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Synthesis of Borondipyrromethene (BODIPY)-Labeled Sphingosine Derivatives by Cross-metathesis Reaction

Carsten Peters, Andreas Billich, Michael Ghobrial, Klemens Högenauer, Thomas Ullrich, and Peter Nussbaumer*

Novartis Institutes for BioMedical Research, Brunnerstrasse 59, A-1235 Vienna, Austria

peter.nussbaumer@novartis.com

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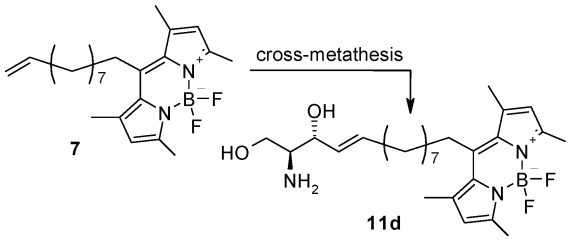

A new efficient and flexible synthesis of fluorescently labeled sphingosine derivatives from commercially available Garner aldehyde (**8**) is described. For this, appropriate alkenylated borondipyrromethene (BODIPY) dyes were synthesized and used for the first time in a cross-metathesis reaction, the key step of the approach. The labeled sphingosines with appropriate chain length were accepted as substrates by sphingosine kinases (SPHKs), yielding the corresponding phosphorylated products. One of these derivatives (**11d**) was identified as the first reported selective substrate for SPHK-1.

Members of the family of sphingolipids not only are essential structural components of higher eukaryotic cells but also serve as extra- and intracellular mediators in signal transduction.[1] Research on the identification and elucidation of sphingolipid pathways is in need of tool compounds. In particular, the introduction of suitable tags or labels into the backbone of sphingolipids for localization, metabolism, and binding studies is of high interest. Recently, we reported on sphingosine analogues with different fluorescent functionalities, linkers, and chain lengths in the backbone,[2] finally resulting in the establishment of the first cell-free, nonradioactive sphingosine kinase (SPHK) assay using the 7-nitro-4-benzofurazanamine- (NBD-) substituted sphingosine **1** (Figure 1).[3]

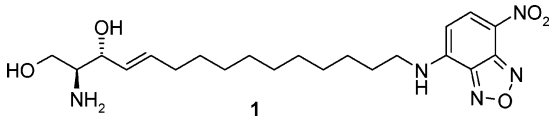

FIGURE 1. NBD-labeled sphingosine **1** used as substrate for a nonradioactive SPHK assay.

Searching for a shorter and more flexible synthesis of labeled sphingolipids, we then devised a highly efficient method to introduce various functionalities such as photoaffinity tags, biotin, and the NBD fluorophore, starting from the natural products as precursors.[4] To extend the scope of this methodology, in particular for fluorescence labeling of natural and unnatural sphingosine and ceramide derivatives in the alkenyl chain, we now report on an alternative flexible synthetic approach starting from commercially available Garner aldehyde. The key step is a cross-metathesis reaction establishing the *E*-double bond of the sphingosine backbone. Total syntheses of unlabeled sphingosine and ceramide by cross-metathesis were already described[5] but all required a multistep preparation of the respective headgroup precursor. In addition to the previously used NBD dye, we aimed at introducing the 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (borondipyrromethene; BDP or BODIPY[6]) group as fluorophore, which is known to have excellent fluorescence properties (high fluorescence quantum yield and photostability) combined with compact size and good lipophilic properties,[7] at varying distances from the sphingosine head group. Finally, we tested the labeled sphingosines for their phosphorylation potential by SPHKs in order to assess whether the introduced fluorophores are tolerated by a natural receptor.

We used three different routes to generate ω-alkenyl-substituted dyes (Scheme 1) as one set of the olefinic components for the cross-metathesis reaction. The NBD derivative **2** was prepared by reaction of undecenylamine with 4-chloro-7-nitrobenzofurazane (Scheme 1A). For an efficient entry into alkenylated BDPs bearing an all-carbon linker, we developed the route shown in Scheme 1B: starting from ω-alkenoic acids **3a,b**, the alkenylated pyrroles **4a,b** were obtained according to a procedure reported by Fürstner et al.[8] These alkenylpyrroles were then reacted with 2,4-dimethylpyrrole-5-carboxaldehyde in an Ehrlich-type reaction to yield dipyrromethenes **5a,b**. The BF_2 bridge was introduced by use of borontrifluoride in the presence of DBU,[9] furnishing the alkenylated BDP dyes **6a,b** in excellent yield.

