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A Modular Synthesis of Alkynyl-Phosphocholine Headgroups for Labeling Sphingomyelin and Phosphatidylcholine

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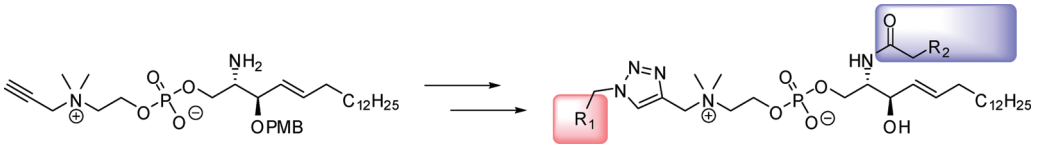

A general route to phospho- and sphingolipids that incorporate an alkyne in the phosphocholine headgroup is described. The strategy preserves the ammonium functionality of the phosphocholine and can be easily modified to introduce desired functional groups at the *N*-acyl chain. The targets accessible with this strategy provide a bioorthogonal handle for postsynthetic introduction of fluorophores or other labeling agents with aqueous phase chemistry. We report the synthesis of sphingomyelin derivatives that incorporate a fluorophore and an alkyne. The modified sphingolipids retain activity as substrates for sphingomyelinase, making these compounds viable probes of enzymatic activity. Importantly, the strategy allows modification of the lipid across the phosphodiester, making the alkyne a potential probe of sphingomyelinase activity.

Introduction

Sphingomyelin is a critical component of the plasma membrane bilayer in mammalian cells. Importantly, the molecule is a substrate for several enzymes, known as sphingomyelinases (SMase), such as the acidic and neutral sphingomyelinases.[1−3] These enzymes convert sphingomyelin to the signaling molecule ceramide upon cleavage of the phosphodiester.[3,4] Synthesis of various sphingomyelin analogues has been reported previously in the literature.[5,6] The subcellular activity of acidic sphingomyelinases (ASM) is poorly understood, and new fluorescent derivatives that can be used to examine the location and rate of enzyme activity may help elucidate their function in live cells.[7] Therefore, synthetic strategies to introduce bioorthogonal labels into these molecules are needed.[8]

Although stereoselective routes to the core of the lipid are known,[9,10] modifications to naturally isolated lipids have been more frequently applied in labeling strategies. Existing strategies capable of generating fluorescently modified sphingomyelin include modification of the aminoacyl chain[11,12] and cross-metathesis.[13,14] Recently, Lampkins et al. have demonstrated the applicability of labeling in diacylglycerol lipids by installing an azide handle at the acyl chain of the lipid, followed by attachment of alkynyl fluorophores.[15] To the

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best of our knowledge, the only example that uses a biocompatible label to modify a lipid headgroup is the introduction of an azide at the *sn*-1 position of diacylglycerol; however, this method has not yet been adapted to phosphocholyl lipids or sphingolipids.[16] Therefore, there are no existing strategies that allow for labeling of phosphocholyl lipids under aqueous conditions or in the presence of other biomolecules. We propose that strategies which introduce a chemical handle at the cholyl headgroup could overcome this limitation and will be of great utility in biological systems where the cleavage of this group is of interest.

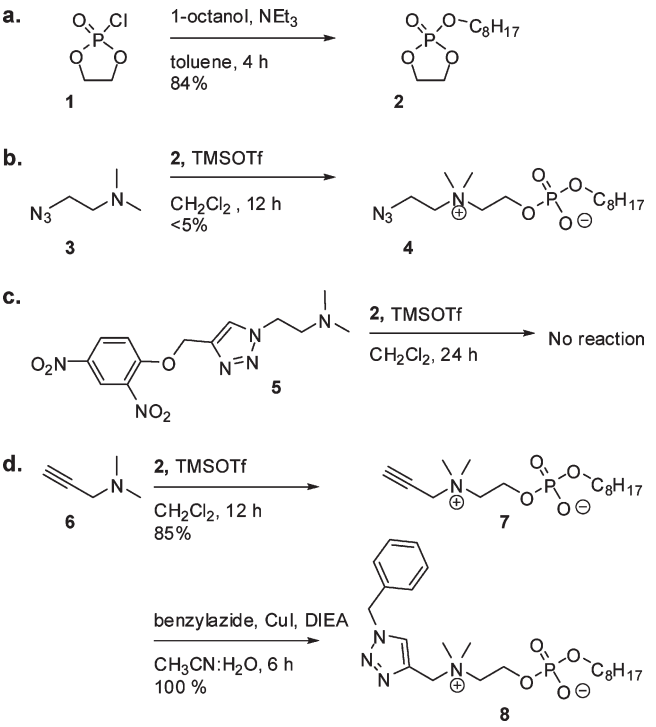
General methods for the modification of the sphingolipid headgroup are relatively sparse. Sphingomyelin analogues that contain modified headgroups have been produced as secondary amines,[17] and related derivatives are commercially available. At least one strategy has been able to generate a modification of the quaternary ammonium group found in the native ligand.[18] We chose to pursue a general method for producing sphingomyelin analogues that contain a label in the phosphocholine headgroup while preserving the ammonium group of the native compound.

