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# Constrained azacyclic analogues of the immunomodulatory agent FTY720 as molecular probes for sphingosine 1-phosphate receptors

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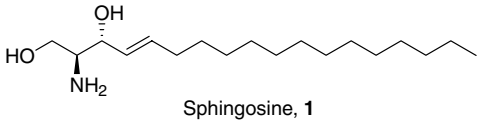
**Abstract**—Constrained azacyclic analogues of FTY720 were prepared starting with D- and L-pyroglutamic acids. One enantiomer was shown to be a substrate for sphingosine kinase 2, being phosphorylated 4-fold more efficiently than FTY720. Among the corresponding phosphates, two were found to have unusual specificity in binding to S1P receptors: while being inactive on S1P1 and S1P3, they acted as potent agonists on S1P4 and S1P5. The phosphates may be useful to explore the biology and binding site of these receptors.

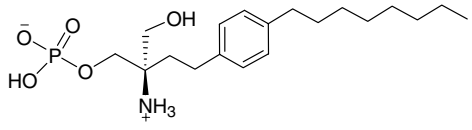

FTY720 is an orally active immunomodulatory agent with a novel mode of action, sequestering lymphocytes from blood and spleen into secondary lymphoid organs and thereby preventing their migration to sites of inflammation.[1] In addition to the effects on lymphocyte recirculation, the drug preserves vascular integrity by enhancing endothelial barrier function.[2] FTY720 is currently in clinical phase III trials for the treatment of multiple sclerosis.

Given its structural analogy to sphingosine (**1**), FTY720 is phosphorylated in vivo by sphingosine kinases (SPHKs), in particular the type 2 enzyme.[3,4] The phosphate FTY720-P (**2**) binds to four of the five G protein-coupled sphingosine 1-phosphate receptors (S1P1-5).[5] Most importantly it acts as a high-affinity agonist at S1P1 on thymocytes and lymphocytes, thereby inducing aberrant internalization of the receptor. This renders the cells unresponsive to the serum lipid sphingosine 1-phosphate, depriving them from an obligatory signal to egress from lymphoid organs.[1]

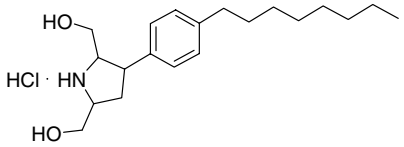
The recent interest in synthetic analogues of FTY720 and the lack of information about the structure of S1P receptors encouraged us to design molecular probes for this system. The approach chosen to achieve this goal was to synthesize constrained analogues of **2** which would bear limited conformational freedom, thus allowing us to gain more insight into the requirements of the active site of the S1P receptors. The synthesis of a set of different phosphate ester isomers of the constrained azacyclic prototype **3** and their respective biological activities are reported here.



Sphingosine, **1**



FTY720-P, **2**



**3**

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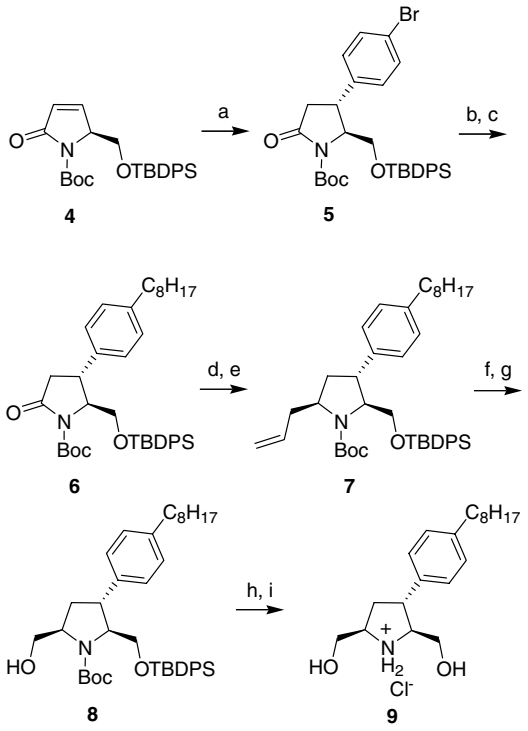
There are many reported examples of 1,4-additions to the readily available α,β-unsaturated lactam **4**, obtained from L-pyroglutamic acid.[6] However, we were not successful in directly inserting the 1-bromo-4-octylphenyl unit using diaryl lithiocuprate, or copper-catalyzed Grignard addition, even in the presence of a strong Lewis acids.[7] However, the cuprate prepared from the mono-lithiated form of 1,4-dibromobenzene could be added to the α,β-unsaturated lactam **4**, giving the *p*-bromophenyl adduct **5** in excellent yield (Scheme 1). The octyl chain was then added by Suzuki coupling with 1-octyne followed by hydrogenation to obtain **6**. Sonogashira coupling gave lower yields. Next, a *C*-allylation at C-5 was planned by addition to an *N*-acyliminium ion obtained from the *O*-Me aminal of **6**. Reduction of **6** with Super-Hydride was very prone to β-elimination, affording the ene-carbamate as soon as the reaction mixture was quenched.[8] This difficulty was circumvented by reducing the lactam to the hemiaminal and directly quenching the reaction mixture with PPTS in MeOH. This afforded the crude *O*-Me aminal, from which the *N*-acyliminium ion was generated by the action of $BF_3{\cdot}OEt_2$.[9] Attempts to add different vinyl organocopper reagents proved unsuccessful, affording mostly the ene-carbamate in every case. The titanium-catalyzed addition of allyltrimethylsilane[10] afforded product **7** in satisfactory yields. Only one isomer was obtained and the stereochemistry was assigned at a later stage by $^1$H-NOESY NMR analysis. The terminal alkene **7** could then be isomerized following the Roy protocol[11] to obtain the 2-propenyl derivative which was then cleaved by ozonolysis and the resulting aldehyde was reduced to yield the alcohol **8**. An aliquot of this alcohol was then completely deprotected by sequential treatment with TBAF, followed by HCl in 1,4-dioxane to afford the diol **9** which was isolated as the hydrochloride salt. This diol was then assayed against sphingosine kinases to test whether it was recognized as a substrate by the enzyme, thereby confirming its analogy to FTY720.