* Corresponding author: fax +43-1-80166345.

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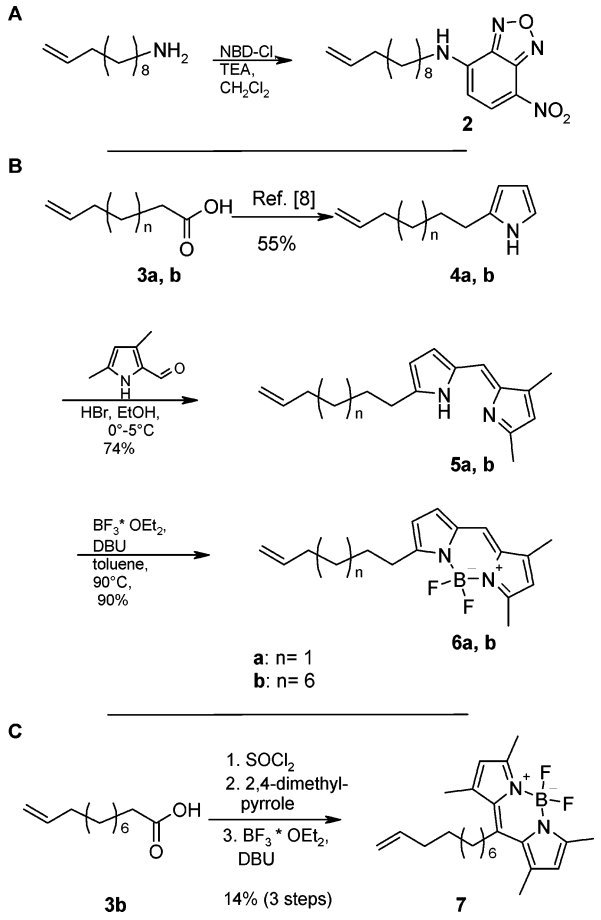
SCHEME 1. Synthesis of Alkenyl-Substituted Fluorescent Labels

