

## Highly Efficient and Regioselective Phosphorylation of Sphingolipids by Phase-Transfer Catalysis\*

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(Received May 22nd, 2007)

Phosphorylation of *D-erythro*-sphingosine and its N-BOC or N-palmitoyl derivatives with trimethyl phosphite was carried out in 72–92% yield at room temperature for 20 min in a biphasic system comprised of dichloromethane/aqueous solutions of NaOH or K<sub>2</sub>CO<sub>3</sub> using 1,2-dibromotetrachloroethane as a source of halogen and cetyl pyridinium bromide as a phase-transfer catalyst. These are the first reported examples of a highly selective *O*- and *N*-phosphorylation of sphingolipids by the phase-transfer catalysis. Our studies show that the developed phosphorylation protocol works as a modular process, in which the synthetic outcome is controlled by a type of the used base, catalyst and solvent system.

**Key words:** phosphorylation, trimethyl phosphite, 1,2-dibromotetrachloroethane, phase-transfer catalysis, sphingosine, ceramide, sphingosine 1-phosphate, ceramide 1-phosphate

Phosphoryl-group transfer is a fundamental reaction in cell biology and, as a model to follow, still represents a formidable task for synthetic organic chemistry because of the difficulty of installing this group at key desired sites within complex natural products [1,2].

All critical cellular processes such as energy utilization, ribonucleic acids synthesis and maintenance, signal transduction as well as phospholipid biosynthesis rely on enzymes that utilize oxygen or nitrogen nucleophiles as acceptors of the  $\gamma$ -phosphate of adenosine 5'-triphosphate [3].

To date, however, selectivity in laboratory syntheses of polyfunctional phosphates is achieved by application of the multiple step protective group strategies and utilization of P(III) and P(V) chemistry under a stringent reaction conditions [4–7]. This approach is well-documented in the synthesis of complex phospholipids, however, sphingophospholipids (SPLs) have been not explored in depth in comparison to their glycerol counterparts [8–12].

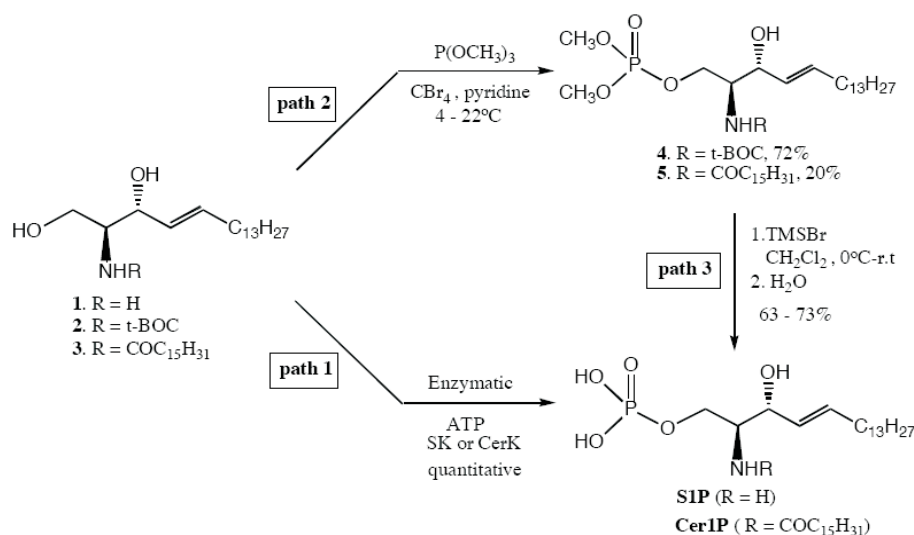
An accelerated interest in SPLs is mostly generated by the simplest metabolite, *D-erythro*-sphingosine 1-phosphate (S1P; Scheme 1), which is abundantly present in blood platelets and plays a critical role in blood vessel formation [13]. S1P is a natural

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\* Dedicated to Prof. Jacek Młochowski on the occasion of his 70<sup>th</sup> birthday.

ligand to extra-cellular EDG receptors, which control important biological functions in cells including calcium mobilization, regulation of growth, cytoskeletal organization, differentiation, migration and angiogenesis [14,15]. S1P has been implicated in pathophysiology of immunological and inflammatory disorders, wound healing, atherosclerosis and cancer [16–19]. Moreover, another recently identified and investigated endogenous SPL, *D-erythro*-ceramide 1-phosphate (Cer1P, Scheme 1), proved complementary to S1P profile of biological activity. Cer1P regulates cell survival and inflammatory responses by controlling eicosanoids synthesis *via* direct activation of cPLA<sub>2</sub>α [20].

