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SYNTHESIS OF ENANTIOMERICALLY PURE PHOSPHOROTHIOLATE ASSAY SUBSTRATE FOR PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C

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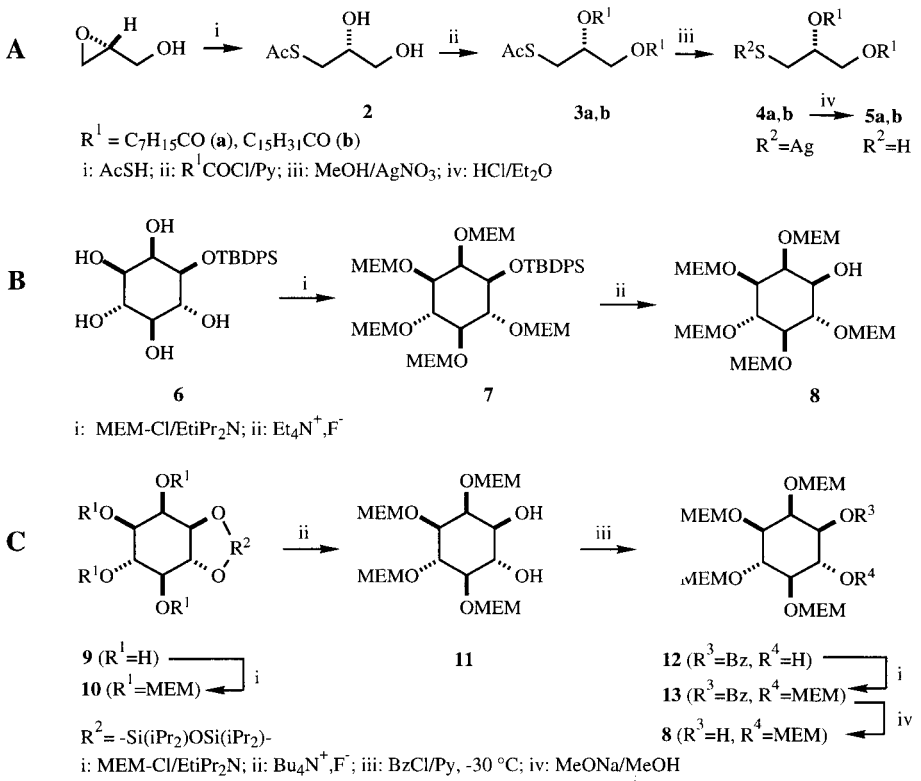
Abstract: An optimized synthesis of enantiopure (2*R*)-1,2-dioctanoyloxy- and (2*R*)-1,2-dipalmitoyloxypropane-thiophospho-(1D-*myo*-inositol) is reported starting from *R*-glycidol and the readily available 1D-1-*tert*-butyldiphenylsilyl-*myo*-inositol. The key synthesis of the phosphothiolester bond is carried out by the phosphoramidite chemistry.

Phosphatidylinositol-specific phospholipase C (PI-PLC) is one of the key enzymes involved in cellular signal transduction.[1] Studies of the role of this enzyme in signaling events, and investigation of its chemical mechanism necessitate precise measurement of activity under a variety of conditions. A number of assay substrates have been synthesized toward this end[2] including radioactive[3], fluorescent,[4] and chromogenic[5,6] analogs of phosphatidylinositol. Among these, the phosphorothiolate analogs[6] of PI are especially convenient for kinetic studies,[7] and are particularly noteworthy since they allow a continuous assay and feature a relatively minor modification of the natural substrate, therefore they are suitable for probing structure-function relationship of the PI-PLC active site.[8] Due to the interfacial nature of the enzyme reaction[5,9] and the aggregated nature of the natural substrate, the assay substrate should be synthesized in the optically pure form to eliminate uncertain effects of the unnatural diastereomers on the property of the water-lipid interface. The preliminary study from another laboratory[10] suggested that despite a relative nonstereospecificity of PI-PLC with regard to the structure and configuration of the hydrophobic component of the substrate,[11] the inclusion of the full structure of the diacyloxypropyl moiety in the thiol analog can increase the rate of the enzymatic cleavage several-fold. This communication presents an expedient route to such optically pure assay substrates. In contrast to previous syntheses using phosphotriester[6c] or Arbuzov[6a,d,10] chemistries we employed the phosphoramidite methodology, widely used for synthesis of oligonucleotides[12] including thiol analogs. The difficulty with regard to synthesis of the thiol analog of PI, as compared to synthesis of thiol analogs of oligonucleotides, is due to the presence of the alkali-labile diacyloxypropyl residue, limiting the range of reagents suitable for protection-deprotection of inositol and phosphate moieties.

