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Bioorganic & Medicinal Chemistry Letters
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Bioorganic & Medicinal Chemistry Letters 14 (2004) 1555–1558

Fluorescence-labeled sphingosines as substrates of sphingosine kinases 1 and 2

Peter Ettmayer,* Andreas Billich, Thomas Baumruker, Diana Mechtcheriakova, Heide Schmid and Peter Nussbaumer

Novartis Institute for Biomedical Research, Brunnerstrasse 59, Vienna A-1235, Austria

Received 1 October 2003; revised 27 November 2003; accepted 23 December 2003

Abstract—Fluorescently labeled D-*erythro*-sphingosines have been successfully synthesized in 9 and 12 steps starting from commercially available Garner aldehyde. Determining the influence of the nature, position and linkage of the label on the in vitro phosphorylation rate by sphingosine kinases 1 and 2 resulted in the identification of a pyrene- and a NBD-labeled sphingosine which are both phosphorylated with efficiency comparable to the natural substrate.

Sphingosine-1-phosphate (SPP) is an important signaling molecule with both intra- and extracellular actions (reviewed in refs 1–3). SPP is formed from sphingosine (SP) by sphingosine kinases (SPHKs); we recently reported on the kinetics of sphingosine phosphorylation by the two human isoforms (SPHK-1 and -2).[4] For studies on the function and subcellular localization of both SP and SPP, fluorescently labeled, bioactive SP and SPP analogues would be ideal tools. While sphingomyelin and ceramide derivatives with various fluorescent labels in the *N*-acyl side chain are frequently used, there are only two reports on ceramides with such a modification in the SP backbone.[5] While this work was in progress, Hakogi et al. published a 17-step synthesis of NBD-sphingosine starting from *N*-benzyl-4-methoxycarbonyl oxazolidinone.[6] This labeled SP was then successfully converted to NBD-SPP using recombinant murine SPHK-1.

Our initial studies on the phosphorylation efficiency of various SP analogues with intact head group by SPHK-1 indicated a strong dependence on the length and functionalization of the linear saturated tridecanyl (C13) chain of the SP backbone (Fig. 1). Aiming at the identification of a backbone-labeled SP derivative with bioequivalence to natural SP, we varied the position and the chemical linkage of different fluorescent moieties within the C13 chain (Fig. 1).

We now report the 9 and 12-step synthesis of pyrene-, nitrobenzo-2-oxa-1,3-diazole- (NBD), and dansyl-labeled SP derivatives starting from commercially available Garner aldehyde (Aldrich). Using recombinant human SPHK-1 and -2, we describe the substrate properties of these labeled SPs by quantifying the conversion rate to the corresponding phosphates.

In analogy to the synthesis by Kozikowski et al.,[7] the first backbone components were prepared from 3-bromopropanol and 10-bromodecanol, respectively (**3**, $n = 3$, 10; Scheme 1). Protection of the hydroxyl groups catalyzed by PPTS provided the THP ethers **4**. Reaction with lithium acetylide ethylene diamine complex in DMSO provided the THP protected alkynols **5**. Addition of the lithium anions of **5** to *N*-Boc-(*S*)-2,2-dimethyl-oxazolidine-4-carbaldehyde (Garner aldehyde) under non-chelating conditions (THF/HMPT) at low temperature yielded exclusively (>20:1) the anti-stereoisomers[8]

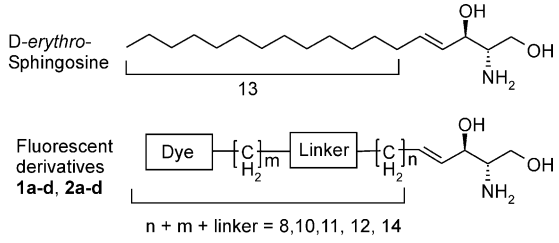

Figure 1. Design of fluorescently labeled sphingosine derivatives.

