Synthesis of Inositol Phosphodiesters by Phospholipase C-Catalyzed Transesterification†

Karol S. Bruzik,*,‡ Zhiwen Guan,‡ Suzette Riddle,§ and Ming-Daw Tsai§

Contribution from the Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, Chicago, Illinois 60612-7231, and Departments of Chemistry and Biochemistry, The Ohio State University, Columbus, Ohio 43210

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Abstract: Transesterification of primary alcohols with inositol 1,2-cyclic phosphate (IcP) in the presence of phosphatidylinositol-specific phospholipase C (PI-PLC) resulted in the formation of *O*-alkyl inositol 1-phosphates. The starting IcP was obtained in a single step by PI-PLC catalyzed cleavage of phosphatidylinositol from the soybean phospholipid. The transesterification reaction was performed with a series of 20 structurally diverse hydroxyl compounds, ranging in the structural complexity from methanol to the serine-containing Ser-Tyr-Ser-Met tetrapeptide, to give the corresponding phosphodiesters with 20−80% yields, depending mainly on the solubility of alcohols in water. The rates of transesterifications, and of the competing hydrolysis of IcP to inositol 1-phosphate (IP), were relatively insensitive to the alcohol structure. With polyhydroxyl compounds such as glycerol and hexitols, the enzyme displayed complete preference toward formation of the inositol phosphate derivatives of the primary hydroxyl groups. On the other hand, PI-PLC did not discriminate between primary hydroxyl groups in different environments and showed low stereoselectivity with racemic alcohols featuring a chiral center at the β-position. The *O*-alkyl inositol phosphates formed were readily separable from the hydrolytic product, IP, by the anion-exchange chromatography, and were fully characterized by means of ^{1}H and ^{31}P NMR and electrospray MS. Our results provide a new, simple, and efficient two-step synthetic route to substituted *O*-alkyl inositol phosphates from inexpensive starting materials. The reported reaction was successfully applied to synthesis of complex inositol phosphate derivatives, as illustrated by inositol phosphoesters of mono- and oligosaccharides, nucleosides and peptides. The synthetic usefulness of this reaction, however, is not limited to the examples shown. Because transesterification activity of phospholipase C has not been reported before, its mechanism is discussed in a broad context of mechanisms of phosphoesterases.

Introduction

Development of new synthetic methods leading to inositol phosphates is of considerable interest in view of their role as second messengers in the transduction of cellular signals.[1,2] The current state of art allows synthesis of all known naturally occurring inositol phosphates and phospholipids, and many of their analogs with almost any desired pattern of the phosphodiester and phosphomonoester substitution of inositol.[3−10] Despite significant advances, however, most of the described synthetic methods still take more than 10 steps from inositol or any other precursor. The biggest effort in these syntheses is expended on elaborate protection−deprotection schemes, necessary to achieve the desired regio- and stereoselective phosphorylation pattern of inositol framework. Creating the molecular diversity needed for studies of structure−activity relationship of inositol-related enzymes and receptors,[5,7] while feasible, is extremely labor intensive. In the past we have embarked on devising chemical syntheses of phosphoinositides applicable toward a goal of a broader application.[8−10] In this paper we report on the first enzymatic synthesis of *O*-alkyl inositol phosphates starting from the corresponding alcohols, and the inexpensive and readily available soybean phospholipid, using phosphatidylinositol-specific phospholipase C (PI-PLC†).

The action of PI-PLC on phosphatidylinositol (PI, **1**), or its phosphorylated derivatives, produces the mixture of the corresponding 1,2-cyclic phosphate (IcP, **2**) and inositol 1-phosphate (IP, **3**) (Scheme 1) or their 4- and 5-phosphorylated congeners.[8,11−13] The bacterial species of PI-PLC cleave the non-phosphorylated phosphatidylinositols (PI and glycosyl-PI) to afford initially the cyclic phosphate[11,14] as a sole product. This enzyme also catalyzes the subsequent slow hydrolysis of IcP into inositol 1-phosphate.[11,14] In our earlier report[8] we have

† Abbreviations: ESMS, electrospray mass spectrometry; glycero-3-phospho-(1-*myo*-inositol), Gro-IP; HPAEC, high performance anion exchange chromatography; IcP, inositol 1,2-cyclic phosphate; IP, inositol 1-phosphate; MOPS, morpholinepropanesulfonic acid; PAD, pulse amperometric detection; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; SDC, sodium deoxycholate; Tris, tris(hydroxymethylene)aminomethane.

‡ University of Illinois at Chicago.

§ The Ohio State University.

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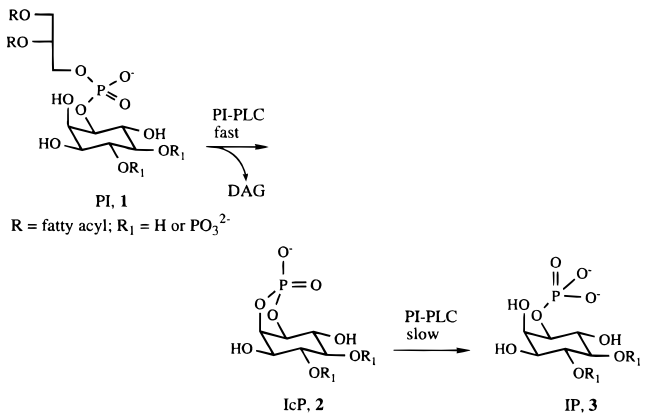
Scheme 1



provided evidence that in spite of the apparently different behavior of mammalian and bacterial enzymes, reflected by simultaneous vs sequential formation of the cyclic and acyclic products, respectively, both enzymes utilize analogous transesterification mechanisms, involving attack of the inositol 2-hydroxyl group on the phosphorus atom as a key step of the phospholipid cleavage.

Reverse reactions of esterases, proteases, and glucosidases are widely used in the synthesis of esters, peptides, and oligosaccharides, respectively. In contrast, examples of synthetic applications of phosphoesterases are limited to the transphosphatidylation activity of phospholipase D[15−18] and transesterification activity of pancreatic ribonuclease,[19,20] snake venom phosphodiesterase,[21,22] and alkaline phosphatase.[23] This report is intended to fill this gap by exploring the synthetic potential of PI-PLC toward synthesis of the diverse series of inositol phosphodiesters.

