2H; $-COCH_2-$), 2.04 (qua, $J = 7.1$ Hz, 2H; $=C-CH_2-$), 1.27–1.40 (m, 22H), 1.13 (t, $J = 7.6$ Hz, 3H; $-CH_3$), 0.94 (t, $J = 6.8$ Hz, 3H; $-CH_3$); ^{13}C NMR (CD_3OD): δ 177.1 (C=O), 135.1 (–CH=), 131.5 (–CH=), 73.0 (–CH(OH)–), 65.5 ($-OCH_2-$), 55.9(–CH(NH)–), 34.6, 33.8, 33.5, 31.2, 31.1, 31.0, 30.9, 30.8, 30.7, 24.1, 14.8, 10.9; ^{31}P NMR (CD_3OD): δ 2.77; ESI-MS$^-$: 434.2 [M^-]; HRMS: calcd for [M + Na] 458.2642, found 458.2643.

N-[(1*S*,2*R*,3*E*)-2-hydroxy-1-[(phosphonooxy)methyl]-3-heptadecenyl]-(15*Z*)-tetracosenamide (C24:1-ceramide 1-phosphate, **2d**), 74% yield:

^{1}H NMR (CD_3OD): δ 5.70 (dt, $J = 15.2 + 6.7$ Hz, 1H; H-4), 5.45 (dd, $J = 15.2 + 7.6$ Hz, 1H; H-3), 5.30–5.39 (m, 2H; (*Z*)-CH=CH–), 4.10–4.23 (m, 2H), 3.84–3.91 (m, 2H), 2.13–2.24 (m, 2H; $-COCH_2-$), 1.98–2.07 (m, 6H), 1.54–1.63 (m, 2H), 1.25–1.40 (m, 54H), 0.90 (t, $J = 6.8$ Hz, 6H; $2 \times -CH_3$); ^{13}C NMR (CD_3OD): δ 176.3 (C=O), 135.1 (–CH=), 131.6 (–CH=), 131.3 ($2 \times$ –CH=), 72.8 (–CH(OH)–), 65.5 ($-OCH_2-$), 55.9 (–CH(NH)–), 37.8, 33.9, 33.5, 33.4, 31.3, 31.2, 31.1, 31.0, 30.9, 30.8, 30.7, 28.5, 27.5, 24.1, 14.9; ^{31}P NMR (CD_3OD): δ 3.11; ESI-MS$^-$: 726.4 [M^-]; HRMS: calcd for [M + H] 728.5953, found 728.5951.

N-[(1*S*,2*R*,3*E*)-2-hydroxy-1-[(phosphonooxy)methyl]-3-heptadecenyl]-5-[(3*aS*,4*S*,6*aR*)-hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl]-pentanamide (biotin-C5-ceramide 1-phosphate, **2e**), 53% yield:

^{1}H NMR (CD_3OD/d_6-DMSO): δ 5.70 (dt, $J = 15 + 6.7$ Hz, 1H; H-4), 5.47 (dd, $J = 15.2 + 7.5$ Hz, 1H; H-3), 4.46–4.52 (m, 1H), 4.28–4.34 (m, 1H), 4.07–4.27 (m, 2H), 3.91–4.00 (m, 2H), 3.17–3.25 (m, 1H), 2.92 (dd, $J = 5 + 12.6$ Hz, 1H), 2.70 (d, $J = 12.6$ Hz, 1H), 2.22 (t, $J = 7.4$ Hz, 2H; $-COCH_2-$), 2.03 (qua, $J = 7$ Hz, 2H; $=C-CH_2-$), 1.23–1.78 (m, 28H), 0.90 (t, $J = 6.8$ Hz, 3H; $-CH_3$); ^{13}C NMR (CD_3OD/d_6-DMSO): δ 174.3 (C=O), 133.4 (–CH=), 129.7 (–CH=), 71.1 (–CH(OH)–), 64.1 ($-OCH_2-$), 61.9, 60.2, 55.5, 54.0, 39.7, 35.5, 32.0, 31.7, 29.4, 29.3, 29.0, 28.9, 28.4, 28.0, 25.4, 22.3, 13.1; ^{31}P NMR (CD_3OD): δ 3.11; ESI-MS$^-$: 604.4 [M^-]; HRMS: calcd for [M + Na] 628.3156, found 628.3157.