* Corresponding author. Tel.: +43-1-86634-378; fax: +43-1-86634-383; e-mail: peter.ettmayer@pharma.novartis.com

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doi:10.1016/j.bmcl.2003.12.099

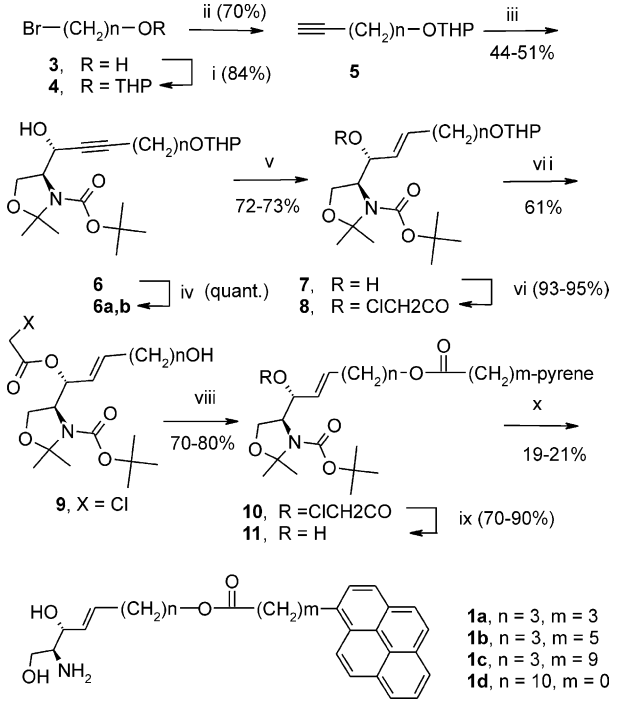
6. Enantiomerical purity of 6 was assessed as >95% de via the Mosher esters 6a,b.[9]

Birch reduction using excess Li in liquid NH_3 gave the *E*-olefins 7. Chloroacetyl protection of the allylic hydroxyl group was followed by selective removal of the THP protecting group using *p*-TsOH in a 3:1 mixture of acetone and methanol to provide the omega hydroxy sphingosine derivatives 9. Acylation of 9 using EDCHCl/DMAP-activated 4-pyrenylbutyric acid, 6-pyrenylhexanoic acid, 10-pyrenyldecanoic acid and pyrene carboxylic acid yielded the protected SP derivatives 10. The photosensitivity of the pyrene label required protection from bright daylight, while dark room conditions were not needed. Quantitative removal of the chloroacetyl group using aqueous ammonium hydroxide in methanol was followed by the acidic deprotection of the amino alcohol to generate the pyrene labeled sphingosines 1a–d.[10] While 10% TFA in dichloromethane was found to be superior to ethyl acetate/1N HCl for the removal of the acetonide and the Boc protecting group, simultaneous partial cleavage of the ester bond could not be completely avoided. However, the breakdown products were easily removed by preparative reversed-phase chromatography (RP-HPLC).

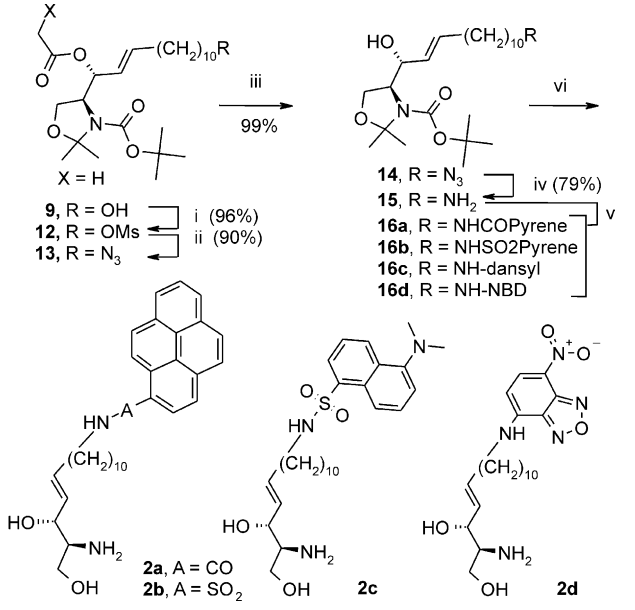
For attaching the fluorescence label via an omega amino group instead of the hydroxyl group in order to generate a more stable amide linker (Scheme 2), the head group protected ω-hydroxy sphingosine 9 ($n = 10$) was converted into the mesylate 12 and reacted with NaN_3 in DMF to give the azide 13.

