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Phosphorylation by Sphingosine Kinase 2 is Essential for in vivo Potency of FTY720 Analogues

Klemens Högenauer,*[a] Andreas Billich,[a] Charles Pally,[b] Markus Streiff,[b] Trixie Wagner,[b] Karl Welzenbach,[b] and Peter Nussbaumer[a]

FTY720 is a novel orally active immunomodulator currently in phase III trials for the treatment of multiple sclerosis.[1] The drug has a unique mode of action: it inhibits lymphocyte egress out of lymphoid organs. Consequently, levels of immunoreactive T-cells are depleted in the peripheral tissue.[2] FTY720 is stereoselectively phosphorylated (Figure 1) to the

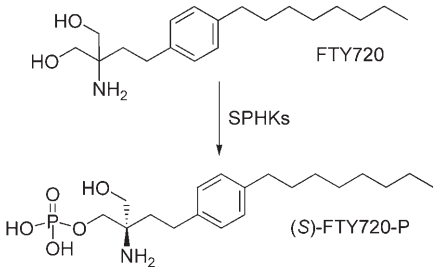

Figure 1. FTY720 is phosphorylated by SPHKs.

active principle (*S*)-FTY720-phosphate which binds to four of the five known sphingosine-1-phosphate receptors (S1P1–5).[3] It has been convincingly demonstrated that the immunomodulatory effect is primarily mediated by S1P1.[2,4]

Sphingosine kinases (SPHKs) have been identified as the enzymes that catalyze the mono-O-phosphorylation of the amino alcohol head group of FTY720. We and others have found that SPHK2 is much more efficient than SPHK1 in phosphorylation of FTY720 (Table 1).[5] Indeed, in vivo experiments have demonstrated that SPHK2 is essential for the lymphopenia induced by FTY720.[6] Although apoptosis induced by FTY720 at higher concentrations has been shown to be receptor independent,[7] a recent publication suggests that SPHK2 is also essential for this pathway.[8]

The finding that only one of the prochiral hydroxy groups in FTY720 is phosphorylated in vivo suggests that the enzymatic phosphorylation reaction can be tuned by offering various stereochemical features in the substrate. For the chiral analogues **1a** and **1b** (Figure 2)[3a,9] it has already been demonstrated

Table 1. In vivo and in vitro data.

Compd	Lymphocyte depletion ED_{50} [mg kg^{-1}][a]	S1P1 [γ-^{35}S]GTP EC_{50} [nM][b]	Phosphorylation rate [%][c] SPHK1	SPHK2
Sphingosine	>3	–	100	100
S1P	–	0.9	–	–
FTY720	0.09	–	0.6	13.0
(*S*)-FTY720-P	–	0.14	–	–
(*R*)-FTY720-P	–	133	–	–
1a	0.03	–	4.2	25.0
1a-P	–	0.85	–	–
1b	>3	–	<0.04	<0.04
1b-P	–	271	–	–
2a	0.07	–	<0.04	20.0
2a-P	–	0.5	–	–
3a	>1	–	<0.04	1.25
3a-P	–	0.07	–	–
2b	>1	–	<0.04	0.4
3b	>1	–	<0.04	0.2
11	>1	–	<0.04	<0.04

[a] Determined in rat model at 6 h; only amino alcohols were tested. [b] All compounds shown are full agonists; only phosphates were tested, as amino alcohols are less potent agonists by several orders of magnitude.[9b] [c] Values given are relative to the rate for D-sphingosine (mean value of two determinations).[17]

that induction of lymphopenia correlates with SPHK phosphorylation efficiency as well as with the potency as agonists of the corresponding phosphates toward S1P1.[3a,5a] For potent S1P1 agonists that do not induce lymphopenia, a difference in SPHK activity has already been suspected as a possible reason for their lack of in vivo efficacy.[10] Herein we report the synthesis and systematic investigation of FTY720-like amino alcohols featuring two stereocenters in the head group in order to establish the relative importance of SPHK phosphorylation compared with potency toward S1P1 for lymphopenia induction in vivo.