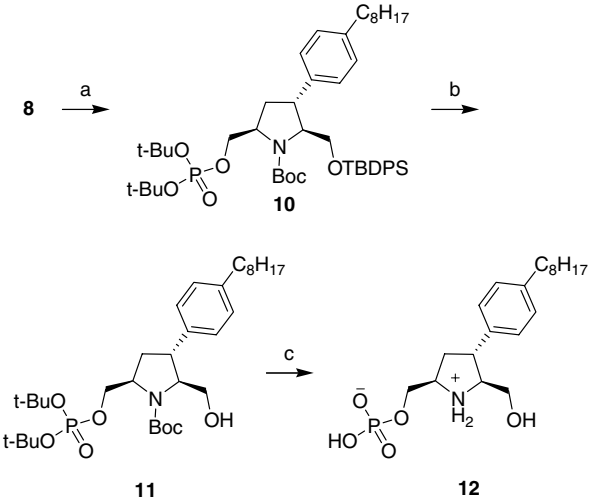



**Scheme 1.** Reagents and conditions: (a) i—1,4-dibromobenzene, *n*-BuLi, $Et_2O$, −20 °C; ii—$CuBr{\cdot}Me_2S$, −20 °C; iii—TMSCl, α,β-unsaturated lactam, −78 °C to rt, 94%; (b) i—1-octyne, catecholborane, 70 °C; ii—aryl bromide, $Pd(PPh_3)_4$, $NaHCO_3$, DME, reflux, 64%; (c) $H_2$, Pd/C, EtOAc, rt, 100%; (d) i— Super-Hydride, THF, −78 °C; ii—PPTS, MeOH, −78 °C to rt; (e) allyltrimethylsilane, $TiCl_4$, DCM, −78–0 °C, 63% (2 steps); (f) Grubbs 2nd gen. cat. *N*-allyltritylamine, *i*-$Pr_2NEt$, PhMe, reflux, 69%; (g) i—$O_3$, DCM/MeOH, −78 °C; ii—$Me_2S$, rt; iii—$NaBH_4$, MeOH, 0 °C, 73%; (h) TBAF, THF, rt, 100%; (i) HCl, 1,4-dioxane, rt, 68%.

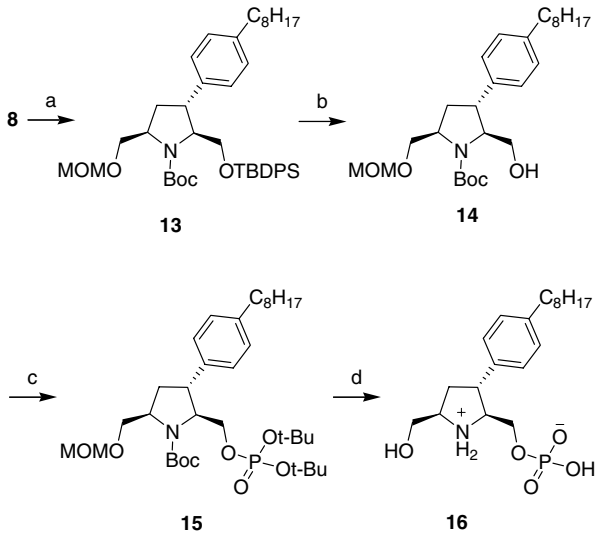
Next, we phosphorylated the end-differentiated intermediate **8** at each of its hydroxymethyl groups individually. For the analogue bearing the phosphate at C-5, the primary alcohol in **8** was phosphorylated using phosphoramidite chemistry, followed by oxidation (Scheme 2). The TBDPS group was then cleaved by treatment with TBAF to afford **11**. The ester was cleaved to give the monophosphate **12**.