Results

Discovery of the Reverse Transesterification Catalyzed by PI-PLC. Hydrolysis of 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1-*myo*-inositol) (DPPI, **1a**)[8] dispersed as mixed micelles with sodium deoxycholate (SDC) in 70 mM Tris-HCl buffer (pH 7.0) catalyzed by PI-PLC from both *Bacillus thuringiensis* or *Bacillus cereus* provided IcP (**2**, δ_{31P} 16.1 ppm, Figure 1A). The monitoring of the further hydrolytic cleavage of IcP catalyzed by PI-PLC from either source revealed formation of inositol 1-phosphate (IP, **3**, δ 3.4 ppm) as expected, and two unidentified products giving rise to signals at −0.3 and −0.5 ppm (Figure 1B). Since both enzyme preparations contained significant amounts of glycerol, the control reaction was performed using the dialyzed enzyme from *B. thuringiensis* in 70 mM MOPS-Na buffer at pH 7.0, which produced IP as an exclusive product (Figure 1C). In another experiment, the

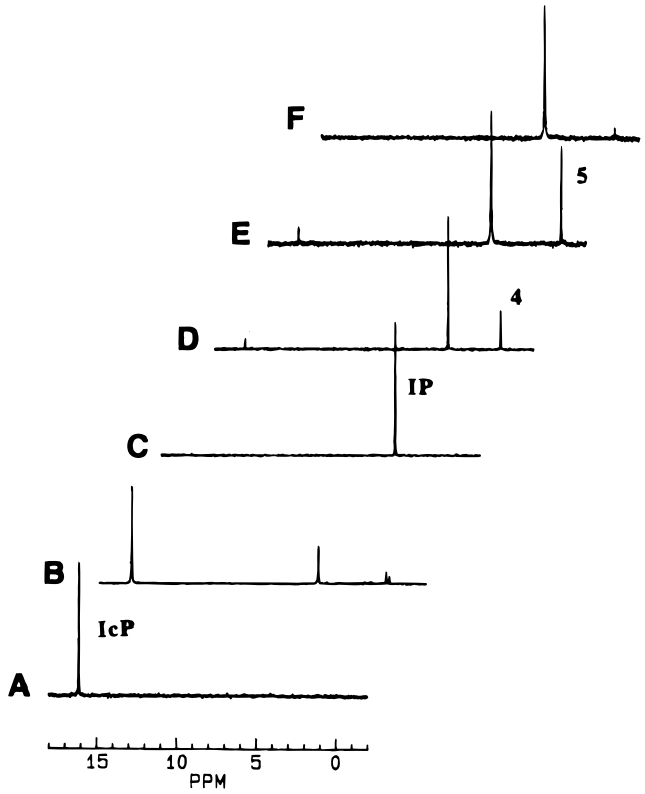


Figure 1. ^{31}P NMR spectra of reaction mixtures of IcP and PI-PLC under the following conditions: (A) IcP (10 mM) in Tris-HCl (70 mM) and EDTA (10 mM) buffer; (B) sample of DPPI (10 mM) dispersed with sodium deoxycholate (0.1 M) in Tris-HCl (0.2 M) 17 h after addition of PI-PLC from *B. cereus* (3.5 μg); (C) sample of IcP (10 mM) in 70 mM MOPS-Na, 10 mM EDTA treated with dialyzed PI-PLC; (D) same as trace C, but in the presence of 0.5 M glycerol; (E) same as the trace C, but in the presence of 0.5 M Tris-HCl; and (F) same as C, but after 16 days. All experiments were performed at pH 7.0.

treatment of IcP with PI-PLC from *B. thuringiensis* in MOPS buffer, and in the presence of 0.5 M glycerol, afforded IP and a product giving rise to the signal at −0.3 ppm (Figure 1D). These products were resolved by the anion-exchange chromatography on Sephadex QAE, and their identity was determined by NMR, MS, and high performance anion-exchange chromatography (HPAEC). The comparison of spectral and chromatographic data of this compound with those of the product of deacylation of phosphatidylinositol, unequivocally established its structure as glycero-3-phospho-(1-*myo*-inositol) (Gro-IP, **4**). The treatment of IcP with the dialyzed *B. thuringiensis* PI-PLC in the presence of 0.5 M Tris-HCl buffer at pH 7.0 resulted in the formation of a product giving rise to the signal at −0.5 ppm (Figure 1E), in addition to IP. Purification of this product and its analysis by ^{1}H NMR and MS determined its structure as 1-*O*-(2-amino-2-hydroxymethylene-3-hydroxypropyl) 1-*myo*-inositol phosphate (**5**). Hence, both products **4** and **5** are formed as a result of the phospholipase C catalyzed transesterification of glycerol or Tris, respectively, through the ring opening of the five-membered cyclic phosphate. In the blank experiments, incubation of IcP with each of the alcohols in the absence of enzyme over several days produced no reaction.

Driving Force for Transesterification. The hydrolysis of the cyclic five-membered phosphodiesters is energetically more

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(24) ^{31}P NMR chemical shifts are somewhat sensitive to the pH and buffer conditions. The chemical shifts of signals in the spectra shown in Figure 1 recorded for buffered samples differ, therefore, from those reported in the Experimental Section, obtained for samples of pure phosphodiesters dissolved in D_2O only by 0.5−1 ppm.

Table 1. $^1H-^1H$ and $^{31}P-^1H$ Coupling Constants for IcP, IP, and Inositol[a]

compd	H1-H2	H2-H3	H3-H4	H4-H5	H5-H6	H6-H1	P-H1	P-H2
IcP	4.8	3.8	10.1	10.1	9.4	8.3	20.1	ca. 0
IP	2.7	2.7	9.7	8.9	8.9	9.6	8.6	
inositol	2.7	2.7	9.8	8.8	8.8	9.8		

[a] All coupling constants are for aqueous solutions at neutral pH; 0.2 Hz digital resolution.

Table 2. Maximum Yields of *O*-Alkyl Inositol Phosphates and Their Physicochemical Data

alcohol [M]	maximum yield [%]	$\delta^{31}P$ [ppm] (multiplicity)	ES MS [*m*/*z*]	retention time[a] [min]
methanol [6.0]	70	1.1 (qtr)	273.6	3.7
n-propanol[c]	63	0.29	301	nd
butanol [0.6]	15	0.86	315.5	4.0
allyl [1.4]	33	0.63	299.4	4.0
ethane-1,2-diol [4.5]	74	0.14 (q)	303.4	4.5
(DL)-propane-1,2-diol [6.0]	46	0.07 (q)	317.7	3.6
hexane-1,6-diol [2.0]	55		359.8	3.5
glycerol [4.0]	81	0.14	333.8	5.8
D-mannitol [1.5]	30	0.44 (dtr)	423.0	12.11
L-iditol [0.15]	24	0.63	423.0	10.1
3-aminopropanol [6.0]	40	0.68	317.0	2.5
Tris buffer [0.5]	50	−1.56		nd
choline [0.5]	nd	−1.06 (q)	329.9	nd
(DL)-serine [0.67]	18	−0.74, −0.81	346	19.0
pentaethylene glycol[d]	nd	0.49	479	1.9
biotinylpentaethylene glycol[e]	nd	0.44	706	*b*
glucose [3.0]	25	0.06	421	15.0
uridine [0.4]	15	−0.47	485	2.7
Ser-Tyr-Ser-Met [0.04]	10	nd	727	15.8, 16.2

