Substrate Stereospecificity of Phosphatidylinositol-Specific Phospholipase C from *Bacillus cereus* Examined Using the Resolved Enantiomers of Synthetic *myo*-Inositol 1-(4-Nitrophenyl phosphate)†

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ABSTRACT: The substrate stereospecificity of phosphatidylinositol-specific phospholipase C from *Bacillus cereus* is examined using the resolved optical isomers of synthetic *myo*-inositol 1-(4-nitrophenyl phosphate), a chromogenic substrate for the phospholipase. The synthetic route employs mild acid-labile protecting groups and separation of the substituted *myo*-inositol enantiomers as the (–)-camphanyl ester diastereomers. Measurements of the initial rates of cleavage of the D and L enantiomers of the nitrophenyl substrate by phosphatidylinositol-specific phospholipase C from *B. cereus* show that this enzyme is essentially stereospecific for the D enantiomer. Under identical conditions, the rate of cleavage of the L isomer is less than 0.2% of that observed for the D isomer. The same is observed for the highly homologous enzyme from *Bacillus thuringiensis*. There is no measurable inhibition by the L enantiomer of the *B. cereus* enzyme acting on the D enantiomer, even when the molar ratio of L:D is 5, indicating that binding of the L enantiomer to the phospholipase is negligible. Thus, the enzyme active site is exquisitely sensitive to the stereochemistry of the *myo*-inositol group of the substrate.

Phosphatidylinositol-specific phospholipase C (PI-PLC)[1] (EC 3.1.4.10) catalyzes cleavage of the phosphodiester bonds of inositol phospholipids, producing diacylglycerol and inositol phosphates. PI-PLCs are ubiquitous enzymes found intracellularly in eukaryotes (Rhee et al., 1989), as ectoenzymes at the cell surface of certain mammalian cultured cells (Ting & Pagano, 1990; Volwerk et al., 1992), and as secreted enzymes in the culture media of an increasing number of microbes (Griffith et al., 1991; Low, 1981; Taguchi & Ikezawa, 1987; Leimeister-Wächter et al., 1991; Jäger et al., 1991). Intracellular PI-PLCs are calcium ion-dependent and are involved in phosphoinositide signal transduction pathways producing the second messengers diacylglycerol and inositol trisphosphate following activation by G-proteins (Smrcka et al., 1991; Moriarty et al., 1990) or by tyrosine phosphorylation (Nishibe et al., 1990). The physiological role of the calcium-dependent PI-PLC found at the external cell surface has yet to be defined. However, in mouse fibroblasts its activity is cell density dependent, suggesting a possible role in signalling mechanisms related to density-dependent growth control (Ting & Pagano, 1991; Volwerk et al., 1992).

In contrast to the mammalian enzymes, bacterial PI-PLCs have no metal ion dependence and do not cleave the phosphorylated forms of PI, i.e., phosphatidylinositol 4-phosphate and 4,5-bisphosphate. However, the bacterial phospholipases are unique in their ability to cleave, in addition to PI, the glycosyl-PI moieties of membrane protein anchors (for a recent review, see Ikezawa, 1991). There is some evidence suggesting a possible role of the bacterial PI-PLC as a virulence factor (Camilli et al., 1991; Mengaud et al., 1991; Marques et al., 1989). Calcium-independent phospholipases C specific for glycosyl-PI have been purified from *Trypanosoma brucei* (Fox et al., 1986) and rat liver (Fox et al., 1987). The latter has been implicated in insulin action (Fox et al., 1987).

Delineating the functional role of the various PI-PLCs within their biological context requires a detailed understanding of the mechanism of action of these enzymes. With this purpose in mind we have focussed on the design and chemical synthesis of a series of inositol phospholipid analogues for enzymological studies of PI-PLCs. An approach of this type can be very fruitful as is indicated by the progress made in understanding the mechanism of action of another lipolytic enzyme, phospholipase A_2 (for example, see Dennis, 1987; De Haas et al., 1990; Gelb et al., 1991). Earlier we reported the synthesis and application of PI analogues as inhibitors of bacterial PI-PLC (Shashidhar et al., 1990a–c), as well as novel fluorescent (Shashidhar et al., 1991a) and chromogenic (Shashidhar et al., 1991b) substrate analogues suitable for the continuous assay and kinetic analysis of PI-PLCs. However, the fluorescent substrate, racemic *myo*-inositol 1-(2-naphthyl phosphate), was found to be cleaved very slowly by PI-PLC from *Bacillus cereus* (Shashidhar et al., 1991a), as was a racemic thiophosphate-containing PI analogue (Hendrickson et al., 1991). The chromogenic substrate, racemic *myo*-inositol 1-(4-nitrophenyl phosphate), on the other hand, proved to be rapidly cleaved by *B. cereus* PI-PLC (Shashidhar et al., 1991b), thus providing a good starting point for further development of synthetic substrates.

