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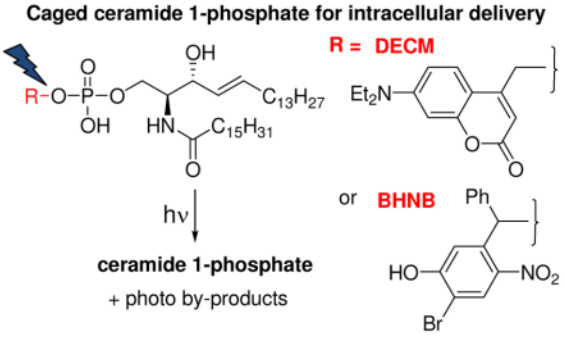
Caged Ceramide 1-Phosphate Analogues: Synthesis and Properties

Ravi S. Lankalapalli[†], **Alberto Ouro**[‡], **Lide Arana**[‡], **Antonio Gómez Muñoz**[‡], and **Robert Bittman**[*,†]
Department of Chemistry and Biochemistry, Queens College of The City University of New York, Flushing, New York 11367-1597, and Department of Biochemistry and Molecular Biology, University of the Basque Country, Bilbao, Spain

Abstract



Sphingolipid phosphate analogues bearing 7-(diethylamino)coumarin (DECM) and 4-bromo-5-hydroxy-2-nitrobenzhydryl (BHNB) groups in a photolabile ester bond were synthesized. The ability of the "caged" ceramide 1-phosphate analogues to release the bioactive parent molecule upon irradiation at 400–500 nm was demonstrated by stimulation of macrophage cell proliferation.

"Caging" is a strategy employed in biochemistry, neurobiology, and physiology for the in vitro investigation of the cellular activity of cell-impermeable bioactive molecules.[1] The ionic groups in charged, hydrophilic molecules are temporarily masked by a covalent link to a photolabile moiety, thereby facilitating cell delivery and bypass of cell-surface receptors. The bioactive molecule is released in the cytosol on photolysis using light that does not damage cellular components. The first caged biomolecules reported, the intracellular messengers cyclic-AMP[2a] and ATP,[2b] incorporated *o*-nitrobenzyl phosphate esters as the photolabile moiety. Subsequently, the 1-(2-nitrophenyl)ethyl (NPE) group and derivatives containing additional hydroxy or methoxy substituents were used as caging addends.[1b,2c] Since the NPE chromophore has only weak absorption above 350 nm, cages with more attractive photophysical properties have been developed. For example, derivatives of coumarins[3] and quinolines[4] are rapidly uncaged using two-photon activation with IR laser light in tiny excitation volumes and may be used in vivo, since the photolytic conditions are noninvasive and permit spatial and temporal control of the uncaging process.

robert.bittman@qc.cuny.edu.
[†]CUNY
[‡]University of the Basque Country

Supporting Information Available: Experimental procedures and NMR spectra for the reported compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

The phosphorylated sphingolipid metabolites sphingosine 1-phosphate (S1P) and ceramide 1-phosphate (C1P) are mediators of a multitude of cellular activities.[5] Most of the activities of S1P arise from its action as an extracellular signaling molecule following binding to a family of five G-protein coupled receptors at the cell surface.[6] In contrast to S1P, C1P functions as a lipid messenger mainly at the intracellular level.[7] Natural C1P is not taken up readily by cells in culture unless it is dispersed in organic solvents such as EtOH/dodecane (49:1), which may allow incorporation of other extracellular compounds. C1P regulates diverse cellular functions, including arachidonic acid release, mast cell degranulation, $Ca2^{+}$ mobilization, translocation of lipid-metabolizing enzymes, vesicular trafficking, cell proliferation, and cell survival.[8] Our investigations of cellular responses to intracellular C1P have been limited to the addition of unnatural short-chain amide analogues of C1P, such as *N*-acetyl- and *N*-octanoyl-C1P, as no caged C1P derivative has yet been reported. In fact, the only caged sphingolipid derivatives that have been reported are S1P analogues that contain the NPE chromophore.[9]

We report here the synthesis of caged S1P (**1**, **2**) and C1P (**1a**, **2a**) analogues in which the phosphate headgroup is esterified to a 7-(diethylamino)coumarin (DECM) or 4-bromo-5-hydroxy-2-nitrobenzhydryl (BHNB) group (Figure 1). We assessed the ability of caged C1P conjugates **1a** and **2a** to deliver C1P into mammalian cell cultures on exposure to visible light. Photochemical uncaging of C1P may be particularly useful in cells that cannot be transfected with specific enzymes as in primary bone marrow derived macrophage (BMDM) cell types, [10] and to distinguish between intracellular and cell surface receptor-mediated activities.