[a] Carbopack PA 1, 4 × 250 mm, isocratic 0.1 NaOH in NaOAc gradient 40−200 mM in 30 min. [b] Product not visible by HPAEC. [c] Propanol concentration 50% v/v. [d] Concentration 22% w/v. [e] Concentration 26% w/v; nd, not determined.

favorable in comparison with hydrolysis of the acyclic phosphodiesters by 4.6 kcal/mol.[25a] The origin of this free enthalpy difference, once thought to arise from dioxaphospholane ring strain,[25a] has now been ascribed to the differential solvation energies of the substrate and the product.[25b] In the case of a five-membered cyclic phosphate fused to a six-membered ring, as in the case of IcP, the energy difference should be enhanced due to an additional torsional strain of the cyclohexyl ring produced by the bridging of the axial and equatorial hydroxyl groups of inositol by the phosphate function. This strain would be relieved upon ring opening giving rise to a greater driving force for the ring opening. The examination of the vicinal $^1H-^1H$ coupling constants in IcP and IP and inositol (Table 1) revealed that there are significant differences between these parameters in IcP and IP. The increase in H1−H2, H2−H3 couplings and the decrease in H1−H6 coupling constants in IcP indicate flattening of the cyclohexyl ring in its C3−C2−C1−C6 fragment. For example, the C3−C2−C1−C6 torsional angle is changed from −57° in IP to −47° in IcP (Figure 2), and the C2−C1−C6−C5 angle is similarly decreased from 57° to 51°. In contrast, IP displays very similar couplings to those of the unphosphorylated inositol, indicating that the distortion of the six-membered ring is due to the presence of the five-membered ring, and not phosphorylation.

Consistently, the half-life of IcP at pH 1 is less than 1 min,[13] and IcP hydrolysis is already detectable at pH 4.0, suggesting that ring opening of IcP could be a useful phosphorylation reaction, if an alcohol could be substituted for water. In fact, the treatment of tetra-*n*-butylammonium salt of IcP with butanol

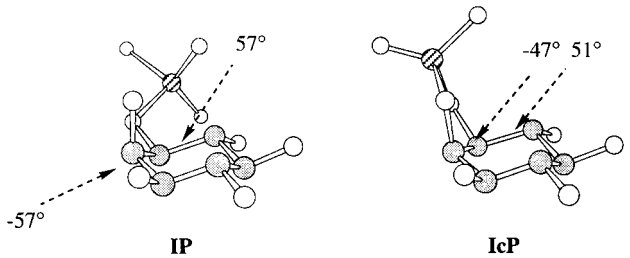

Figure 2. MM2 optimized conformations of IP and IcP. The numerical values designate C3−C2−C1−C6 and C2−C1−C6−C5 torsional angles.

in anhydrous DMSO in the presence of boron trifluoride resulted in the formation of the mixture of the corresponding *O*-butyl inositol 1- and 2-phosphates. The low yield (15%) of the acyclic diesters and the low regioselectivity of this reaction indicated, however, that the utility of IcP as a nonenzymatic phosphorylating agent is doubtful (see also ref 26).

Enzymatic Synthesis of IcP. For transesterification reaction of alcohols with IcP to be of any synthetic utility, the latter starting material has to be readily available. The earlier reported syntheses of the racemic IcP,[27] and its phosphorothioate analog,[28] from inositol have been ruled out as a source of this material, due to a greater difficulty in synthesis of the enantiomerically pure IcP. Alternatively, we have considered naturally occurring phosphoinositides as a starting material. The crude phosphoinositide, containing PI as a major component, can be readily obtained in the multigram quantity from the inexpensive soybean phospholipid by the precipitation with methanol from its chloroform solution.[29] The treatment of the sodium deoxycholate (SDC) dispersion of this phosphoinositide fraction with phospholipase C followed by the detergent removal via chloroform−methanol extraction at the pH 5.0, and further anion-exchange chromatography afforded IcP contaminated with only small amounts of the glycosylated IcP. In a typical preparation, treatment of 20 g of phosphoinositide fraction with only 20 μg of PLC afforded 1.7 g of IcP. This procedure can be further simplified by omitting the use of the detergent. The treatment of the heterogeneous ether−water dispersion of the soybean phospholipid with PI-PLC resulted in the exhaustive cleavage of phosphatidylinositol from the mixture. The removal of the unhydrolyzed phospholipid by extraction with chloroform−methanol, and the anion-exchange chromatography of the water-soluble product using ammonium carbonate step-gradient afforded pure IcP.

Time Course of Transesterification of Alcohols with IcP. A representative time course of the enzymatic transesterification of an alcohol with IcP catalyzed by PI-PLC, exemplified by the reaction of IcP with 1,6-hexanediol, is shown in Figure 3. Formation of the *O*-1-(6-hydroxyhexyl) 1-*myo*-inositol phosphate (**6**) was followed by HPAEC on Carbopack PA1 column using pulse-amperometric detector (PAD) for monitoring peak elution. The disappearance of the IcP signal (t_R 10.5 min) was accompanied by the formation of the phosphodiester **6** at 3.5 min and IP (t_R 13.5 min). At longer reaction times, the concentration of IP increased mainly at a cost of the acyclic diester product, while the concentration of IcP declined only very slowly. At the very long reaction times (several days) IP

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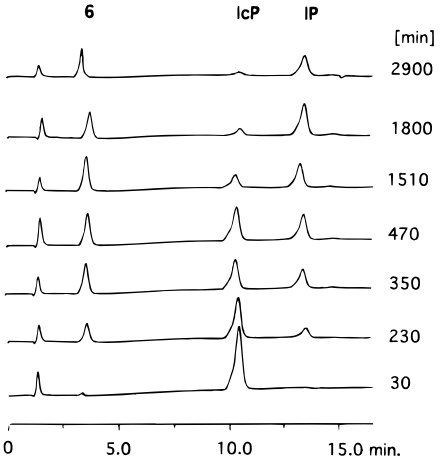
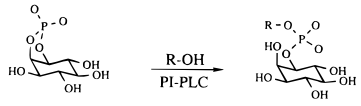
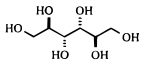
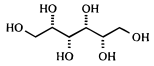
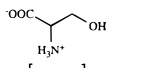
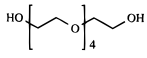
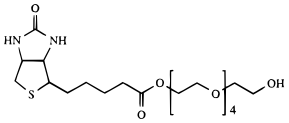
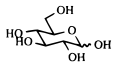
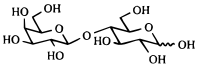
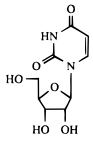
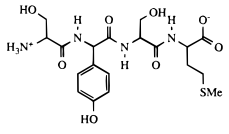


Figure 3. Time course of transesterification of hexane-1,6-diol with IcP in the presence of PI-PLC at room temperature followed by HPAEC-PAD. Reaction conditions: [diol], 2 M; [IcP], 0.4 M; PI-PLC, 15 μg. Chromatographic conditions: Carbopack PA1 column 4.6 × 250 mm with 4.6 × 50 mm guard column was eluted with isocratic NaOH (100 mM) and sodium acetate gradient (40−200 mM in 30 min).