Here we examine for the first time the enantioselectivity of PI-PLC from *B. cereus* with respect to the chirality of the *myo*-inositol moiety of the substrate. For this purpose the resolved D and L enantiomers of the chromogenic substrate, *myo*-inositol 1-(4-nitrophenyl phosphate), were synthesized. We find that the phospholipase is highly selective both in the cleavage and the binding of these optical isomers.

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[1] Abbreviations: PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; NPIP, *myo*-inositol 1-(4-nitrophenyl phosphate); BSA, bovine serum albumin; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; PPTs, pyridinium *p*-toluenesulfonate; TBAF, tetrabutylammonium fluoride; s, singlet; d, doublet; t, triplet; m, multiplet; dd, doublet of doublet.

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MATERIALS AND METHODS

PI-PLC from *B. cereus* or *Bacillus thuringiensis* was isolated as previously reported (Koke et al., 1991). The amount of enzyme used in experiments was as determined by Bradford's method (Bradford, 1975). ^{1}H NMR spectra were recorded on a Nicolet QE-300 spectrometer, optical rotations were determined using a Perkin-Elmer 141 polarimeter, and melting points are uncorrected. Magnesium sulfate was used as drying agent, and all column chromatography was performed using 200–425-mesh silica gel with the elution of products monitored by TLC. TLC was conducted on Kieselgel 60 F_{254} plates, and spots were rendered visible either by exposing the plates to UV light or by spraying with a solution of 5% (w/v) vanillin, 5% (v/v) concentrated sulfuric acid in ethanol and heating the plates in an oven to 140 °C.

1-O-(tert-Butyldiphenylsilyl)-4-O-camphanyl-2,3:5,6-di-O-isopropylidene-D-*myo-inositol* (**1**) *and 1-O-(tert-Butyldiphenylsilyl)-4-O-camphanyl-2,3:5,6-di-O-isopropylidene*-L-*myo-inositol* (**2**). To a solution of racemic 1-*O*-(*tert*-butyldiphenylsilyl)-2,3:5,6-di-*O*-isopropylidene-*myo*-inositol (1.16 g, 2.33 mmol) (Ward & Young, 1988) and dry pyridine (0.28 mL, 3.46 mmol) in anhydrous dioxane (13 mL) was added (–)-camphanic acid chloride (0.73 g, 3.37 mmol), and the mixture was stirred at room temperature for 4 h. Further (–)-camphanic acid chloride (0.24 g, 1.12 mmol) and pyridine (0.09 mL, 1.15 mmol) were added, and the reaction mixture was stirred for 16 h. Diethyl ether (70 mL) was added, and the organic layer was extracted with aqueous sodium chloride (20 mL) and then water (20 mL). The dried organic phase was evaporated and the residue purified by column chromatography to yield a mixture of the diastereomers **1** and **2** (1.44 g, 91%). The diastereomers were separated by preparative HPLC utilizing a 25 × 2.14 cm Dynamax 60A column (SiO_2, 8 μm) with 7% tetrahydrofuran/hexane as solvent. The less polar isomer **1** was obtained as a white solid and recrystallized from hexane: mp 179–179.5 °C, $[\alpha]^{20}_D$ +4.2° (c = 1 in MeCN) (Young et al., 1990, mp 179–180 °C, $[\alpha]^{20}_D$ +4.4° (c = 1.54 in MeCN)); ^{1}H NMR ($CDCl_3$) δ 0.97 (s, 3 H), 1.04 (s, 3 H), 1.11 (s, 9 H), 1.12 (s, 3 H), 1.20 (s, 3 H), 1.38 (s, 3 H), 1.49 (s, 3 H), 1.63 (s, 3 H), 1.70 (s, 1 H), 1.90 (s, 1 H), 2.06 (s, 1 H), 2.46 (m, 1 H), 3.28 (dd, J = 9.3, 10.8 Hz, 1 H), 3.95 (m, 2 H), 4.04 (m, 1 H), 4.19 (dd, J = 9.3, 10.0 Hz, 1 H), 5.33 (dd, J = 6.3, 10.8 Hz, 1 H), 7.36–7.46 (m, 6 H), 7.78–7.84 (m, 4 H).

The more polar isomer **2** was obtained as a white solid and recrystallized from hexane: mp 189–190 °C, $[\alpha]^{20}_D$ –10.2° (c = 1 in MeCN) (Young et al., 1990, mp 192–193.5 °C, $[\alpha]^{20}_D$ –9.0° (c = 0.62 in MeCN)); ^{1}H NMR ($CDCl_3$) δ 0.93 (s, 3 H), 0.99 (s, 3 H), 1.08 (s, 9 H), 1.09 (s, 3 H), 1.26 (s, 3 H), 1.36 (s, 3 H), 1.46 (s, 3 H), 1.60 (s, 3 H), 1.65 (s, 1 H), 1.89 (s, 1 H), 2.04 (s, 1 H), 2.40 (m, 1 H), 3.25 (dd, J = 9.3, 11.1 Hz, 1 H), 3.85 (dd, J = 4.5, 6.9 Hz, 1 H), 3.91 (dd, J = 3.9, 4.5 Hz, 1 H), 4.00 (dd, J = 3.9, 9.6 Hz, 1 H), 4.15 (dd, J = 9.3, 9.6 Hz, 1 H), 5.36 (dd, J = 6.9, 11.1 Hz, 1 H), 7.34–7.44 (m, 6 H), 7.78–7.84 (m, 4 H).

