Supporting Information

Synthesis and Molecular Recognition of Phosphatidylinositol-3-methylenephosphate

Joanna Gajewiak,[†] Yong Xu,[†] Stephanie A. Lee,[‡] Tatiana G. Kutateladze,[‡] and Glenn D. Prestwich*[,†]

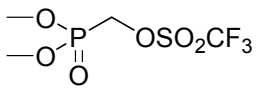
[†]Department of Medicinal Chemistry, The University of Utah, 419 Wakara Way, Suite 205, Salt Lake City, Utah, 84108-1257 USA

Phone: +1-801-585-9051. Fax: +1-801-585-9053. Email: gprestwich@pharm.utah.edu

[‡]Department of Pharmacology, University of Colorado Health Sciences Center, Aurora, CO 80045 USA

Table of Contents

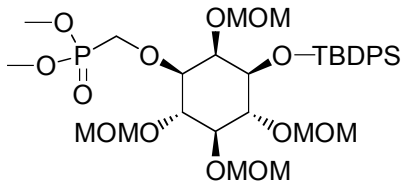
General. Chemicals were purchased from Aldrich and Acros Chemical Corporation and used without prior purification. Solvents were purchased anhydrous CH_2 THF. Reactions requiring anhydrous conditions were carried out in oven-dried glassware (2 h, 120 ^{o}C) under inert atmosphere (N_2 or Ar) unless otherwise indicated. Concentration *in vacuo* refers to the use of rotary evaporator for solvent removal, and purification on SiO_2 refers to flash chromatography (FC) on silica gel (Whatman 230~400 mesh ASTM silica gel). TLC was done using precoated silica gel aluminum sheets (EM SCIENCE silica gel $60F_{254}$. NMR spectra were recorded on a Varian INOVA 400 at 400 MHz (^{1}H), 101 MHz (^{13}C) or 162 MHz (^{31}P) at ambient temperature. Chemical shifts are reported in ppm relative to those of internal chloroform peaks (δ_H 7.24), and (δ_C 77.0) and to CD_3OD peaks (δ_H 4.78) and (δ_C 49). For ^{31}P NMR, 85% H_3PO_4 (δ = 0ppm) was used as an internal standard. Optical rotations were obtained at ambient temperature. Low- and high-resolution spectra were obtained on HP5971A MSD and Finnigan MAT95 double focusing mass spectrometer (MS) instruments, respectively. Symbols: s, singlet; dd, doublet of doublets; m, multiple; p, quintuplet; q, quartet; t, triplet. Coupling constants (*J*) are all reported in Hz. The synthesis of compounds **3** and **4** was previously described[19,20] and the procedures are not given in this supporting information.



5

Dimethyl phosphonomethyltriflate (5). Dimethyl hydroxymethylphosphonate (1.8 g, 12.8 mmol) was diluted with anhydrous CH_2Cl_2 (21 mL) and 2,6-lutidine (2.5 mL, 14.8 mmol) was added and the reaction was conducted for 3h. Then the reaction mixture was washed with H_2O, 1M HCl, again with H_2O, then dried with Na_2SO_4, concentrated *in vacuo* and used without

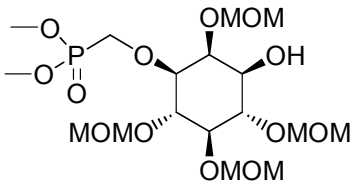
further purification. R_f 0.67 (ethyl acetate: ethanol, 9:1); ^{1}H NMR ($CDCl_3$) δ: 4.62 (d, 2H, J = 8.8), 4.18-4.10 (d, 6H, J = 11.2); ^{13}C NMR ($CDCl_3$) δ: 118.4 (q, J = 321.1), 66.3, 64.6, 64.16 (d, J = 6.2); ^{31}P NMR ($CDCl_3$) δ: 15.9; ^{19}F NMR ($CDCl_3$) δ: -74.6;