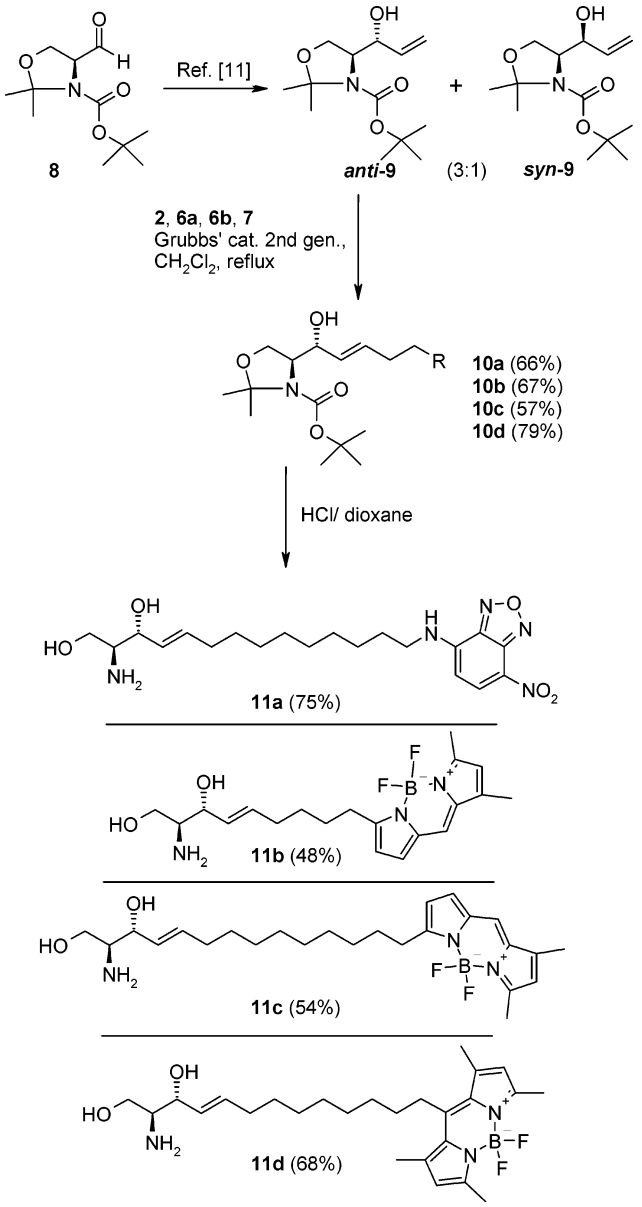


In our third approach, the symmetrical BDP derivative **7** was prepared in analogy to a procedure described by Li et al.[10] (Scheme 1C). The synthesis also started from the ω-alkenoic acid **3b**, which was transformed into the corresponding acyl chloride and subsequently used for the formation of the dipyrromethene by reaction with 2,4-dimethylpyrrole. This intermediate was used without purification for the introduction of the BF_2 bridge, furnishing the functionalized dye **7**. The yield of this one-pot reaction sequence is significantly lower than those described above for the asymmetrical analogues, but the method provides a simple and fast access to (alkenylated) BDP dyes.

These functionalized dyes were then used to introduce the label into the backbone of sphingosine by cross-metathesis reaction, which had been proven to generate the newly formed double bond with high *E*-stereo selectivity.[4] To allow the labeling of both natural and nonnatural sphingosine derivatives, we developed an efficient, modular total synthesis with the allylic alcohol **9** as key component for the cross-metathesis reaction (Scheme 2). The allylic alcohol **9** is easily accessible from the commercially available Garner aldehyde (**8**),[11] and the resulting 3:1 (anti/syn) mixture of diastereomers could be readily separated by preparative HPLC.

The cross-metathesis reactions of *anti*-**9** with alkenylated NBD and BDP analogues **2**, **6a,b**, and **7** (Scheme 2) proceeded

SCHEME 2. Synthesis of NBD- and BDP-Labeled Sphingosines 11a−d



smoothly. After purification by flash chromatography, the labeled Boc-/acetonide-protected sphingosines **10a−d** were obtained in good yields. Deprotection was achieved by reaction with 4 M HCl in dioxane, and the resulting sphingosines **11a−d** were purified by RP-HPLC. The fluorophore proved to be highly stable under the cross-metathesis conditions and did not seem to reduce the activity of the catalyst. Only during the deprotecting step did we observe slight decomposition of the BDP dye. The byproducts could be easily separated and were not isolated.

The attachment of the labels to the natural biomolecule was accomplished via a carbon−carbon bond, avoiding the usual introduction of an additional polar functional linker, such as an amide or carbamate, and thus minimizing the impact by the structural modification on the biological properties. To demonstrate that the side-chain modifications are tolerated in a biological setting, we tested sphingosine derivatives **11a−d** for phosphorylation by human recombinant SPHK-1 and -2.[12] Both enzymes convert sphingosine into sphingosine 1-phosphate,

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TABLE 1. Phosphorylation Rates[a] of Synthesized Compounds by SPHK-1 and -2

compound	rate (nmol min^{-1} mg^{-1}) SPHK-1	SPHK-2
D-*erythro*-sphingosine	41 ± 4	25 ± 3
1	21 ± 2	12 ± 2
11a	20 ± 5	13 ± 2
11b	2.5 ± 0.8	0.8 ± 0.2
11c	12 ± 3	7 ± 1
11d	12 ± 2	0.5 ± 0.1

[a] Rates are shown ± standard deviation.

which is an important signaling molecule in the regulation of diverse physiologic functions, including angiogenesis, lymphocyte recirculation, vascular permeability, and heart rate. The phosphorylation rates observed with the test compounds and the natural substrate sphingosine as standard are summarized in Table 1. In addition, the previously reported compound **1** was used as reference.