Direct chemo- and regioselective phosphorylation of *D-erythro*-sphingosine (**1**) or *D-erythro*-C16-ceramide (**2**) at the C1 position is a challenge which only the enzyme sphingosine kinase (SK) or ceramide kinase (CerK) can accomplish under physiological conditions (Scheme 1, path 1) [21,22]. In order to avoid formation of a complex mixture of several of the 13 possible linear and cyclic mono- di- and tri-phosphate esters and phosphoramidates of **1**, protection of the 2-amino-group was necessary (Scheme 1, path 2) [23].



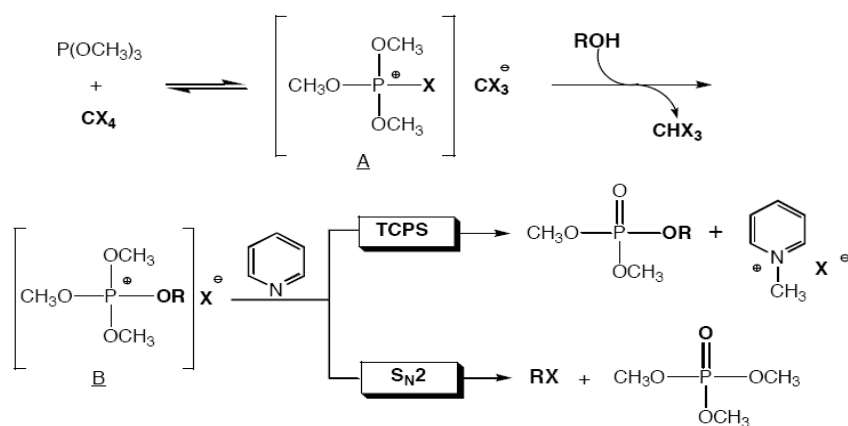
**Scheme 1.** Enzymatic and laboratory synthesis of *D-erythro*-sphingosine 1-phosphate (S1P) and *D-erythro*-ceramide 1-phosphate (Cer1P).

To date, S1P, Cer1P and their analogs have been the subject of a few synthetic approaches; but, so far a practical method for their preparation has not been elaborated [8,24–28]. Recently, we and others found that a three-component phosphorylation system (TCPS, Scheme 1, path 2), comprised of trimethyl phosphite, carbon tetrabromide and pyridine was a satisfactory method for regioselective incorporation of the phosphoryl group into the sphingolipid system only when **3** was used as a substrate [23,29,30]. However, our further investigations in this area revealed that this

version of the TCPS protocol had some limitations, which preclude its wider application as a practical method for a large scale preparation of N-BOC-protected sphingosine 1-phosphate ester (**4**) and its close and distant SPL analogs.

First, the yield of the reaction was extremely sensitive to traces of moisture and the remains of other solvents in the lipid samples or in pyridine (*vide infra*, Table 1). Secondly, there was a low yield of the phosphorylation reaction with other less reactive sphingolipid substrates, *i.e.*, ceramides [23]. Finally, optimization and scaling up of this protocol to prepare **4** or **5** on a large scale was not economically feasible, due to the formation of unwanted 1,3-O-diphosphate ester side-byproducts in significant quantities. Additionally, the process was inconvenient and hazardous because of pyridine and problems associated with its disposal.

The analysis of the mechanism postulated by Oza and Corcoran for phosphorylation of alcohols with P(III) reagents performed in the presence of halogenoalkanes and bases as well as other mechanistic studies of X-philic reactions implied that three coherent factors might eliminate the described above shortages and limitations (Scheme 2) [31–34]. First, faster formation of the intermediate phosphonium salt **A** and its successive transformation to the mixed ester derivative **B** following an efficient removal of the halogen counter ion from the proximity of the polar head of the sphingolipid should significantly improve its effectiveness toward the exclusive introduction of the phosphate ester group on the primary hydroxyl group. Second, the use of an inert solvent, not forming donor/acceptor hydrogen bonds with the lipid, may increase the rate of exchange of the alcohol proton on the phosphonium salt. Third, a strong inorganic base may work more efficiently than a nitrogen-based Lewis base to deprotonate primary alcohol and mixed-ester phosphonium salt **B**. *This goal can be achieved if (i) the used halogenoalkane is more reactive than CBr<sub>4</sub>, (ii) the reaction is performed under a liquid-liquid two-phase catalyzed conditions and, (iii) an efficient phase-transfer (PT) catalyst, capable of sufficiently disperse sphingolipid at the liquid-liquid interface, is found.*



**Scheme 2.** Postulated mechanism for the phosphorylation reaction of alcohol with trimethyl phosphite and carbon tetrahalide in pyridine solution (see ref. 31 and 32).