Primary alcohols **1a** and **1b** were prepared as reported previously by using a Schöllkopf auxiliary-based approach.[9a,11] For the synthesis of these two compounds as well as the secondary alcohols **2a** and **3a**, the N-Boc-protected amino alcohol **4** served as the precursor (Scheme 1). Using the protocol described by Ley et al.,[12] this alcohol was oxidized to the aldehyde **5**. The addition of methyl magnesium bromide resulted

[a] *Dr. K. Högenauer, Dr. A. Billich, Dr. P. Nussbaumer*
Novartis Institutes for Biomedical Research
Brunner Strasse 59, 1235 Wien (Austria)
Fax: (+43) 1-80166-354
E-mail: klemens.hoegenauer@novartis.com

[b] *C. Pally, Dr. M. Streiff, Dr. T. Wagner, Dr. K. Welzenbach*
Novartis Institutes for Biomedical Research
4002 Basel (Switzerland)

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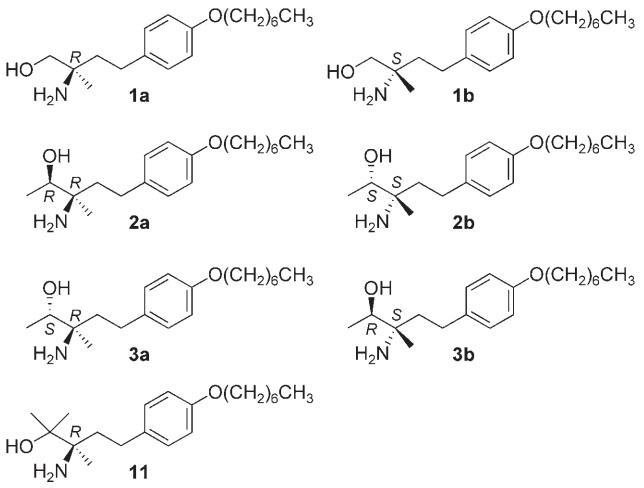
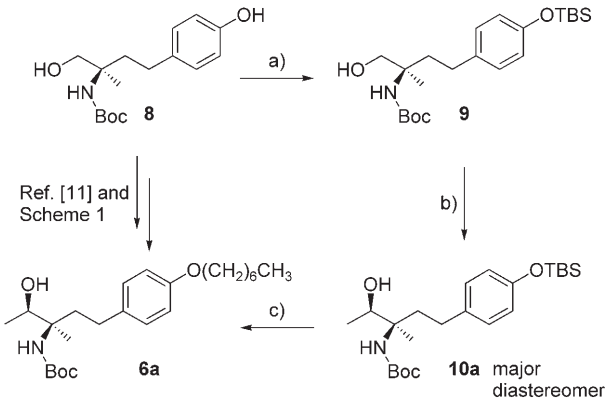
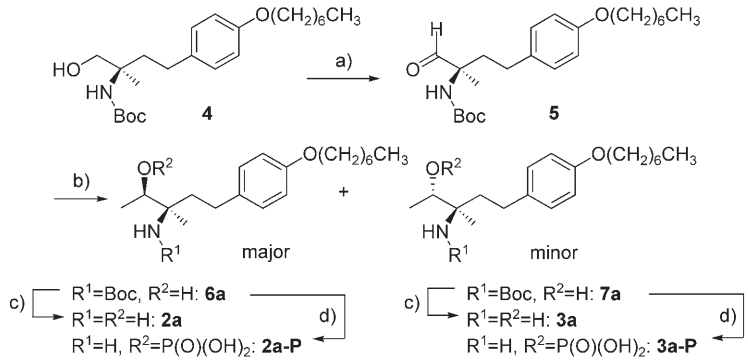


Figure 2. Absolute configurations of the amino alcohols tested.



Scheme 2. Reagents and conditions: a) 1) *tert*-butyldimethylsilyl chloride (TBSCl, 2.5 equiv), imidazole, *N*,*N*-dimethylformamide, RT, 2) $Sc(OTf)_3$ (cat.), CH_3CN, H_2O, RT, 86% over 2 steps; b) 1) TPAP (cat.), NMO, molecular sieves (4 Å), CH_2Cl_2, RT, 96%, 2) MeMgBr, Et_2O, 0 °C→RT, 94% (d.r. = 2.3:1), 3) preparative HPLC; c) 1) HF (aq., 48%), CH_3CN, RT, 29%, 2) $CH_3(CH_2)_6OMs$, K_2CO_3, EtOH, 60 °C, 50%.