The NBD-labeled sphingosine **11a**, which differs from **1** only by lacking one methylene group in the aliphatic chain between the double bond and the label, shows the same phosphorylation rate as **1** with about 50% efficiency compared to sphingosine. The BDP-labeled sphingosines were also accepted as substrates by the sphingosine kinases; however, different conversion rates were observed. Derivative **11c** with a C9 aliphatic chain between the double bond and the label was equally well phosphorylated by both enzymes with about 30% efficiency relative to sphingosine (rates of 12 nmol min^{-1} mg^{-1} compared to 41 for SPHK-1 and 7 nmol min^{-1} mg^{-1} compared to 25 for SPHK-2), not very different from the NBD-labeled analogues. In contrast, BDP-sphingosine **11b** with a shorter chain length (C4 chain between double bond and label) was substantially less phosphorylated by both enzymes. These results confirm our initial findings with pyrene-labeled sphingosine derivatives, where we also observed a strong dependence on the chain length.[2] Analogue **11d**, bearing the symmetrical BDP substituent, showed a different profile. While SPHK-1 converted this substrate with comparable efficiency (rate of 12 nmol min^{-1} mg^{-1}) to **11c**, phosphorylation by SPHK-2 was 15- and 50-fold less efficient relative to analogue **11c** and sphingosine, respectively. The result with SPHK-1 is somewhat surprising because the overall length of the molecule is shorter than that of sphingosine and the labeled analogues **1**, **11a**, and **11c**. Apparently, the BDP dye is sufficiently lipophilic to mimic the missing alkyl chain present in sphingosine. In addition, with **11d** we identified a labeled substrate that is selectively phosphorylated by SPHK-1 over SPHK-2. So far, only substrates with selectivity for SPHK-2 are known (e.g., FTY720 and D,L-*threo*-dihydrosphingosine[13]).

We developed two routes to introduce labels into the alkyl backbone of sphingolipids and synthesized the first sphingosine analogues with BDP dyes incorporated into the alkenyl chain. The key step in the syntheses is the cross-metathesis reaction using second-generation Grubbs' catalyst. To our knowledge, this is the first study using BDP analogues in a metathesis reaction. Our results emphasize the broad applicability of this reaction and the robustness of the BDP dye which remained unaffected by the reaction conditions. Biological examinations revealed that BDP-labeled sphingosines **11c** and **11d** were efficiently phosphorylated by SPHK-1 and **11c** was also phosphorylated by SPHK-2, indicating some bioequivalence to the natural substrate sphingosine. On the basis of these initial results and the fact that BDP is an ideal dye for fluorescence spectroscopy, these compounds will represent useful tools for further biological investigations and elucidation of lipid signaling.

Experimental Section

4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacen-3-hex-5-ene (6a). Under inert atmosphere, dipyrromethene (110 mg, 0.43 mmol) **5a** was dissolved in dry toluene and treated with DBU (194 μL, 1.3 mmol) and $BF_3{\cdot}OEt_2$ (273 μL, 2.16 mmol) at room temperature. Then the reaction mixture was placed into a preheated oil bath at 90 °C for 30 min. After cooling to room temperature, the reaction mixture was quenched by adding water and then dichloromethane was added. The organic layer was separated and washed thoroughly with water and brine (1×) and dried over Na_2SO_4, and removal of the solvent yielded a black oily solid, which was purified by flash column chromatography to furnish a dark red oil; yield 130 mg (0.43 mmol, 99%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.53 (quin, J = 7.7 Hz, 2H), 1.76 (dt, J = 7.7 Hz, 2H), 2.11 (q, J = 7.4 Hz, 2H), 2.25 (s, 3H), 2.55 (s, 3H), 2.98 (t, J = 7.5 Hz, 2H), 4.94 (dd, J = 10.2, 1.1 Hz, 1H), 5.02 (dd, J = 17.2, 1.9 Hz, 1H), 5.82 (ddt, J = 10.3, 6.8, 6.6 Hz, 1H), 6.08 (s, 1H), 6.28 (d, J = 3.8 Hz, 1H), 6.90 (d, J = 3.8 Hz, 1H), 7.06 (s, 1H); ESI-MS$^+$ 324.2 [M + Na]. HRMS: M calcd for $C_{17}H_{21}BF_2N_2Na$ 324.1694, found 324.1695.