Removal of the acetyl group using sodium methanolate in methanol yielded 14 which was smoothly reduced with PPh_3 in 20% aqueous THF at 60 °C to afford the corresponding terminal amine 15. The fluorescent labels were introduced by acylating 15 with carbonyl diimidazole (CDI)-activated pyrene carboxylic acid, pyrenylsulfonyl chloride, dansyl chloride and NBD-Cl, respectively. Deprotection of 16a–d in TFA containing 10% water followed by preparative RP-HPLC provided the target structures 2a–d[11] in 61, 72, 79, and 64% yield (over the last 2 steps).

The new fluorescently labeled SP analogues 1a–d and 2a–d were used as substrates for human recombinant SPHK-1 and -2;[12] in fact all of them were converted to phosphorylated derivatives as visualized by the incorporation of radiolabeled phosphate upon incubation with [γ-32P]ATP and the enzymes. The rates of phosphorylation were determined and are reported in Table 1 relative to the natural substrate SP. Using SPHK-1, the pyrene-labeled derivative 1a with the shortest backbone chain (sum of atoms in the chain between label and head group $m+n+$linker$=8$) was about 20 times less efficiently converted to the corresponding phosphate than SP. Increasing the chain length by two CH_2-groups (1b) improved the phosphorylation rate by a factor of two, whereas further backbone elongation by additional 4 CH_2-groups (1c, $m+n+$linker$=14$ atoms) resulted in inferior substrate turnover by SPHK-1 (about 40-fold less as compared to SP). To investigate whether the introduction of the bulky label or the presence of the ester group in the middle of the sphingolipid backbone was responsible for the lack of efficient conversion



Scheme 1. Variation of the label position. Reagents and conditions: (i) DHP, PPTS, CH_2Cl_2, rt; (ii) Li–acetylide ethylene diamine complex, DMSO, 10 °C->rt; (iii) (a) *n*-BuLi, THF, −20 °C, 2 h; (b) HMPT, Garner aldehyde, −78 to −20 °C; (iv) **6a**: (*S*)-(+)- and **6b**: (*R*)-(−)-α-methoxy-α-trifluoro-methylphenylacetyl chroride, pyr, CH_2Cl_2, 16 h, rt; (v) NH_3, Li, THF, 5 h, −60 °C; (vi) chloroacetyl chloride, pyr, CH_2Cl_2, rt; (vii) *p*TsOH, acetone/methanol 3/1, 1 h, rt; (viii) pyrene-(CH_2)m-COOH, CH_2Cl_2, EDC·HCl, DMAP, rt; (ix) NH_4OH, MeOH, rt; (x) 10%TFA in CH_2Cl_2, prep RP-HPLC.



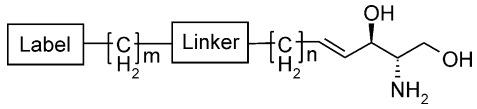
Scheme 2. Variation of the label. Reagents and conditions: (i) CH_3SO_2Cl, Et_3N, CH_2Cl_2, rt; (ii) NaN_3, DMF, 50 °C; (iii) CH_3ONa, MeOH, rt; (iv) $(Ph)_3P$, 10% aqu. THF, 60 °C; (v) imidazol-1-yl-pyren-1-yl-methanone, THF, Et_3N, rt; or pyrene-1-sulfonylchloride, Et_3N, THF, rt; or dansyl chloride, Et_3N, THF, rt; or NBD-Cl, Et_3N, THF; (vi) 10% aqu. TFA, rt.

to the phosphates, we moved the ester linker to the end of a decanyl chain (**1d**, $m+n+\text{linker}=12$). For this derivative almost equal phosphorylation by SPHK-1 relative to natural SP was observed proving that the bulky pyrene moiety in **1d** is tolerated by the enzyme. Interestingly, in **1d** the lipophilic label is an appendix to the C13 chain of SP and does not function as mimicry of a portion of the aliphatic hydrocarbon chain in SP. Using SPHK-2, relative phosphorylation rates for **1a–d** were higher than using SPHK-1, but the ranking of the substrate efficiency is the same for both enzymes, with **1d** being comparable to SP for both enzymes.