1-O-(tert-Butyldiphenylsilyl)-2,3:5,6-di-O-isopropylidene-D-*myo-inositol* (**3**). To a solution of **1** (0.370 g, 0.546 mmol) in ethanol (30 mL) was added potassium hydroxide (1.03 g, 18.3 mmol) in ethanol (45 mL), and the mixture was stirred for 30 min until no starting material was observed by TLC. Glacial acetic acid (1.06 mL, 18.3 mmol) was added, and the solvent was removed in vacuo. Aqueous sodium bicarbonate (30 mL) was added, the aqueous solution was extracted with diethyl ether (30 mL), and the dried organic phase was evaporated. The residue was purified by column chromatography to yield **3** as a white solid, which was recrystallized from hexane (0.255 g, 94%): mp 127–128 °C (Young et al., 1990, mp 127–128 °C); ^{1}H NMR ($CDCl_3$) δ 1.07 (s, 9 H), 1.27 (s, 3 H), 1.42 (s, 3 H), 1.49 (s, 3 H), 1.55 (s, 3 H), 2.31 (d, J = 2.7 Hz, 1 H), 3.13 (dd, J = 8.7, 10.5 Hz, 1 H), 3.72 (dd, J = 4.8, 6.6 Hz, 1 H), 3.84–3.94 (m, 2 H), 4.02–4.05 (m, 2 H), 7.34–7.44 (m, 6 H), 7.79–7.85 (m, 4 H).

1-O-(tert-Butyldiphenylsilyl)-2,3:5,6-di-O-isopropylidene-L-*myo-inositol* (**4**). Camphanate ester **2** (0.317 g, 0.47 mmol) yielded the required alcohol **4** (0.220 g, 94%), mp 126–127 °C (Young et al., 1990, mp 127–129 °C) using a procedure similar to that for **3**: ^{1}H NMR ($CDCl_3$) δ 1.07 (s, 9 H), 1.27 (s, 3 H), 1.42 (s, 3 H), 1.49 (s, 3 H), 1.55 (s, 3 H), 2.32 (d, J = 2.7 Hz, 1 H), 3.13 (dd, J = 8.7, 10.8 Hz, 1 H), 3.72 (dd, J = 4.8, 6.6 Hz, 1 H), 3.83–3.93 (m, 2 H), 4.02–4.05 (m, 2 H), 7.34–7.46 (m, 6 H), 7.79–7.86 (m, 4 H).

1-O-(tert-Butyldiphenylsilyl)-4-O-(4-methoxytetrahydropyran-4-yl)-2,3:5,6-di-O-isopropylidene-D-*myo-inositol* (**5**). To a solution of **3** (0.255 g, 0.51 mmol) and pyridinium *p*-toluenesulfonate (0.031 g, 0.123 mmol) in dry dichloromethane (3 mL) containing 4A molecular sieves was added 5,6-dihydro-4-methoxy-2*H*-pyran (0.580 g, 5.18 mmol), and the mixture was stirred for 1 h. Further 5,6-dihydro-4-methoxy-2*H*-pyran (0.580 g, 5.18 mmol) was added and the reaction mixture stirred at room temperature for 16 h. The solids were filtered off, and diethyl ether (20 mL) was added and extracted with saturated aqueous sodium bicarbonate solution (10 mL) and water (10 mL). The organic layer was dried and evaporated in vacuo to yield a residue which was purified by column chromatography to afford **5** as a viscous gum (0.247 g, 79%): ^{1}H NMR ($CDCl_3$) δ 1.07 (s, 9 H), 1.26 (s, 3 H), 1.36 (s, 3 H), 1.42 (s, 3 H), 1.55 (s, 3 H), 1.70–2.00 (m, 4 H), 3.11 (dd, J = 8.7, 10.5 Hz, 1 H), 3.27 (s, 3 H), 3.60–3.77 (m, 5 H), 3.88–4.03 (m, 4 H), 7.34–7.43 (m, 6 H), 7.79–7.87 (m, 4 H).

1-O-(tert-Butyldiphenylsilyl)-4-O-(4-methoxytetrahydropyran-4-yl)-2,3:5,6-di-O-isopropylidene-L-*myo-inositol* (**6**). Alcohol **4** (0.220 g, 0.44 mmol) was protected in a fashion similar to that for **5** to give the required product **6** (0.262 g, 97%) containing a trace impurity: ^{1}H NMR ($CDCl_3$) δ 1.05 (s, 9 H), 1.24 (s, 3 H), 1.34 (s, 3 H), 1.40 (s, 3 H), 1.53 (s, 3 H), 1.68–1.95 (m, 4 H), 3.09 (dd, J = 9.0, 10.5 Hz, 1 H), 3.25 (s, 3 H), 3.56–3.76 (m, 5 H), 3.87–4.02 (m, 4 H), 7.31–7.41 (m, 6 H), 7.77–7.84 (m, 4 H).