Currently, phase-transfer catalysis (PTC) is a well-established synthetic method benefiting reactions by enhancing their rate [35]. The advantages of using the PTC technique are mild reaction conditions, its operational simplicity giving high selectivity toward the required product, high conversion of the reactants and the use of inexpensive and environmentally acceptable reagents [36–39]. To date, PTC has been successfully applied to phosphorylate phenolates and a short chain alkyl and aromatic alcohols as well as amines under the Atherton-Todd reaction conditions [40,41].

In this manuscript, we present a successful application of the PTC method for the regioselective phosphorylation of sphingolipids.

## RESULTS AND DISCUSSION

In order to find the proper halogenoalkane/solvent system for the PTC version of the TCPS protocol, we reinvestigated phosphorylation of model sphingolipids **1** and **3**, applying a combination of a varied key reaction components under homogeneous conditions (Table 1; Scheme 3, paths 1 and 2).

Surveying various polyhalogenoalkanes and using 4-(dimethylamino)pyridine (4-DMAP) [42] or imidazole (IMD) [43,44] as bases in lieu of pyridine [31], we found superior halogen donor properties of 1,2-dibromotetrachloroethane (DBTCE) [32] when the phosphorylation reaction was performed in dichloromethane (DCM) solution (Table 1).

In contrast to other applied polyhalogenoalkanes, DBTCE gave a high yield of **4** if used in halogenated solvents for a shorter time without formation of unwanted **6** (entries 8–13). This strongly enhanced reactivity of DBTCE can apparently be attributed to the facilitated formation of the by-product pair:  $\text{CCl}_2=\text{CCl}_2/\text{Br}^-$  from hexahalogenoethane, compared to the less efficient leaving group  $\text{CX}_3^-$  generated from tetrahalogenomethane [45].

Moreover, DBTCE in comparison to  $\text{CBr}_4$  gave also better results in pyridine, particularly when 1-O-monophosphorylated product **4** was sought (80% yield; entry 5), but not in the synthesis of its diphosphorylated analog **6** (28% yield, entry 6). Interestingly, DBTCE worked also effectively for the unprotected amino alcohol **1** when IMD was used in lieu of 4-DMAP, because 1-O-regioisomer **7** was formed as a sole product in 38% (Table 1; entry 15, Scheme 3).

We were delighted to find that phosphorylation of model sphingolipids **1–3** and **8** proceeded with DBTCE as well under a liquid-liquid two-phase catalyzed conditions (Table 2; Scheme 2, paths 3 and 4). Thus, the desired 1-O-phosphoester **4** was isolated in 70% yield within 30 min, when trimethyl phosphite was added to aqueous NaOH/DCM mixture of **3**, DBTCE and 20 mol % of tetrabutylammonium iodide (entry 2).

**Table 1.** Survey of various reactants and the influence of the reaction parameters on the formation of mixed phosphate esters under TCPS-1 conditions<sup>a</sup>.

Entry	SPL	P(OCH <sub>3</sub> ) <sub>3</sub> equiv	Solvent	Oxidant (equiv)	Base (equiv)	Time (h)	Temp (°C)	Yield (%) <sup>b</sup> of			
								4	6	6	7
1	3	1.4	pyridine	CBr <sub>4</sub> (1.2)	pyridine	2	4-22	72	6	–	–
2	3	4	pyridine	CBr <sub>4</sub> (1.2)	pyridine	4	4-22	8	75	–	–
3	3	1.4	pyr.-water 20:1, v/v	CBr <sub>4</sub> (1.2)	pyridine	2.5	4-22	5	–	–	–
4	3	1.4	pyr.-DCM 20:1, v/v	CBr <sub>4</sub> (1.2)	pyridine	2.5	4-22	10	–	–	–
5	3	1.6	pyridine	DBTCE (2)	pyridine	2.5	4-22	80	3	–	–
6	3	4	pyridine	DBTCE (2)	pyridine	4	4-22	45	28	–	–
7	3	1.5	DCM	C <sub>2</sub> Cl <sub>2</sub> Br <sub>2</sub>	4-DMAP (4)	2.5	4-22	–	NR	–	–
8	3	1.5	DCM	CBr <sub>4</sub> (1.2)	4-DMAP (4)	4	4-22	12	~2	–	–
9	3	1.5	DCM	DBTCE (2)	4-DMAP (4)	4	4-22	55	–	–	–
10	3	1.5	DCM	DBTCE (2)	4-DMAP (4)	2.5	15-22	70	–	–	–
11	3	1.5	CHCl <sub>3</sub>	DBTCE (2)	4-DMAP (4)	2.5	15-22	62	–	–	–
12	3	1.5	CCl <sub>2</sub> =CCl <sub>2</sub>	DBTCE (2)	4-DMAP (4)	4	15-22	32	–	–	–
13	3	1.5	toluene	DBTCE (2)	4-DMAP (4)	2.5	15-22	5	–	–	–
14	1	1.5	DCM	DBTCE (2)	4-DMAP (4)	0.5	15-22	–	CRM	–	–
15	1	2	DCM	DBTCE (2)	IMD (4)	0.5	15-22	–	–	–	38