Scheme 2



Alcohol	Product	Alcohol	Product
CH_3-OH	**7**		
CH_3CH_2-OH	**8**		
$CH_3CH_2CH_2-OH$	**9**		
$CH_2=CHCH_2-OH$	**10**		
$HOCH_2CH_2-OH$	**11**		
$HOCH_2(CH_2)_4CH_2-OH$	**6**		
$CH_3-CH(OH)-CH_2-OH$	**12**		
			**4**
			**13**
			**14**
$H_2NCH_2CH_2CH_2-OH$	**15**		
$HOCH_2-C(CH_2OH)(NH_2)-CH_2-OH$	**5**		
$(CH_3)_3N^+CH_2CH_2-OH$	**16**		
	**17**		
	**18**		
			**19**
			**20**
			**21**
			**22**
			**23**

was an exclusive product (not shown, but see Figure 1F for analogous reaction). Due to the breakdown of the acyclic phosphodiesters at the longer reaction times, their optimal yields in reactions of various alcohols were assured by monitoring the reactions by HPAEC-PAD, reverse phase HPLC, or ^{31}P NMR and stopping the reaction when the concentration of an acyclic diester reached a highest point.

Alcohol Specificity of PI-Specific Phospholipase C. In order to determine structural specificity of the enzyme we have examined a series of reactions of IcP with primary and secondary alcohols (Scheme 2). The results are summarized in Table 2 and in Figure 4A,B. The reactions of alcohols with IcP in the presence of PLC resulted in the formation of the corresponding acyclic phosphodiesters **4**−**23** with the yields generally exceeding 20%, but in some cases greater than 70%. The highest

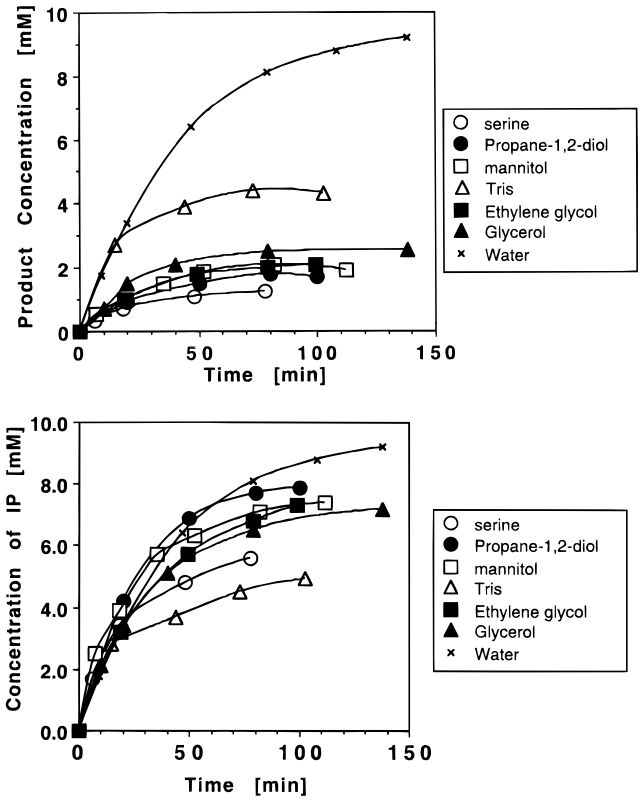


Figure 4. (A) Rates of formation of *O*-alkyl inositol phosphates from IcP with various alkanols in the presence of PI-PLC. Reaction conditions: initial concentration of alkanols 0.5 M, [IcP] 10 mM, PI-PLC 3.5 μg. All reactions were followed by ^{31}P NMR using ca. 30 min accumulation time for each spectrum. The experimental points denoted refer to the middle of the accumulation period of the spectrum. (B) Rate of formation of IP in the presence of 0.5 M alkanols.

yields were obtained in cases of simple alcohols, where their high molar concentrations were possible to achieve. The yields in some cases were limited by the poor solubility of the alcohols in water. The transesterification reaction was found to be of a general scope, with most alcohols participating at a comparable efficiency (Figure 4A). For example, the calculated alcohol efficiency parameter, defined as the ratio of the mole fraction of the *O*-alkyl inositol phosphate in the product mixture to the mole fraction of alcohol in the starting alcohol/water solution, was as follows: methanol (6), mercaptoethanol (12), propanol (17), serine (18), propane-1,2-diol (21), ethylene glycol (25), glycerol (28), mannitol (29), and Tris (57). The rates of the formation of the acyclic diesters with such alcohols as ethylene glycol, glycerol, mannitol, and serine were very close indicating that the reaction is quite insensitive to the nature of substituents at the β-position such as hydroxyl, amino, tetraalkylammonium, carboxyl, and sulfhydryl group. The only exception among alcohols studied was Tris, which produced ca. 2-fold higher yield of the product. As shown in Figure 4B the presence of alcohols and their structural variation had little effect on the initial rates of the competing IcP hydrolysis, indicating that none of the alcohols studied was bound in the IcP site.

Although the range of alcohol structures accepted by the enzyme was quite broad, we did note a few examples where we were unable to obtain products of transesterification. Reactions with secondary alcohols, isopropyl alcohol, 2-butanol, and cyclohexanol did not afford the corresponding phosphodiesters, nor did the presence of such alcohols affect the rate of the hydrolysis of IcP (results not shown). Likewise, reactions with long chain primary alcohols such as dodecanol and hexadecanol also failed to afford the product. The attempt to

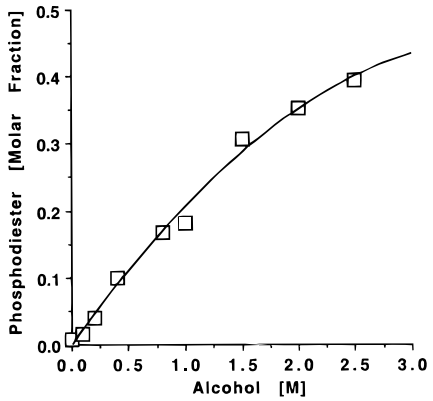


Figure 5. Dependence of the yield of diester **6** on the concentration of hexanediol. The reactions were monitored by HPAEC as described in Figure 2. The yields of **6** are expressed as a molar fraction of all inositol components after 2 h. The reaction conditions are the same as in Figure 3, except for alcohol concentration.

increase the alcohol solubility in water by adding the hydrophilic function, as in ω-hydroxyhexanoic acid, produced a negative result, as well. On the other hand, the addition of 0.5 M inositol did not provide the acyclic diester product, but it almost completely inhibited the hydrolysis of IcP, indicating inositol binding in the IcP site as previously reported.[30] Other unsuccessful attempts included alcohols with added electrophilic reactivity, such as in bromoethanol and glycidol.