4-O-(4-Methoxytetrahydropyran-4-yl)-2,3:5,6-di-O-isopropylidene-D-*myo-inositol* (**7**). To a solution of silyl ether **5** (0.177 g, 0.29 mmol) in anhydrous tetrahydrofuran (3 mL) was added tetrabutylammonium fluoride (1.0 M solution in tetrahydrofuran, 1.80 mmol), and the reaction mixture was stirred at room temperature for 20 h. Water (10 mL) and diethyl ether (15 mL) were added and separated, and the organic phase was dried. The solvent was evaporated and the residue purified by column chromatography to afford **7** as a white solid which was recrystallized from hexane (0.079 g, 73%): mp 120–121 °C, $[\alpha]^{20}_D$ +30.2° (c = 1 in MeCN) (Young et al., 1990, mp 122–122.5 °C, $[\alpha]^{20}_D$ +34.9° (c = 0.53 in MeCN)); ^{1}H NMR ($CDCl_3$) δ 1.35 (s, 3 H), 1.41 (s, 3 H), 1.42 (s, 3 H), 1.55 (s, 3 H), 1.75–1.97 (m, 4 H), 2.29 (d, J = 6.3 Hz, 1 H), 3.30 (s, 3 H), 3.35 (m, 1 H), 3.59–3.84 (m, 5 H), 3.93–4.05 (m, 2 H), 4.13 (dd, J = 5.4, 5.7 Hz, 1 H), 4.46 (dd, J = 4.8, 5.1 Hz, 1 H).

4-O-(4-Methoxytetrahydropyran-4-yl)-2,3:5,6-di-O-isopropylidene-L-*myo-inositol* (**8**). Silyl ether **6** (0.100 g, 0.163 mmol) was deprotected in a manner identical to that for **5**, affording **8** after purification as a white solid which was recrystallized from hexane (0.051 g, 83%): mp 121–122 °C,

$[\alpha]^{20}_D$ −35.8° (c = 1, in MeCN) (Young et al., 1990, mp 122–123 °C, $[\alpha]^{20}_D$ −38.7° (c = 0.284 in MeCN)); ^{1}H NMR ($CDCl_3$) δ 1.35 (s, 3 H), 1.41 (s, 3 H), 1.42 (s, 3 H), 1.55 (s, 3 H), 1.75–2.00 (m, 4 H), 2.29 (d, J = 8.7 Hz, 1 H), 3.30 (s, 3 H), 3.33 (m, 1 H), 3.60–3.84 (m, 5 H), 3.95–4.05 (m, 2 H), 4.13 (dd, J = 5.4, 5.7 Hz, 1 H), 4.46 (dd, J = 4.8, 5.1 Hz, 1 H).

*4-O-(4-Methoxytetrahydropyran-4-yl)-2,3:5,6-di-O-isopropylidene-*D*-myo-inositol 1-(4-Nitrophenyl phosphate)* (**9**). To a solution of 4-nitrophenyl phosphorodichloridate (0.086 g, 0.33 mmol) in dry pyridine (2 mL) was added pentaprotected inositol **7** (0.042 g, 0.11 mmol), and the mixture was stirred at room temperature for 16 h. Water (0.3 mL) was added and stirring continued for 1 h. Chloroform (7 mL) was added and the organic phase separated, dried, and evaporated. The residue was purified by column chromatography to yield the pyridinium salt of **9** as a white solid (0.062 g, 86%): mp 186–189 °C dec; ^{1}H NMR (CD_3CN/DMSO-d_6) δ 0.99 (s, 3 H), 1.27 (s, 3 H), 1.28 (s, 3 H), 1.34 (s, 3 H), 1.62–1.80 (m, 4 H), 3.15 (s, 3 H), 3.30 (t, 1 H), 3.35–3.50 (m, 2 H), 3.52–3.64 (m, 2 H), 3.80–4.01 (m, 3 H), 4.45 (t, 1 H), 4.56–4.66 (m, 1 H), 7.25 (t, 2 H), 7.34 (d, J = 9.0 Hz, 2 H), 7.64 (t, 2 H), 8.09 (d, J = 9.0 Hz, 2 H), 8.50 (d, 1 H).

*4-O-(4-Methoxytetrahydropyran-4-yl)-2,3:5,6-di-O-isopropylidene-*L*-myo-inositol 1-(4-Nitrophenyl phosphate)* (**10**). Reaction of **8** (0.033 g, 0.09 mmol) with 4-nitrophenyl phosphorodichloridate (0.068 g, 0.27 mmol) in dry pyridine (1.5 mL) using the same procedure as for **9** afforded the pyridinium salt of **10** a white solid (0.047 g, 82%), mp 192–196 °C dec.