<sup>a</sup>Reaction conditions: (i) for the entries 1 and 3–5 see the reported procedure in ref. 30; for the entries 2 and 6 see the Standard Procedure B in the Experimental Part, (ii) for the entries 8–13 oxidant and 4-DMAP were added to a stirred mixture of 3 and P(OCH<sub>3</sub>)<sub>3</sub>, (iii) for the entries 14 and 15 see the Standard Procedure C. Experiments performed on 0.25 mmol scale, except as indicated in the text or listed in the Experimental Part.

<sup>b</sup>Isolated yields. Composition of the reaction mixtures and the purity of products was confirmed by TLC, <sup>31</sup>P- and <sup>1</sup>H-NMR. Abbreviations: NR, no reaction; CRM, complex reaction mixture.

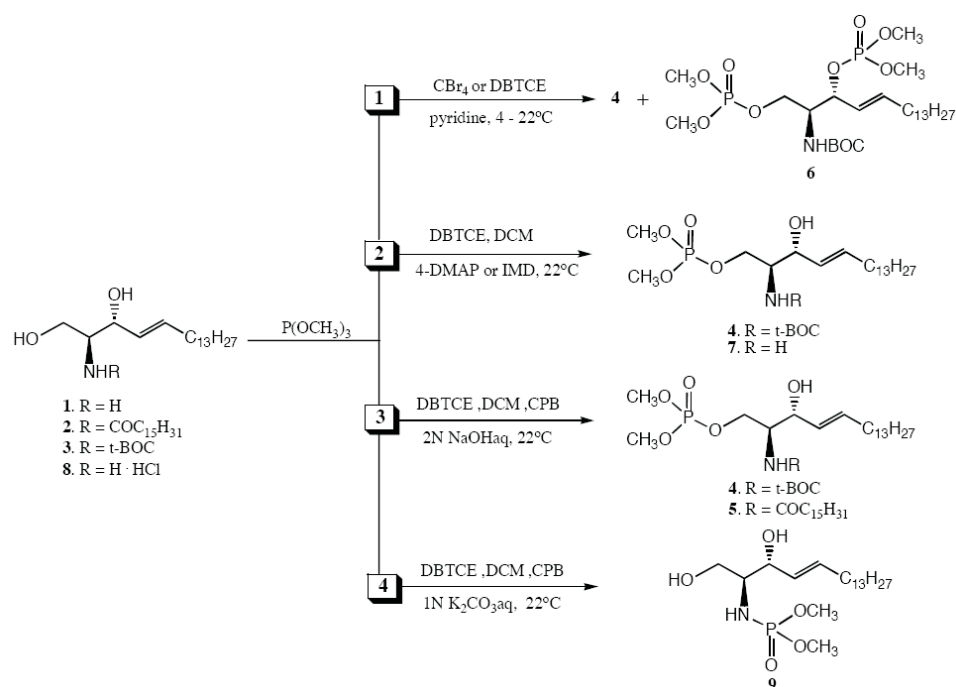
Survey of various PT-catalysts and reaction conditions revealed that combination of 20 mol % cetylpyridinium bromide (CPB) and 2N aqueous NaOH represented the most effective set of the catalyst/base co-reactants. Under these conditions, and when the base was added to the reaction mixture as a final reactant, phosphorylation of **3** proceeded with an excellent yield of 92% (entry 12).

**Table 2.** Survey of various reactants and the influence of the reaction parameters on yield of phosphate esters **4**, **5** and phosphoramidate **9** formation under PTC conditions<sup>a</sup>.