In order to optimize transesterification conditions we have investigated the dependence of the yield of the phosphodiester upon the alcohol concentration (Figure 5). The yield of the diester **6** linearly increased with the concentration of hexane-1,6-diol and started leveling off only at concentrations above 2 M, indicating weak binding affinity of the alcohol to PI-PLC.

Regio- and Stereospecificity of PLC Catalyzed Transesterification. With compounds featuring multiple hydroxyl groups such as mannitol and glycerol there is a possibility of two types of product isomerism due to phosphorylation of the primary vs secondary hydroxyl group in mannitol, and the pro-*S* vs the pro-*R* hydroxyl group in glycerol. In all cases of polyols examined we have seen only phosphorylation of the primary hydroxyl group, as evidenced by observation of either a quartet or a doublet of triplets (not shown) in ^{1}H-coupled ^{31}P NMR spectra. Phosphorylation of the primary hydroxyl group is also evident from the analysis of the ^{1}H NMR spectra. The reaction of IcP with glycerol afforded two isomers giving rise to very closely spaced ^{31}P NMR signals with 45:55 intensity ratio[31] (Figure 6A). The addition to this product of a single isomer of Gro-PI (**4**), obtained by the deacylation of PI, showed the increase of the down-field signal (Figure 6B). Both ^{1}H NMR and ^{1}H$-^1$H COSY spectra were consistent with the presence of two stereoisomers having either the pro-*S* or pro-*R* hydroxyl group phosphorylated.[32] With mannitol and iditol only a single product was formed with the phosphorylated primary hydroxyl group. This is due to the fact that both primary OH groups present in these molecules are homotopic because of the C_2 symmetry. Further experiments with racemic alcohols such as dl-propane-1,2-diol (**12**) and dl-serine (**17**) also provided almost equimolar mixtures of diastereomers (not shown). In summary, while PI-PLC displays a strong preference for the primary hydroxyl groups, it exhibits very low stereoselectivity with regard to configuration at the C-2 of an alcohol. This conclusion is consistent with our earlier results showing that PI cleavage by PI-PLC is nonstereospecific with regard to configuration at the C-2 of the diacylglycerol moiety.[8]

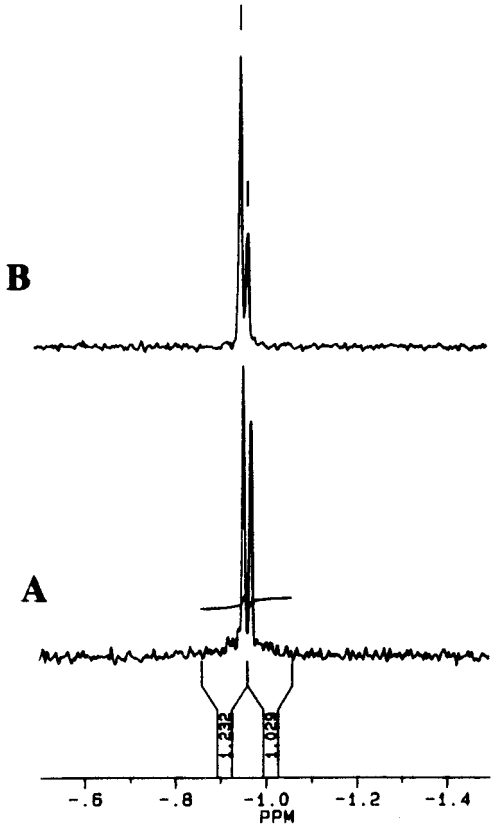


Figure 6. ^{31}P NMR spectra of Gro-PI (**4**) (A and B). Spectrum A shows the product obtained by transesterification of glycerol with IcP. Spectrum B was acquired after adding the diasteromerically pure Gro-PI, obtained by deacylation of PI, to the sample shown in trace A.

Application of PI-PLC Catalyzed Transesterification to Synthesis of Complex Inositol Phosphodiesters. The potential of the PI-PLC-catalyzed transesterification reaction as applied to derivatization of structurally complex compounds is illustrated by the syntheses discussed below: (A) 6-glucosylphosphoinositol (**20**), (B) 5′-uridyl phosphoinositol (**22**), and (C) inositol 1-phosphate derivative of Ser-Tyr-Ser-Met tetrapeptide (**23**).

(A) Monitoring of the transesterification of glucose (the mixture of α- and β-anomers) with IcP by HPAEC-PAD indicated formation of a new product giving rise to the peak at 15.4 min, in addition to those of IcP and IP (Figure 7A). Purification of this product by the preparative anion-exchange chromatography and analysis by ^{1}H, ^{31}P, and ESMS confirmed the structure of the product as 6-glucosyl phosphoinositol. The product **20** constituted a mixture of both α- and β-anomers as indicated by the presence of two doublets at 5.20 ppm (3.8 Hz) and at 4.62 ppm (8.0 Hz), respectively, in ^{1}H NMR spectrum. The two anomeric products are not resolved by HPAEC and ^{31}P NMR. An analogous reaction of lactose with IcP produced a complex mixture of two α- and two β-isomers (**21**), due to the random phosphorylation of the 6-hydroxyl groups in the galactose and glucose rings. The obtained mixture of lactose-phosphoinositols gave rise to a broad ^{31}P NMR signal at 0.5 ppm, and two HPAEC peaks at 13.6 min and 14.0 min. This

(30) Heinz, D. W.; Ryan, M.; Bullock, T. L.; Griffith, O. H. *EMBO J.* **1995**, *15*, 3855−3863.

(31) The two ^{31}P NMR signals of Gro-IP obtained by transesterification of glycerol are observed only when high resolution spectra of the Gro-IP sample are obtained, typically requiring sample treatment with Chelex resin or EDTA. This signal doubling was not observable in the spectra shown in Figure 1.

(32) The ^{1}H NMR spectrum of this diastereomeric mixture (giving rise to a ^{31}P NMR spectrum shown in Figure 6A) displays additional splitting of glycerol H-1′$_A$ and H-1′$_B$ protons (by ca. 2.7 Hz (A) and 1 Hz (B), respectively). Other resonances remain identical to those of the pure *sn*-3-Gro-1-IP.

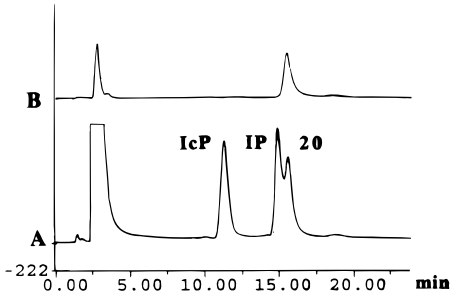


Figure 7. (A) Formation of glucose inositol phosphate as observed by HPAEC-PAD on Carbopack PA1. Identities of peaks are t_R 2.8 min, glucose, t_R 11.2 min, IcP; t_R 15 min, IP; t_R 15.5 min, compound **20**. (B) HPAEC of the purified **20**. Chromatographic conditions are the same as in Figure 3.