Since the phosphocholine group found in sphingomyelin is also a component of phosphatidylcholine choline lipids, strategies for generating analogues of this headgroup should prove instructive. Previous methods to modify the cholyl group have relied on phosphoramidate chemistry.[19] Alkylammonium labels have also been introduced to phosphatidylcholyl analogues using enzyme-catalyzed methods.[20] We sought to develop an alternative modular strategy for generating a modified trialkylammonium phosphocholine group amenable to postsynthetic modification using fluorophores or labeling reagents. A critical goal of our strategy is to develop compounds which exploit bioorthogonal reactions compatible with aqueous phase chemistry.[8] We have developed a phosphorylchloride strategy that provides access to trialkylammonium sphingolipids incorporating alkyne functionality at the lipid headgroup. This strategy allows for reaction of the headgroup alkyne with an azide via a click reaction. The strategy is general and has been applied to both sphingolipids and phospholipids, and we demonstrate routes to the corresponding sphingomyelin and phosphatidylcholine choline analogues. A fluorophore group was introduced into a sphingomyelin analogue to demonstrate the feasibility of the approach. We also confirm that these modified derivatives retain substrate activity for a sphingomyelinase enzyme.

Results and Discussion

To keep perturbations of the native structure to a minimum, we considered small functional groups that could be used to introduce a desired label. We considered alkyne−azide coupling using copper-catalyzed Huisgen 1,3-dipolar addition to a modified alkylammonium group of a phospho- or sphingolipid substrate (Scheme 1).[21] We envisioned these derivatives could be made through a modification of known methods that exploit cyclic chlorophosphate (2-chloro-2-oxo-1,2,3-dioxaphospholane) **1** via phosphorylation and subsequent nucleophilic opening of the cyclic phosphate triester intermediate **2**.[22−25] Our initial attempts to generate an azidophosphocholine were impeded by the instability of the *N*,*N*-dimethylazidoethylamine nucleophile, **3**. Elevated temperature and activation with TMSOTf only provided minor quantities of the desired product, **4**. This observation is perhaps unsurprising considering that **3** can be used as a hypergolic fuel.[26] The purification of the minor product was difficult because of high concentrations of triflate salt impurities. We attempted to avoid this issue by first converting the azidoamine to the corresponding triazole **5** (see the Supporting Information), followed by subsequent amination. Unfortunately, no product was observed from this reaction, probably due to the increased steric bulk of nucleophile **5**. Therefore we changed our strategy by introducing the alkyne handle instead of azide for use as a click reaction substrate (Scheme 1d).

SCHEME 1. Synthesis of Model Alkyne Ammonium Analogues



Nucleophilic opening of the phosphate triester **2** by *N*,*N*-dimethylpropargylamine, **6**, gave the desired phosphodiester, **7**, in good yield. We then confirmed that copper-catalyzed reaction with benzylazide could provide the 1,2,3-triazole derivative, **8**, in excellent yield. On the basis of this validation of our strategy, we turned our attention to the synthesis of alkyne-modified

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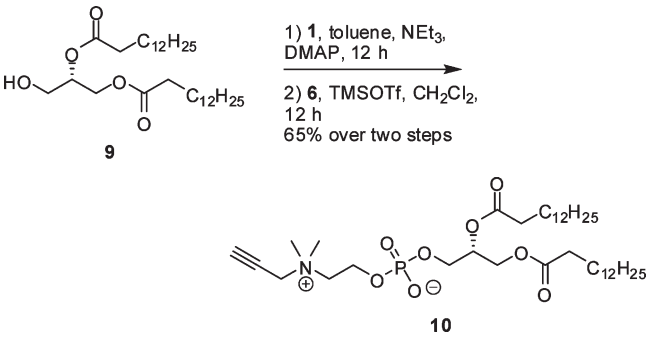
SCHEME 2. Synthesis of Alkyne-Modified Phosphatidylcholine