Scheme 1. Reagents and conditions: a) tetra-*N*-propylammonium perruthenate (TPAP, cat.), *N*-methylmorpholine-*N*-oxide (NMO), molecular sieves (4 Å), CH_2Cl_2, RT, 90%; b) 1) MeMgBr, Et_2O, 0 °C→RT, 76% (d.r. = 3:1), 2) preparative HPLC; c) saturated HCl in MeOH, RT, quant.; d) 1) $(tBuO)_2PN(iPr)_2$, tetrazole, THF, RT, then H_2O_2, RT, 56–70%, 2) saturated HCl in MeOH, RT, quant.

in the formation of two epimeric secondary alcohols **6a** and **7a** (d.r. = 3:1) which were separated by preparative HPLC. Acidic deprotection of **6a** and **7a** led to amino alcohols **2a** and **3a**, respectively. The same synthetic sequence was applied to obtain the enantiomeric series (**2b** and **3b**). The phosphates **1ab-P**, **2a-P** as well as **3a-P** were prepared via the bis-*tert*-butyl phosphate esters followed by HCl deprotection.[11]

To prove the absolute and relative configuration of all stereoisomers, it was hoped that a crystalline derivative could be synthesized. For this purpose, TBS ether **9** was prepared via bis-silylation of diol **8**, followed by selective cleavage of the silyl alkyl ether (Scheme 2).[13] Alcohol **9** was oxidized, and the resulting aldehyde was treated with methyl magnesium bromide to give two separable epimeric alcohols (d.r. = 2.3:1). The major diastereomer **10a** could be crystallized, and the absolute configuration was determined to be 2*R*,3*R* by single-crystal X-ray diffraction (Figure 3).[14] Desilylation and subsequent alkylation of **10a** gave ether **6a**. This correlation sequence then allowed us to unambiguously assign the relative and absolute configuration of amino alcohols **2ab** and **3ab**. The formation of secondary alcohols **6a** and **10a** as major diastereomers in the respective Grignard additions is consistent with the expected outcome according to the chelate-controlled Felkin–Anh transition-state model.

The biological results are summarized in Table 1. As reported earlier, SPHK2 is much more efficient than SPHK1 in phosphorylating FTY720 to the active principle. Consistent with the selective phosphorylation, the lymphopenia-inducing phosphate (*S*)-FTY720-P is a much more potent S1P1 agonist than the enantiomer (*R*)-FTY720-P.[3c] For the chiral amino alcohols, **1a** is active in transiently decreasing peripheral lymphocyte counts in vivo, with an ED_{50} value even lower than FTY720, whereas the enantiomer **1b** did not show in vivo activity up to a 100-fold higher dose. These results agree fully with the observation that phosphate **1a-P** is a nanomolar agonist of S1P1, whereas the enantiomer **1b-P** is a much weaker S1P1

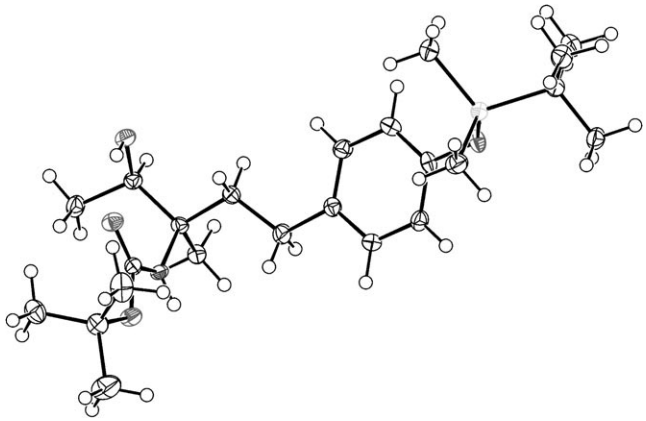


Figure 3. Structure of crystalline alcohol **10a** (atomic displacement ellipsoids drawn at the 50% probability level; hydrogen atoms drawn as spheres of arbitrary radius).[15] For the *R*,*R* diastereomer shown, the Flack *x* parameter is refined to 0.04(2).[16]

agonist. With respect to phosphorylation by SPHK activity, only the in vivo active compound **1a** is converted in vitro. As for FTY720, SPHK2 is more efficient than SPHK1 in phosphorylating **1a**, but the difference is only sixfold, and therefore not as pronounced as for FTY720 (25-fold). Based on these data, we speculated that the difference in SPHK2 efficiency might be the prime reason for the greater in vivo activity of **1a** relative to FTY720, and we aimed at other derivatives to show a clear distinction.