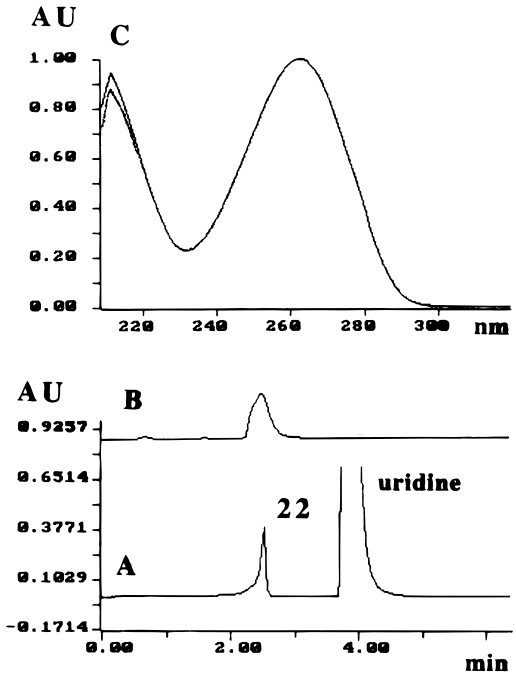


Figure 8. (A) A representative RP-HPLC of the reaction mixture of transesterification of uridine with IcP to form uridine inositol phosphate **22**. (B) HPLC of the purified **22**. See Experimental Section for chromatographic conditions. (C) A superposition of the UV spectra of the eluates at 3.9 and 2.7 min.

experiment demonstrated a rather low regioselectivity of PI-PLC toward the primary hydroxyl groups of oligosaccharides.

(B) The reaction of IcP with uridine was followed by the reverse phase HPLC, monitoring the appearance of the product by the photodiode array UV detector (Figure 8A). The product giving rise to the peak at 2.5 min had a UV spectrum identical to that of uridine itself (t_R 3.9 min) (Figure 8C), indicating the presence of the intact uracyl residue. This product was purified by the anion-exchange chromatography (Figure 8B). The analysis of the product by ^{1}H, ^{31}P NMR, and ES MS confirmed its structure as 5′-uridyl phosphoinositol (**22**).

(C) The Ser-Tyr-Ser-Met tetrapeptide was chosen to test positional specificity of PI-PLC in the phosphorylation of peptide hydroxyl groups in different environments, since it features two nonequivalent serine residues and a phenolic hydroxyl group. The reaction progress was followed by reverse phase HPLC. After 48 h formation of two products giving rise to two peaks of equal intensity at 15.5 (**23a**) and 15.8 min (**23b**) was observed, in addition to that of the starting tetrapeptide at 18.7 min (Figure 9A). Further reaction resulted in the diminution of one of the peaks. Both products **23a** and **23b** had

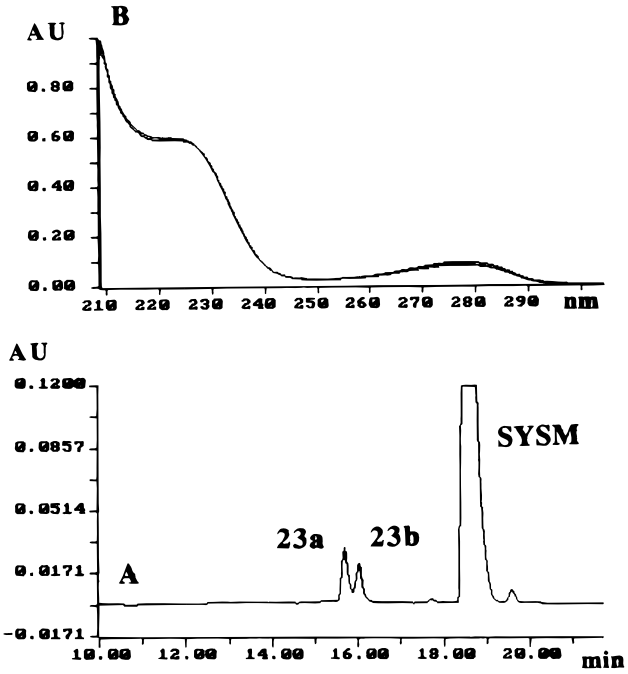


Figure 9. (A) A representative HPLC of the reaction mixture of Ser-Tyr-Ser-Met tetrapeptide with IcP and PI-PLC after 48 h showing phosphorylated products **23a** at t_R 15.4 and **23b** at t_R 15.8 min. Reaction conditions: tetrapeptide 0.04 M, PI-PLC 4 μg. Chromatographic conditions are as described in Experimental Section. (B) An overlay of the UV spectra of the tetrapeptide, the product **23a** and the product **23b**.

identical UV spectra to that of the starting material (Figure 9B), suggesting that the phosphorylation took place at one of the serines rather than at the tyrosine, since phosphorylation of the latter would most likely affect the UV profile of the tetrapeptide. The two isomers were preparatively separated by RP-HPLC and analyzed by ES-MS. The presence of a single inositol phosphate residue in the product was verified by the observation of the molecular ion at *m*/*z* 727 for each isomer. However, the small amounts of material produced precluded assignment of chromatographic peaks to particular positional isomers by using NMR.

Demonstration of Reversibility of PI-PLC-Catalyzed Reaction. The observed transesterifcation reaction is a manifestation of the reversibility of the formation of IcP from PI. This reversibility is further demonstrated by the conversion of the acyclic diesters obtained from IcP back into IcP in the presence of PI-PLC. For example, the reaction of IcP with allyl alcohol in the presence of PI-PLC afforded *O*-allyl inositol phosphate (**10**). The treatment of the purified diester **10** with PI-PLC has led to the mixture of IcP and IP, and eventually to IP after long reaction times. In contrast, we have been unable to detect formation of the phosphodiester from IP, or an equilibrium between IcP and IP, using a sensitive HPAEC-PAD detection method.

Discussion

The results reported above are significant in two aspects: (i) they show a vast potential of PI-PLC for the synthesis of *O*-alkyl inositol phosphodiesters, limited mainly by the hydrophobicity of the primary alkanol; (ii) our results indicate far reaching analogies between various groups of phosphoesterases, which allow certain generalizations to be made.