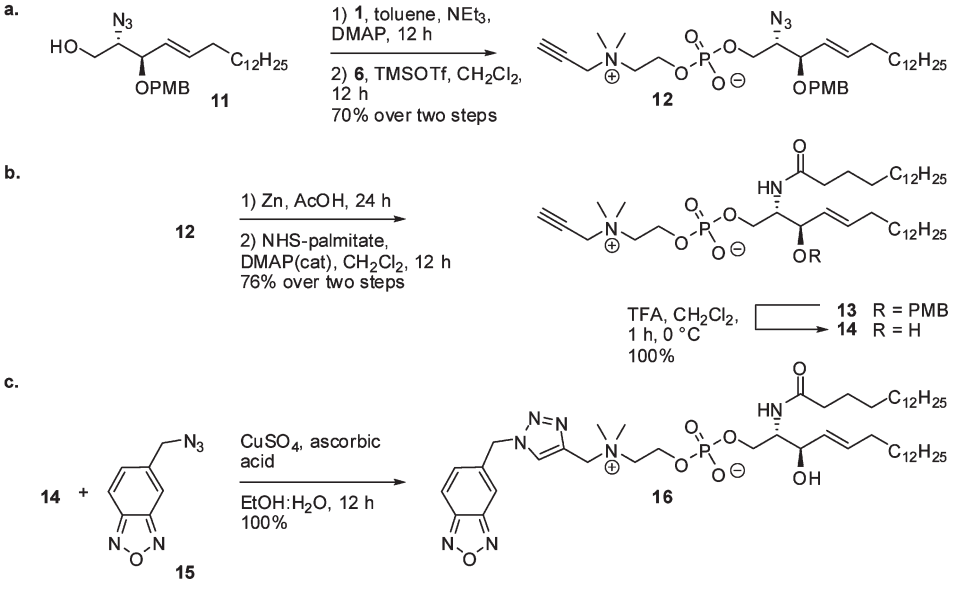
phospho- and sphingolipids. We began the synthesis of alkynyl-phosphatidylcholine, **10**, from commercially available diacylglycerol **9** (Scheme 2). Phosphorylation and amination of the alcohol as before provided the desired alkyne-modified phospholipid, **10**, in moderate yield over two steps. Although this strategy requires no protecting group manipulations, introduction of modified acyl chains in **10** would likely require protection of one of the glycerol side chains.

We proceeded to test a modification of this strategy that allows for elaboration of the acyl chain using sphingolipids. Beginning from a known protected sphingosine base, **11**,[27] we planned to first install the alkyne-modified phosphocholine. Subsequent reduction of an azide would then provide a free amine for acylation with any desired lipid chain. The protected sphingosine base, **11**, was synthesized from commercially available D-*erythro*-sphingosine, using the method of Du et al. with only minor modification (see the Supporting Information).[27] Briefly, sphingosine was converted to 2-azidosphingosine using imidazole-1-sulfonyl azide hydrochloride,[28] followed by protection of the primary hydroxyl group using trityl chloride and protection of the secondary hydroxyl as a *p*-methoxybenzyl (PMB) ether. Cleavage of the trityl ether with BF_3OEt_2 provided compound **11**. We chose to employ the PMB protecting group to avoid migration side reactions often observed in sphingosines with acyl protecting groups.[29] The protected sphingosine, **11**, was reacted under similar conditions to the diacylglycerol derivative used in Scheme 2 for both phosphorylation and amination (Scheme 3). The protected phosphodiester, **12**, was obtained in moderate yield. Intermediate **12** is a versatile starting material that can now be selectively modified at the *N*-acyl chain and the alkynylammonium group. Initial attempts to reduce the azide of **12** to an amine were not successful with hydrogen sulfide or Staudinger reduction conditions.[30] We found the best conditions for reducing the azide were zinc and acetic acid.[31,32] Subsequent acylation with an NHS-activated ester of palmitic acid provided compound **13** in good yield. The deprotection of the PMB ether with ceric ammonium nitrate was incomplete even after extended reaction times. However, we observed quantitative conversion of **13** in the presence of trifluoroacetic acid to give the desired alkynyl-sphingomyelin, **14**, in 53% yield over 5 steps from the protected sphingosine, **11**.[33]

To demonstrate the feasibility of introducing a fluorophore label using the alkyne handle, we chose to attach a benzoxadiazole fluorophore. Benzoxadiazole derivatives are commonly used for labeling of lipids.[34] Their small size and environmental sensitivity are desirable attributes for these applications.[35] Introduction of the fluorophore using a Sharpless−Meldal reaction can be performed in aqueous media with excellent yield.[36] A variety of conditions are known for this reaction, varying in the choice of copper source, reducing agent, base, ligand, and solvent.[36,37] In our hands, reaction of fluorescent benzoxadiazole azide, **15**, with alkyne **14** provided the fluorescent triazole **16** in quantitative yield with copper sulfate and ascorbic acid conditions.

