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Short Communication

Improved synthesis of *myo*-inositol 1-(4-nitrophenyl hydrogen phosphate), a chromogenic substrate for phosphatidylinositol-specific phospholipase C

Aleksey V. Rukavishnikov [a], Tatiana O. Zaikova [a], O. Hayes Griffith [a,b,*], John F.W. Keana [a]

[a] *Department of Chemistry, University of Oregon, Eugene, OR 97403-1229, USA*
[b] *Institute of Molecular Biology, University of Oregon, Eugene, OR 97403-1229, USA*

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Abstract

This paper describes an improved procedure for the synthesis of racemic *myo*-inositol 1-(4-nitrophenyl hydrogen phosphate) (NPIP) a useful substrate for the continuous spectrophotometric assay of phosphatidylinositol-specific phospholipase C (PI-PLC). © 1997 Elsevier Science Ireland Ltd.

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1. Introduction

Chromogenic substrate analogs based on 4-nitrophenol are widely used in enzymology because of the ease of detection of the highly colored nitrophenylate anion (Walsh, 1979; Price and Stevens, 1989). In order to make available the corresponding substrate for phosphatidylinositol-specific phospholipase C (Scheme 1) (PI-PLC), the synthesis and characterization of D,L-*myo*-inositol 1-(4-nitrophenyl hydrogen phosphate) (NPIP) was carried out in our laboratory several years ago

Abbreviations: BzCl, benzoyl chloride; d, doublet; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; Ins(1:2cyc)P, D-*myo*-inositol-1,2-cyclic-phosphate; m, multiplet; NMR, nuclear magnetic resonance; NPIP, D,L-*myo*-inositol 1-(4-nitrophenyl hydrogen phosphate); PI-PLC, phosphatidylinositol-specific phospholipase C; PPTs, pyridinium *p*-toluenesulfonate; Py, pyridine; q, quartet; s, singlet; t, triplet; THF, tetrahydrofuran; TsOH, *p*-toluenesulfonic acid.

* Corresponding author. Fax: +1 541 3464643; e-mail: griffith@oregon.uoregon.edu

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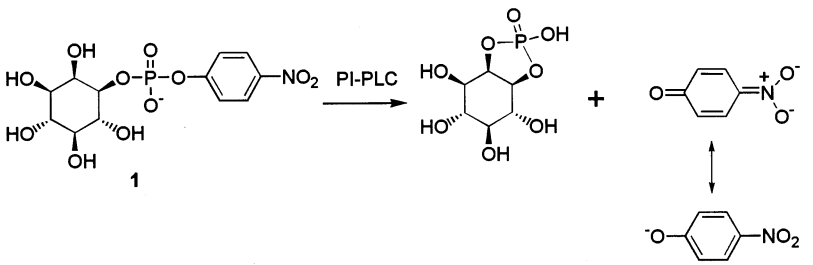


Scheme 1. The cleavage of NPIP to yield Ins(1:2cyc)P and the highly colored nitrophenolate anion

(Shashidhar et al., 1991). The continued interest in the use of NPIP as a substrate for biophysical studies on bacterial PI-PLCs (Ryan et al., 1996; Martin and Wagman, 1996; Bruzik and Tsai, 1994; Leigh et al., 1992) has prompted us to look for ways to improve the original protocol. Here we report procedures that simplify the synthesis and increase the yield of NPIP (Scheme 2).

