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Synthesis and Immobilization of *erythro*-C14-ω-Aminosphingosine-1-phosphate as a Potential Tool for Affinity Chromatography

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A sphingosine-1-phosphate (S1P) analogue containing a terminal alkyl chain amino group is synthesized in a few steps via olefin cross-metathesis of an optically resolved intermediate and subsequent phosphorylation. Regioselective acylation of this intermediate at its N terminus in solution is demonstrated as a model reaction and provides a biologically active derivative. Finally, the ω-amino intermediate is immobilized on an affinity matrix. The choice of a UV-active phosphate protecting group allows for quantification of resin loading after cleavage which amounted to 66 %.

Introduction

Sphingosine-1-phosphate (S1P, **1**, Figure 1) is a bioactive lipid produced from the metabolism of sphingomyelin and glycosphingolipids. It is released from activated platelets and from erythrocytes and is also produced by a number of other cell types in response to growth factors and cytokines.[1a,b] It is believed to act both as an extracellular mediator and as an intracellular second messenger.[2] The cellular effects of **1** (including growth related effects, such as proliferation, differentiation, and cell survival, and cytoskeletal effects, such as chemotaxis, aggregation, adhesion, morphological change, and secretion) are widely associated with its extracellular targets, the S1P receptors.[3] On the other hand, its intracellular actions—including the regulation of Ca^{2+} mobilization and suppression of apoptosis—are poorly understood today. Identification of intracellular molecular targets for **1** is mandatory to elucidate its involvement in unrevealed signaling pathways.

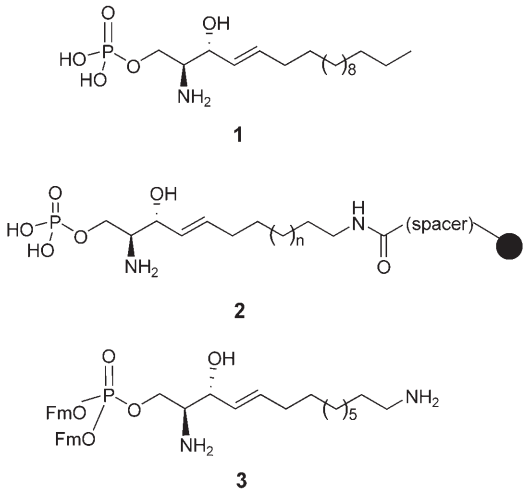

Figure 1. S1P (**1**) and functionalized derivatives for immobilization.

Bioactive small molecules have been used to manipulate biological pathways without knowing the definite molecular target for a long time. The so-called "target fishing" approach has become a very successful tool for target identification and binding studies. As the method of choice, affinity chromatography techniques allow protein separation on the basis of a nonmodifying, reversible interaction between a particular protein (or group of proteins) and a specific ligand coupled to a chromatographic matrix. It offers high selectivity, hence high resolution, and usually high capacity for the protein(s) of interest.[4] An appropriate affinity matrix carrying immobilized **1** could therefore be used to specifically bind and isolate target proteins out of cell or tissue lysates.

Whereas we consider the polar head group of **1**—including the phosphate, amino, and hydroxyl group—to be vital for biological activity, the far end of its fatty acid chain is believed to play the least significant role in target interactions; the latter point is supported by previously disclosed results.[5]

Therefore, the terminus of the alkyl chain of the sphingoid base was chosen to introduce a functional group, that is, a primary amino group, allowing coupling to a resin. Affi-Gel is a