For the synthesis of the DECM-caged S1P **1** and C1P **1a**, (*S*)-Garner aldehyde **3**[11] (Scheme 1) was treated with vinylmagnesium bromide at −78 °C to provide a 6:1 mixture of erythro alcohol **4**[12] and its C3-epimer **4a**. Alcohol **4** was purified by chromatography (hexane/EtOAc 5:1), and the hydroxy group was protected as its MOM ether **5**. The oxazolidine group was selectively deprotected using 1 M HCl in THF at rt to give *N*-Boc-3-*O*-MOM-protected alcohol **6**. An *E*-selective cross metathesis of olefin **6** with 1-pentadecene using Grubbs' second-generation catalyst[13] afforded the 3-MOM ether of (2*S*,3*R*)-*N*-Boc-sphingosine **7**.[14] Sphingosine derivative **7** was treated with *i*-$Pr_2NP(OMe)Cl$ in the presence of DIPEA to provide phosphoramidite intermediate **8**.

7-(Diethylamino)-4-hydroxymethylcoumarin (**9**) was prepared by oxidation of 4-methyl-7-(diethylamino)coumarin with SeO_2, and subsequent reduction with $NaBH_4$.[3d,15] Reaction of alcohol **9** with phosphoramidite **8** in the presence of 1*H*-tetrazole provided a phosphite intermediate, which was oxidized in the same pot with anhydrous TBHP to afford phosphate **10** (Scheme 2). Removal of the protecting groups required some exploration. An initial attempt to deprotect both the Boc and MOM groups with TFA resulted in the loss of the Boc group in 1 h; however, only 50% of the MOM group was removed even after stirring for 3 days at rt. Reaction with Me_3SiBr in CH_2Cl_2, rather than removing the methyl ester of the phosphate, resulted instead in the loss of the coumarin moiety from the lipid. Fortunately, we found that when **10** was heated in 6 M HCl/THF (2:1) at 60 °C for 6 h, all three groups, Boc, MOM, and the phosphate methyl ester, were removed to provide DECM-S1P (**1**), which on neutralization and *N*-acylation using *p*-nitrophenyl palmitate in DMF/CH_2Cl_2[16] gave C1P analogue **1a** in good yield.

To provide caged phospholipids with a high molar absorptivity at longer wavelengths than **1** and **1a**,[17] we prepared BHNB-caged compounds **2** and **2a**. Bromo and nitro groups were selected as the electron-withdrawing groups, with the nitro group positioned ortho to the benzhydryl carbon to facilitate photorelease of the phosphorylated sphingolipid.[18] The first step in the preparation of the BHNB cage was bromination of 4-methoxy-2-methyl-1-nitrobenzene (**11**, Scheme 3). When **11** was heated with NBS overnight at reflux in MeCN, we observed a substantial amount of the starting material along with the product. However, when

a pressure tube was used for this reaction, heating at 140 °C overnight resulted in a mixture of the desired brominated product **12** together with unseparable by-products, and no starting material. We used a known method for the conversion of a methyl group in the ortho position to a nitro group to an aldehyde; thus, reaction of **12** with DMF dimethyl acetal (DMF-DMA), followed by oxidation of the intermediate enamine with $NaIO_4$,[19] successfully converted **12** to aldehyde **13**. After the by-products were removed by chromatography, addition of PhMgBr to **13** afforded the desired caged benzhydryl alcohol **14**. A reaction time of 30 min at −78 °C is critical for this addition reaction, as decomposition of compound **14** was observed after 30 min.