Motivated by this encouraging result, we now kept the decanyl spacer ($n=10$) fixed and investigated the influence of other chemical linkers and dyes. The ester functionality in **1d** might be metabolically labile (although no degradation was observed in the short term in vitro phosphorylation experiments) and was, therefore, replaced by an amide (**2a**). This reduced the phosphorylation efficiency only by factor of 2 and 3 for SPHK-1 and -2, respectively. The exchange of the ester to a sulfonamide linker (**2b**) was less tolerated by SPHK-1 (about 20 times less conversion than with **1d**) than by SPHK-2, which phosphorylated **2b** almost as efficiently as SP. These results indicate pronounced differences in substrate specificity for SPHK-1 versus SPHK-2, which confirms recent results obtained with FTY-720.[4]

So far the pyrene label was used, which among available fluorescence dyes should result in the least disturbance of the highly lipophilic character of the SP backbone. Next, we tested two other labels containing polar functionalities. The dansyl-labeled analogue **2c** was not well tolerated by both kinases. The inefficient conversion of **2c** can only partially be attributed to the sulfonamide linkage because it is not well recognized by both SPHK-1 and SPHK-2 in contrast to pyrene-labeled sulfonamide derivative **2b**. The NBD-labeled sphingosine derivative **2d** ($m+n+\text{linker}=11$), where the dye is attached to the aliphatic backbone via an aromatic amino function, was well phosphorylated by both SPHK-1 and SPHK-2 (about 2 times slower than SP). Thus, also an NBD-label is tolerated by the enzymes despite its polar functionalities, which is in agreement with the recent finding by Hakogi et al.[6] who used an NBD-labeled SP derivative shorter than **2d** by one CH_2 group.

Table 1. Rate of phosphorylation of labeled sphingosine derivatives by SPHK-1 and 2. Values are given relative to the rate for D-sphingosine[12]



Compd	Label	M	Linker	*n*	SPHK-1	SPHK-2
Sphingosine	H	0	—	13	'1'	'1'
1a	Pyrene	3	COO	3	0.048	0.14
1b	Pyrene	5	COO	3	0.078	0.83
1c	Pyrene	9	COO	3	0.024	0.065
1d	Pyrene	0	COO	10	0.83	1.25
2a	Pyrene	0	CONH	10	0.28	0.59
2b	Pyrene	0	SO_2NH	10	0.051	0.83
2c	Dansyl	0	SO_2NH	10	0.063	0.026
2d	NBD	0	NH	10	0.5	0.48

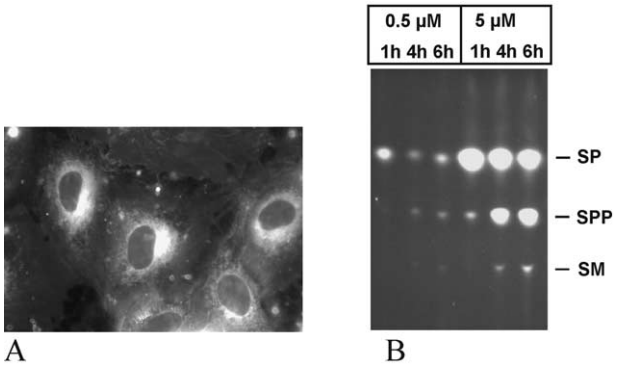


Figure 2. (A) Rapid incorporation of **1d** into endothelial cells (HUVEC). Cells were incubated for 15 min with 1 µM of **1d** under normal growth conditions. (B) The pattern of metabolism of **1d** assayed by thin-layer chromatography: conversion of **1d** to pyrene-labeled metabolites that co-migrate with sphingosine-1-phosphate (SPP) and sphingomyelin (SM).