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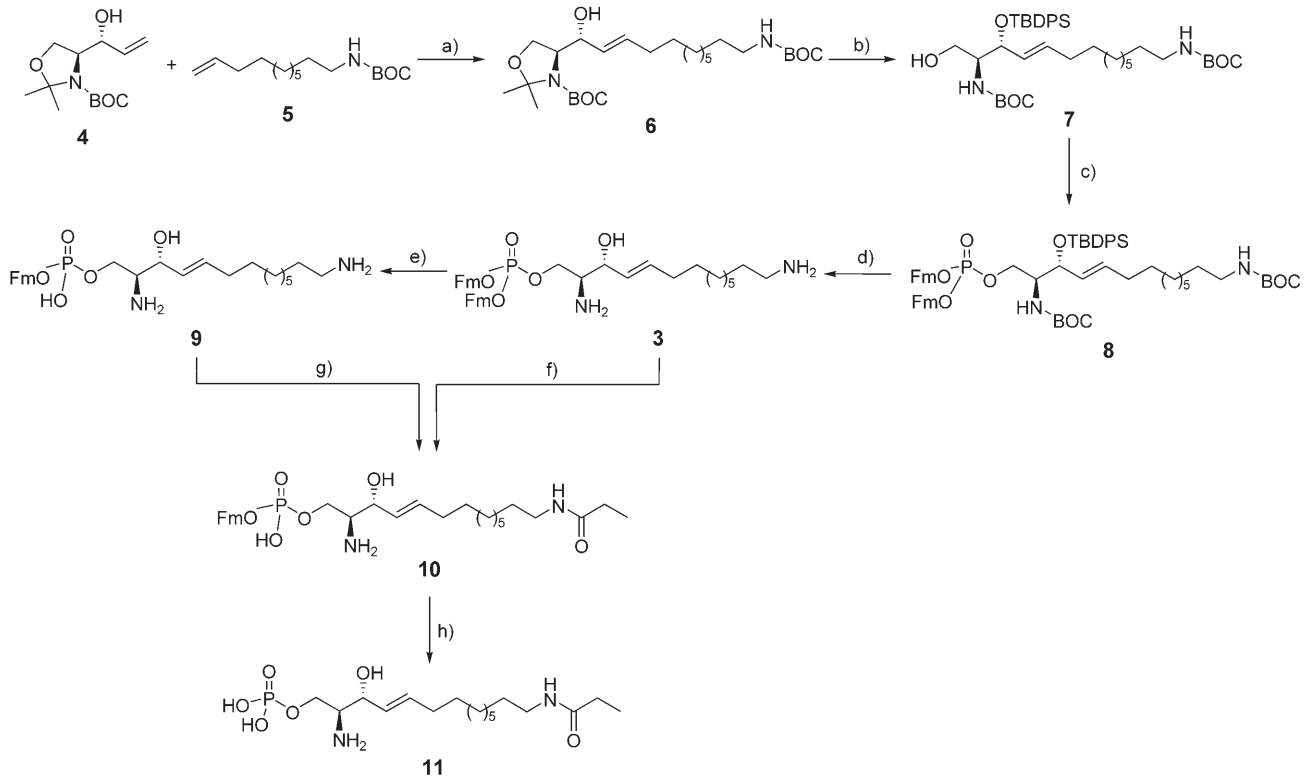
widely used activated agarose-based affinity support that offers rapid, high-efficiency coupling for all ligands with a primary amino group by virtue of its surface-bound activated *N*-hydroxysuccinimide (NHS) esters.[6] In this first approach we opted for a chain length of nine C atoms (**2**, $n=5$) between the double bond and the amino group, as previous studies had demonstrated that a C_9 alkyl chain was appropriate to retain biological activity of backbone-functionalized sphingosine analogues to a satisfactory extent.[7] Furthermore it was necessary to introduce a phosphate moiety with appropriate protective groups to provide ω-aminosphingosine-1-phosphate **3**. Ideally, these protective groups should be UV active and cleavable after immobilization, thus providing a quantitative measure of gel loading. We chose fluorenylmethyl (Fm), a protective group which is widely used in phosphate chemistry and can be easily cleaved under mild basic conditions.[8] Thus, compound **3** featuring a double Fm-protected phosphate and a terminal alkyl amino group, was synthesized.