After alcohol **14** was converted to phosphoramidite **15** with *i*-$Pr_2NP(OMe)Cl$ (Scheme 3), coupling with sphingosine derivative **16**[9b] (Scheme 4) in the presence of 1*H*-tetrazole afforded **17**. Phosphite **17** was purified by flash chromatography prior to oxidation because the corresponding phosphate co-eluted with excess alcohol **16**. Oxidation of **17** with TBHP afforded phosphate **18** in 70% yield over three steps. Several reagents (BBr_3 in CH_2Cl_2 at −78 °C, 33% HBr in AcOH at 0 °C, 1 M HCl/THF (1:1) at rt, and TMSBr in CH_2Cl_2) to remove the protecting groups in **18** were screened unsuccessfully. Use of excess LiI in pyridine at reflux was successful, resulting in *O*-demethylation of both the anisole methyl group and phosphate methyl ester groups of **18**, affording **19** in 90% yield. Reaction of **19** with 3 M HCl/MeOH/CH_2Cl_2 (1:2:1) at gentle reflux for 2.5 h provided BHNB-caged S1P **2**. N-Acylation with *p*-nitrophenyl palmitate gave C1P analogue **2a** in 77% yield.[16]

Figure 2 shows a comparison of the effects of adding growth factors,[20] *N*-palmitoyl-C1P (30 or 50 μM, as an aqueous dispersion), **1a** (2.5 μM in EtOH), and **2a** (1.0 μM in EtOH) to RAW264.7 (panel A) or primary BMDM (panel B). Compounds **1a** and **2a** stimulated cell division, as determined by the MTS assay,[21] at 2.5 and 1.0 μM, respectively, on exposure of the cells to light. To achieve the same relative level of RAW264.7 and BMDM cell growth with exogenous C1P, optimum concentrations of 30 and 50 μM[8b] were required, respectively. **1a**, at 2.5 μM, was as potent as exogenous C1P at 30 μM (see Supporting Information). The putative photo by-product[22] formed on photolysis of **1a** (compound **9**) did not stimulate cell proliferation, nor did an analogue of the photo by-product **20** formed on photolysis of **2a**.[23] No growth stimulation was observed in the dark.

In conclusion, **1a** and **2a**, the first caged analogues of C1P, were prepared and found to release C1P intracellularly, as demonstrated by stimulation of macrophage proliferation at significantly lower concentrations than exogenous C1P. These compounds, and their N-acyl variants, are likely to find utility as research tools for investigating the intracellular activities of this important lipid second messenger.

Experimental Section

(4*S*,5*R*,1″*E*)-*tert*-Butyl-4-((((4′-bromo-5′-hydroxy-2′-nitrophenyl)(phenyl)methoxy) (hydroxy) phosphoryloxy)methyl)-2,2-dimethyl-5-(pentadec-1″-enyl)oxazolidine-3-carboxylate (19)

A solution of **18** (48 mg, 0.056 mmol) and anhydrous LiI (150 mg, 1.12 mmol) in dry py (5 mL) was heated at reflux overnight. The reaction was quenched with saturated aq NH_4Cl solution (15 mL), and the product was extracted with CH_2Cl_2 (3 × 15 mL), dried (Na_2SO_4), concentrated, and purified by chromatography ($CHCl_3$/MeOH 3:1) to afford **19** (37 mg, 90%): R_f 0.60 ($CHCl_3$/MeOH 3:1); ^1H NMR (CD_3OD) δ 0.78 (t, 3H, J = 6.6 Hz), 1.18 (m, 31H), 1.38 (m, 6H), 1.90 (m, 2H), 3.79 (m, 3H), 4.40 (m, 1H), 5.42 (m, 1H), 5.69 (m, 1H), 6.99 (m, 1H), 7.23 (m, 6H), 7.53 (m, 1H), 8.20 (m, 1H); ^{31}P NMR (CD_3OD) δ −2.92, −2.03; ESI-HRMS $[M+Na]^+$ $C_{39}H_{58}{}^{81}BrN_2O_{10}PNa$ calcd for *m/z* 849.2889, found 849.2894.

(2*S*,3*R*,4*E*)-2-Amino-3-hydroxyoctadec-4-enyl-(4′-bromo-5′-hydroxy-2′-nitrophenyl)(phenyl) methyl Hydrogen Phosphate (2)