With sphingomyelin derivatives **14** and **16** in hand, we proceeded to test the activity of these compounds as substrates for a sphingomyelinase enzyme. Previous reports of sphingomyelin analogues modified at the alkylammonium group have observed that these compounds remain substrates for the enzyme.[18] Several methods are known for the assay of sphingomyelinase activity, which include the use of radioactivity,[18,38] coupled enzyme assays,[39] or thin-layer chromatography (TLC).[40] Experiments using commercially available coupled assays that rely on

SCHEME 3. Alkyne- and Triazole-Modified Sphingomyelin



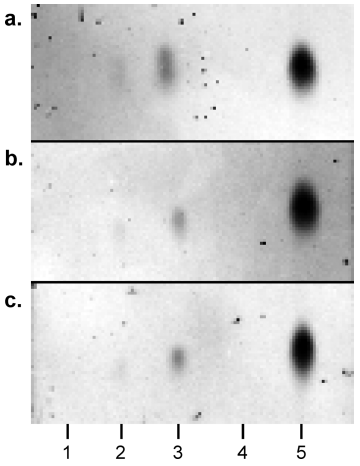


FIGURE 1. Enzymatic cleavage of sphingomyelin analogues. SMase substrates sphingomyelin (a), **14** (b), and **16** (c) were incubated for 7 or 24 h with the enzyme. The region of the TLC plate shown is at the R_f of the expected product of SMase hydrolysis, ceramide (R_f 0.33). Lanes on the plate were substrate in buffer (1); substrate, buffer, and enzyme at 7 h (2); substrate, buffer, and enzyme at 24 h (3); buffer (4); and ceramide (5). The intensities of the spots in lane 3 relative to the control (a) were 50% (b) and 70% (c), respectively.

choline oxidase activity gave inconsistent results in our hands, presumably due to the structural modifications of the choline group. We therefore employed a TLC-based assay. Cleavage of the native sphingomyelin, alkyne **14**, and triazole **16** was compared over a 7−24 h period (Figure 1). The intensity of the spots indicating product formation from compounds **14** and **16** is 0.5 and 0.7, relative to the native sphingomyelin substrate. These results strongly support that both modified sphingomyelin derivatives are substrates for the enzyme, although they appear to be less active than the native substrate. Future work will determine the kinetic parameters of these substrates and test the viability of the substrates for sphingomyelinase enzymes from other sources.

Conclusions

We describe here the development of a general route for sphingomyelin analogues that contain modifications of the *N*-acyl lipid chain and an *N*,*N*-dimethyl-*N*-propargylammonium headgroup. As shown in Scheme 3, this method can be used to introduce fluorescent groups into the lipid. Importantly, modification of the headgroup with an alkyne or triazole moiety does not prevent cleavage of the phosphodiester by sphingomyelinase. This strategy is unique in that it allows for postsynthetic modification of the lipid through the aqueous phase-exposed headgroup. Current work in our group is aimed at developing fluorogenic versions of compound **16** containing both a fluorophore and an intramolecular quencher group. These routes will exploit compound **12** as the key intermediate.

Experimental Section

(2-Azidoethyl)[2-(hydroxyoctyloxyphosphoryloxy)ethyl]dimethylammonium (4). NEt_3 (0.36 mL, 2.6 mmol) was added slowly under stirring to an ice cold solution of 2-chloro[1,3,2]dioxaphospholane 2-oxide **1** (285 mg, 2 mmol) and freshly distilled 1-octanol (260 mg, 2 mmol) in anhydrous toluene (5 mL). The reaction was stirred at room temperature for 4 h. The solution was filtered through a pad of Celite to remove the $NEt_3 \cdot HCl$ salts and the solvent was evaporated under reduced pressure. The colorless oily substance **2** was obtained. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.87 (t, J = 6.8 Hz, 3H), 1.2−1.42 (m, 10H), 1.64−1.74 (m, 2H), 4.09−4.16 (m, 2H), 4.30−4.48 (m, 4H). ^{31}P NMR (162 MHz, $CDCl_3$) δ 18.69. Compound **2** was immediately dissolved in anhydrous CH_2Cl_2 (10 mL) and TMSOTf (0.7 mL, 4 mmol) and 2-azidoethyldimethylamine (900 mg, 10 mmol) were added at 0 °C. The reaction mixture was allowed to warm to room temperature and maintained there for 12 h. The reaction mixture was diluted with $CHCl_3$ (10 mL) and neutralized with sat. $NaHCO_3$. The organic layer was dried with Na_2SO_4 and concentrated, giving a sticky solid. ^{1}H NMR (400 MHz, CD_3OD) δ 0.89 (t, J = 7.2 Hz, 3H), 1.26−1.44 (m, 10H), 1.58−1.67 (m, 2H), 3.22 (s, 6H), 3.62−3.72 (m, 4H), 3.81−3.90 (m, 2H), 3.91−4.10 (m, 2H), 4.03−4.11 (m, 2H). EIMS 351 (M^+ + H), 373 (M^+ + Na).