Results and Discussion

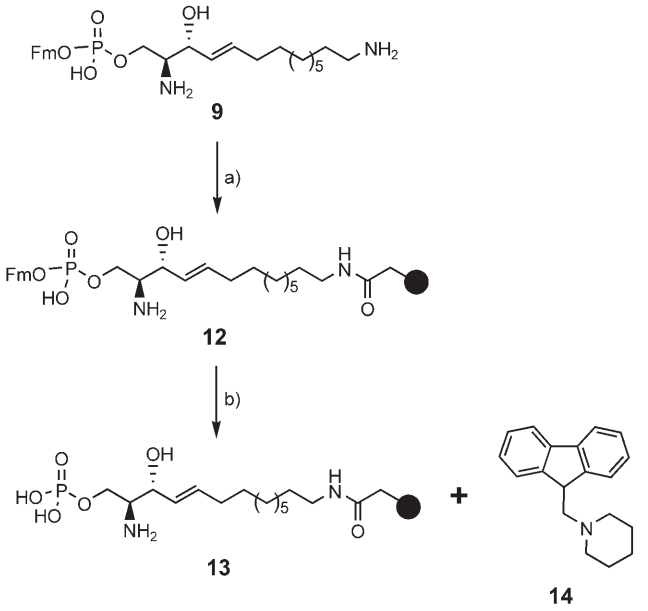
Three independent total syntheses of protected ω-aminosphingosines (with different chain lengths) have been reported.[5,9,10] Opting for a short and easily reproducible route, we used a method which we recently described for efficient synthesis of backbone-labeled sphingolipids.[7,11] Olefin cross metathesis of allyl alocohol **4**[12] and *N*-Boc-undec-10-enylamine (**5**)[13] under standard conditions using Grubb's catalyst (2nd generation) gave **6** in excellent yields (Scheme 1) under exclusive formation of the (*E*)-double bond, as previously observed with similar analogues by us[7,11] and others.[14] The secondary alcohol was protected as *tert*-butyl-diphenylsilyl (TBDPS) ether prior to cleavage of the acetonide by aqueous acetic acid[15] to afford **7**. Other silyl ethers, such as *tert*-butyl-dimethylsilyl (TBDMS) ether, proved too labile under these conditions. Conversion to Fm-protected phosphate **8** was achieved by a one-pot treatment with Fm-substituted diisopropylphosphoramidate followed by oxidation of the resulting trivalent phosphorus to pentavalent phosphorus.[16] The high stability of the Fm groups towards acidic hydrolysis allowed for concomitant cleavage of the TBDPS group and both N-Boc groups with a mixture of HF and HCl to give **3**. The terminal amino group of **3** was expected to react regioselectively with the reactive groups of the selected affinity matrix,[9] that is, Affi-Gel 10 loaded with 15 μmol mL^{-1} of NHS esters linked to the gel beads by decapeptide spacers. To test whether conversion would occur in solution phase, **3** was reacted with *N*-succinimidyl propionate. It was observed that under weakly basic conditions using diisopropylethylamine (DIPEA, Hünig's base) one of the two Fm groups was rapidly cleaved whereas the second one remained intact even after prolonged exposure. The reaction resulted exclusively in the formation of **10**. A control experiment showed that in the absence of acylation reagent, rapid single Fm cleavage also occurred to give **9**. Impor-



Scheme 1. Solution-phase synthesis: a) Grubb's catalyst (2nd generation), DCM, 40 °C, 4 h, 86%; b) TBDPS-chloride, imidazole, DMF, rt, 18 h; aq. AcOH, 50 °C, 4 h, 73%; c) bis-(9H-fluoren-9-ylmethyl)-diisopropylphosphoramidate, 1*H*-tetrazole, *t*BuOOH, DCM/MeCN, rt, 5 h, 86%; d) 4*N* HCl/dioxane, aq. HF, MeCN, 60 °C, 12 h, 26%; e) DIEA, NMP, rt, 5 h, 90%; f) *N*-succinimidyl propionate, DIEA, DMF, rt, 24 h, 47%; g) *N*-succinimidyl propionate, DIEA, DMF, rt, 2 h, 68%; h) piperidine, DMF, rt, 1 h, 44%.

tantly, it could be demonstrated that the free phosphate OH group in **9** did not impede N-acylation, as the compound could be almost quantitatively converted into **10** under the same conditions as applied before and, therefore, **9** proved to be the suitable substrate for immobilization.