A solution of **19** (25 mg, 0.030 mmol) in 3 M HCl/MeOH/CH_2Cl_2 (1:2:1, 4 mL) was stirred at gentle reflux (50–60 °C) for 2.5 h. The reaction mixture was diluted with CH_2Cl_2 (30 mL) and washed with brine (10 mL). The organic layer was dried (Na_2SO_4) and concentrated. Column chromatography ($CHCl_3$/MeOH 6:1, then 3:1), followed by removal of suspended silica gel by filtration of a solution of **2** in $CHCl_3$ through a 0.45-μm syringe filter, afforded **2** (12 mg, 60%) as a yellow solid: R_f 0.35 ($CHCl_3$/MeOH 3:1); ^{1}H NMR (CD_3OD) δ 0.89 (t, 3H, *J* = 7.0 Hz), 1.28 (m, 22H), 2.03 (m, 2H), 2.95 (m, 1H), 3.59 (m, 1H), 3.75 (m, 1H), 4.07 (m, 1H), 5.28 (dd, 1H, *J* = 6.5, 15.4 Hz), 5.70 (dt, 1H, *J* = 6.7, 14.7 Hz), 7.02 (m, 1H), 7.25 (m, 5H), 7.44 (m, 1H), 8.25 (m, 1H); ^{13}C DEPT-45 NMR (CD_3OD) δ 14.5, 23.7, 30.2, 30.4, 30.5, 30.6, 30.7, 30.8, 33.1, 33.3, 57.0, 63.3, 70.6, 76.4, 116.8, 127.8, 128.8, 129.0, 129.2, 129.3, 129.4, 131.8, 136.3, 136.8; ^{31}P NMR (CD_3OD) δ −1.41, −1.20; ESI-HRMS $[M-H]^-$ $C_{31}H_{45}BrN_2O_8P$ calcd for *m/z* 683.2096, found 683.2095; UV: λ_{max} 406 nm (ε = 12,000 $M^{-1}cm^{-1}$), 278 nm (ε = 6,294 $M^{-1}cm^{-1}$) in 50% aq EtOH, 50% 10 mM Tris, pH 7.4.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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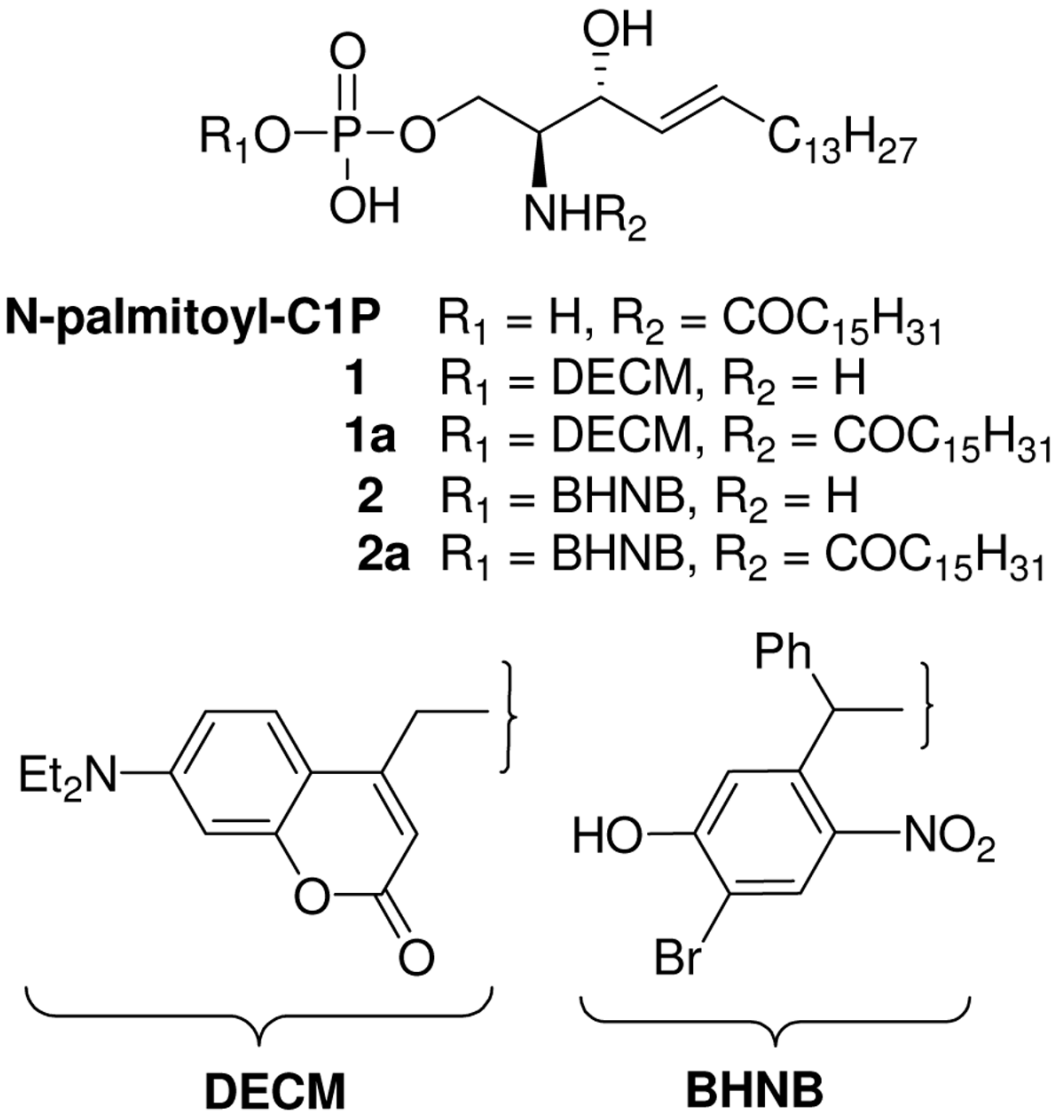