For the analogue bearing the phosphate at C-2 of the molecule, it was first necessary to protect the primary alcohol in **8**. The MOM protecting group was chosen on the basis that it could later be removed at the same time as the *tert*-butyl groups by acidic treatment. The TBDPS group in **13** was cleaved to afford the alcohol **14** (Scheme 3). The alcohol was phosphorylated following the same conditions as specified above to afford **15**. Acidic cleavage of the *tert*-butyl groups then afforded the regioisomeric monophosphate **16**. The yield of this last step was modest because some partially unprotected analogue was also formed.

The same transformations were performed starting from D-pyroglutamic acid, affording the enantiomeric pyrrolidines **17**, **18**, and **19**, respectively (Fig. 1).




**Scheme 2.** Reagents and conditions: (a) i—$(t\text{-BuO})_2PNEt_2$, 1*H*-tetrazole, THF, rt; ii—*m*-CPBA, DCM, −78 °C to rt, 84%; (b) TBAF, THF, rt, 84%; (c) HCl, 1,4-dioxane, rt, 51%.




**Scheme 3.** Reagents and conditions: (a) MOMCl, *i*-$Pr_2NEt$, DMAP, DCM, rt, 100%; (b) TBAF, THF, rt, 87%; (c) i—$(t\text{-}BuO)_2PNEt_2$, 1*H*-tetrazole, THF, rt; ii—*m*-CPBA, DCM, −78 °C to rt, 86%; (d) HCl, 1,4-dioxane, rt, 39%.

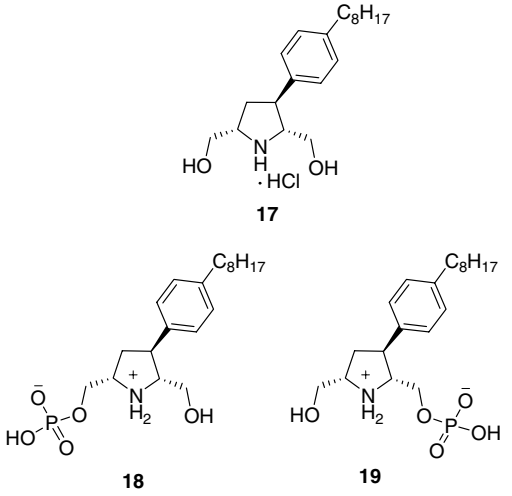



**Figure 1.**

The cyclic analogues of FTY720, **9** and **17**, were used as substrates for human recombinant SPHK1 and SPHK2. The rates of phosphorylation were determined by assessing the incorporation of radiolabeled phosphate upon incubation with [γ-$^{32}$P]ATP and the appropriate enzymes.[12,13] Results are reported in Table 1 relative to the natural substrate sphingosine. While phosphorylation of **17** was not detectable, the enantiomer **9** was phosphorylated by both enzymes. Such enantioselectivity is not unprecedented, since SPHKs phosphorylate D-*erythro*-sphingosine, but not L-*erythro*-sphingosine, and phosphorylation only takes place at the pro-*S* hydroxy group of FTY720, not the pro-*R* moiety.[14,15] The reaction product of **9** formed by the SPHKs co-migrated with **12** and **16** (which were not separable by TLC), indicating monophosphate formation. Phosphorylation of **9** by SPHK1 was slow as compared to sphingosine, but about twice as efficient as FTY720. In contrast, **9** was a good substrate for SPHK2, with a phosphorylation rate only 2-fold lower as compared to sphingosine, but about 4-fold higher than for FTY720. The more efficient phosphorylation of **9** by SPHK2 as compared to SPHK1 is in line with prior observations of broader substrate use by SPHK2.[16,17] Since SPHK2 is known to be the major enzyme responsible for FTY720 phosphorylation in animals,[3,4] **9** can be likewise expected to be rapidly phosphorylated in vivo.