6

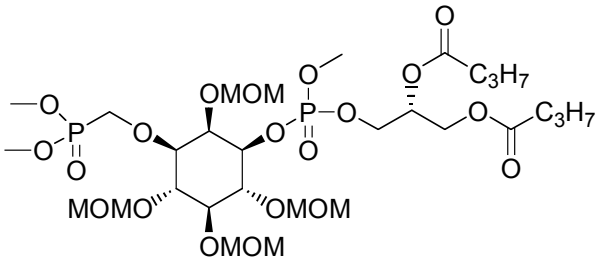
1D-1-*O*-(*tert*-Butyldiphenylsilyl)-3-(dimethyl methylenephosphonate)-2,4,5,6-*O*-*tetrakis* (methoxymethylene)-*myo*-inositol (6). To the solution of **2** (126 mg, 0.212 mmol) in anhydrous THF (3 mL), colled to -78^{o}C, *n*BuLi (0.16 mL, 0.254 mmol) was added under Ar atmosphere. The reaction mixture was stirred for 1h at -78^{o}C and then **5** (70 mg, 0.254 mmol) in THF (0.5 mL) was added and the reaction was stirred for another 1h at -78^{o}C. Then it was allowed to warm up to 0^{o}C for another 1h 30 min. After TLC showed consumption of the starting material, the reaction was quenched with sat. NH_4Cl and extracted with CH_2Cl_2. The combined organic phases wee dried with Na_2SO_4, concentrated and purified using FC with hexanes: acetone (7:3) to produce 97 mg (0.135 mmol) of the final compound **6** in 64%. R_f 0.31 (hexanes: acetone, 6:4); $[\alpha]^{20}_D$ = + 32.5 (*c* 0.64, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ: 7.74 (d, 2H, J = 6.8), 7.67 (d, 2H, J = 6.4), 7.45-7.36 (m, 6H), 5.03 (d, 1H, J = 6.4), 4.93 (d, 1H, J = 6.4), 4.85 (d, 2H, J = 6.4), 4.75 (s, 2H), 4.52 (d, 1H, J = 6.8), 4.43 (d, 1H, J = 6.8), 4.00 (t, 1H, J = 9.6), 3.77 (t, 1H, J = 9.6), 3.70-3.64 (m, 7H), 3.46 (d, 6H, J = 10), 3.40-3.29 (m, 5H), 3.21 (s, 3H), 3.07 (s, 1H), 3.04-2.98 (m, 1H), 2.76-2.73 (m, 1H), 1.08 (s, 9H); ^{13}C NMR ($CDCl_3$) δ: 135.9 (s), 135.7 (s), 134 (s), 132.7 (s), 130.1 (s), 129.8 (s), 127.9 (s), 127.7 (s), 98.8 (s), 98.5 (s), 98.1 (s), 97.2 (s), 82.14 (s), 82.0

(s), 78.6 (s), 78.2 (s), 73.9 (s),73.3 (s), 62.6 (s), 61.0 (s), 56.5 (d, J = 7.6), 56.3 (s), 55.3 (s), 52.5 (dd, J = 3.0, 6.2), 27.1 (s), 19.0 (s); ^{31}P NMR ($CDCl_3$) δ: 24.55; LRMS (MALDI) *m/z* 739.3 (M + Na). HRMS (MALDI) for $C_{33}H_{53}NaO_{13}PSi$ found: 739.2847, calcd: 739.2891.