Figure 1.
Structures of C1P, DECM- and BHNB-Caged S1P Analogues **1** and **2**, and C1P Analogues **1a** and **2a**.

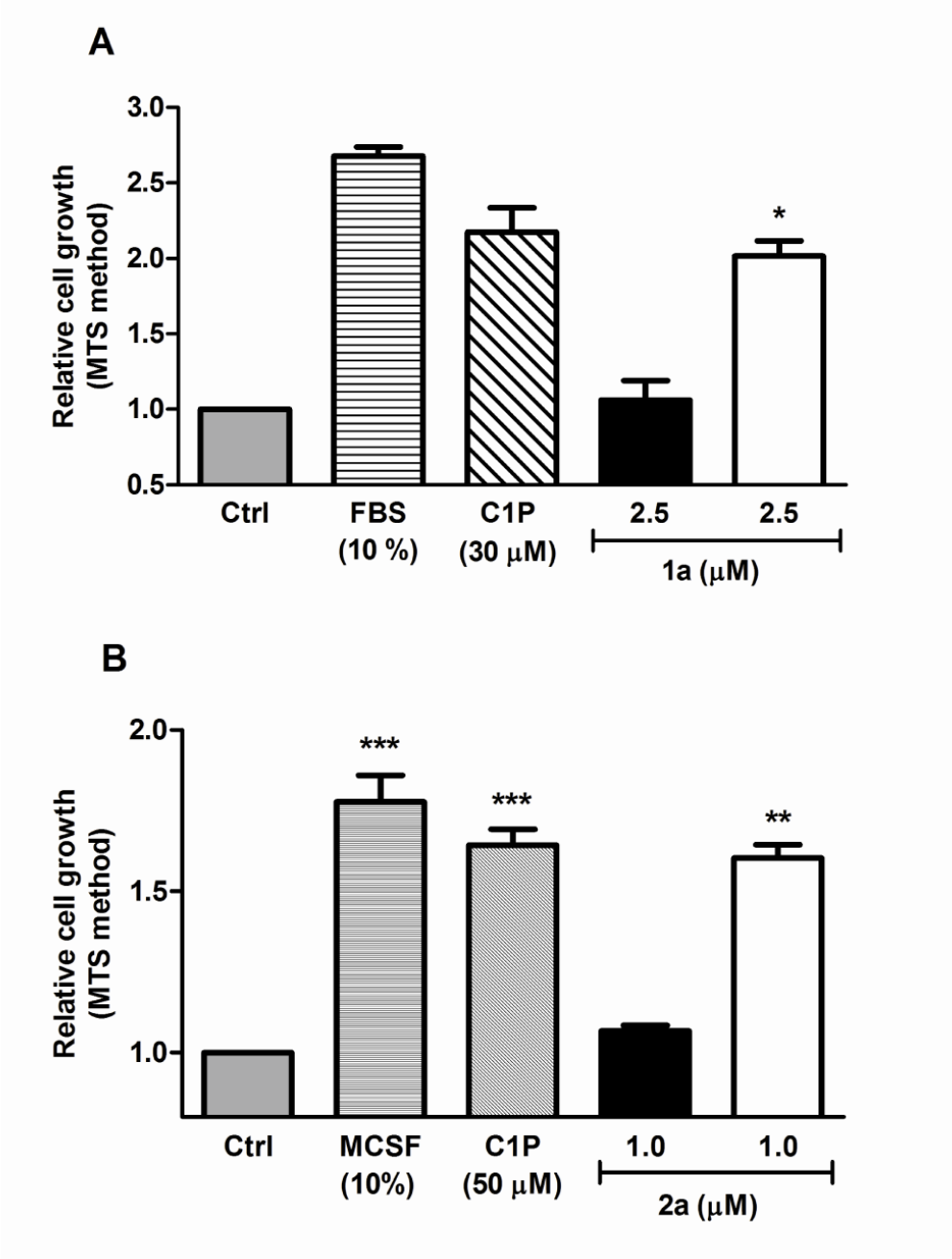