[2-(Hydroxyoctyloxyphosphoryloxy)ethyl]dimethylprop-2-ynylammonium (7). Compound **2** (47 mg, 0.2 mmol) was dissolved in CH_2Cl_2 (10 mL) and TMSOTf (0.07 mL, 0.4 mmol) and *N*,*N*-dimethylpropargylamine **6** (33 mg, 0.4 mmol) were added at 0 °C. The reaction mixture was allowed to warm to room temperature and maintained there for 12 h. The reaction mixture was diluted with $CHCl_3$, neutralized, and extracted with H_2O (10 mL). The organic layer was dried with Na_2SO_4 and evaporated under reduced pressure to an oil. The crude product was purified by column chromatography with Iatrobeads (CH_2Cl_2/MeOH, 4:1 then CH_2Cl_2/MeOH/H_2O, 55:45:3) to obtain **7** (54 mg, 85%) as a colorless oil. IR (KBr neat) 3380, 3310, 3255, 2926, 2855, 2121, 1653, 1468, 1235, 1072 cm^{-1}. ^{1}H NMR (400 MHz, CD_3OD) δ 0.88 (t, J = 7.2 Hz, 3H), 1.20−1.40 (m, 10H), 1.55−1.66 (m, 2H), 3.25 (s, 6H), 3.52 (t, J = 2.8 Hz, 1H), 3.69−3.74 (m, 2H), 3.85 (dt, J = 6.8, 6.4 Hz, 2H), 4.22−4.30 (m, 2H), 4.42 (d, J = 2.8 Hz, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ 14.4, 23.7, 26.9, 30.41, 30.43, 31.9 (d, J_{C-P} = 7.3 Hz), 33.0, 52.0, 56.4, 60.1 (d, J_{C-P} = 4.6 Hz), 67.0 (d, J_{C-P} = 6.1 Hz), 65.3 (d, J_{C-P} = 6.9 Hz), 72.4, 83.2. ^{31}P NMR (162 MHz, CD_3OD) δ 1.13. ESIMS calcd for $C_{15}H_{31}NO_4P$ $[M]^+$ 320.1985, found 320.1984.

(1-Benzyl-1*H*-[1,2,3]triazol-4-ylmethyl)[2-(hydroxyoctyloxyphosphoryloxy)ethyl]dimethylammonium (8). Alkyne **7** (20 mg, 0.06 mmol) and benzyl azide (9.9 mg, 0.07 mmol) were dissolved in CH_3CN:H_2O (4 mL, 1:1). CuI (2.3 mg, 0.01 mmol) and *N*,*N*-diisopropylethylamine (16 mg, 0.12 mmol) were added. The reaction mixture was stirred for 6 h at room temperature. The reaction mixture was then diluted with $CHCl_3$ (5 mL); the organic layer was separated, dried with Na_2SO_4, and concentrated under reduced pressure. Purification by column chromatography was performed with Iatrobeads (CH_2Cl_2/MeOH, 4:1 then

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CH_2Cl_2/MeOH/H_2O, 60:40:3), giving **8** (27 mg, 100%) as a colorless oil. IR (KBr neat) 3352, 2922, 2855, 1600, 1431, 1051 cm^{-1}. ^1H NMR (500 MHz, CD_3OD) δ 0.88 (t, J = 6.8 Hz, 3H), 1.22−1.40 (m, 10H), 1.55−1.63 (m, 2H), 3.17 (s, 6H), 3.54−3.60 (m, 2H), 3.82−3.90 (m, 2H), 4.24−4.34 (m, 2H), 4.73 (s, 2H), 5.66 (s, 2H), 7.35−7.40 (m, 5H), 8.35 (s, 1H). ^{13}C NMR (125 MHz, CD_3OD) δ 14.4, 23.7, 26.9, 30.40, 30.42, 31.8 (d, J_{C-P} = 6.7), 31.9, 32.9, 52.1, 55.2, 60.2, 60.4, 64.9, 67.0, 129.3, 129.7, 129.8, 130.1, 136.4, 137.1. ^{31}P NMR (202 MHz, $CDCl_3$) δ 3.7 (m). ESIMS calcd for $C_{22}H_{37}N_4O_4PNa$ $[M + Na]^+$ 475.2444, found 475.2448.