Thus, reaction of **9** (2 equiv) with Affi-Gel 10 in NMP in the presence of excess DIPEA produced immobilized product **12** (Scheme 2). Following a procedure reported by Shannon et al.,[17] treatment with excess piperidine/DMF then led to cleavage of the Fm group to give free phosphate **13** and quantitative formation of the piperidine-dibenzofulvene



Scheme 2. Immobilization of **9** and cleavage of the protective group: a) Affi-Gel 10, DIEA, NMP, rt, 5 h; b) piperidine/DMF (1:1), NMP, rt, 5 h.

adduct **14** which could be identified by LC/MS. To determine the percentage of sphingosine phosphate bound to the gel, the concentration of **14** was determined by UV spectroscopy measuring absorbance at 301 nm and using the known extinction coefficient for the dibenzofulvene chromophore.[17] According to this calculation, the effective loading was 66%, corresponding to 10 μmol mL^{-1} gel. Under the chosen deprotection conditions, unreacted NHS esters on the resin were concomitantly blocked during repeated washing with piperidine.

Prior to initiating target fishing experiments with **13**, it had to be established whether an amino-functionalized sphingosine-1-phosphate displayed sufficient biological activity. To this end, **10** was deprotected with piperidine to afford phosphoric acid **11** (Scheme 1), the structure of which was confirmed by HR-MS, ^{1}H, ^{13}C, and ^{31}P NMR. **11** was tested and compared with the natural analogue S1P (**1**) for agonist-induced changes in intracellular Ca^{2+} in CHO cells expressing the human sphingosine-1-phosphate receptors S1P-1 (formerly Edg-1), S1P3 (Edg-3), S1P4 (Edg-6), and S1P5 (Edg-8).[18] The compound behaved as a full agonist at S1P1, S1P3, and S1P5, and as a partial agonist at S1P4. In general, its potency was 1–3 orders of magnitude lower than that of the natural substrate, S1P (Table 1). Importantly, **11** gave EC_{50} values at submicromolar concentrations on all four receptors. Moreover, **11** was also functionally active in human endothelial cells where it induced phosphorylation

Table 1. Potency (pEC_{50}) values of S1P and 11 at human S1P receptors.

Compd.	S1P-1 (pEC_{50})[a,b]	S1P-3 (pEC_{50})	S1P-4 (pEC_{50})	S1P-5 (pEC_{50})
1 (S1P)	10.2 ± 0.1	9.75 ± 0.50	8.53 ± 0.47	8.95 ± 0.60
11	7.21 ± 0.04	8.12 ± 0.07	6.82 ± 0.42	7.94 ± 0.21

[a] pEC_{50}: −log molar concentration of compounds resulting in 50% of maximal Ca^{2+} mobilization. [b] Average value of three to six experiments.

of extracellular-signal regulated kinases (ERK1/2) at about only 30-fold higher concentrations than S1P.[19] Based on these biological data, **13** appears to be a suitable tool for target-fishing experiments.

Conclusions

The present synthetic route provided Fm-protected *erythro*-C14-ω-aminosphingosine-1-phosphate (**9**) in only a few steps from commercially available materials. **9** was successfully acylated and deprotected in solution to furnish **11**, mimicking the immobilization procedure. **11** was shown to act as a full or partial agonist at four sphingosine-1 receptors and to induce ERK-phosphorylation in a cellular system, proving that the biological activity of natural S1P can be retained to a satisfactory extent. With these results in hand, we subjected **9** to immobilization on an affinity gel. Quantitative measurement of the cleaved protective group by UV spectroscopy resulted in a calculated gel loading of 66%. These data give confidence that the immobilized sphingosine-1-phosphate derivative **13** will be indeed suitable for target-fishing affinity chromatography. Results will be reported in due course.

Experimental Section

All commercial reagents were purchased from Sigma–Aldrich unless stated otherwise. Garner's aldehyde was purchased from Epsilon Chimie, *N*-succinimidyl propionate from Wako Pure Chemical Industries, Ltd., and Affi-Gel 10 was obtained from Bio-Rad Laboratories, Inc. Normal phase chromatography was performed using Biotage SP1 or SP4 equipment. Reversed phase chromatography was carried out on a Gilson Abimed system. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a 400 MHz or 500 MHz Bruker Avance spectrometer and chemical shifts are reported in δ units (ppm) relative to tetramethylsilane and phosphoric acid, respectively, as internal standards. HR-MS spectra were recorded on a Bruker Daltonics 9.4T APEX-III FT-MS.