Synthesis of the starting materials is shown in Scheme 1. (2*R*)-Glycidol was treated with thiolacetic acid at 4 °C for 1 week to give exclusively the thiolacetate **2**. Further acylation of the crude thiolester **2** with octanoyl chloride afforded the triester **3a** (86%). The regioselectivity of the deacetylation of the triester **3a** with several nucleophilic reagents was investigated. The aminolysis of **3a** with NH_4OH/dioxane or butylamine/toluene gave ca. 50% yields of the thiol **4a**, with a competing cleavage of the octanoyl groups as a side-reaction; the KCN-catalyzed ethanolysis (KCN/95% EtOH) afforded even lower yields, and no reaction was observed with TBAF/methanol. In sharp contrast, a complete regioselectivity of the deacetylation was achieved using silver-catalyzed methanolysis.[13] The reaction of the triester **3a** with methanol in the presence of silver nitrate produced the corresponding silver thiolate **4a** as a solid precipitate (91%). The subsequent neutralization of the thiolate with dry HCl in ether produced the pure thiol **5a** (80%). The product thus produced was less susceptible to air oxidation than the analogous product obtained by the alkali-catalyzed deacetylation.

Scheme 1

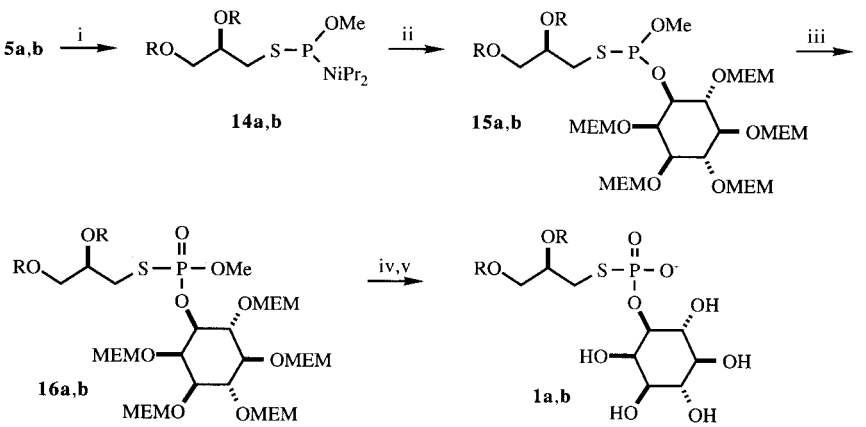


1D-(1-*tert*-Butyldiphenylsilyl)-*myo*-inositol (**6**, Scheme 1B), obtained in three steps from inositol,[14] was exhaustively protected with methoxyethoxymethylene (MEM) chloride in the presence of diisopropylethylamine in DMF at 50 °C to afford the fully protected derivative **7** (82%), which after desilylation with tetraethylammonium fluoride gave 2,3,4,5,6-pentaMEM-*myo*-inositol **8** (94%). Alternatively, the alcohol **8** was prepared from the 1D-[1,6-(1,1,3,3-tetraisopropyldisiloxanedi-1,3-yl)]-*myo*-inositol[14] (**9**, Scheme 1C) by the exhaustive protection with MEM-Cl in DMF/EtiPr$_2$N, the subsequent removal of the silyl groups in the fully protected derivative **10** with TBAF/THF to give the diol **11** (86% in two steps), the regioselective low-temperature benzoylation of the diol **11** at the 1-position to give the benzoate **12**, MEMylation of the alcohol **12** to give the derivative **13** (80%), and its alkaline methanolysis to give the alcohol **8** (85%).

The two components, **5** and **8**, were coupled using the standard phosphoramidite chemistry as shown in Scheme 2. Thus, the treatment of the thiol **5a** with *P*-chloro-*N,N*-diisopropyl-*O*-methyl-phosphoramidite resulted in the formation of the thiophosphoramidite **14a** in a quantitative yield, as demonstrated by two ^{31}P NMR signals of the crude product at 166.5 and 166.4 ppm.[15] This product was treated without purification[16] with the solution of the alcohol **8** and tetrazole in anhydrous THF during 24 h to afford essentially quantitatively the thiophosphite **15a** as a mixture of diastereomers (^{31}P NMR, δ 196.9 and 191.9 ppm). The subsequent oxidation of this mixture with a solution of N_2O_4 in chloroform proceeded with a relatively low yield (45%) to give the ca. 1:1 mixture of diastereomers **16a** (^{31}P NMR δ 29.6 and 29.04 ppm). A higher yield was obtained using tetrabutylammonium periodate as an oxidant[17] (65%). The triester **16a** was