{2-[(2,3-Bis(tetradecanoyloxy)propoxy)hydroxyphosphoryloxy]-ethyl}dimethylprop-2-ynylammonium (10). A solution of freshly distilled 2-chloro-1,3,2-dioxaphospholane 2-oxide **1** (56 mg, 0.39 mmol) in dry toluene (1 mL) was added to 1,2-dimyristoyl-*sn*-glycerol **9** (68 mg, 0.13 mmol) and DMAP (catalytic) in dry toluene (5 mL). The reaction mixture was cooled to 0 °C. NEt_3 (40 mg, 0.39 mmol) was added dropwise to the reaction mixture and allowed to stir at room temperature for 12 h. TLC (hexane/EtOAc, 3:1) showed complete disappearance of starting material and formation of a new spot at R_f 0.07. The reaction mixture was filtered through a pad of Celite to remove salts and concentrated under reduced pressure. The crude compound was dissolved in dry CH_2Cl_2 (10 mL) followed by addition of 1-dimethylamino-2-propyne **6** (110 mg, 1.3 mmol) in CH_2Cl_2 (2 mL). The reaction was cooled to 0 °C and TMSOTf (117 mg, 0.52 mmol) was added dropwise over 5 min, then the reaction mixture was allowed to stir at room temperature for 12 h. TLC (CH_2Cl_2/MeOH/H_2O, 70:30:1) showed the disappearance of the starting material and formation of a new spot at R_f 0.1. Concentration in vacuo and purification by column chromatography with Iatrobeads (CH_2Cl_2/MeOH, 9:1 then CH_2Cl_2/MeOH/H_2O, 70:30:1) gave **10** (60 mg, 65% from **9**) as a colorless oil. $[\alpha]^{25}_D$ +4.12 (*c* 0.5, $CHCl_3$). IR (KBr neat) 3398, 3250, 2918, 2850, 2127, 1738, 1467, 1255, 1065 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, J = 7.0 Hz, 6H), 1.25−1.45 (m, 40H), 1.55−1.64 (m, 4H), 1.85 (br s, 4H), 2.28 (t, J = 7.5 Hz, 2H), 2.30 (t, J = 7.5 Hz, 2H), 2.86 (t, J = 2.5 Hz, 1H), 3.44 (s, 6H), 3.90 (br s, 2H), 3.91−4.03 (m, 2H), 4.15 (dd, J = 12.0, 7.0 Hz, 1H), 4.35 (br s, 2H), 4.41 (dd, J = 12.0, 3.0 Hz, 1H), 4.68 (br s, 2H), 5.20−5.26 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.1, 22.7, 24.9, 25.0, 29.21, 29.23, 29.3, 29.4, 29.5, 29.6, 29.7, 32.0, 34.1, 34.2, 34.4, 52.0, 55.3 59.1 (d, J_{C-P} = 5.4 Hz), 63.0, 63.5, 64.5, 70.6 (d, J_{C-P} = 4.3), 71.8, 81.2, 173.3, 173.6. ^{31}P NMR (202 MHz, $CDCl_3$) δ 0.77 (m). ESIMS calcd for $C_{38}H_{72}NO_8PNa$ $[M + Na]^+$ 724.4887, found 724.4884.

(2-{[2-Azido-3-(4-methoxybenzyloxy)octadec-4-enyloxy]hydroxyphosphoryloxy}ethyl)dimethylprop-2-ynylammonium (12). Compound **12** was prepared from azido sphingosine **11** (172 mg, 0.38 mmol) employing the same procedure as described for **10**. Purification by column chromatography with Iatrobeads (CH_2Cl_2/MeOH, 9:1 then CH_2Cl_2/MeOH/H_2O, 55:45:3) gave **12** (168 mg, 70% from the azido sphingosine **11**). $[\alpha]^{25}_D$ −22.78 (*c* 2.6, $CHCl_3$). IR (KBr neat) 3389, 2924, 2853, 2101, 1613, 1514, 1467, 1249, 1073 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 0.87 (t, J = 7.0 Hz, 3H), 1.20−1.35 (m, 20H), 1.34−1.44 (m, 2H), 2.08 (apparent q, J = 6.5 Hz, 2H), 2.91 (s, 1H), 3.33 (s, 6H), 3.66−3.72 (m, 1H), 3.76−3.72 (m, 6H), 4.0−4.08 (m, 2H), 4.22−4.34 (m, 3H), 4.49 (d, J = 11.5 Hz, 1H), 4.61 (br s, 2H), 5.37 (dd, J = 15.5, 7.5 Hz, 1H), 5.71 (dt, J = 15.5, 6.5 Hz, 1H), 6.84 (d, J = 8.5 Hz, 2H), 7.21 (d, J = 8.5 Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.1, 22.7, 29.1, 29.3, 29.4, 29.5, 29.7, 29.72, 31.9, 32.4, 51.4, 55.2, 55.3, 59.2 (J_{C-P} = 3.7 Hz), 63.9 (J_{C-P} = 4.5 Hz), 65.0 (J_{C-P} = 4.1 Hz), 65.4 (d, J = 7.0 Hz), 69.5, 72.2, 79.4, 81.2, 113.8, 125.8, 129.3, 130.1, 138.1, 159.1. ^{31}P NMR (201 MHz, $CDCl_3$) δ 0.54. ESIMS calcd for $C_{33}H_{55}N_4O_6P$-Na $[M + Na]^+$ 657.3751, found 657.3745.