We selected the pyrene labeled sphingosine **1d** to examine the uptake, subcellular distribution and metabolism (conversion to the phosphate) in human endothelial cells (HUVEC). Fluorescently labeled SP **1d** was rapidly incorporated into the cells within 5 min after addition, and showed predominant distribution to the endoplasmic reticulum and to the Golgi apparatus after 15–30 min of incubation (Fig. 2A). Furthermore, **1d** was converted intracellularly into two products as shown by thin-layer chromatography[13] (Fig. 2B): the major metabolite co-migrated with sphingosine-1-phosphate (SPP) and a minor product with sphingomyelin (SM). The pattern of metabolic conversion of **1d** in HUVEC was similar to that of [^{3}H]-sphingosine (data not shown). These data are in line with previously reported rapid uptake and metabolism of sphingosine[14] as well as of an NBD-labeled sphingosine derivative[6] by cultured cells.

In summary, we have developed novel, readily accessible, fluorescently labeled sphingosine derivatives, which can serve as valuable tools for many biological investigations. Compounds with two different dye labels that are phosphorylated by both SPHK-1 and-2 with comparable efficiency to the natural substrate SP (**1d**, **2d**), as well as a compound which is quite selectively converted by SPHK-2 (**2b**), are now available. In addition, we have identified different substrate specificities for SPHK-1 and SPHK-2 confirming recent results obtained with FTY-720.[4]