D-*myo-Inositol 1-(4-Nitrophenyl phosphate)* (**11**). 4-Nitrophenyl phosphate **9** (0.062 g, 0.09 mmol) was suspended in 1:4 acetic acid/water (5 mL) and stirred for 16 h at room temperature. The reaction mixture was extracted with diethyl ether (3 × 5 mL) and the aqueous solution lyophilized to give **11** as a white solid (0.030 g, 84%): mp 184–186 °C dec; ^{1}H NMR (D_2O) δ 3.34 (t, J = 9.3 Hz, 1 H), 3.56 (dd, J = 2.4, 9.9 Hz, 1 H), 3.68 (t, J = 9.3 Hz, 1 H), 3.80 (t, J = 9.6 Hz, 1 H), 4.12 (dt, J = 2.4, 9.3 Hz, 1 H), 4.29 (br s, 1 H), 7.42 (d, J = 9.0 Hz, 2 H), 8.29 (d, J = 9.0 Hz, 2 H).

L-*myo-Inositol 1-(4-Nitrophenyl phosphate)* (**12**). 4-Nitrophenyl phosphate **10** (0.047 g, 0.07 mmol) was deprotected in a manner identical to that for **9** to give **12** as a white solid (0.024 g, 87%): mp 182–184 °C dec; ^{1}H NMR (D_2O) δ 3.35 (t, J = 9.3 Hz, 1 H), 3.56 (dd, J = 2.4, 9.9 Hz, 1 H), 3.65 (t, J = 9.3 Hz, 1 H), 3.80 (t, J = 9.6 Hz, 1 H), 4.10 (dt, J = 2.4, 9.3 Hz, 1 H), 4.27 (br s, 1 H), 7.40 (d, J = 9.0 Hz, 2 H), 8.27 (d, J = 9.0 Hz, 2 H).

PI-PLC Activity Assay. The activity of bacterial phospholipase C toward D- and L-NPIP was measured spectrophotometrically as previously reported for racemic NPIP (Shashidhar et al., 1991b). All measurements were done at pH 7.0, which was found to be the optimum for enzyme activity and assay sensitivity, while nonenzymatic hydrolysis was negligible at pH 7.0 and below. Stock solutions of substrates were prepared and the concentrations determined as previously described (Shashidhar et al., 1991b). Assays were performed at ambient temperature (22 °C), and the buffer used was 100 mM Hepes/NaOH containing 1 mM EDTA. The presence of EDTA was required to correct for the 30–50% lower activity of substrate samples prepared from intermediates that had undergone HPLC separation compared to substrate samples prepared from intermediates that had not been subjected to HPLC. It is possible that during the HPLC procedure traces of metal ions are introduced that are not removed during subsequent synthetic steps and that are strongly inhibitory to the phospholipase. Inhibition of PI-PLC by metal ions has been observed previously (Sundler et al., 1978).

RESULTS AND DISCUSSION

Synthesis of D-*myo-Inositol 1-(4-Nitrophenyl phosphate* (**11**) *and* L-*myo-Inositol 1-(4-Nitrophenyl phosphate)* (**12**). There are several methods available in the literature (for example, see Reitz, 1991) for the separation of substituted *myo*-inositol enantiomers. During the course of the synthesis of racemic *myo*-inositol 1-(4-nitrophenyl phosphate) it was found that this compound was sensitive to strong acids and bases, thus precluding deprotection of the *myo*-inositol under these conditions (Shashidhar, 1991b). Also protecting groups that require removal by catalytic hydrogenation cannot be used because of concurrent reduction of the aromatic nitro group to the amine. Thus the key intermediates in the synthesis of the resolved D- and L-*myo*-inositol 4-nitrophenyl phosphate diesters were the pentaprotected inositols **7** and **8** in which the isopropylidene and 4-methoxytetrahydropyran-4-yl protecting groups could be removed by mild acid hydrolysis.

Utilization of a recently published HPLC method (Young et al., 1990) for the separation of substituted *myo*-inositol racemates required the synthesis of racemic 1-*O*-(*tert*-butyldiphenylsilyl)-4-*O*-camphanyl-2,3:5,6-di-*O*-isopropylidene-*myo*-inositol (**1**, **2**). This was prepared from *myo*-inositol via the protected 2,3:5,6-di-*O*-isopropylidene-*myo*-inositol (Gigg et al., 1985), which was then regioselectively silylated in the 1-position using *tert*-butyldiphenylsilyl chloride (Ward & Young, 1988) and converted to the mixture of **1** and **2** by reaction with (−)-camphanic acid chloride (Young et al., 1990) (Scheme I).