2. Experimental

2.1. General methods

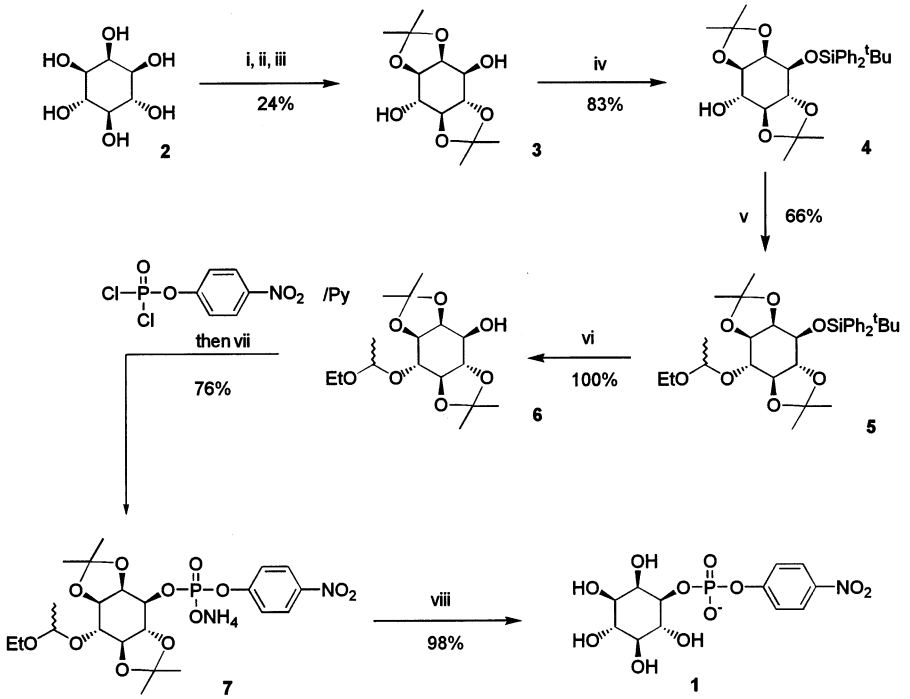
Commercial reagents were used without further purification. Melting points were taken on a Uni-Melt Thomas Hoover melting point apparatus and uncorrected. Dichloromethane was distilled from P_2O_5. Di-iso-propylidene-*myo*-inositol **3** was prepared from *myo*-inositol **2** as described (Gigg et al., 1985). Column chromatography was performed on Mallinckrodt silica gel 150 (60–200 mesh). Analytical thin layer chromatography was performed on aluminum-backed silica gel 60 F_{254} plates and visualization was effected with an ultraviolet lamp or by spraying with alcohol solution of vanillin (5%) and H_2S0_4 (5%) followed by heating at 60–70°C. ^{1}H NMR spectra were recorded on a 300 MHz Varian Inova instrument; chemical shifts are reported in delta units (δ) referenced to residual proton signals of the deuterated solvents ($CHCl_3$, δ7.26; CH_3SOCH_2D, δ2.49; HOD, δ4.80).

2.2. 3-O-[(1,1-dimethylethyl)diphenylsilyl]-1,2:4,5-bis-O-(1-methylethylidene)-D,L-myo-inositol **4**

To a solution of diol **3** (3.17 g, 12.2 mmol) and imidazole (1.27 g, 18.9 mmol) in pyridine (Py) (30 ml) *tert*-butyldimethylsilyl chloride (3.74 g, 13.6 mmol) was added while stirring and cooling at −15°C. The mixture was left at −20°C for 48 h. Methanol (2 ml) was added to the solution, the solvents were then evaporated in a vacuum. The residue was treated with water (20 ml). The product was extracted with ether (3 × 20 ml). The combined ether extracts were washed with brine, dried over Na_2SO_4 and evaporated to dryness by azeotropic evaporation with toluene (2 × 15 ml) to give alcohol **4** as a viscous oil (5.04 g, 83%) that solidified at room temperature (RT). Melting point (m.p.) was 143–146°C (from ethyl acetate). This compound was essentially pure by NMR (greater then 90%) and was used for the next step as received. Literature m.p. 142–145°C (Ward and Young, 1988). ^{1}H NMR ($CDCl_3$): 1.07, s, 9H; 1.27, s, 3H; 1.41, s, 3H; 1.48, s, 3H; 1.54, s, 3H; 3.13, dd (J 8.7, 9.9 Hz), 1H; 3.66–3.76, m, 1H; 3.80–3.96, m, 2H; 3.97–4.10, m, 2H; 7.20–7.56, m, 6H; 7.80, d (J 9.0 Hz), 2H; 7.84, d (J 9.0 Hz), 2H.