N-[(1*S*,2*R*,3*E*)-2-hydroxy-1-[(phosphonooxy)methyl]-3-heptadecenyl]-6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-hexanamide (NBD-C6-ceramide 1-phosphate, **2f**), 76% yield:

^{1}H NMR (CD_3OD): δ 8.53 (d, $J = 8.9$ Hz, 1H; Ar–H), 6.37 (d, $J = 8.9$ Hz, 1H; Ar–H), 5.70 (dt, $J = 15.3 + 6.7$ Hz, 1H; H-4), 5.45 (dd, $J = 15.2 + 7.3$ Hz, 1H; H-3), 3.97–4.18 (m, 4H), 3.48–3.57 (m, 2H; $-CH_2-NH-$), 2.20–2.28 (m, 2H; $-COCH_2-$), 2.00 (qua, $J = 7$ Hz, 2H; $=C-CH_2-$), 1.79 (qui, $J = 7.5$ Hz, 2H), 1.68 (qui, $J = 7.6$ Hz, 2H), 1.48 (qui, $J = 7.6$ Hz, 2H), 1.20–1.38 (m, 22H), 0.88 (t, $J = 7$ Hz, 3H; $-CH_3$); ^{13}C NMR (CD_3OD): δ 174.7 (C=O), 138.4 (Ar–C), 135.0 (–CH=), 131.0 (–CH=), 99.4 (Ar–C), 72.6 (–CH(OH)–), 66.2, 55.0, 44.2, 36.7, 33.4, 33.0, 30.8. 30.7, 30.5, 30.4, 30.3, 28.8, 27.6, 26.6, 23.7, 14.4; ^{31}P NMR (CD_3OD): δ 2.77; ESI-MS$^-$: 654.4 [M^-]; HRMS: calcd for [$M - H$] 654.3273, found 654.3279.

3. Results and discussion

We found only one report of direct acylation of S1P where excess acetic anhydride and triethylamine were used to generate C2–C1P (Gijsbers et al., 1999). The same group also prepared tritium labeled *N*-acetyl- and *N*-hexanoyl-sphinganine 1-phosphate from sphinganine 1-phosphate by this method (De Ceuster et al., 1995). However, in both cases the 3-hydroxy group in the sphingosine/sphinganine backbone was also acylated and the ester intermediates had to be selectively cleaved. Moreover, we expected complications in the reaction and product purification when using longer, less soluble fatty acid derivatives. In our first experiments, we attempted to use the *N*-hydroxysuccinimide ester of octadecanoic acid to achieve selective acylation, since such activated esters are known to react highly preferentially with an amino over a hydroxy group. As solvent, we chose pyridine because S1P is very sparingly soluble in other organic solvents including dimethylformamide and dimethylsulfoxide. At room temperature, no conversion of S1P could be detected either in the presence or absence of triethylamine; only at 60 °C for 48 h some product formation was observed (TLC and MS detection). When 4-dimethylaminopyridine (DMAP) was used as catalyst, very slow conversion of S1P was seen already at room temperature, whereas a reasonable reaction time (15 h) for nearly complete conversion was observed at 60 °C. However, for full consumption of the starting material at least 2 equivalents of hydroxysuccinimide ester had to be applied and under these conditions also some double-acylated product (*N*- and 3-*O*-acylation) was generated. This by-product and unreacted materials could be separated from the desired C18-ceramide phosphate (**2a**, Scheme 1) by trituration with ethyl acetate followed by chromatography (silica gel, *n*-BuOH:MeOH:NH_4OH:H_2O = 8:1:1:1), but we were not satisfied with the procedure.