(2-{[2-Hexadecanoylamino-3-(4-methoxybenzyloxy)octadec-4-enyloxy]hydroxyphosphoryloxy}ethyl)dimethylprop-2-ynylammonium (13). To the solution of azide **12** (40 mg, 0.06 mmol) in AcOH:H_2O/5:1 (5 mL) was added zinc powder (50 mg, 0.75 mmol) in three portions during the 24 h reaction time. After complete conversion observed by TLC (CH_2Cl_2/MeOH/H_2O, 55:45:3), a new spot was observed at R_f 0.1, then the reaction mixture was filtered through Celite and concentrated in vacuo. The remaining AcOH was removed by azeotrope with toluene (three times). The crude compound was dissolved in CH_2Cl_2 (10 mL). DMAP (catalytic) and the NHS ester of palmitic acid (33 mg, 0.09 mmol) were added and the solution was allowed to stir at room temperature for 12 h; the mixture was then concentrated in vacuo and purified by column chromatography with Iatrobeads (CH_2Cl_2/MeOH, 9:1 then CH_2Cl_2/MeOH/H_2O, 60:40:3) to give **13** (38 mg, 76% from **12**). $[\alpha]^{25}_D$ −14.52 (*c* 0.9, $CHCl_3$). IR (KBr neat) 3291, 2953, 2917, 2850, 2124, 1644, 1515, 1467, 1247, 1071 cm^{-1}. ^1H NMR (600 MHz, $CDCl_3$) δ 0.88 (t, J = 7.2 Hz, 6H), 1.20−1.42 (m, 46H), 1.48−1.60 (m, 2H), 2.0−2.16 (m, 4H), 2.80 (s, 1H), 3.28 (br s, 6H), 3.72 (br s, 2H), 3.78 (s, 3H), 3.90 (dd, J = 8.4, 7.8 Hz, 1H), 3.96−4.02 (m, 1H), 4.08−4.18 (m, 2H), 4.18−4.28 (m, 3H), 4.48 (d, J = 11.4 Hz, 1H), 4.54 (br s, 2H), 5.38 (dd, J = 15.6, 8.4 Hz, 1H), 5.67 (dt, J = 15.6, 6.6 Hz, 1H), 6.72 (d, J = 7.8 Hz, 1H, NH), 6.84 (d, J = 8.4 Hz, 2H), 7.22 (d, J = 8.4 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.3, 22.9, 26.1, 29.5, 29.6, 29.7, 29.8, 29.9, 30.0, 32.1, 32.6, 37.2, 51.8, 53.0 (d, J_{C-P} = 5.3 Hz), 55.5, 59.2 (d, J_{C-P} = 3.7 Hz), 64.3 (d, J_{C-P} = 4.2 Hz), 64.5 (d, J_{C-P} = 5.0 Hz), 70.0, 72.0, 79.9, 81.4, 113.9, 127.5, 129.4, 131.2, 137.0, 159.2, 173.2. ^{31}P NMR (162 MHz, $CDCl_3$) δ 1.14. ESIMS calcd for $C_{49}H_{87}N_2O_7PNa$ $[M + Na]^+$ 869.6143, found 869.6144.