4,4-Difluoro-1,3,5,7-tetramethyl-8-(dec-9-en)-4-bora-3a,4a-diaza-*s*-indacene (7). Undecenyl acid (1.0 g, 5.3 mmol) was dissolved in dry CH_2Cl_2 and dry *N*,*N*-dimethylformamide (DMF) (40 μL, 0.5 mmol) was added. Afterward oxalyl chloride was added carefully and the solution was stirred for 60 min after the evolution of gas subsided. After coevaporation with toluene and drying in high vacuum, the residue was dissolved in dry CH_2Cl_2 and treated with a solution of 2,4-dimethylpyrrole (1.4 mL, 13.3 mmol) in CH_2Cl_2. The resulting reaction mixture was refluxed overnight; meanwhile, the color of the solution turned to dark red. Afterward the reaction mixture was concentrated in vacuo, treated with *n*-pentane, and stored overnight in the freezer. The supernatant was discarded and the oily residue was dissolved in dry toluene without further purification. To this solution were added DBU (2.4 mL, 15.9 mmol) and $BF_3{\cdot}OEt_2$ (3.4 mL, 26.6 mmol) at room temperature, and the reaction mixture was transferred afterward for 30 min into an oil bath preheated at 90 °C. After cooling to room temperature, the reaction mixture was quenched by addition of water, and then CH_2Cl_2 was added. The organic layer was separated, washed thoroughly with water and brine (1×), and dried over Na_2SO_4, and removal of the solvent yielded a black oil, which was purified by flash column chromatography to furnish a dark red oil; yield 296 mg (0.77 mmol, 14.4%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.25−1.67 (m, 12H), 2.05 (q, J = 7.6 Hz, 2H), 2.41 (s, 3H), 2.51 (s, 3H), 2.90−2.98 (m, 2H), 4.93 (dd, J = 10.3, 1.2 Hz, 1H), 5.01 (dd, J = 17.0, 2.0 Hz, 1H), 5.80 (ddt, J = 10.1, 6.7, 6.6 Hz, 1H), 6.04 (s, 1H); ^{13}C NMR (100 MHz) δ 14.8, 16.8, 28.9, 29.3, 29.4, 29.7, 29.8, 30.8, 32.3, 34.1, 114.6, 122.0, 131.8, 139.5, 140.7, 147.1, 154.1; ESI-MS$^+$ 409.2 [M + Na]. HRMS: M calcd for $C_{23}H_{33}BF_2N_2Na$ 408.2633, found 408.2633.

(*S*)-4-[(*E*)-(*R*)-1-Hydroxy-12-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacen-3)-dodec-2-enyl]-2,2-dimethyloxazolidine-3-carboxylic Acid *tert*-Butyl Ester (10c). According to the general procedure, Garner allylic alcohol (*anti*-**9**) (60.5 mg, 0.24 mmol) was reacted with C11-alkenyl-BDP **6b** (175 mg, 0.47 mmol) and second-generation Grubbs' catalyst (19 mg, 0.024 mmol): yield 80 mg (0.13 mmol, 57%); orange oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.28 (s, 6H), 1.32−1.68 (m, 12H), 1.49 (s, 9H), 1.72 (quin, J =

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7.7 Hz, 2H), 2.04 (q, J = 7.4 Hz, 2H), 2.24 (s, 3H), 2.56 (s, 3H), 2.96 (t, J = 7.8 Hz, 2H), 3.78−4.24 (m, 4H), 5.45 (dd, J = 15.3, 6.1 Hz, 1H), 5.45 (td, J = 15.3, 6.1 Hz, 1H), 6.08 (s, 1H), 6.28 (d, J = 4.0 Hz, 1H), 6.89 (d, J = 4.0 Hz, 1H), 7.06 (s, 1H); ESI-MS$^+$ 624.8 [M + Na].

(*S*)-4-[(*E*)-(*R*)-1-Hydroxy-11-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene-8)-undec-2-enyl]-2,2-dimethyloxazolidine-3-carboxylic Acid *tert*-Butyl Ester (10d). According to the general procedure, Garner allylic alcohol (*anti*-**9**) (30 mg, 0.11 mmol) was reacted with alkenylated BDP **7** (90.1 mg, 0.22 mmol) and second-generation Grubbs' catalyst (2 mg, 0.002 mmol): yield 60.2 mg (0.09 mmol, 79%); orange oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.25−1.39 (m, 8H), 1.42−1.51 (m, 15H), 1.52−1.67 (m, 4H), 2.01−2.08 (m, 2H), 2.41 (s, 6H), 2.51 (s, 6H), 2.89−2.95 (m, 2H), 3.75−4.34 (m, 5H), 5.44 (dd, J = 15.4, 5.9 Hz, 1H), 5.68−5.78 (m, 1H), 6.04 (s, 2H); ^{13}C NMR (100 MHz) δ 14.3, 16.3, 26.3, 28.4, 29.0, 29.1, 29.6, 30.3, 30.8, 31.8, 32.2, 62.6, 64.8, 74.6, 79.6, 81.0, 94.4, 98.6, 121.5, 127.3, 128.3, 129.2, 131.4, 133.1, 140.2, 146.6, 153.6; ESI-MS$^+$ 638.4 [M + Na].