The most limiting factor we experienced in these initial experiments was the very poor solubility of S1P which did not allow much variation of reaction conditions. Therefore, we planned to apply *in situ* intermediate protection of the phosphate group, still avoiding a multi-step sequence, and opted for a silyl protecting group which would be cleaved during work-up. For amino acids this strategy had been known for a long time (Birkofer et al., 1959) and during our investigations the application to phosphinate amino acids was reported (Li et al., 2007). The *in situ* silylation was achieved by reacting S1P with excess of neat *N*,*O*-bis(trimethylsilyl)acetamide (BSA).

Within minutes, S1P dissolved completely and after evaporation under high vacuum the silylated S1P also dissolved well in dry dichloromethane. Now the conversion with octadecanoyl chloride (**1a**) in the presence of diisopropylethylamine (DIEA) proceeded smoothly at room temperature without formation of the double-acylated product and with nearly complete consumption of S1P (the silylated intermediates could not be analysed due to their instability). The only remaining issue was the isolation and purification of the ceramide phosphate **2a** since aqueous treatment to remove the silyl protecting groups resulted in a very poorly soluble crude product and simple precipitation with acetone from dichloromethane/methanol solution did not provide pure material. The optimised procedure we developed was to directly load the crude reaction mixture together with the solvent on a RP-18 reversed phase chromatography samplet and to perform the chromatographic purification on reversed phase material without any work-up. The presence of water in the starting eluent was sufficient to cleave the silyl protecting groups and we never isolated a silylated S1P or C1P derivative by applying this procedure. Thus, the final one-pot procedure became very simple, i.e. silylation of S1P, concentration, acylation and finally chromatographic purification.

The actual goal was to develop a method allowing broad variation of the *N*-acyl residue. Therefore, we reacted the *in situ* generated silylated S1P with additional fatty acid chlorides **1b** and **1d** (Scheme 1). The corresponding products **2b** and **2d** were again obtained in high purity and good yield (82% and 74%, respectively). Then we applied the hydroxysuccinimide ester of propanoic acid (**1c**) as a model reagent, in combination with DMAP as catalyst to guarantee fast conversion. In this reaction we also obtained the desired ceramide phosphate derivative (**2c**) in high yield (86%) demonstrating that less activated acid derivatives can be used as acylation reagents without the need for more drastic reaction conditions. This result is in contrast with the observation in an initial experiment that acylation of (non-silylated) S1P in pyridine with the hydroxysuccinimide ester of octadecanoic acid was only achieved at 60 °C. Next, we investigated the synthesis of C1P derivatives featuring two different labels in the fatty acid residue (biotin and the fluorescent dye 7-nitro-4-benzofurazanamine (NBD); Scheme 1). The use of biotin chloride (**1e**) gave product **2e** in moderate yield (53%), which was mainly caused by the very poor solubility of the product and not due to a lower conversion. The introduction of the NBD-label was accomplished by using the activated ester **1f**, again in good yield (76%), confirming that under the optimised conditions with the *in situ* silylation, the conversion of hydroxysuccinimide esters occurs readily at room temperature. Compound **2f** is a useful tool compound to complement metabolism studies with the corresponding, commercially available NBD-C6-ceramide.

In summary, the intermediate *in situ* silylation of S1P increases its solubility in organic aprotic solvents dramatically and simultaneously renders the amino group readily accessible for acylation with structurally diverse acid chlorides and hydroxysuccinimide esters. The cleavage of the silyl protecting groups during the chromatographic purification avoids handling of poorly soluble crude products. All reactions studied proceeded well with moderate to good yields (Scheme 1) and we never observed a double-acylated product, as the 3-hydroxy group of the sphingosine backbone was also intermediately silylated under the described conditions (confirmed by NMR experiments of the silylated S1P). Occasionally, the isolated products **2** contained some residual DIEA which could be easily removed under high vacuum. Thus, this new method provides quick and easy access to ceramide 1-phosphate derivatives with various acyl residues.

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