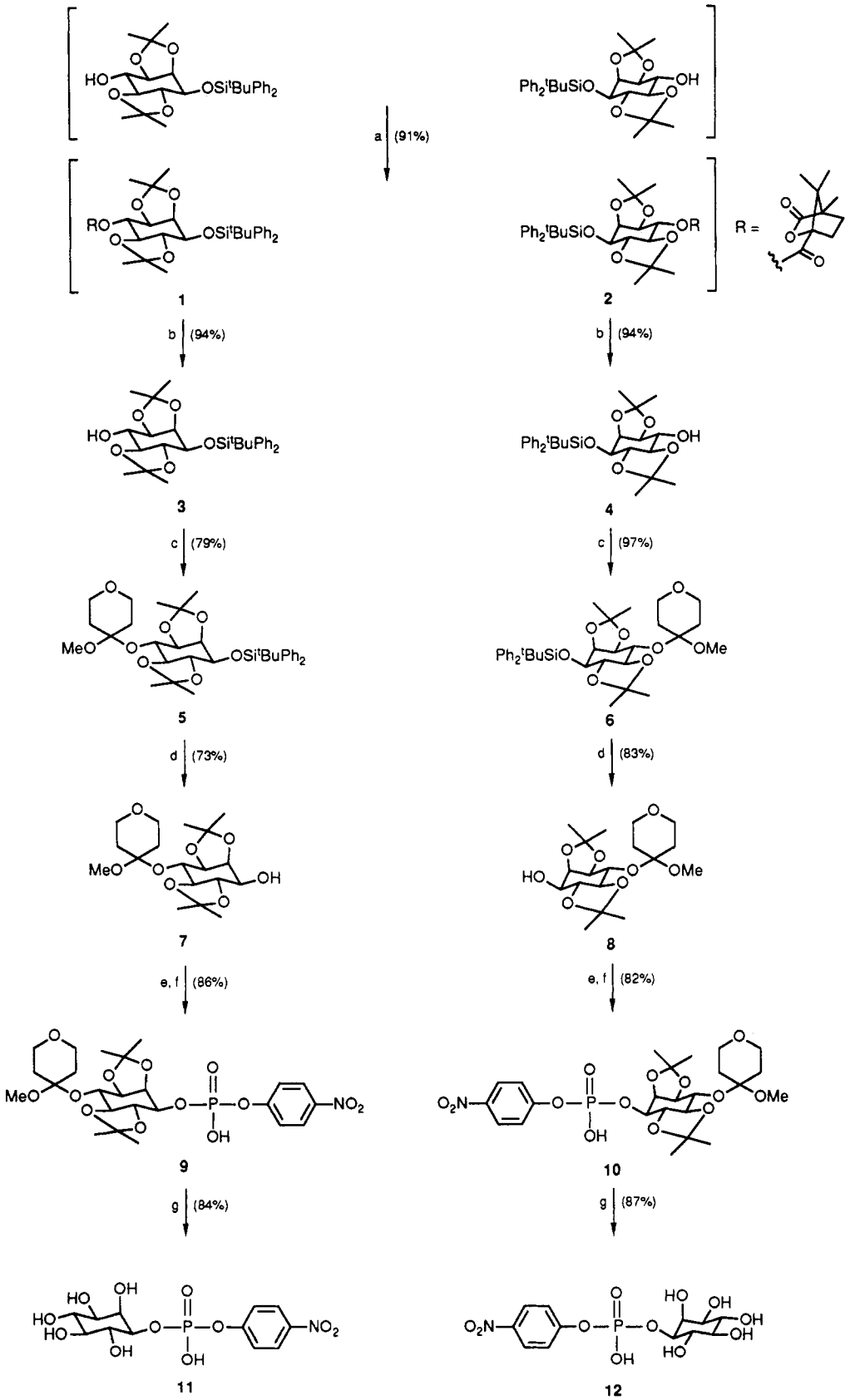
The less polar D isomer (retention time 52 min, 7% tetrahydrofuran/hexane) was obtained in pure form after one HPLC separation while the more polar L isomer (retention time 59 min) required rechromatography to remove a 5% impurity of the less polar isomer that remained after the initial separation. The absolute stereochemistry of the less polar diastereomer has been shown previously by X-ray crystallography to be 1-*O*-silyl-D-*myo*-inositol derivative **1** (Young et al., 1990).

The separated esters **1** and **2** were hydrolyzed with potassium hydroxide in ethanol to give the respective alcohols **3** and **4**. These were converted to their 4-methoxytetrahydropyran-4-yl derivatives **5** and **6**, which were subsequently desilylated using TBAF to give the chiral intermediates **7** and **8**. Condensation of these with 4-nitrophenyl phosphorodichloridate in pyridine (Shashidhar et al., 1991b) gave the expected phosphorochloridates, which on hydrolysis afforded the phosphodiesters **9** and **10**. The inositol protecting groups were removed by reaction with 1:4 acetic acid/water at room temperature to yield the required chiral *myo*-inositol 1-(4-nitrophenyl phosphates), D-NPIP (**11**) and L-NPIP (**12**) (Scheme I).