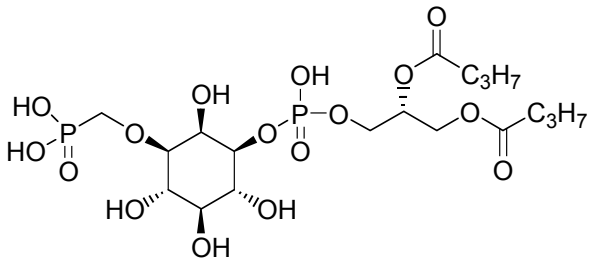
7

1D-3-(Dimethyl methylenephosphonate)-2,4,5,6-*O-tetrakis*(methoxymethylene)-*myo*-inositol (7). A solution of **6** (83 mg, 0.116 mmol) in THF (1 mL) was treated with Bu_4NF at rt. The reaction was stirred overnight, concentrated and purified using FC with acetone: hexanes 7:3 yielding the colorless oil in 93% yield (51.6 mg, 0.108 mmol). R_f 0.25 (acetone: hexanes, 7:3); $[\alpha]^{20}_D$ = - 22.8 (*c* 0.35, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ: 4.80 (d, 2H, J = 6.0), 4.76-4.72 (m, 4H), 4.69-4.64 (m, 2H), 4.09-4.08 (m, 1H), 3.98-3.92 (m, 1H), 3.82 (t, 1H, J = 9.6), 3.72 (d, 3H, J = 3.2), 3.69 (d, 3H, J = 3.6), 3.60-3.55 (m, 1H), 3.37-3.34 (m, 12H), 3.33-3.31 (m, 1H), 3.25-3.22 (m, 1H); ^{13}C NMR ($CDCl_3$) δ: 98.6 (S), 98.2 (s), 97.7 (s), 83.1 (s), 82.4 (s), 82.3 (s), 78.9 (s), 77.4 (s), 74.6 (s), 70.7 (s), 56.3 (s), 56.1 (s), 55.9 (s), 55.6 (s), 52.8-52.6 (m); ^{31}P NMR ($CDCl_3$) δ: 24.54; LRMS (MALDI) *m/z* 501.2 (M + Na); HRMS (MALDI) for $C_{17}H_{35}NaO_{13}P$ found: 501.1708, calcd: 501. 1713.



8

1D-*O*-(1,2-di-*O*-butanoyl-*sn*-(2*S*)-glycerol-3-*O*-methylphosphono)-3-(dimethyl methylenephosphonate)-2,4,5,6-*O*-*tetrakis*(methoxymethylene)-*myo*-inositol (8). To a solution of alcohol **7** (45 mg, 0.094 mmol) in anhydrous THF (1.5 mL) was added 1*H*-tertrazole (65 mg, 0.94 mmol) and *N*,*N*-diisopropyl-*O*-methyl-*O*-(di-butanoyl-*sn*-(2*S*)-glycerol)-phosphonamidite (148 mg, 0.376 mmol). The mixture was stirred overnight. Then oxidation was performed with $(n\text{-}C_4H_9)_4NIO_4$ (203 mg, 0.47 mmol) at -20°C for 1h. The reaction mixture was warmed up to rt for additional 30 min. the solution was diluted with CH_2Cl_2 and washed with 10% $NaHSO_3$. The organic layer was concentrated and the residue was chromatographed on SiO_2 (gradient: acetone: hexanes 4:6 – 7:3) to yield **8** 61% (45 mg, 0.057 mmol) as yellowish oil. R_f 0.41 (acetone: hexanes 7:3); $[\alpha]^{20}_D$ = - 11.21 (*c* 0.33, $CHCl_3$). ^{1}H NMR ($CDCl_3$) δ: 5.28-5.21 (m, 1H), 4.86-4.80 (m, 5H), 4.76-4.70 (m, 3H), 4.34-4.29 (m, 2H), 4.26-4.11 (m, 3H), 4.10-4.85 (m, 5H), 3.82-3.75 (m, 9H), 3.43-3.37 (m, 13H), 3.28-3.25 (m, 1H), 2.32-2.25 (m, 4H), 1.67-1.60 (m, 4H), 0.94-0.90 (m, 6H); ^{13}C NMR ($CDCl_3$) δ: 172.9 (s), 172.6 (s), 98.7 (s), 98.4 (s), 97.5 (s), 82.1 (m), 78.9 (s), 77.2 (s), 77.1 (s), 76.5 (m), 73.1 (s), 69.3 (m), 65.7 (d, *J* = 5.4), 65.4 (d, *J* = 5.4), 64.5 (s), 62.8 (s), 61.4 (d, *J* = 5.3), 56.6 (s), 56.5 (s), 55.7 (d, *J* = 3.3), 54,7 (d, *J* = 6.3), 54.5 (d, *J* = 6.2), 52.8 (d, *J* = 6.2), 35.9 (s), 35.7 (s), 18.2 (s), 13.5 (m); ^{31}P NMR ($CDCl_3$) δ: 24.33, 0.91 (d, *J* = 34.8); LRMS (MALDI) *m/z* 809.3 (M + Na); HRMS (MALDI) for $C_{29}H_{56}NaO_{20}P_2$ found: 809.2669, calcd: 809.2738.