Entry	SPL	P(OCH <sub>3</sub> ) <sub>3</sub> equiv	Catalyst (20 % mol)	Base	Time (min)	Temp (°C)	Yield (%) <sup>b</sup> (product)
1	<b>3</b>	4	TBAI	5% NaOH	30	22	35 ( <b>4</b> )
2	<b>3</b>	4	TBAI	10% NaOH	30	22	70 ( <b>4</b> )
3	<b>3</b>	4	– <sup>c</sup>	10% NaOH	30	22	NR
4	<b>3</b>	4	TBAI	20% NaOH	30	22	60 ( <b>4</b> )
5	<b>3</b>	4	TBAI	2N NaOH	30	22	72 ( <b>4</b> )
6	<b>3</b>	4	TBAF	2N NaOH	30	22	59 ( <b>4</b> )
7	<b>3</b>	4	TBAC	2N NaOH	30	22	45 ( <b>4</b> )
8	<b>3</b>	4	TBAB	2N NaOH	30	22	75 ( <b>4</b> )
9	<b>3</b>	4	TEBAC	2N NaOH	30	22	55 ( <b>4</b> )
10	<b>3</b>	6	CTMAB	2N NaOH	30	22	80 ( <b>4</b> )
11	<b>3</b>	6	CPC	2N NaOH	30	22	60 ( <b>4</b> )
12	<b>3</b>	6	CPB	2N NaOH	20	22	92 ( <b>4</b> )
13	<b>3</b>	6	CPB	2N NaOH	30	4–10	65 ( <b>4</b> )
14	<b>3</b>	6	CPB	2N NaOH	30	15–18	82 ( <b>4</b> )
15	<b>4</b>	4	CPB	2N NaOH	30	22	NR
16	<b>1</b>	6	CPB	1N K <sub>2</sub> CO <sub>3</sub>	20	22	72 ( <b>9</b> )
17	<b>8</b>	6	CPB	1N K <sub>2</sub> CO <sub>3</sub>	20	22	80 ( <b>9</b> )
18	<b>2</b>	6	CPB	2N NaOH	20	22	72 ( <b>5</b> )

<sup>a</sup>Isolated yields. <sup>b</sup>Experiments were performed according to the Standard Procedure A, except entries 1–3 and 16, where P(OCH<sub>3</sub>)<sub>3</sub> was added to the reaction mixtures as the last reactant. Composition of the reaction mixtures and the purity of products were confirmed by TLC, <sup>31</sup>P- and <sup>1</sup>H-NMR. <sup>c</sup>No PT-catalyst. Abbreviations: NR, no reaction; TEAB: tetrabutylammonium bromide; TBAF: tetrabutylammonium fluoride; TBAC: tetrabutylammonium chloride; TBAI: tetrabutylammonium iodide; TEBAC: tetraethylbenzylammonium chloride; CTMAB: cetyltrimethylammonium chloride; CPC: cetylpyridinium chloride; CPB: cetylpyridinium bromide.

Usage of a stronger base or decreasing the temperature did not improve the conversion rate of **3** to **4**. This result can be related to a possible side-by reactions because of the halogenation at the C1 position and a partial hydrolysis of the formed phosphate methyl ester [46]. Satisfactory result was also obtained with ceramide **2**. Its PTC phosphorylation provided the desired 1-O-phosphate methyl ester **5** in 72% yield (Table 2, entry 18; Scheme 3, path 3).



**Scheme 3.** Phosphorylation of sphingolipids with trimethyl phosphite performed under varied reaction conditions.

Finally, PTC phosphorylation of unprotected sphingosine **1**, or its hydrochloride salt **8**, resulted in phosphoramidate **9** in 72% or 80% yield, respectively (Table 2, entries 16 and 17; Scheme 2, path 4). This was confirmed by the presence of the resonance signal at  $-6.5$  ppm in its  $^{31}\text{P}$  NMR spectrum, indicating that phosphorous atom is located in the phosphoramidate group [47]. Previously synthesized esters, *i.e.*, regioisomer **7** and N-protected analogs **4**, **5** or **6**, showed  $^{31}\text{P}$  resonance signals at  $-18.1$ ,  $-16.4$ ,  $-17.8$  or  $-16.2$  and  $17.2$  ppm, respectively [23,48].

The observed complete O- or N-selectivity in the phosphorylation reactions of the amino alcohol **1** is not totally surprising. These peculiar differences agree with the reported trend that Lewis bases activate phosphitylation reagents for their O-selective action under anhydrous conditions, while strong bases activate them under aqueous conditions to attack first the more nucleophilic center to yield an N-phosphorylated product [42,48–51].

Moreover, we identified two new factors, which govern the efficiency of the PT-catalyzed phosphorylation of sphingolipids: size and type of the N-based quaternary salt. Better PT-catalysts are surfactants having: (i) a one long hydrocarbon chain and a compact small cationic heads, *i.e.*, C16-pyridinium > C16-trimethylammonium > triethylbenzylammonium > tetrabutylammonium and, (ii) bromide as a counter ion. These observations are in accordance with the reported trend that larger lipophilic

quaternary ammonium salts are more effective catalysts than their short-chain homologues when oxidation of hydrophobic ligands is performed by PTC under aqueous conditions [52].