**Table 1.** Rates of phosphorylation of **9** and **17**

| Compound | SPHK1 (%) | SPHK2 (%) |
|---|---|---|
| Sphingosine | 100.0 | 100.0 |
| FTY720 | 0.6 | 13.0 |
| **9** | 1.3 | 55.0 |
| **17** | <0.1 | <0.1 |

Values are given relative to the rate for D-sphingosine.[13]

The calcium release assay[18] was used to evaluate the biological activity of the phosphorylated derivatives **12**, **16**, **18**, and **19**. In this assay, compounds were added to cells overexpressing human S1P-receptors. The cells were loaded with a $Ca^{2+}$ specific fluorescent probe. Activation of the S1P receptors promoted a concentration dependent increase of intracellular $Ca^{2+}$, that results in an increase of fluorescence. FTY720(R) bound to and activated four (S1P1, S1P3, S1P4, and S1P5) of the five known S1P receptors.[14] Compounds **12**, **16**, **18**, and **19** were not capable to mediate $Ca^{2+}$ release in cells expressing S1P1 and S1P3 receptors, in contrast to FTY720P(R) which was a very potent agonist at these two receptors (Table 2). Compounds **12** and **16** were potent agonists at S1P4 and S1P5, whereas compound **19** was less potent but it still showed nM affinity toward both S1P4 and S1P5. Compound **18** was a weaker agonist in the S1P4 and S1P5 assays (Table 2). The profile of the azacyclic compounds is strikingly different than that of FTY720(*R*) and the endogenous agonist sphingosine 1-phosphate. The azacyclic compounds are capable to access the agonist binding pocket of S1P4 and S1P5, while their constrained structure prevents them from activating the S1P1 and S1P3 receptors. Among the two possible phosphorylated derivatives of compound **9** (the preferred substrate by SHPKs, Table 1), compound **12** was the most potent, whereas compound **16** showed only weak activity.

Apart from S1P1, other S1P receptors have gained attention as potential targets for pharmacological inter-

**Table 2.** Calcium release mediated by compounds **12**, **16**, **18**, and **19** in CHO cells expressing human S1P receptors

| Compound | S1P1 | S1P3 | S1P4 | S1P5 |
|---|---|---|---|---|
| Sphingosine-P | 0.55 | 1.6 | 7.9 | 0.91 |
| FTY720-P(*R*) | 0.68 | 1.7 | 18.6 | 2.9 |
| **12** | >1000 | >1000 | 7.4 | 10.2 |
| **16** | >1000 | >1000 | 347 | 133.8 |
| **18** | >1000 | >1000 | 16.8 | 5.8 |
| **19** | >1000 | >1000 | 33.1 | 21.3 |

The given values are $EC_{50}$ in nM. Compounds **12**, **16**, **18**, and **19** are full agonist at S1P4 but partial agonist at S1P5.

vention in disease as well.[2] While potent S1P1 selective compounds have recently been described,[19,20] selective agents in particular for S1P4 and S1P5 receptors are not known. Therefore, compounds **12** and **18** could be interesting probes to explore the effects of pharmacological intervention with S1P4 and S1P5, using compounds devoid of activity on S1P1. In addition they also could be used to assist molecular modeling of the binding site of these receptors.

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13. The phosphorylation reactions were performed essentially as described in Ref. 12. Briefly, the cytoplasmic fraction of recombinant HEK-293 cells overexpressing human SPHK1 or SPHK2 was incubated at 30 °C in total volumes of 100 μl with D-erythro-sphingosine, FTY-720 or derivatives **9** or **17** (20 μM; added from stock solutions in DMSO), 1 mM ATP, and 2 μCi [$\gamma$-$^{32}$P]ATP in 50 mM Hepes buffer (pH 7.4) containing 15 mM $MgCl_2$, 0.005% Triton X-100, 10 mM KCl, 10 mM NaF, and 1.5 mM semicarbazide. Following incubations for different time points up to 2 h, lipids were extracted and separated by thin-layer chromotography (TLC) plates (Merck) using butanol/acetic acid/water 3:1:1 as mobile phase Radiolabeled derivatives were visualized and quantified using a Molecular Dynamics Storm PhosphorImager (Sunnyvale, CA). The calculated phosphorylation efficiency is reported as value relative to sphingosine (=100%) for which the rate was 41 and 25 nmol/min/mg with SPHK1 and SPHK2, respectively.
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18. The calcium flux assay using FLIPR (fluorometric imaging plate reader) was performed essentially as described in Ref. 12. Briefly, CHO cells expressing the recombinant human receptors were plated in black Costar plate (384 well, 12,500 cells) in αMEM containing 10% FCS and cultured for 20–24 h at 37 °C in a $CO_2$ incubator. After the removal of the culture medium cells were incubated in HBSS medium containing 2 μM Fluo4AM (molecular probes) and 5 mM probenicid for 1 h at 37 °C, rinsed with HBSS buffer/2.5 mM probenicid, and overlaid with the same medium (50 μl). The plates were transferred to the FLIPR. After measuring the baseline for 40 s, 25 μl of the agonist solution in HBSS was added and the fluorescence was measured at intervals of 2 s for 3–5 min. Calculations of the $EC_{50}$ were performed using a nonlinear regression fit program provided in the Origin 7 RS2 software package (Origin LabCorporation).
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