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9. Characteristic NMR data for the Mosher esters. **6a**,**b** ($n=3$): ^{1}H NMR (DMSO-d_6, 500 MHz, 330 K) δ: 3.43 (s; 3H, **6a**-OCH_3), 3.54 (s, 3H; **6b**-OCH_3). ^{19}F NMR (DMSO-d_6, 377 MHz, 299K) δ −0.194 (s; **6a**-CF_3), −0.192 (s; **6b**-CF_3).
10. Data for the pyrene labeled sphingosine **1d**: $[\alpha]_D^{20}$ −4.2 ($c=1.0$, CH_3OH); ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 9.16 (d, $J=9$ Hz; 1H), 8.51 (d, $J=9$ Hz; 1H), 8.15–7.80 (m; 7H), 5.78 (bs; 1H), 5.42 (bs; 1H), 4.56 (bs; 1H); 4.41 (t, $J=9$ Hz; 2H); 3.97–3.60 (m; 2H), 3.50 (bs; 3H), 1.96 (bs; 2H), 1.84–1.77 (m; 2H), 1.50–1.20 (m; 14H). Electrospray MS: 502.3 (M + H)+, $C_{32}H_{39}NO_4$, calcd 501.3.
11. Data for the pyrene labeled sphingosine **2a**: $[\alpha]_D^{20}$ −2.4 ($c=1.0$, CH_3OH); ^{1}H NMR ($CDCl_3$ + DMSO-d_6, 400 MHz) δ: 8.55 (d, $J=9$ Hz; 1H), 8.28–8.04 (m; 8H), 7.60 (bs; 1H), 5.80 (dt, $J=7$, 15 Hz; 1H), 5.45 (dd, $J=6$, 15 Hz; 1H), 4.42 (bs; 1H); 3.83–3.75 (m; 2H), 3.57 (dt, $J=7$, 6 Hz; 2H), 3.20 (bs; 1H), 2.04 (dt, $J=7$, 6 Hz; 2H), 1.77–1.70 (m; 2H), 1.52–1.26 (m; 14H). Electrospray MS: 501.3 (M + H)+, $C_{32}H_{40}N_2O_3$, calcd 500.3. Data for the pyrene labeled sphingosine **2b**: $[\alpha]_D^{20}$ −2.2 ($c=1.0$, CH_3OH); (CD_3OD) δ: 8.94 (d, $J=9.4$ Hz; 1H), 8.56 (d, $J=8.2$ Hz; 1H), 8.60–8.00 (m; 7H), 5.72 (dt, $J=6.6$, 15.3 Hz; 1H), 5.34 (dd, $J=6.8$, 15.3 Hz; 1H), 4.18 (t; $J=5.6$ Hz; 1H), 3.69/3.56 (AB-System, $J=4.0$, 11.6, 8.3 Hz; 2H), 3.08 (m; 1H), 2.72 (t, $J=6.7$ Hz; 2H), 2.93 (m; 2H), 1.23–1.07 (m; 4H), 1.07–0.55 (m; 12H). Electrospray MS: 537.3 (M + H)+, $C_{31}H_{40}N_2O_4S$, calcd 536.3. Data for the dansyl labeled sphingosine **2c**: $[\alpha]_D^{20}$ −1.8 ($c=2$, CH_3OH); (CD_3OD) δ: 8.46 (d, $J=8.5$ Hz; 1H), 8.27 (d, $J=8.7$ Hz; 1H), 8.11 (d, $J=7.4$ Hz; 1H), 7.51–7.46 (m; 2H), 7.19 (d, $J=7.5$ Hz; 1H), 5.75 (dt, $J=6.4$ Hz, 16.2 Hz; 1H), 4.30 (dd, $J=5.4$, 6.8 Hz; 1H), 4.19 (t, $J=5.3$ Hz; 1H), 3.69/3.57 (AB-system, $J=11.6$ Hz, 4.0, 8.4 Hz; 2H), 3.11 (m; 2H), 2.80 (s; 6H), 2.73 (t, $J=6.9$ Hz; 2H), 2.00 (q, $J=6.9$ Hz, 2H), 1.33–1.22 (m; 2H), 1.22–0.90 (m; 14H). Electrospray MS: 506.3 (M + H)+, $C_{27}H_{43}N_3O_4S$, calcd 505.3. Data for the NBD labeled sphingosine **2d**: $[\alpha]_D^{20}$ −3.8 ($c=1.0$, CH_3OH); ^{1}H NMR (CD_3OD, 400 MHz) δ: 8.43 (d, $J=9$ Hz; 1H), 6.25 (d, $J=9$ Hz; 1H), 5.74 (dt, $J=15$, 7 Hz; 1H), 5.36 (dd, $J=15$, 7 Hz; 1H), 4.18 (dd, $J=6$, 7 Hz; 1H); 3.69 (dd, 4, 12 Hz; 1H); 3.56 (dd, 8, 12 Hz; 1H); 3.44 (bs; 2H), 3.12–3.03 (m; 1H), 2.00–1.95 (m; 2H), 1.72–1.64 (m; 2H); 1.40–1.25 (m; 14H). Electrospray MS: 436.2 (M + H)+, $C_{21}H_{33}N_5O_5$, calcd 435.2.
12. The phosphorylation reactions were performed essentially as described in ref 4. Briefly, the cytoplasmic fraction of recombinant HEK-293 cells overexpressing human SPHK-1 or-2 was incubated at 30 °C in total volumes of 100 μL with SP derivatives (20 μM; added from stock solutions in DMSO), 1 mM of ATP, and 2 μCi [γ-^{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). Radiolabeled SPP derivatives were visualized and quantified using a Molecular Dynamics Storm PhosphorImager (Sunnyvale, CA). The rate of phosphorylation was calculated and is reported for the SP derivatives as value relative to the rate for sphingosine (for which the rate was 41 and 25 nmol/min/mg with SPHK-1 and -2, respectively).
13. Thin-layer chromatography was performed on silica plates, using either butanol/acetic acid/ water 3:1:1 or $CHCl_3$/MeOH/H_2O/NH_4OH ($28^w/_w$%) 200:150:29:1 as mobile phase. In these systems sphingosine and **1d** co-migrate. Identity of the metabolites with pyrene-labeled SPP and SM was established by co-migration with **1d-phosphate** (prepared with recombinant SPHK-1) and commercially available tritium-labeled SPP and SM as standards.
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