In the series based on the *R*-configured quaternary stereocenter, alcohol **2a** showed an ED_{50} value of 0.07 mg kg^{-1} in transiently decreasing peripheral lymphocyte counts in vivo, which is in a range similar to that of **1a** and FTY720. In contrast, the diastereomer **3a** did not show any activity up to a dose of 1 mg kg^{-1}. In agreement with the high in vivo activity of the parent amino alcohol **2a**, phosphate **2a-P** is a potent S1P1 receptor agonist. Surprisingly, phosphate **3a-P** turned out to be an even more potent agonist than **2a-P** and (*S*)-FTY-P, although the parent amino alcohol **3a** did not induce lymphopenia. This apparent contradiction between in vitro and in vivo potency can be rationalized by the data obtained from in vitro phosphorylation by SPHKs. Whereas neither **2a** nor **3a** are substrates for SPHK1, both diastereomers are phosphorylated by SPHK2, albeit at different rates. The conversion rate for **3a** to **3a-P** is low (~15-fold slower than for **2a**→**2a-P**) suggesting that SPHK2 efficiency is the limiting factor for generating the phosphates and hence the observed difference in efficacy in vivo. We also tested the sterically congested dimethyl alcohol **11**, which was shown to be inactive in vivo as well as inactive in both SPHK phosphorylation assays.

Amino alcohols **2b** and **3b** (based on the *S*-configured quaternary stereocenter) did not induce lymphopenia. This confirms that the absolute configuration of the quaternary stereocenter is the main structural parameter for in vivo efficacy in this series. Both compounds were very slowly converted into their corresponding phosphates by SPHK2, and no product was detected upon incubation with SPHK1. Based on these data, we did not synthesize and test the corresponding phosphates to study their binding affinity.

In summary, we could demonstrate that the introduction of a second stereocenter in the amino alcohol head group of FTY720-like compounds has a strong effect on the phosphorylation efficiency and selectivity by SPHKs and on transiently decreasing peripheral lymphocyte counts in vivo. Amino alcohol **2a** is a new FTY720 analogue that is phosphorylated efficiently and selectively by SPHK2 and which displays high activity in the lymphocyte depletion model in vivo. With this compound, we observed increased isotype selectivity (**1a**: sixfold for SPHK2 versus SPHK1, **2a**: >500-fold). Comparison of the SPHK2 phosphorylation rates of the epimeric compounds **2a** versus **3a** and of the potency of the corresponding phosphates **2a-P** and **3a-P** as S1P1 agonists strongly suggests that phosphorylation by SPHK2 is the determining factor for generating the corresponding phosphates and hence, for transiently decreasing peripheral lymphocyte counts in vivo. The pair **2a**/**3a** is also the final proof that SPHK1 activity is not necessary for in vivo potency. Although it is possible that differences in PK and/or dephosphorylation by phosphatases are also contributing factors, there is now strong evidence that SPHK2 phosphorylation capacity ultimately limits in vivo potency of FTY720-type amino alcohols. A low SPHK2 phosphorylation rate will probably explain a lack of lymphopenia for many published amino alcohols for which potent S1P1 agonism of the corresponding phosphates has been described.

Keywords: biologically active compounds · FTY720 · immunomodulators · medicinal chemistry · sphingosine kinase