## CONCLUSIONS

In summary, we have uncovered a novel method for N- and O-regioselective phosphorylation of sphingolipids using phase-transfer catalysis. The described protocol, called TCPS-PTC-2, is convenient, rapid, mild, clean, and delivers dimethyl phosphate monoesters or phosphoryl amides of sphingolipids with high yields. Simplified versions of the TCPS protocol called TCPS-1, *i.e.*, performed under homogenous conditions, can be used to prepare other mono- or polyphosphorylated sphingolipid derivatives.

In our laboratory studies and experiments are ongoing to investigate in detail the mechanism of these reactions and the scope and the limitations of the developed TCPS protocols in regard to the remaining stereoisomers of **1**, **2** or **3** and congeners.

We believe that the developed PTC method, comprised of  $P(OCH_3)_3$ , DBTCE, 2N  $NaOH_{aq}$ /DCM and lipophilic pyridinium salt, may serve as a laboratory benchmark of modular process of lipid phosphorylation and find wide application.

## EXPERIMENTAL

**General:** All solvents and general reagents were purchased from Aldrich and used without prior purification. *D-erythro*-Sphingolipids were prepared from L-serine as described previously [53–55]. Analytical thin layer chromatography (TLC) was performed using EMD Reagent 0.25 mm silica 60-F<sub>254</sub> plates. Flash chromatography was performed using EM Silica Gel 60 (230–400 mesh) with the indicated eluent system. Melting points were determined in open capillaries on an electrothermal IA 9200 melting point apparatus and are reported uncorrected. Optical rotation data were acquired using a Jasco P-1010 polarimeter. <sup>1</sup>H-, <sup>13</sup>C- and <sup>31</sup>P-NMR spectra were recorded using a Bruker AVANCE 500 MHz spectrometer equipped with Oxford Narrow Bore Magnet. Chemical shifts are given in ppm on the  $\delta$  scale from an internal standard of residual chloroform ( $\delta = 7.26$ ); <sup>31</sup>P, 10% aqueous solution of methylenediphosphonic acid ( $\delta = 0$ ). Mass spectral data were recorded with a positive electrospray ionization (ESI) mode on Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer. Samples were infused in methanol solution with an ESI voltage of 4.5 kV and capillary temperature of 200°C [56].

### Standard Procedure A (TCPS-PTC-2)

**D-erythro-2N-t-Butoxycarbonyl-sphingosine 1-phosphate dimethyl ester (4).** 2N  $NaOH_{aq}$  (24 ml) was added dropwise over 1 min at 22°C to a vigorously stirred mixture of *D-erythro*-N-t-butoxycarbonyl-sphingosine (**3**, 400 mg, 1.0 mmol), 1,2-dibromotetrachloroethane (97%, 652 mg, 2.0 mmol), cetylpyridinium bromide monohydrate (98%, 82 mg, 0.20 mmol) and trimethyl phosphite (99.999%, 0.783 ml, 6.0 mmol) in DCM (40 mL). The reaction mixture was stirred for an additional 20 min. After being diluted with DCM (20 ml), the mixture was washed with water and brine. The organic layer was dried over anhydrous  $MgSO_4$  and filtered. The solvent was removed under reduced pressure and the residue was purified by flash chromatography (ethyl acetate/hexane, 4:1 v/v) to give pure **4** as a pale yellow oil (467 mg, 92%). This material solidified when refrigerated (+4°C) overnight. Analytical sample of **4** was obtained by crystallization from cold anhydrous acetone-hexane (1:4, v/v, -5°C) as a white powder, m.p. 38–39°C [lit. [23] 36–37°C];  $[\alpha]_D^{21} = +4.1$  [lit. [23]  $[\alpha]_D^{24} = +4.3$ ],  $[\alpha]_{365}^{21} = +6.8$  ( $c = 1.0$ ,  $CHCl_3$ ). Remaining data are identical to the literature [23].

**D-erythro-C16-Ceramide 1-phosphate dimethyl ester (5).** This compound was prepared from D-erythro-C16-ceramide (**2**, 538 mg, 1.0 mmol) according to the standard procedure A. The reaction mixture was stirred for 20 min, and the desired phosphate ester **5** was obtained after flash chromatography (CHCl<sub>3</sub>/MeOH/conc. NH<sub>4</sub>OH, 12:1:0.1, v/v/v) as a white solid (465 mg, 72%). Analytical sample of **5** was obtained by crystallization from *n*-hexane-ethyl acetate (4:1, v/v; white powder); m.p. 63–65°C [lit. [23] 61–62°C]; [α]<sub>D</sub><sup>22</sup> = –3.5, [α]<sub>365</sub><sup>22</sup> = –14.3 (c = 1.0, CHCl<sub>3</sub>). Remaining data are identical to the literature [23].