readily purifiable by the routine silica gel chromatography. The final deprotection of the triester **16a** could be achieved in one step using ethanethiol/BF_3 etherate to yield the phosphorothiolate **1a**.[18] Better results, however, were obtained using a two step procedure: (i) dealkylation of the triester **16a** with anhydrous trimethylamine followed by (ii) the exhaustive cleavage of the MEM groups with ethanethiol/BF_3 etherate. Synthesis of the dipalmitoyl derivative **1b** proceeded analogously starting from the dipalmitoylthiol **5b**.

Scheme 2



Assay of PI-PLC with Thiolesters 1a and 1b

Both substrates **1a** and **1b** were used in a continuous assay of PI-PLC from *B. thuringiensis*. As expected, the thiolester **1b** showed a higher V_{max} than the analogous single chain substrate, hexadecylthiophosphoinositol,[6a,b] when used in the presence of a zwitterionic detergent, hexadecylphosphocholine (HDPC). In contrast to **1b**, the substrate **1a** displayed a linear kinetics of cleavage without the use of detergent[19] with ca. 6-fold higher V_{max} than that of **1b** (Figure 1). We therefore conclude that **1a** is a preferred substrate for the continuous assay of the bacterial PI-PLC.

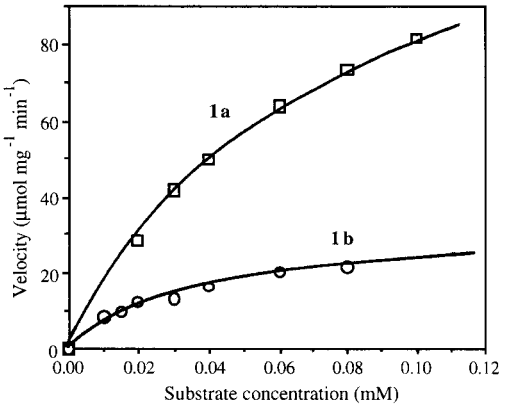


Figure 1. Concentration dependence of the cleavage rates of **1a** and **1b** by PI-PLC from *B. thuringiensis*. Assay conditions: **1b**; 4:1 ratio of HDPC to **1b**; 1 mM 4,4'-dithiobispyridine; 50 mM MOPS buffer, pH 7.2, 1.12 µg/mL PI-PLC, 1 mL total assay volume, temperature 25 °C. **1a**; no HDPC was used, other conditions were the same as above. The calculated V_{max} for **1a** and **1b** were 167 and 25 µmol mg^{-1} min^{-1}, respectively. The corresponding apparent K_m values were 75 and 33 µmol, respectively,[20] The cmc of **1a** measured by a rhodamine incorporation method was 35 µmol. No significant cleavage rate with either substrate was observed in the absence of enzyme.

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15. The application of the racemic chlorophosphoramidite for phosphitylation of thiols **5** results in the formation of ca. 1:1 mixtures of diastereomers of all **14**-**16** derivatives, giving rise to duplication of signals in the ^{31}P NMR spectra. The chirality at the phosphorus atom is removed during demethylation of the thioltriester **16**.

16. The attempts to purify the thiophosphoramidite **15a** by the silica gel chromatography, analogously to the oxygen bearing phosphoramidites, has failed due to its poor hydrolytic stability.

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18. **1a**: ^{1}H NMR (400 MHz, CD_3OD) δ 5.3 (m, 1H), 4.46 (dd, 1H), 4.23 (tr, 1H), 3.98 (ddd, 1H), 3.78 (m, 2H), 3.63 (m, 2H), 3.38 (m, 2H), 3.04 (dd, 2H), 2.32 (tr, 4H), 1.61 (m, 4H), 1.31 (m, 16H), 0.91 (tr, 6H); ^{31}P NMR (CD_3OD) δ 19.83 ppm; ESMS: *m/z* 601 ($M+H^+$). **1b**: ESMS: *m/z* 825 $M+H^+$); other data were essentially identical to those of **1a**, except for the integration of the hydrocarbon protons in the ^{1}H NMR spectrum.

19. The substrate **1b** could not be tested without detergent due to its insolubility in the aqueous phase. In contrast, the substrate **1a** dispersed with HDPC displayed significant decrease of the cleavage rate following the initial few percent of conversion.

20. The K_m value for the substrate **1a** appears to be slightly smaller than that for vesicular dimirystoyl thiol analog (0.2 mM) reported recently.[10]

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