Synthetic Utility of IcP-Alcohol Transesterification Catalyzed by IcP. We have demonstrated that the transesterification reaction catalyzed by PI-PLC can be used to synthesize a wide

variety of substituted *O*-alkyl phosphoinositols. The enzymatic synthesis is advantageous over chemical synthesis in that (i) it uses a readily available soybean phospholipid as a starting material, (ii) no protection/deprotection steps of alcohols are required in order to accomplish synthesis of the phosphodiester linkage with many polyfunctional alcohols, and (iii) the reaction can be performed with a wide range of primary alcohols and polyols with a high degree of selectivity between primary and secondary hydroxyl groups. It is thus possible with minimal effort to produce a series of polyfunctional alkyl phosphoinositols for studies of specificity of inositol-dependent enzymes or enzyme inhibition. The procedure also eliminates the need for synthesis of appropriately protected inositol derivatives to be used in the phosphoester group assembly. It should, furthermore, be applicable for one-step incorporation of fluorescent and radioisotope labels into inositol phosphodiesters to be used as molecular probes in biological studies. Many applications of the reaction may be possible as a need for a particular structure arises.

To prove the applicability of this reaction to syntheses of more complex compounds we have used PLC to attach inositol phosphate residue to carbohydrates, nucleosides, and peptides. With hexoses and ribonucleosides only the primary 6-hydroxyl and 5′-hydroxyl groups, respectively, were phosphorylated. In contrast, the reaction was very regio-nonspecific with regard to primary hydroxyl groups in different environments. This was demonstrated by phosphorylation of the lactose and Ser-Tyr-Ser-Met tetrapeptide. The results indicate that the enzyme can phosphorylate indiscriminately hydroxymethylene groups of serine and hexoses, but not the tyrosine hydroxyl. It is conceivable that this reaction could be extended to attach inositol phosphate moiety to solvent-exposed serines of larger peptides or even proteins, and various oligosaccharides. Although we have failed to phosphorylate the long chain fatty alcohols, the phosphorylation of the non-hydrophobic long-chain alcohols such as pentaethylene glycol (PEG) can be achieved. Such alcohols can be used as flexible linkers to attach inositol phosphate to various functionalities. In the past various radioactive and fluorescently labeled inositol phosphate derivatives have been synthesized and used as molecular probes.[3,5a,6,7,57−59] In the current work, we have synthesized the biotinylated PEG inositol phosphate (**19**) to be used as a ligand for an affinity chromatography of proteins with affinity for the inositol phosphate residue. An extension of this experiment for synthesis of a fluorescently labeled inositol phosphodiesters can be easily envisioned.

Factors Affecting Efficiency of Transesterification. The above described transesterification reaction is a reverse reaction of PI-PLC, in which different alcohols substitute for the diacylglycerol. The competing hydrolysis of IcP can be also considered as a reverse reaction, with water molecule substituting for the diacylglycerol. The feasibility of the transesterification reaction as a source of *O*-alkyl inositol phosphates depends on three factors: (i) kinetic competition between diacyl glycerol, externally added alcohol and water molecules; (ii) the ability of a product of the reverse reaction to serve as a substrate for the forward reaction; and (iii) the equilibrium constant between *O*-alkyl inositol phosphate (RO-PI), and ROH and IcP. The importance of these factors is discussed below.

(i) The fact that the rate of IcP hydrolysis is close to the rate of formation of RO-PI with alcohol concentration in the 0.5 M range (Figure 4A) suggests that alcohol can efficiently compete with water for the diacylglycerol binding site. This is reflected by the magnitude of the efficiency parameter in the range of 6−57 for several studied alcohols, showing that alcohol is a more efficient substrate than water. We cannot address the problem of the competition between the diacyl glycerol and an alcohol since we have been using the purified IcP, rather than the PI, as a substrate. The relatively weak binding affinity of an alcohol substrate seems to be a common property of not only PI-PLC but also other phosphoesterases as discussed in the next section. This low binding affinity results probably from the optimization of the catalysis for the forward reaction, in which the tight alcohol binding would cause product inhibition.

(ii) PI-PLC catalyzes the cleavage of PI to IcP with k_{cat} approaching 1200 s^{-1},[14] but the subsequent hydrolysis of IcP to IP is at least 500 times slower.[11] The cleavage of the non-hydrophobic inositol phosphodiesters synthesized in this work is still several-fold slower than the hydrolysis of IcP. The large reduction in the cleavage rate of such non-hydrophobic RO-PI is probably due to an absence of the interfacial activation of PI-PLC with non-hydrophobic substrates (low k_{cat}).[33] Unlike with other phospholipases, the observed interfacial activation of PI-PLC, however, is relatively weak.[33] For example, the cleavage of dihexanoyl-PI with the bacterial and mammalian δ-type PI-PLC is activated only 2-[33b] and 5-fold,[33c] respectively, at the critical micelle concentration. In contrast, the nonaggregating dibutyroyl-PI is completely resistant to PI-PLC-δ,[33c] glycerophosphoinositol 4-phosphate is resistant to PI-PLC-α,[34] and Gro-IP is almost fully resistant to bacterial PI-PLC[35] (see also this work). It, thus, appears that both bacterial and mammalian enzymes display three regions of activity depending on the substrate hydrophobicity: (i) very low activity with hydrophilic substrates bearing an equivalent of eight methylene residues or less in their alcohol leaving group; (ii) ca. 20−50% activity with more hydrophobic substrates below cmc; and (iii) full activity with hydrophobic substrates above cmc. The large gap in reactivity between non-hydrophobic substrates such as Gro-IP, or dibutyroyl-PI, and dihexanoyl-PI below cmc can be explained by the enzyme-induced aggregation of dihexanoyl-PI at submicellar concentration, but not Gro-IP, nor dibutyroyl-PI. Since the binding of an acyclic alkyl inositol phosphate is likely to be comparable to that of PI,[34] it is most probable that the reduction in the k_{cat} is responsible for the resistance of non-hydrophobic alkyl inositol phosphates to cleavage by PI-PLC.

(iii) The thermodynamic equlibrium between the cyclic phosphodiester and monoester is clearly shifted in favor of the monoester[36] due to favorable solvation factors, and the earlier discussed ring strain (see Results). Similarly, the equilibrium between IcP and RO-PI should be shifted toward RO-PI due to the ring strain present in IcP.

Transterification as a Common Property of Phosphoesterases which Form Ester Intermediates. Phosphatidylinositol-specific phospholipase C is a member of a large group of phosphoesterases which employ a transesterification mechanism for the phosphoester bond cleavage.[37] These enzymes can be broadly divided into two groups: (I) those which use the intrinsic serine/threonine/tyrosine hydroxyl group of the active site as a nucleophile, exemplified by phospholipase D,[15−18,38,39] snake venom phosphodiesterase,[21,22] and alkaline