[1] a) T. Baumruker, A. Billich, V. Brinkmann, *Expert Opin. Invest. Drugs* **2007**, *16*, 283; b) L. Kappos, J. Antel, G. Comi, X. Montalban, P. O'Connor, C. H. Polman, T. Haas, A. A. Korn, G. Karlsson, E. W. Radue, *N. Engl. J. Med.* **2006**, *355*, 1124.
[2] V. Brinkmann, J. G. Cyster, T. Hla, *Am. J. Transplant.* **2004**, *4*, 1019.
[3] a) V. Brinkmann, M. D. Davis, C. E. Heise, R. Albert, S. Cottens, R. Hof, C. Bruns, E. Prieschl, T. Baumruker, P. Hiestand, C. A. Foster, M. Zollinger, K. R. Lynch, *J. Biol. Chem.* **2002**, *277*, 21453; b) S. Mandala, R. Hajdu, J. Bergstrom, E. Quackenbush, J. Xie, J. Milligan, R. Thornton, G. J. Shei, D. Card, C. Keohane, M. Rosenbach, J. Hale, C. L. Lynch, K. Rupprecht, W. Parsons, H. Rosen, *Science* **2002**, *296*, 346; c) R. Albert, K. Hinterding, V. Brinkmann, D. Guerini, C. Müller-Hartwieg, H. Knecht, C. Simeon, M. Streiff, T. Wagner, K. Welzenbach, F. Zecri, M. Zollinger, N. Cooke, E. Francotte, *J. Med. Chem.* **2005**, *48*, 5373.
[4] N. Cooke, F. Zécri, *Annu. Rep. Med. Chem.* **2007**, *42*, 251.
[5] a) A. Billich, F. Bornancin, P. Devay, D. Mechtcheriakova, N. Urtz, T. Baumruker, *J. Biol. Chem.* **2003**, *278*, 47408; b) T. Sanchez, T. Estrada-Hernandez, J. H. Paik, M. T. Wu, K. Venkataraman, V. Brinkmann, K. Claffey, T. Hla, *J. Biol. Chem.* **2003**, *278*, 47281; c) S. W. Paugh, S. G. Payne, S. E. Barbour, S. Milstien, S. Spiegel, *FEBS Lett.* **2003**, *554*, 189.
[6] a) B. Zemann, B. Kinzel, M. Müller, R. Reuschel, D. Mechtcheriakova, N. Urtz, F. Bornancin, T. Baumruker, A. Billich, *Blood* **2006**, *107*, 1454; b) Y. Kharel, S. Lee, A. H. Snyder, S. L. Sheasley-O'Neill, M. A. Morris, Y. Setiady, R. Zhu, M. A. Zigler, T. L. Burcin, K. Ley, K. S. K. Tung, V. H. Engelhard, T. L. Macdonald, S. Pearson-White, K. R. Lynch, *J. Biol. Chem.* **2005**, *280*, 36865; c) M. L. Allende, T. Sasaki, H. Kawai, A. Olivera, Y. D. Mi, G. van Echten-Deckert, R. Hajdu, M. Rosenbach, C. A. Keohane, S. Mandala, S. Spiegel, R. L. Proia, *J. Biol. Chem.* **2004**, *279*, 52487.
[7] V. Brinkmann, C. Wilt, C. Kristofic, Z. Nikolova, R. P. Hof, S. Chen, R. Albert, S. Cottens, *Transplant. Proc.* **2001**, *33*, 3078.
[8] A. S. Don, C. Martinez-Lamenca, W. R. Webb, R. L. Proia, E. Roberts, H. Rosen, *J. Biol. Chem.* **2007**, *282*, 15833.
[9] a) K. Hinterding, R. Albert, S. Cottens, *Tetrahedron Lett.* **2002**, *43*, 8095; b) V. Brinkmann, K. R. Lynch, *Curr. Opin. Immunol.* **2002**, *14*, 569.
[10] F. W. Foss, Jr., J. J. Clemens, M. D. Davis, A. H. Snyder, M. A. Zigler, K. R. Lynch, T. L. Macdonald, *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4470.
[11] K. Hinterding, S. Cottens, R. Albert, F. Zecri, P. B. Buehlmayer, C. Spanka, V. Brinkmann, P. Nussbaumer, P. Ettmayer, K. Hoegenauer, N. Gray, S. F. Pan, *Synthesis* **2003**, 1667.
[12] S. V. Ley, J. Norman, W. P. Griffith, S. P. Marsden, *Synthesis* **1994**, 639.
[13] S. V. Ankala, G. Fenteany, *Tetrahedron Lett.* **2002**, *43*, 4729.
[14] Crystallographic data (excluding structure factors) for **10a** have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 671713.
[15] A. L. Spek, *J. Appl. Crystallogr.* **2003**, *36*, 7.
[16] H. D. Flack, *Acta Crystallogr. Sect. A* **1983**, *39*, 876.
[17] P. Ettmayer, A. Billich, T. Baumruker, D. Mechtcheriakova, H. Schmid, P. Nussbaumer, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1555.

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