2.3. 3-O-[(1,1-dimethylethyl)diphenylsilyl]-6-O-(D,L-1-ethoxyethyl)-1,2:4,5-bis-O-(1-methylethylidene)-D,L-myo-inositol **5**

To a solution of alcohol **4** (9.0 g, 18 mmol) and ethyl vinyl ether (34.57 ml, 0.36 mol) in dry methylene chloride (90 ml), pyridinium *p*-toluenesulfonate (PPTs) (1.97 g, 7.88 mmol) was added while stirring at RT. The mixture was kept at RT for 30 min. Then the solution was diluted with ether (120 ml), washed with 5% $NaHCO_3$ (40 ml), brine (30 ml) and dried over Na_2SO_4. The solvents



Scheme 2. Synthesis of NPIP; i – $(MeO)_2CMe_2$/TsOH/DMF; ii – B_2Cl/Py/DMF; iii – NaOH/MeOH; iv – $^tBuPh_2SiCl$/imidazole/Py; v – ethyl vinyl ether/PPTs/CH_2Cl_2; vi – $^nBu_4NF$/THF; vii – H_2O/Py; viii – AcOH/H_2O.

were evaporated in vacuo to give the silyl derivative **5** as an oil.

The crude product was purified by column chromatography on 250 g of silica gel. The impurities were eluted with 10:1 hexane/ether mixture. Silyl derivative **5** was eluted with 10:2 hexane/ether mixture. The eluate was evaporated in vacuum to give desired product **5** (diastereomeric mixture) as a clear oil, solidified at RT (6.8 g, 66%). M.p. 146–147°C, ^1H NMR ($CDCl_3$): 1.06, s, 18H; 1.16, t (J 6.9 Hz), 3H; 1.17, t (J 6.9 Hz), 3H; 1.23–1.30, m, 12H; 1.36, s, 3H; 1.38, s, 3H; 1.43, s, 3H; 1.44, s, 3H; 1.54, s, 3H; 3.06–3.16, m, 2H; 3.42–3.57, m, 2H; 3.62–4.08, m, 12H; 4.93–5.01, m, 2H; 7.20–7.43, m, 12H; 7.78–7.85, m, 8H.

2.4. 6-O-(D,L-1-ethoxyethyl)-1,2:4,5-bis-O-(1-methylethylidene)-D,L-myo-inositol **6**

To a solution of silyl derivative **5** (6.8 g, 12 mmol) in THF (7 ml), was added 1 M solution of tetrabutylammonium fluoride (17 ml). The resulting solution was kept at RT for 20 h, then THF was evaporated. The residue was partitioned between ethyl ether (30 ml) and water (20 ml). The ether layer was removed, and the aqueous solution was washed with ether (2 × 15 ml). The combined organic extracts were washed with brine, dried over Na_2SO_4 and evaporated to yield alcohol **6** as a mixture with *tert*-butyldiphenylsilyl fluoride that was used for the next step without any purification. ^1H NMR ($CDCl_3$): 1.18, t (J 6.9 Hz), 3H; 1.19, t (J 6.9 Hz), 3H; 1.32–1.37, m, 12H; 1.40, s, 3H; 1.41, s, 3H; 1.43, s, 3H; 1.44, s, 3H; 1.53, s, 3H; 1.54, s, 3H; 3.26–3.36, m, 2H; 3.40–4.12, m, 12H; 4.43, t (J 4.5 Hz), 1H; 4.44, t (J 4.5 Hz), 1H; 4.97–5.05, m, 2H. (signals of $^tBuPh_2SiF$: 1.06, s; 7.32–7.37, m, 7.72–7.75, m).

2.5. 6-O-(D,L-1-ethoxyethyl)-1,2:4,5-bis-O-(1-methylethylidene)-D,L-myo-inositol 3-(4-nitrophenyl hydrogen phosphate) ammonium salt **7**