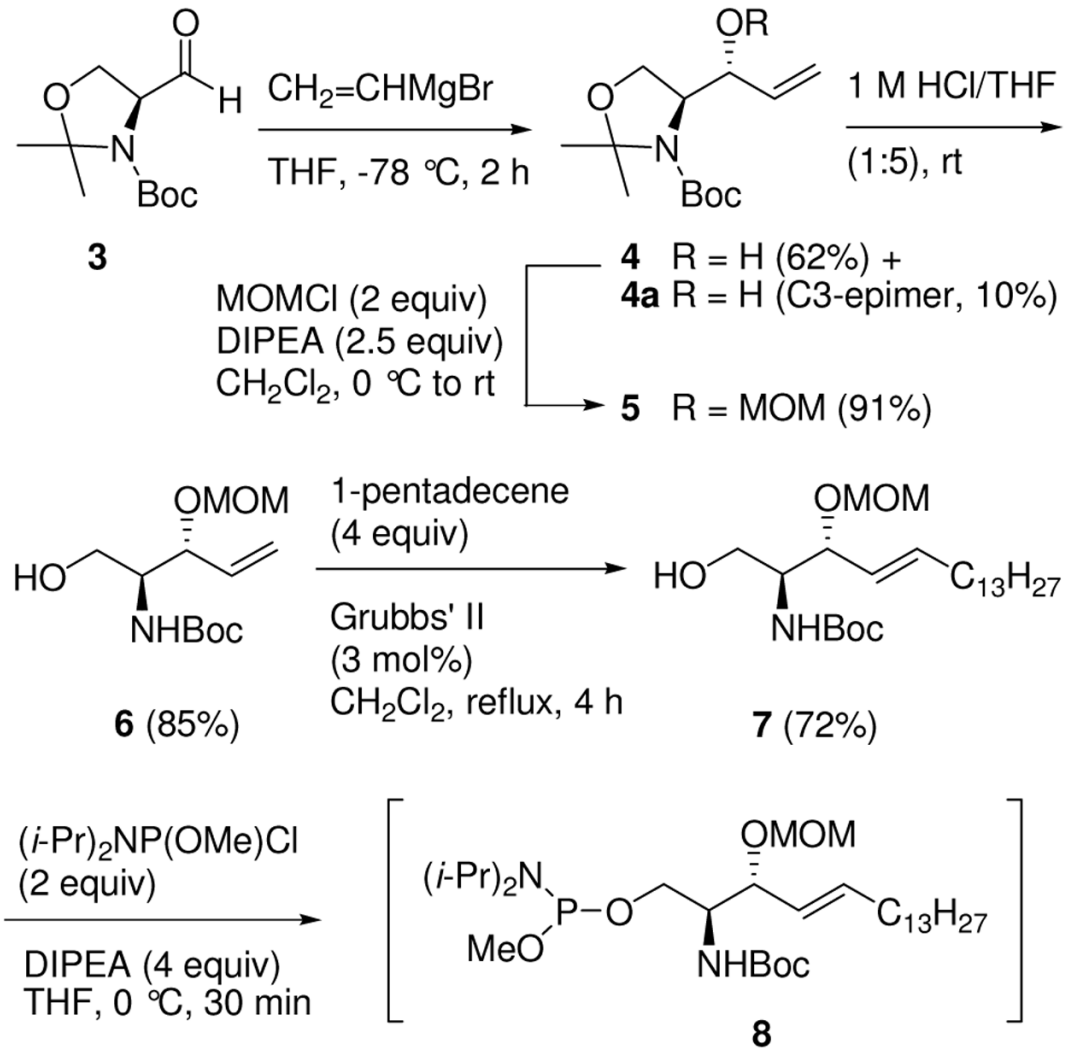
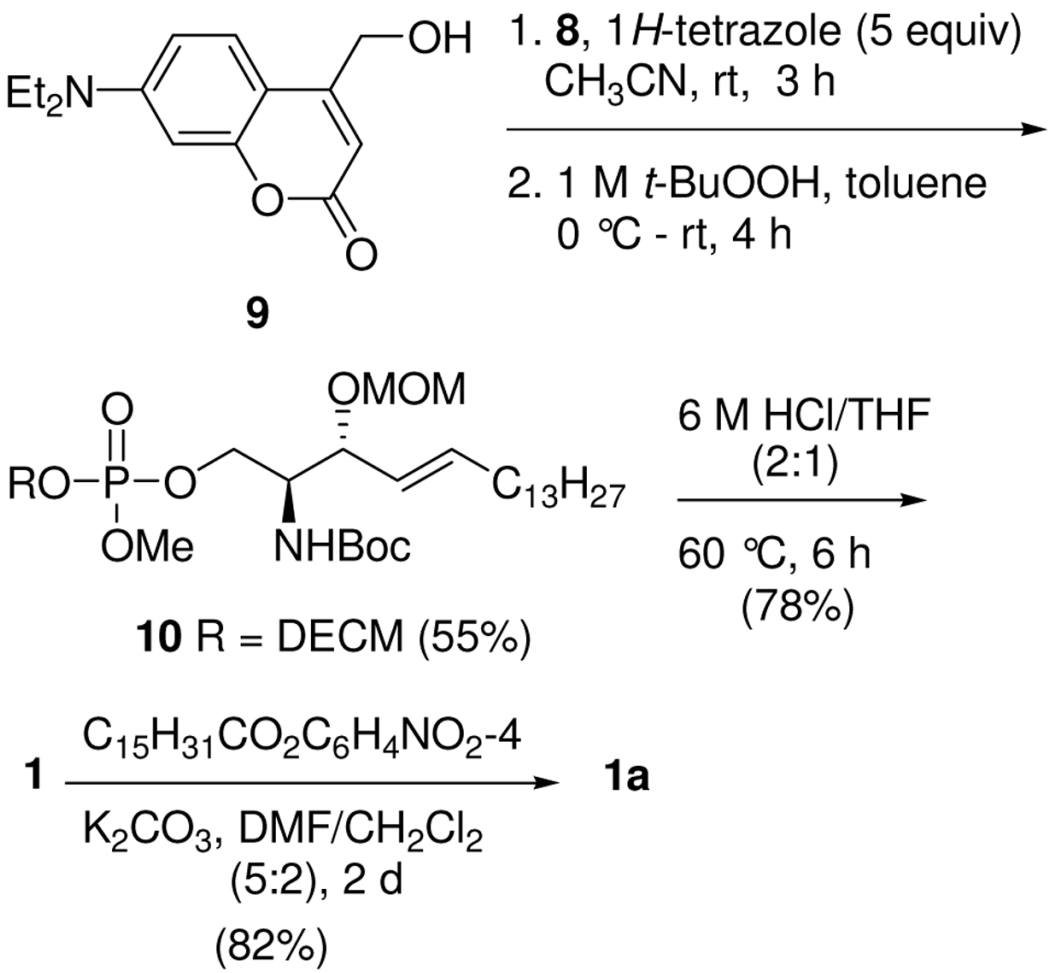