As reported earlier, NPIP is stable upon storage as a solid or frozen in mildly acidic aqueous solutions at −20 °C (Shashidhar et al., 1991b). Because the final enantiomerically pure products were obtained by lyophilization from aqueous solutions, preparations generally contained 10–20% water (by weight). Therefore, the concentrations of D- or L-NPIP stock solutions were routinely determined spectrophotometrically following complete alkaline hydrolysis as described previously (Shashidhar et al., 1991b).

Activity of PI-PLC toward D- *and* L-*NPIP.* The activity of PI-PLC from *B. cereus* toward the pure optical isomers of

Scheme I[a]



[a] (a) (–)-Camphanic acid chloride, pyridine, dioxane; (b) KOH, ethanol; (c) 5,6-dihydro-4-methoxy-2*H*-pyran, PPTs, dichloromethane; (d) TBAF, THF; (e) 4-nitrophenyl phosphorodichloridate, pyridine; (f) water; (g) 4:1 water/acetic acid.

NPIP was measured by monitoring spectrophotometrically the enzyme-catalyzed release of the intensely colored 4-nitrophenolate anion (Scheme II). Under the assay conditions used here, the other product formed is inositol 1,2-(cyclic)-monophosphate (Scheme II). Previously we reported that PI-PLC from *B. cereus* cleaves its substrates in two steps: inositol 1,2-(cyclic)monophosphate is produced in the first step, which is converted to inositol 1-monophosphate in the

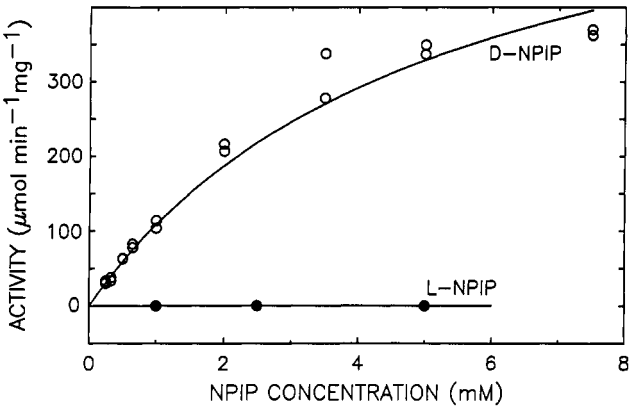
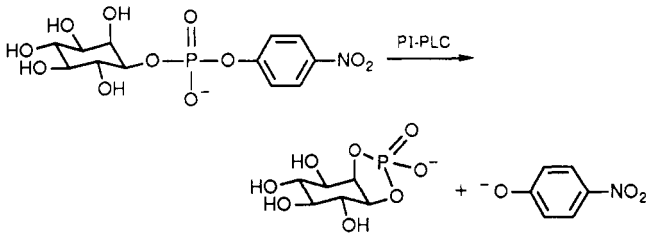


FIGURE 1: Velocity versus substrate concentration for the cleavage of the D (open symbols) and L (closed symbols) enantiomers of *myo*-inositol 1-(4-nitrophenyl phosphate) by PI-PLC from *B. cereus*. Initial rates were determined in duplicate at pH 7.0 (100 mM Hepes/NaOH containing 1 mM EDTA) and 22 °C by monitoring the production of nitrophenol spectrophotometrically at 397 nm. The molar extinction coefficient for nitrophenol used was 7700 M^{-1} cm^{-1} (Shashidhar et al., 1991b). The curved line is calculated from the Michaelis–Menten equation, $v = V_{max}S(K_M + S)^{-1}$, where v is the velocity and S is the substrate concentration, with V_{max} = 650 μmol min^{-1} mg^{-1} and K_M = 5.0 mM.

Scheme II: Cleavage of D-NPIP Catalyzed by PI-PLC



second step (Volwerk et al., 1990; Shashidhar et al., 1991a,b). However, the second reaction requires much higher enzyme concentrations and much longer reaction times than used in the present study. Figure 1 shows plots of the specific enzyme activity as a function of the concentration of D-NPIP or L-NPIP. In these experiments a strict proportionality between the initial rate and the enzyme concentration was always observed. From the data for D-NPIP, values for the kinetic parameters V_{max} and K_M were estimated by nonlinear least squares analysis and by extrapolation of a double-reciprocal plot of velocity versus substrate concentration. Values for these parameters that gave a reasonable fit (Figure 1) to the experimental data were V_{max} = 650 (±50) μmol min^{-1} (mg of protein)$^{-1}$ and K_M = 5 (±1) mM. Thus, the specific activity for the D-NPIP is about 35% of that for the natural substrate PI solubilized in sodium deoxycholate, where the apparent V_{max} is ca. 1800 μmol min^{-1} (mg of protein)$^{-1}$ (Koke et al., 1991). This observed activity for the synthetic D-NPIP enantiomer is between 1 and 2 orders of magnitude greater than that of the synthetic substrate, racemic *myo*-inositol 1-(*S*-hexadecyl phosphorothioate) (Hendrickson et al., 1991; V_{max} = 16.9 (±0.6) μmol min^{-1} (mg of protein)$^{-1}$), and 4 orders of magnitude greater than that of racemic *myo*-inositol 1-(2-naphthyl phosphate) (Shashidhar et al., 1991a; specific enzyme activity about 0.044 μmol min^{-1} (mg of protein)$^{-1}$ at a substrate concentration of 0.8 mM).