(*S*)-4-((*E*)-(*R*)-12-tert-Butoxycarbonylamino-1-hydroxy-dodec-2-enyl)-2,2-dimethyl-oxazolidine-3-carboxylic acid tert-butyl ester (6): Grubb's catalyst (2nd generation) (396 mg, 0.466 mmol) is added to a degassed solution of **4**[20] (1.20 g, 4.66 mmol) and **5**[13]

(5.03 g, 18.7 mmol) in dichloromethane (80 mL). The mixture is stirred at reflux temperature in an argon atmosphere for 5 h. After evaporation of the solvent, the residue is purified by silica gel chromatography (cyclohexane/ethyl acetate 1:3). 2.00 g (4.01 mmol, 86% yield) of **6** is obtained. ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 6.70$ (t, 1H, $J = 5.2$ Hz), 5.49 (td, 1H, $J = 15.4$, 6.4 Hz), 5.38 (dd, 1H, $J = 15.4$, 6.7 Hz), 4.96 (bs, 1H), 3.60–3.95 (m, 4H), 2.86 (qua, 2H, $J = 6.7$ Hz), 1.88–1.99 (m, 2H), 1.39 (s, 9H), 1.35 (s, 9H), 1.15–1.34 ppm (m, 20H); HR-MS (*m/z*): calcd. 499.3791, found 499.3741 (MH).

[(*E*)-(12R,13S)-13-tert-Butoxycarbonylamino-12-(tert-butyl-diphenyl-silanyloxy)-14-hydroxy-tetradec-10-enyl]-carbamic acid tert-butyl ester (7): *tert*-Butyldiphenylsilyl chloride (987 mg, 3.6 mmol)is added dropwise to a solution of **6** (1.51 g, 3.0 mmol), imidazole (306 mg, 4.5 mmol), and 4-(dimethylamino)pyridine (37 mg, 0.3 mmol) in *N*,*N*-dimethylformamide (50 mL). The mixture is stirred in an argon atmosphere at 55 °C for 18 h, quenched with 1N HCl and extracted exhaustively with ethyl acetate. The collected organic layers are washed with brine, dried (sodium sulfate), filtered, and evaporated. The residue is taken up in acetic acid (100 mL) containing 10% water and stirred at 50 °C for 4 h. The mixture is quenched with aqueous sodium bicarbonate and extracted exhaustively with ethyl acetate. The combined organic extracts are washed with brine, dried (sodium sulfate), filtered, and evaporated. The residue is purified by silica gel chromatography (cyclohexane/ethyl acetate 2:3). 1.52 g (2.18 mmol, 73% yield) of **7** is obtained. ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 7.59$ (dt, 4H, $J = 7.7$, 1.3 Hz), 7.32–7.46 (m, 6H), 6.73 (t, 1H, $J = 5.4$ Hz), 6.21 (d, 1H, $J = 9.1$ Hz), 5.30 (dd, 1H, $J = 15.4$, 8.3 Hz), 4.97 (td, 1H, $J = 15.4$, 6.5 Hz), 4.42 (t, 1H, $J = 5.4$ Hz), 4.17 (dd, 1H, $J = 8.0$, 6.2 Hz), 3.52–3.63 (m, 1H), 3.34–3.49 (m, 2H), 2.87 (qua, 2H, $J = 6.5$ Hz), 1.64 (bs, 2H), 1.36 (s, 9H), 1.33 (s, 9H), 0.98–1.25 (m, 14H), 0.96 ppm (s, 9H); HR-MS (*m/z*): calcd. 697.4606, found 697.4606 (MH).