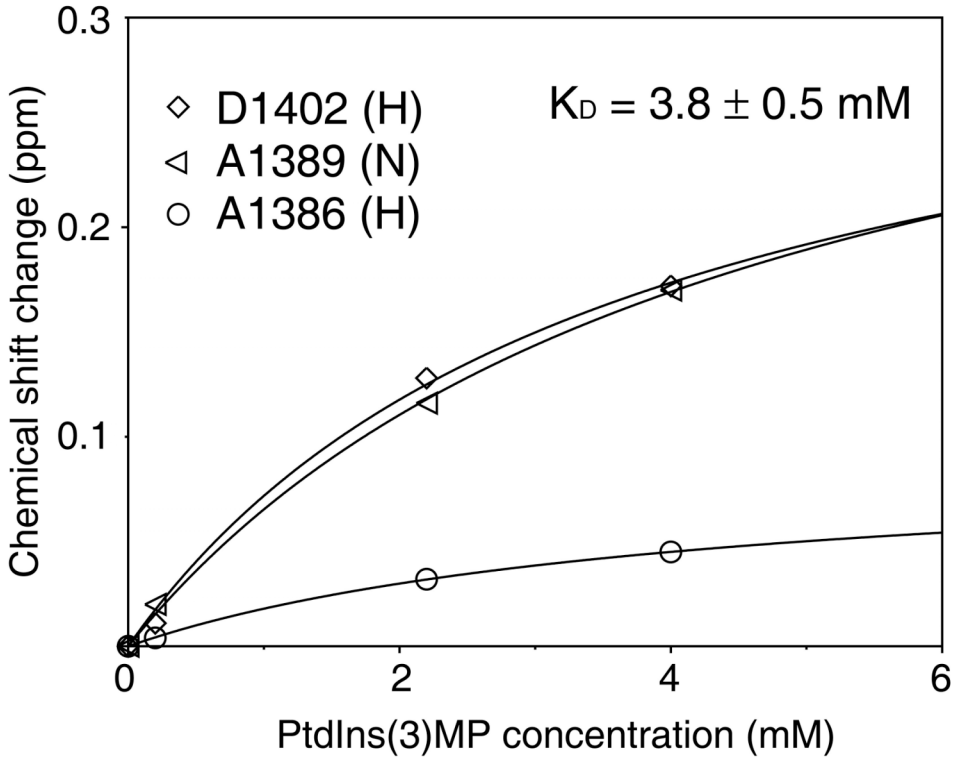
9

1D-*O*-(1,2-di-*O*-butanoyl-*sn*-(2*S*)-glycerol-3-phospho)-3-(methylenephosphonate)-*myo*-inositol (9). Compound **8** (20 mg, 0.025 mmol) was dried overnight under high vacuum and then reacted with fresh TMSBr (67 µl, 0.51 mmol) in anhydrous CH_2Cl_2 (1 mL) for 1h. Then it was concentrated *in vacuo* for 5h and finally subjected to hydrolysis with 90% aqua solution of CH_3OH (1 mL). After 30 min it was concentrated, dried *in vacuo* for 1h, dissolved in H_2O and passed through the short DOWEX 50WX8-400 [H^+] column and then lyophilized. The glassy film was obtained in 50% yield (7 mg, 0.019 mmol). R_f 0.27 ($CHCl_3$: CH_3OH: H_2O, 9:7:2). ^1H NMR ($CDCl_3$: **CD_3OD** as a lock solvent, 3:1) δ: 5.57 (p, 1H, J = 4.8), 4.71-4.67 (m, 2H), 4.53-4.47 (m, 3H), 4.34-4.24 (m, 2H), 4.16-4.07 (m, 3H), 3.59-3.51 (m, 2H), 2.66-2.59 (m, 4H), 2.00-1.91 (m, 4H), 1.28-1.23 (m, 6H); ^{13}C NMR ($CDCl_3$: **CD_3OD** as a lock solvent, 3:1) δ: 174.2, 173.7, 82.4, 82.2, 78.4, 78.4, 77.5, 74.4, 71.8, 70.7 (d, J = 5.4), 69.5 (d, J = 8.4), 67.1 (s), 66.0 (s), 64.8 (d, J = 5.4), 63.8 (s), 61.7 (s), 35.6 (d, J = 12.3), 17.9 (s), 13.0 (d, J = 4.6); ^{31}P NMR ($CDCl_3$: CD_3OD, 3:1) δ: 24.4, 3.53; LRMS (MALDI) *m/z* 591.1 (M + Na), 613.1 (M – H + 2Na) 635.1 (M - 2H + 3Na); HRMS (MALDI) for $C_{18}H_{34}NaO_{16}P_2$ found: 591.1214, calcd: 591.1220.

Protein Expression and Purification. The human EEA1 FYVE domain (residues 1325-1410) and the yeast Vam7 PX domain (residues 2-122) were expressed and purified as described.[13]