**D-erythro-Sphingosine 2-N-phosphoryl dimethyl ester (9).** This compound was prepared from D-erythro-sphingosine hydrochloride (**5**, 336 mg, 1.0 mmol) using 1N K<sub>2</sub>CO<sub>3(aq)</sub> (20 ml) according to the standard procedure A. The reaction mixture was stirred for 20 min, and the desired phosphoramidate **9** was obtained after flash chromatography (CHCl<sub>3</sub>/MeOH/conc. NH<sub>4</sub>OH, 25:4:0.1, v/v/v) as a white solid (325 mg, 80%). Analytical sample of **7** was obtained by crystallization from *n*-hexane-ethyl acetate (3:1, v/v; white microcrystalline powder). TLC *R*<sub>f</sub> = 0.41 (CHCl<sub>3</sub>/MeOH, 10:1, v/v); m.p. 61–62°C; [α]<sub>D</sub><sup>22</sup> = –1.5, [α]<sub>365</sub><sup>22</sup> = –5.5 (c = 1.0, CHCl<sub>3</sub>). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 5.77 (dtd, 15.4, 6.2, 1.2 Hz, 5-H), 5.49 (ddt, 15.4, 6.8, 1.1 Hz, 4-H), 4.28 (t, 4.9 Hz, 3-H), 3.85 (dd, 11.5, 4.2 Hz, 1-Ha), 3.75 (d, 2.2 Hz, OCH<sub>3</sub>), 3.73 (d, 2.2 Hz, OCH<sub>3</sub>), 3.67 (dd, 11.5, 4.2 Hz, 1-Hb), 3.55 (t, 10.1 Hz, NH), 3.15 (m, 2-H), 2.05 (q, 7.0 Hz, C(6)H<sub>2</sub>), 1.35 (m, C(7)H<sub>2</sub>), 1.24 (m, 20H), 0.86 (t, 7.0 Hz, CH<sub>3</sub>); <sup>31</sup>P NMR (CDCl<sub>3</sub>, CH<sub>2</sub>[P(O)(OH)<sub>2</sub>]<sub>2</sub>) δ –6.5; ESI MS (MeOH) *m/z* 837.0 ([2M + Na]<sup>+</sup>, 100), 814.8 (2M<sup>+</sup>, 97), 738 (15), 631 (15), 408.0 (MH<sup>+</sup>, 27), 389.9 ([MH–H<sub>2</sub>O]<sup>+</sup>, 10); Calcd. for C<sub>20</sub>H<sub>42</sub>NO<sub>5</sub>P *m/z* 407.3.