[(*E*)-(1*S*,2*R*)-1-[Bis-(9H-fluoren-9-ylmethoxy)-phosphoryloxymethyl]-13-tert-butoxycarbonylamino-2-(tert-butyl-diphenyl-silanyloxy)-tridec-3-enyl]-carbamic acid tert-butyl ester (8): A solution of diisopropyl-phosphoramidous acid bis-(9H-fluoren-9-ylmethyl) ester[16] (3.06 g, 5.87 mmol) is added to a solution of **7** (1.52 g, 2.18 mmol) in dichloromethane/acetonitrile (1:1) (40 mL) followed by dropwise addition of 1*H*-tetrazole (410 mg, 5.85 mmol) in dichloromethane/acetonitrile (1:1) (20 mL). The mixture is stirred in an argon atmosphere at ambient temperature for 5 h. *tert*-Butylhydroperoxide (3M in toluene, 1.1 mL, 3.3 mmol) is added dropwise, and the mixture is stirred for another 45 min. After quenching with water and extraction with dichloromethane, the combined organic extracts are dried (sodium sulfate), filtered, and evaporated. The residue is purified by silica gel chromatography (cyclohexane/ethyl acetate 1:1). 2.13 g (1.88 mmol, 86% yield) of **8** is obtained. ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 7.85$ (t, 4H, $J = 6.8$ Hz), 7.47–7.56 (m, 8H), 7.20–7.40 (m, 14H), 6.71 (t, 1H, $J = 5.5$ Hz), 6.62 (d, 1H, $J = 8.4$ Hz), 5.21 (dd, 1H, $J = 15.4$, 8.6 Hz), 4.92 (td, 1H, $J = 15.4$, 6.3 Hz), 4.14–4.20 (m, 2H), 4.12 (d, 4H, $J = 4.2$ Hz), 4.02–4.06 (m, 1H), 3.91 (t, 1H, $J = 5.5$ Hz), 3.78 (bs, 2H), 2.86 (qua, 2H, $J = 6.5$ Hz), 1.56 (qua, 2H, $J = 6.1$ Hz), 1.35 (s, 9H), 0.92–1.32 (m, 14H), 1.25 (s, 9H), 0.89 ppm (s, 9H); HR-MS (*m/z*): calcd. 1155.5654, found 1155.5655 (MNa).

Phosphoric acid (E)-(2S,3R)-2,14-diamino-3-hydroxy-tetradec-4-enyl ester bis-(9H-fluoren-9-ylmethyl) ester (3): 8 (2.00 g, 1.77 mmol) is treated with HCl (4N in dioxane, 25 mL) and aqueous HF (40%, 6 mL) in a plastic vessel. The mixture is stirred at 60 °C for 12 h. After evaporation of the solvents, the crude product is purified by reversed phase HPLC (C18, acetonitrile/water containing 0.1% TFA). 320 mg (0.46 mmol, 26% yield) of **3** (bis-trifluoroacetate) is obtained. ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.09$ (bs, 3H), 7.85 (t, 4H, $J = 7.6$ Hz), 7.72 (bs, 3H), 7.56 (dd, 2H, $J = 7.5$, 4.0 Hz), 7.52 (t, 2H, $J = 7.2$ Hz), 7.40 (t, 2H, $J = 7.5$ Hz), 7.37 (t, 2H, $J = 7.7$ Hz), 7.23–7.30 (m, 4H), 5.70 (bs, 1H), 5.64 (td, 1H, $J = 15.4$, 6.8 Hz), 5.33 (dd, 1H, $J = 15.4$, 6.2 Hz), 4,22–4.30 (m, 4H), 4.18 (t, 1H, $J = 5.7$ Hz), 4.17 (t, 1H, $J = 5.9$ Hz), 3.87–3.98 (m, 2H), 3.26 (bs, 2H), 2.73 (sext, 2H, $J = 5.8$ Hz), 1.91 (qua, 2H, $J = 6.6$ Hz), 1.48 (quin, 2H, $J = 7.4$ Hz), 1.15–1.27 ppm (m, 12H); HR-MS (*m/z*): calcd. 695.3608, found 695.3607 (MH).

Phosphoric acid (E)-(2S,3R)-2,14-diamino-3-hydroxy-tetradec-4-enyl ester 9H-fluoren-9-ylmethyl ester (9): A solution of **3** (bis-trifluoroacetate) (290 mg, 0.31 mmol) and diisopropylethylamine (0.102 mL, 0.60 mmol) in *N*-methylpyrrolidone (10 mL) is shaken at room temperature for 5 h. An aliquot of the mixture (2 mL) is concentrated and purified by reversed phase HPLC (C18, acetonitrile/water containing 0.1% TFA). 43 mg (56 μmol, 90% yield) of **9** (bis-trifluoroacetate) is obtained. ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.29$ (bs, 3H), 7.86 (d, 2H, $J = 7.5$ Hz), 7.29 (bs, 3H), 7.67 (d, 2H, $J = 7.5$), 7.40 (t, 2H, $J = 7.2$ Hz), 7.31 (t, 2H, $J = 7.5$ Hz), 5.63 (td, 1H, $J = 15.4$, 6.8 Hz), 5.40 (dd, 1H, $J = 15.4$, 6.2 Hz), 4.18 (t, 1H, $J = 6.7$ Hz), 4.13 (t, 1H, $J = 3.9$ Hz), 3.97–4.10 (m, 3H), 3.70–3.79 (m, 2H), 3.13 (bs, 2H), 2.66–2.74 (m, 2H), 1.98 (qua, 2H, $J = 6.5$ Hz), 1.48 (quin, 2H, $J = 7.5$ Hz), 1.15–1.27 ppm (m, 12H); HR-MS (*m/z*): calcd. 517.2826, found 517.2826 (MH).