Figure 2.

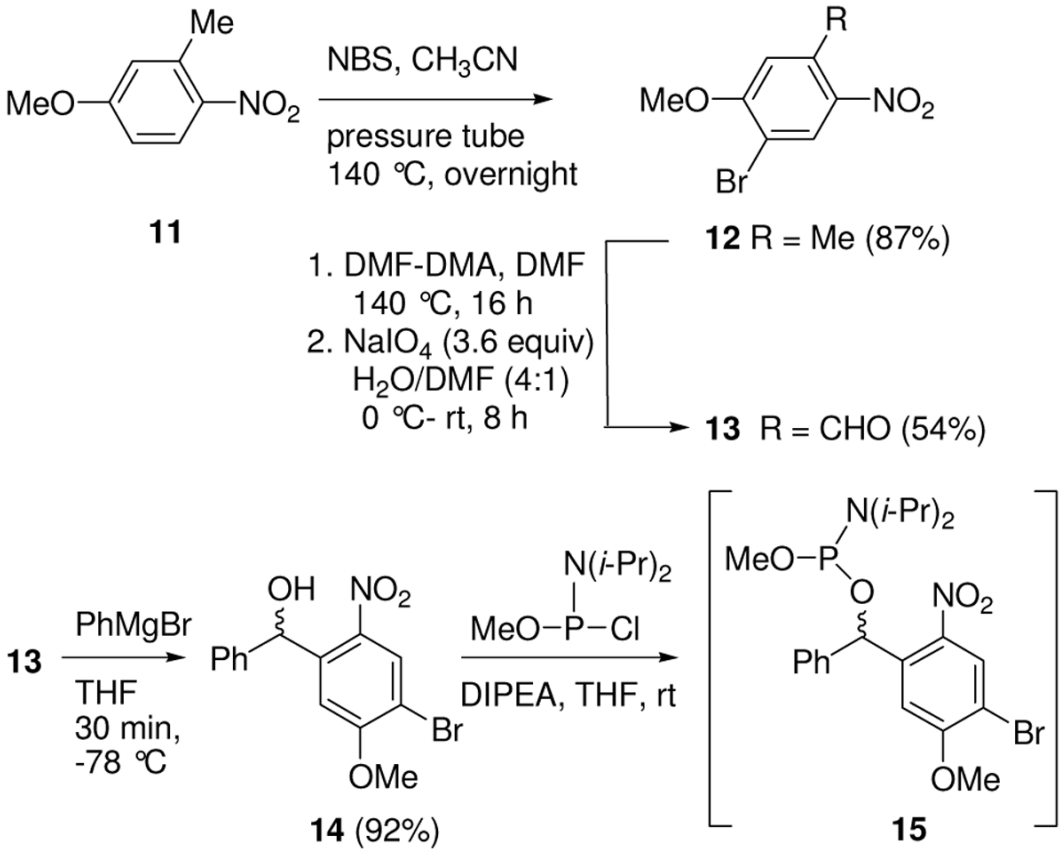
Delivery of **1a** and **2a** into RAW 264.7 macrophages (panel A) and primary BMDM (panel B) stimulates cell growth. Open bars: Cells were exposed to 400–500 nm light in a transilluminator (see Supporting Information). Filled bars: Cells were incubated in the dark with the compounds. The cells were incubated for 48 h in the absence of FBS (A) or with 1.5% MCSF for 24 h (B) prior to the addition of the compounds. Mean ±S.D. of 3 independent experiments.



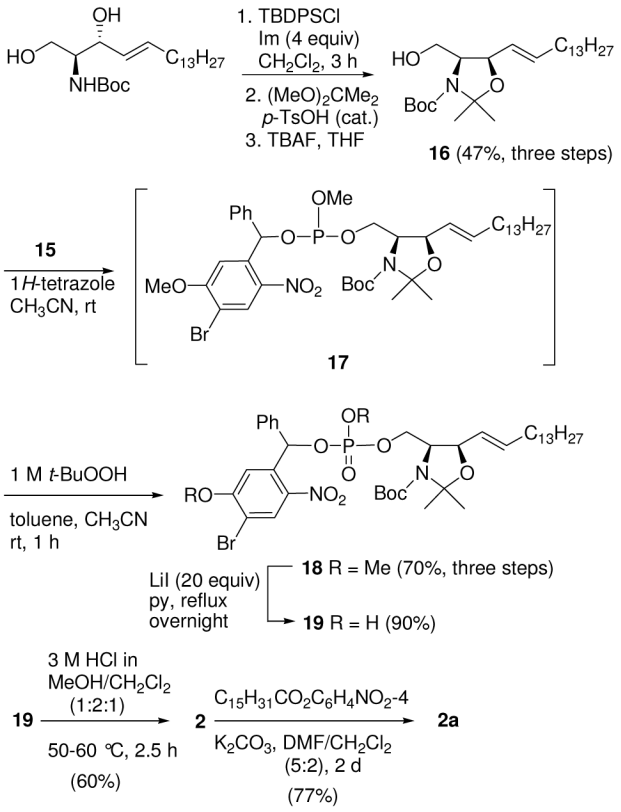
Scheme 1.
Synthesis of Phosphoramidite **8**



Scheme 2.
Synthesis of DECM-C1P Analogue **1a**



Scheme 3.
Synthesis of Benzhydryl Alcohol **14** and Phosphoramidite **15**



Scheme 4.
Synthesis of BHNB-caged C1P Analogue **2a**