#### Standard Procedure B (TCPS-1)

**D-erythro-2-N-t-Butoxycarbonyl-sphingosine 1,3-diphosphate dimethyl ester (6).** Trimethyl phosphite (99.999%, 0.522 ml, 4.0 mmol) was added dropwise over 2 min to a well-stirred and cooled to +4°C solution of D-erythro-N-t-butoxycarbonyl-sphingosine (400 mg, 1.0 mmol) and carbon tetrabromide (99%, 400 mg, 1.2 mmol) in dry pyridine (99.8%, 4.0 ml). After the addition was completed, the cooling bath was removed, and the reaction mixture was stirred at room temperature for 4 h. The mixture was diluted with ethyl acetate (25 ml), washed with an ice-cold 1N HCl (51 ml) and stirred for 5 min at room temperature. The mixture was transferred into a separatory funnel and the organic phase was separated, washed with water, and saturated aqueous NaHCO<sub>3</sub> (2×10 ml) and brine (2×10 ml). Each of the separated aqueous layers was collected in a separate flask and individually extracted with ethyl acetate (2×5 ml). The combined organic extracts were dried over anhydrous MgSO<sub>4</sub>, filtered, and concentrated under reduced pressure yielding a crude product. This material was purified by a three-step flash column chromatography applying three different solvent systems. Elution of the first column with pure ethyl acetate provided monophosphate ester **4** (41.0 mg, 8% yield). Further elution of this column with ethyl acetate-ethanol solvent system (95:5, v/v) gave fractions containing impure diphosphate ester **6**. This material was re-purified on a second column using chloroform-methanol (14:1, v/v) to give pure **6** as a colorless oil (480 mg, 78%). This material solidified when refrigerated (+4°C) overnight resulting in a white solid. TLC *R*<sub>f</sub> = 0.12 (ethyl acetate), *R*<sub>f</sub> = 0.77 (CHCl<sub>3</sub>/MeOH, 10:1, v/v); m.p. 29–31°C; [α]<sub>D</sub><sup>23</sup> = –9.9, [α]<sub>365</sub><sup>23</sup> = –32.2 (c = 1.0, CHCl<sub>3</sub>). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 5.83 (dtd, 15.5, 6.4, 1.3 Hz, 5-H), 5.44 (ddt, 15.5, 6.4, 1.2 Hz, 4-H), 5.11 (d, 6.4 Hz, NH), 4.78 (q, 7.0 Hz, 3-H), 4.20 (m, 1H, 1-Ha), 4.14 (m, 1H, 2-H), 3.79 (d, 2.0 Hz, 3H, 1-O-P(O)(OCH<sub>3</sub>)), 3.77 (d, 2.0 Hz, 3H, 1-O-P(O)(OCH<sub>3</sub>)), 3.75 (d, 11.1 Hz, 3H, 3-O-P(O)(OCH<sub>3</sub>)), 3.70 (d, 11.1 Hz, 3H, 3-O-P(O)(OCH<sub>3</sub>)), 3.67 (dd, 11.5, 4.2 Hz, 1-Hb), 2.05 (q, 7.0 Hz, 2H, C(6)H<sub>2</sub>), 1.41 (s, 9H, t-BOC), 1.35 (m, 2H, C(7)H<sub>2</sub>), 1.24 (m, 20H), 0.86 (t, 7.1 Hz, CH<sub>3</sub>); <sup>13</sup>C NMR δ 138.5 (C-4), 124.8 (C-5), 80.0 (C-(CH<sub>3</sub>)<sub>3</sub>), 79.1 (C-3), 66.1 and 66.0 (C-1 and C-2), 54.7, 54.5 and 54.1 (m, 4 x OCH<sub>3</sub>), 32.5 (C-6), 32.1 (C-7), 29.84 (m), 29.8, 29.7, 29.5, 29.4, 29.0, 28.5 (3 x C-(CH<sub>3</sub>)<sub>3</sub>), 22.9, 14.3 (–CH<sub>2</sub>CH<sub>3</sub>); <sup>31</sup>P NMR (CDCl<sub>3</sub>, CH<sub>2</sub>[P(O)(OH)<sub>2</sub>]<sub>2</sub>) δ –16.2 and –17.2; ESI MS (MeOH) *m/z* 1252.8 ([2M + Na]<sup>+</sup>, 45), 638.1 ([M + Na]<sup>+</sup>, 100), 433.9 (25); Calcd. for C<sub>27</sub>H<sub>55</sub>NO<sub>10</sub>P<sub>2</sub> *m/z* 615.3.

#### Standard Procedure C (TCPS-1)

**D-erythro-Sphingosine 1-phosphate dimethyl ester (7).** Trimethyl phosphite (99.999%, 0.075 ml, 0.6 mmol) was added dropwise over 2 min to a well-stirred and cooled to +15°C mixture of D-erythro-sphingosine (**1**, 90 mg, 0.3 mmol), 1,2-dibromotetrachloroethane (97%, 200 mg, 0.6 mmol), imidazole (82 mg, 1.2 mmol) and DCM (7.0 ml). After the addition of the phosphorylation reagent was completed, the cooling bath was removed, and the reaction mixture was stirred at room temperature for an additional 30 min. The mixture was diluted with DCM (10 ml), washed with water, brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and filtered. The solvent was removed under reduced pressure and the residue was purified by

flash chromatography (CHCl<sub>3</sub>/MeOH/conc. NH<sub>4</sub>OH, 25:4:0.1, v/v/v) to give a pure free base of **7** as a pale yellow oil (47 mg, 38%). This material was transformed to hydrochloride salt by dissolving in an ice-cold ethyl acetate following the addition of 1M HCl solution in diethyl ether at room temperature. Analytical sample of **7**·HCl was obtained by crystallization from anhydrous cold ethyl acetate-hexane (3:1, v/v, -5°C) as a white powder (40 mg, overall 30% yield from **1**). TLC *R<sub>f</sub>* 0.34 (CHCl<sub>3</sub>/CH<sub>3</sub>OH/[(CH<sub>3</sub>)<sub>2</sub>CH]<sub>2</sub>NC<sub>2</sub>H<sub>5</sub>, 10:1:0.01, v/v); m.p. 84–85°C (>90°C decomp. [lit. [23] 83–84°C, decomp. > 88°C]). Remaining data are identical to the literature [23].

#### Acknowledgments

Financial support was provided by the DHHS/NIH/NCI – 1P01CA97132, NIH/NCRR 5P20RR017677 and DHHS/NIH/NIGMS – 2R01GM062887. We thank Hanna Gracz, Ph.D., (NMR Facility, NCSU, Raleigh, NC) for the NMR spectra, Jacek Bielawski, Ph.D., (Lipidomics Core, MUSC, Charleston, SC) for the MS spectra and Jennie Ariail, Ph.D., (CAE, MUSC) for editorial assistance.

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