Phosphoric acid (E)-(2S,3R)-2-amino-3-hydroxy-14-propionylamino-tetradec-4-enyl ester 9H-fluoren-9-ylmethyl ester (10): a) *Preparation from* ***3***: A solution of **3** (bis-trifluoroacetate) (20 mg, 21 μmol) and diisopropylethylamine (15 μL, 85 μmol) in *N*,*N*-dimethylformamide (150 μL) and dichloromethane (1 mL) is treated with *N*-succinimidyl propionate (3.7 mg, 21 μmol). The mixture is stirred in an argon atmosphere at room temperature for 24 h. After evaporation of the solvent, the crude product is purified by reversed phase HPLC (C18, acetonitrile/water containing 0.1% TFA). 6.9 mg (10 μmol, 47%) of **10** (trifluoroacetate) is obtained. b) *Preparation from* ***9***: A solution of **9** (bis-trifluoroacetate) (3.25 mg, 6.17 μmol) and diisopropylethylamine (4.6 μL, 27 μmol) in *N*-methylpyrrolidone (1 mL) is treated with *N*-succinimidyl propionate (1.45 mg, 1.35 μmol). The mixture is stirred in an argon atmosphere at room temperature for 5 h. After evaporation of the solvent, the crude product is purified by reversed phase HPLC (C18, acetonitrile/water containing 0.1% TFA). 2.9 mg (4.2 μmol, 68%) of **10** (trifluoroacetate) is obtained. ^{1}H NMR (500 MHz, CD$_3$OD): $\delta = 7.79$ (d, 2H, $J = 7.5$ Hz), 7.72 (d, 2H, $J = 7.4$), 7.37 (t, 2H, $J = 7.4$ Hz), 7.30 (t, 2H, $J = 7.5$ Hz), 5.80 (td, 1H, $J = 15.2$, 7.2 Hz), 5.40 (dd, 1H, $J = 15.2$, 6.7 Hz), 4.19–4.25 (m, 2H), 4.17 (quin, 2H, $J = 6.7$ Hz), 3.93–4.00 (m, 1H), 3.84–3.92 (m, 1H), 3.27–3.36 (m, 5H), 3.13 (t, 2H, $J = 7.1$ Hz), 2.18 (qua, 2H, $J = 7.6$ Hz), 2.04 (qua, 2H, 7.1 Hz), 1.42–1.50 (m, 2H), 1.32–1.40 (m, 2H), 1.23–1.32 (m, 8H), 1.12 ppm (t, 3H, $J = 7.6$ Hz); HR-MS (*m/z*): calcd. 573.3088, found 573.3087 (MH).