Thus, the DNA fragments encoding residues 1325-1410 of human EEA1 FYVE and residues 2-122 of yeast Vam7 PX were cloned in pGEX-KG and pGEX-2T vectors (Amersham). The ^{15}N-labeled proteins were expressed in *E. coli* BL21 (DE3) pLysS and BL21 Codon Plus RP strains in minimal media supplemented with $^{15}NH_4Cl$ (Cambridge Isotope). Bacteria were harvested by centrifugation after induction with IPTG (0.5 mM) and lysed by French press. The glutathione S-transferase (GST)-fusion FYVE and PX were purified on a glutathione sepharose 4B column (Amersham). The GST tag was cleaved with thrombin (Sigma). The proteins were further purified by FPLC and concentrated in Millipore concentrators (Millipore). The buffers were exchanged into 20 mM d_{11}-Tris (FYVE) or 50 mM potassium phosphate (PX), pH 6.8, 100-200 mM KCl, 1-20 mM perdeuterated dithiothreitol, 50 μM 4-amidinophenylmethane sulfonyl fluoride, 1 mM NaN_3, and 7% ^2H_2O.

NMR spectroscopy and titration of PtdIns(3)MP and PtdIns(3)P. NMR spectra were recorded at 25°C on Varian INOVA 500 MHz spectrometer. The ^1H-^{15}N heteronuclear single quantum coherence (HSQC) spectra of 0.2 mM uniformly ^{15}N-labeled FYVE and PX domains were collected while dibutanoyl-PtdIns(3)MP (up to 4 mM) or dibutanoyl-PtdIns(3)P (up to 1 mM) were added stepwise.



Supplementary Figure 2. Quantitative determination of the affinity of the recombinant FYVE domain for dibutanoyl-PtdIns(3)MP based on ^{1}H and ^{15}N NMR chemical shift changes for three specific amino acid residues. The K_d value was calculated as 3.8 ± 0.5 mM.