(*E*)-(2*S*,3*R*)-2-Amino-14-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacen-3)-tetradec-4-ene-1,3-diol (11c). According to the general procedure, fully protected BDP-labeled sphingosine **10c** (80 mg, 0.13 mmol) was treated with 4 mL of 4 M HCl/dioxane and afterward purified; yield 40 mg (0.07 mmol, 54%); orange lyophilysate; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.18−1.39 (m, 16H), 1.64 (quin, J = 7.7 Hz, 2H), 1.99 (q, J = 7.3 Hz, 2H), 2.23 (s, 3H), 2.45 (s, 3H), 2.82 (t, J = 7.7 Hz, 2H), 2.97−3.06 (m, 1H), 3.45 (dd, J = 11.3, 2.9 Hz, 1H), 3.59 (dd, J = 11.3, 4.1 Hz, 1H), 4.16 (t, J = 5.3 Hz, 1H), 5.12 (br s, 1H), 5.41 (dd, J = 15.4, 6.5 Hz, 1H), 5.45 (br s, 1H), 5.68 (dd, J = 15.4, 6.3 Hz, 1H), 6.26 (s, 1H), 6.39 (d, J = 3.9 Hz, 1H), 7.10 (d, J = 3.9 Hz, 1H), 7.66 (s, 1H), 7.72 (br s, 3H); ^{13}C NMR (100 MHz) δ 11.3, 14.8, 28.6, 28.7, 29.1, 29.2, 29.3, 29.4, 29.5, 29.7, 32.3, 56.2, 63.6, 75.0, 116.9, 119.8, 123.4, 128.5, 129,0, 133.3, 134.6, 134.8, 142.8, 158.9, 160.9; ESI-MS$^+$ 462 [M + H]. HRMS: M calcd for $C_{25}H_{38}BF_2N_3O_2Na$ 483.2954, found 483.2955.

(*E*)-(2*S*,3*R*)-2-Amino-13-(4,4-Difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene-8)-tridec-4-ene-1,3-diol (11d). According to the general procedure, fully protected BDP-labeled sphingosine **10d** (60.4 mg, 0.09 mmol) was treated with 5 mL of 4 M HCl/dioxane and afterward purified; yield 29.2 mg (0.06 mmol, 68%); orange lyophilysate; ^{1}H NMR (400 MHz, MeOD-d_4) δ 1.30−1.46 (m, 9H), 1.48−1.56 (m, 2H), 1.58−1.66 (m, 2H), 2.10 (q, J = 6.9 Hz, 2H), 2.40−2.47 (m, 12H), 2.94−3.01 (m, 2H), 3.18 (quint, J = 4.7 Hz, 1H), 3.66 (dd, J = 13.7, 8.4 Hz, 1H), 3.78 (dd, J = 11.6, 4.0 Hz, 1H), 4.27 (t, J = 5.7 Hz, 1H), 5.43−5.50 (m, 1H), 5.80−5.88 (m, 1H), 6.12 (s, 2H); ^{13}C NMR (100 MHz) δ 13.0, 15.1, 27.9, 28.7, 28.8, 29.0, 29.1, 29.8, 31.7, 31.9, 57.1, 58.0, 69.6, 121.1, 127.1, 131.2, 135.1, 140.7, 147.0, 153.4; ESI-MS$^+$ 476.2 [M + H]. HRMS: M calcd for $C_{26}H_{39}BF_2N_3O_2Na$ 475.3291, found 475.3292.

Biology. Phosphorylation by Sphingosine Kinase 1 and 2. The phosphorylation reactions were performed essentially as described.[12] 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 sphingosine or derivatives **11a**−**d** (20 μM; added from stock solutions in dimethyl sulfoxide, DMSO), 1 mM ATP, and 2 μCi of [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. From the incubation mixtures, samples were drawn at five time points up to 2 h during the quasi-linear phase of the reaction. Lipids were extracted from the samples and separated by thin-layer chromotography (TLC) plates (Merck). Radiolabeled SPP derivatives were visualized and quantified on a Molecular Dynamics Storm PhosphorImager (Sunnyvale, CA). From the data, reaction rates were calculated as nanomoles of phosphorylated derivative formed per minute per milligram of total protein in the enzyme containing lysate.

Supporting Information Available: General experimental procedures and analytical data for compounds **6a**,**b**, **7**, **10a**−**d**, and **11a**−**d**. This material is available free of charge via the Internet at http://pubs.acs.org.

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