Phosphoric acid mono-((E)-(2S,3R)-2-amino-3-hydroxy-14-propionyl-amino-tetradec-4-enyl) ester (11): A solution of **10** (5 mg, 8.70 μmol) in *N*,*N*-dimethylformamide (0.5 mL) is treated with piperidine (5 μL). The mixture is stirred in an argon atmosphere at room temperature for 60 min. After evaporation of the solvent, the crude product is purified by size-exclusion chromatography on Sephadex LH20 using methanol as eluent. 1.5 mg (3.8 μmol, 44%) of **11** is obtained. ^{1}H NMR (500 MHz, CD$_3$OD): $\delta = 5.88$ (td, 1H, $J = 16.0$, 6.0 Hz), 5.51 (dd, 1H, $J = 16.0$, 6.9 Hz), 4.85 (m, 6H), 4.29 (t, 1H, $J = 5.9$ Hz), 4.07–4.13 (m, 1H), 3.96–4.04 (m, 1H), 3.34–3.40 (m, 1H), 3.17 (t, 2H, $J = 7.1$ Hz), 2.20 (qua, 2H, $J = 7.6$ Hz), 2.12 (qua, 2H, 7.3 Hz), 1.47–1.55 (m, 2H), 1.40–1.47 (m, 2H), 1.29–1.39 (m, 10H), 1.13 ppm (t, 3H, $J = 7.6$ Hz); ^{13}C NMR ($CDCl_3$): $\delta = 177.0$, 136.9, 128.2, 70.8, 63.0, 57.6, 40.4, 33.4, 30.6, 30.5, 30.4, 30.4, 30.3, 30.2, 30.1, 28.0, 10.6 ppm; ^{31}P NMR (D$_2$O): $\delta = 2.65$ ppm; HR-MS (*m/z*): calcd. 417.2125, found 417.2124 (MNa).

Resin 12: Affi-Gel 10 (2.06 mL, 31 μmol activated ester) is rinsed four times with *N*-methylpyrrolidone (peptide-grade, 10 mL each). A solution of **9** bis-trifluoroacetate (39 mg, 52 μmol) in *N*-methylpyrrolidone (2 mL) is added, followed by diisopropylethylamine (26 μL, 0.154 mmol) in *N*-methylpyrrolidone (2 mL). The mixture is shaken at room temperature for 5 h. After filtration, resin **12** is rinsed four times with *N*-methylpyrrolidone (5 mL each).

Resin 13: Resin **12** is shaken vigorously with a 1:1 mixture of piperidine/*N*,*N*-dimethylformamide (4 mL) at room temperature for 18 h and then filtered. Washing with piperidine/*N*,*N*-dimethylformamide is repeated until no more cleavage product is detected by HPLC (254 nm). The loaded resin **13** is soaked in *N*-methylpyrrolidone and stored at −20 °C.

Calculation of gel loading: All filtrates containing the piperidine-dibenzofulvene adduct **14** are combined to a total volume (V_t) of 15.5 mL. UV absorbance is measured by UV spectroscopy at 301 nm. Following the Beer–Lambert equation ($E=\varepsilon$*c*d), the concentration (c) of **14** is calculated from the measured extinction (E) and the known extinction coefficient (ε) for the chromophore ($\varepsilon_{301nm}=7800$) to be 20.3 μmol V_t^{-1}. This corresponds to a gel loading of 10 μmol mL^{-1} gel (66% of theoretical loading).

Ligand-induced Ca^{2+} mobilization:[18] CHO overexpressing S1P receptors are plated in 384 well plates. After 24 h the cells are loaded with Fluo4-AM (1.6 μM in HBSS and 2.5 mM probenicid) for 1 h at 37 °C. After washing the cells are transferred to the fluorescent image plate reader (FLIPR) and primed by 10 μM ATP. Pretreatment with ATP allows efficient coupling of S1P receptors to Ca^{2+} mobilization as described for other GPCRs.[21] The agonist is added in different concentrations in HBSS in the presence of 0.1% BSA. Time points are collected as follows: 20 time points (1 or 2 sec) before the addition of the agonist (base line = F_{min}), and at least 60 time points (1 or 2 sec) after the addition of the agonist. This allows the determination of F_{max}, the maximum value of the fluorescence peak. Routinely, 40 000 or 10 000 cells are used for 96 or 384 well plates, respectively. For calculation the $(F_{max}-F_{min})/F_{min}$ is plotted against the log of the concentration of the agonist and the EC_{50} is determined.

Activation of ERK1/2:[19] Human umbilical vein endothelial cells (HUVEC) were incubated in serum-free medium for 6 h and then stimulated for 10 min with graded concentrations in the range of 0.1 to 10 μM of **1** or **11**. Cells were collected for SDS-polyacrylamide gel electrophoresis followed by Western blot analysis on nitrocellulose membranes; membranes were incubated with antibodies directed to phosphorylated ERK1/2 and reprobed with anti-β-actin antibodies to ensure equal loading of samples. Band intensities were measured using AlphaImager 2200 (Alpha Innotech Corporation).

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