{2-[(2-Hexadecanoylamino-3-hydroxyoctadec-4-enyloxy)-hydroxyphosphoryloxy]ethyl}dimethylprop-2-ynylammonium (14). Compound **13** (11 mg, 0.013 mmol) was stirred in TFA:CH_2Cl_2/1:9 (5 mL) for 1 h at 0 °C. TLC (CH_2Cl_2/MeOH/H_2O, 55:45:3) shows complete disappearance of starting material and formation of product at R_f 0.4. The reaction mixture was neutralized with NH_4OH and concentrated in vacuo to give **14** (9.4 mg, 100%). $[\alpha]^{25}_D$ −34.51 (*c* 0.5, $CHCl_3$). IR (KBr neat) 3376, 3268, 2956, 2918, 2850, 2125, 1660, 1468, 1240, 1089 cm^{-1}. ^1H NMR (600 MHz, $CDCl_3$) δ 0.88 (t, J = 7.2 Hz, 6H), 1.20−1.40 (m, 46H), 1.50−1.64 (m, 2H), 1.94−2.02 (m, 2H), 2.10−2.20 (m, 3H), 2.97 (s, 1H), 3.38 (s, 6H), 3.80−4.0 (m, 4H), 4.08 (dd, J = 7.2, 6.6 Hz, 1H), 4.16−4.4 (m, 3H), 4.61 (br s, 2H), 5.46 (dd, J = 15.6, 7.2 Hz, 1H), 5.66 (dt, J = 15.6, 6.6 Hz, 1H), 6.72 (br s, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1, 22.7, 25.9, 26.0, 29.2, 29.3, 29.4, 29.53, 29.58, 29.6, 29.7, 29.8, 29.82, 32.0, 32.6, 36.9, 51.6, 54.5 (J_{C-P} = 3.3 Hz), 55.3, 59.2 (J_{C-P} = 5.4 Hz), 64.4 (J_{C-P} = 4.2 Hz), 65.0, 71.4, 72.0, 81.5, 129.7, 133.6, 173.2. ^{31}P NMR (162 MHz, $CDCl_3$) δ 1.37 (m). ESIMS calcd for $C_{41}H_{79}N_2O_6PNa$ $[M + Na]^+$ 749.5568, found 749.5572.

(1-Benzo[1,2,5]oxadiazol-5-ylmethyl-1*H*-[1,2,3]triazol-4-ylmethyl)-{2-[(2-hexadecanoylamino-3-hydroxyoctadec-4-enyloxy)hydroxyphosphoryloxy]ethyl}dimethylammonium (16). To the solution of alkyne **14** (3.5 mg, 0.005 mmol) in EtOH:H_2O (4 mL, 1:1) were added the azide **15** (1.68 mg, 0.01 mmol), $CuSO_4$ (0.15 mg, 0.001 mmol), and ascorbic acid (0.25 mg, 0.0014 mmol). After 10 min formation of precipitate was observed in the reaction mixture. TLC (CH_2Cl_2/MeOH/H_2O, 55:45:3) showed that both the starting material and the UV active product appear at the same R_f (0.4). Therefore, the reaction was allowed to stir for 12 h to ensure complete conversion. The product was collected by filtration and further purified by column chromatography with Iatrobeads (CH_2Cl_2/MeOH, 9:1 then CH_2Cl_2/MeOH/H_2O, 55:45:3) to obtain **16** (4.55 mg, 100%) as a white solid, mp 118−120 °C. IR (KBr neat) 3364, 3123, 3076, 2925, 2109, 1637, 1541, 1289, 1008, 881, 798, 756 cm^{-1}. ^1H NMR (500 MHz, CD_3OD) δ 0.90 (t, J = 7.0 Hz, 6H), 1.20−1.40 (m, 46H), 1.48−1.64 (m, 3H), 2.01 (dt, J = 7.0, 6.5 Hz, 2H), 2.12−2.22 (m, 2H), 3.20 (s, 6H), 3.54−3.68 (m, 4H), 3.80−3.88 (m, 1H), 3.90−4.14 (m, 4H), 4.33 (br s, 2H), 4.78 (s, 2H), 5.44 (dd, J = 15.0, 7.5 Hz, 1H), 5.7 (dt, J = 15.0, 7.0 Hz, 1H), 6.82−6.88 (br s, 1H, NH), 7.49 (d, J = 9.5 Hz, 1H),

7.85 (s, 1H), 7.93 (d, J = 9.5 Hz, 1H), 8.50 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.4, 23.7, 27.1, 30.41, 30.5, 30.6, 30.7, 30.8, 33.1, 33.4, 37.4, 52.2, 54.4 (J_{C-P} = 3.3 Hz), 55.3, 60.4 (J_{C-P} = 5.4 Hz), 71.5, 72.6, 116.3, 118.3, 130.4, 131.2, 133.2, 135.1, 137.3, 141.0, 150.1, 176.0, ^{31}P NMR (162 MHz, $CDCl_3$) δ 1.04. ESIMS calcd for $C_{48}H_{84}N_7O_7PNa$ $[M + Na]^+$ 924.6062, found 924.6060.

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Supporting Information Available: Experimental procedures for the preparation of compounds **5**, **11**, and **15**, supporting spectral data, and details of enzymatic experiments. This material is available free of charge via the Internet at http://pubs.acs.org.