4-Nitrophenyl phosphorodichloridate (9.16 g,

35.8 mmol) was dissolved in dry Py (100 ml), then the solution of alcohol **6** (crude product from above experiment, 3.95 g, 11.2 mmol) in Py (10 ml) was added while stirring and cooling (ice/water bath). The mixture was stirred for 16 h at ambient temperature, then water (20 ml) was added while cooling (ice/water bath). Stirring was continued for approximately 1 h, then the reaction mixture was diluted with chloroform (250 ml). The aqueous layer was separated and the chloroform solution was dried over anhydrous sodium sulfate. The clear solution obtained was passed through a column of silica gel and the column was further eluted with ethyl ether to remove Py. The column was then eluted with chloroform/methanol/ammonium hydroxide (7:3:0.1) mixture. The solution collected was evaporated in vacuum to obtain the ammonium salt of phosphate **7** (diastereomeric mixture) as a white solid (5.0 g, 76%). ^{1}H NMR (dimethyl sulfoxide (DMSO)-d6): 0.94, s, 6H; 1.04, t (J 7.2 Hz), 6H; 1.16, d (J 4.8 Hz), 3H; 1.17, d (J 5.1 Hz), 3H; 1.28, s, 12H; 1.31, s, 3H; 1.34, s, 3H; 3.99, t (J 5.7 Hz), 2H; 4.04–4.18, m, 2H; 4.35, t (J 4.5 Hz), 2H; 4.48–4.60, m, 2H; 4.82, q (J 5.4 Hz), 1H; 4.87, q (J 5.1 Hz), 1H; 7.34, d (J 9.0 Hz), 2H; 8.11, d (J 9.3 Hz), 2H.

2.6. D,L-myo-Inositol 1-(4-nitrophenyl hydrogen phosphate) ammonium salt *1*

4-Nitrophenyl phosphate **7** (5.0 g, 9.0 mmol) was suspended in 1:4 acetic acid/water solution (230 ml) and stirred at RT for 24 h. The clear solution obtained was extracted with ether (3 × 40 ml) and the aqueous solution was evaporated to dryness by azeotropic evaporation with a 1:1 ethanol-toluene mixture in vacuum to obtain the ammonium salt of NPIP **1** as a white solid (4.5 g, 98%). ^{1}H NMR (D_2O): 3.15, t (J 9.0 Hz), 1H; 3.38, dd (J 2.4, 9.9 Hz), 1H; 3.48, t (J 9.6 Hz), 1H; 3.61, t (J 9.6 Hz), 1H; 3.94, dt (J 2.4, 8.7 Hz), 1H; 4.10, br.s, 1H; 7.22, d (J 9.0 Hz), 2H; 8.09, d (J 9.0 Hz), 2H.

3. Results and discussion

In this revised procedure, two of the steps of the original synthesis of NPIP were improved. As before, the diol **3** was prepared from commercially available *myo*-inositol **2**, using a procedure originally reported by Gigg et al. (1985). Next, instead of protection with benzoyl chloride (BzCl) which we employed previously, the hydroxyl group at the first position was regioselectively protected using *tert*-butyldiphenylsilyl chloride by a modification of the procedure of Ward and Young (1988) to produce the key intermediate **4** in 83% yield. Silylation improved the regioselectivity, producing a higher yield of the penta-protected inositol and eliminating the necessity of chromatographic purification at this step.

In order to protect the last free OH group of alcohol **4**, we selected the readily available and inexpensive ethyl vinyl ether instead of 5,6-dihydro-4-methoxy-2*H*-pyran which was used previously. If desired, the racemic alcohol **4** can be prepared in enantiomerically pure form as reported previously (Leigh et al., 1992) and converted into the D and L enantiomers of NPIP by the present procedure. However, it has been shown that *B. cereus* PI-PLC is essentially stereospecific for the D enantiomer. The L enantiomer is neither a substrate nor an inhibitor (Leigh et al., 1992). Therefore, the racemic mixture can be used in many situations without going to the extra effort of separating the enantiomers.

Continuing from **4**, compound **5** was desilylated with ^{n}Bu$_4$NF to produce the alcohol **6** in quantitative yield. Alcohol **6** was phosphorylated with 4-nitrophenyl phosphorodichloridate as before to yield phosphate **7**. The final deprotection of phosphate **6** gave the desired final product **1** (NPIP). To summarize, the procedure reported here is more convenient, because it eliminates the chromatographic separation step, utilizes a less expensive reagent for protection of the 4-OH, and also results in a higher yield of NPIP.

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