Under these same conditions, the activity of the *B. cereus* PI-PLC toward the L enantiomer of NPIP was less than 0.2% of the activity toward the D enantiomer (Figure 1). For example, at 5 mM L-NPIP the specific activity (observed with a 10-fold excess of enzyme) was 0.47 versus 340 μmol min^{-1} mg^{-1} for the D enantiomer. Similar data were obtained

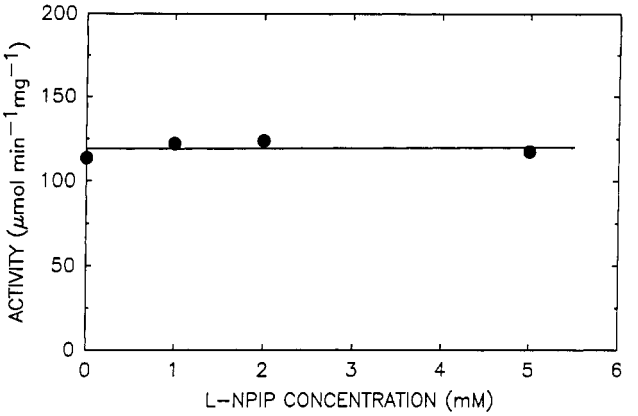


FIGURE 2: Effect of varying concentrations of L-NPIP on the activity of PI-PLC toward D-NPIP. The D-NPIP concentration was kept constant at 1 mM. Conditions were as described in Figure 1.

for the highly homologous enzyme from *Bacillus thuringiensis*. Although the HPLC results suggest that the NPIP enantiomers are optically pure, we cannot exclude the possibility that the minor activity observed with the L isomer is in fact due to the presence of a trace amount of D isomer in the preparation. We conclude that the *Bacillus* PI-PLCs are essentially stereospecific for the D-*myo*-inositol enantiomer of the substrate. This high degree of stereoselectivity is observed with a water-soluble synthetic substrate in which the leaving group (4-nitrophenol) differs substantially from the bulky hydrophobic *sn*-1,2-diacylglycerol group found in naturally occurring phosphatidylinositols.

Previous experiments on racemic mixtures (Shashidhar et al., 1991a,b; Volwerk et al., 1990) did not permit a direct comparison of the relative rates of the two enantiomers, nor did they indicate if the L enantiomers were inhibitory, for instance, by competing with the D enantiomer for binding to the enzyme active site. The question of inhibition by the L enantiomer was investigated by performing assays in which the concentration of the D substrate was kept constant at 1 mM, while the concentration of L-NPIP was varied between 0 and 5 mM. The results (Figure 2) show that there is no inhibition even at a 5-fold molar excess of L-NPIP over D-NPIP. Thus, there is no appreciable binding of the L enantiomer to the active site, indicating that the putative *myo*-inositol binding pocket is highly sensitive to the stereochemistry of the inositol ring system. Previously we found that high concentrations of *myo*-inositol but not *epi*-inositol were inhibitory to the *B. cereus* PI-PLC (Shashidhar et al., 1990b). Both of these observations point to strict geometric constraints on the interaction between the inositol moiety and the protein.

Until now the question of the stereospecificity of bacterial or mammalian PI-PLCs with respect to the chirality of the *myo*-inositol moiety of the substrate has not been addressed. Lin et al. (1989) report the synthesis of a mixture of the (R_p)- and (S_p)-enantiomers of a phosphorothioate analogue of PI containing a D-*myo*-inositol moiety. These derivatives were used to examine the steric course of the phosphodiester bond cleavage catalyzed by PI-PLCs from *B. cereus* and guinea pig uterus but provide no information on the enantioselectivity of these enzymes for the *myo*-inositol residue. Ward & Young (1991) report the synthesis and biological evaluation of a series of inositol phospholipid analogues including the four stereoisomers of dihexadecanoyl phosphatidylinositol, but present no data on the substrate stereospecificity of the guinea pig uterus PI-PLC used in some of their experiments. Our results show that the phosphatidylinositol-specific phospholipase C from *B. cereus* is highly enantioselective not only in the cleavage

but also in the binding of the optical isomers of *myo*-inositol 1-(4-nitrophenyl phosphate). This is the first report describing the synthesis and application of an optically pure chromogenic substrate for the study of PI-PLCs. The same synthetic strategy can be applied to evaluate the stereospecificity of the enzyme versus other nitrophenyl substrates, for example, those which attain a more lipid-like character through introduction of an apolar substituent in the phenyl ring.

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