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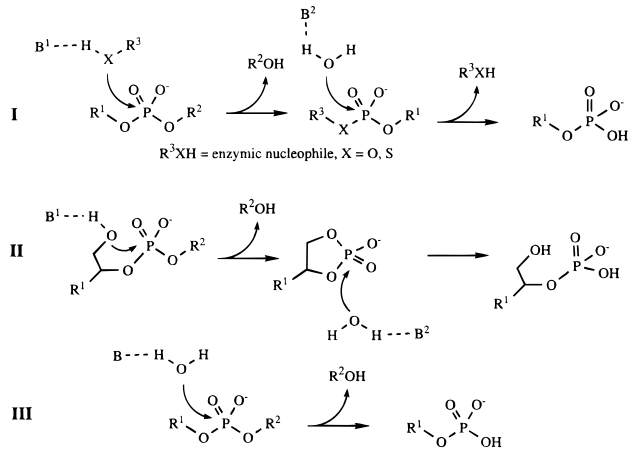
Scheme 3



phosphatase[23] (Scheme 3I), and (II) those which use an internal hydroxyl group of a substrate as an attacking nucleophile, represented by ribonuclease A[40−42] and PI-PLC[8,30] (Scheme 3II). Both groups of enzymes use two consecutive steps of nucleophilic displacement at phosphorus for a complete substrate-product cycle, with formation of a phosphoryl-enzyme intermediate (group I), or a cyclic phosphate (group II), respectively. In addition, a third group of enzymes, represented by inositol 1-phosphate phosphatase,[43,44] and the nonspecific-phospholipase C[45] uses an activated water molecule as a nucleophile for a direct attack at the phosphorus atom (Scheme 3III). To date, of the above three groups of enzymes only the first two have been reported to catalyze transesterification with exogeneous alcohols, most likely involving the reaction of such alcohols with the enzyme-phosphoester intermediate, or the cyclic phosphate. This is a consequence of the transesterification mechanisms used by group I and II enzymes in their natural reactions. For example, in the case of group II enzymes, the externally added alcohol can undergo an exchange with an alcohol of a ternary complex at the end of the first catalytic step (Scheme 4). The exogeneous alcohol can then use the binding site formerly occupied by the natural alkoxy leaving group. In addition, the protonation status of the active site general base−general acid pair is reversed at the end of the first step (i.e., B^1 is protonated, and B^2 is deprotonated, Schemes 3II and 4), as compared to the initial situation, enabling nucleophilic activation of the incoming alcohol. In the resulting transesterification reactions the enzymes therefore catalyze conversion of a diester into a diester, an energetically benign process. In the case of group III enzymes, an exogeneous alcohol molecule would have to be accommodated in place of a water molecule (Scheme 3III), an unlikely scenario due to a greater steric size of an alcohol as compared to water. Alternatively, for the reverse reaction of this group of enzymes, the phosphate monoester product (in the case of diesterase) would have to be converted into a phosphate diester, an energetically disadvantageous process due to a higher solvation energy of a monoester as compared to a diester. The observed transesterification of alcohols by IcP in the presence of PI-PLC falls, thus, into a general property of group I and II phosphoesterases. It is interesting to note the similarities between the transesterification reaction catalyzed by PI-PLC to those of other phosphoesterases.

(A) Snake venom phosphodiesterase catalyzes the cleavage of 3′,5′-dinucleotides and nucleoside triphosphates by a transesterification mechanisms involving formation of the covalent phosphoryl-enzyme intermediate.[46,47] This enzyme also catalyzes transesterification of various primary alcohols with nucleoside triphosphates through alcohol attack at the reactive phosphoryl-enzyme intermediate.[21,22] The rate of transesterification is slow as compared to ATP cleavage and relatively independent of the alcohol structure. In general, the alcohol efficiency parameters for this enzyme[22] are several-fold lower than those for PI-PLC, observed in this work. Another group I enzyme, phospholipase D, has been known to catalyze transesterification (transphosphatidylation) with a broad variety of primary alcohols,[15,48] through formation of the phosphoryl-enzyme intermediate.[38] The recently reported ester group exchange in phosphatidylcholine by PLD in the presence of several hexitols and glucose is proposed to play a role in the diabetic metabolism,[49] while transphosphatidylation of ethanol with phosphatidylcholine could be important in fetal alcohol syndrome.[50]

(B) Ribonuclease A catalyzes transesterification of simple primary alkanols with 2′,3′-cyclic nucleoside phosphates, at a much reduced rate as compared to RNA cleavage.[19] This enzyme is related to the bacterial PI-PLC by the analogous catalytic mechanism,[30,40,51] and by the fact that the cyclic phosphate is the physiological end-product of the RNA cleavage.[40,52] The low rate of the cleavage of 2′,3′-cyclic phosphate, mostly due to reduction in the k_{cat}, results from the absence of a part of the substrate structure needed to bind to the p2 site, to achieve full activity.[53,54] In the case of PI-PLC the analogous rate reduction is brought about by the removal of the hydrophobic chains from the substrate.

In summary, we have shown a potential of PI-PLC in synthesis of alkyl inositol phosphodiesters. One of the possible areas of application of the produced compounds is in studies of structure−activity relationship of inositol-related enzymes such as PI-synthase,[55] or inositol 1-phosphate phosphatase.[7] Both of these enzymes take part in inositol recycling, and thus they could constitute targets of antisignaling drug design.

Experimental Section

^{1}H NMR spectra were recorded in D_2O with a Bruker AM-400, AC-250, AC-200, and a Varian XL-300 spectrometers and were indirectly referenced to TMS. ^{31}P NMR spectra were recorded in D_2O with a Bruker AC-250 and AC-200 spectrometers and were indirectly referenced to 85% phosphoric acid. MS spectra were obtained with a Hewlett Packard 5989B electrospray mass spectrometer. HPLC

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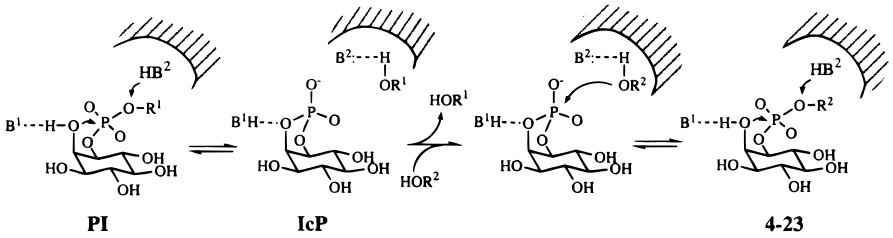
Scheme 4



analyses were performed with a Hitachi L-6200A solvent delivery system, and elutions were analyzed by the Hitachi HPLC Manager software. For separation of nonchromophoric inositol phosphate esters high-performance anion-exchange chromatography (HPAEC) was employed. The Carbopack PA1 column (4.6 × 250 mm) equipped with a guard column (4.6 × 50 mm) (Dionex) was eluted with a linear gradient of sodium acetate (40−200 mM during 30 min) in isocratic 100 mM sodium hydroxide at the 1 mL/min flow rate. Elutions were monitored with the Dionex pulsed amperometric detector (PAD-2) with sensitivity set at 1 μA, and the ionization potentials set as E1 0.05 V, E2 = 0.6 V, E3 = −0.6 V, and the sampling time set at 400 ms. Separation and quantitation of chromogenic samples was performed using the reverse phase chromatography and employing D-4500 UV−vis photo-diode array detector (PDA) set at the 800 ms sampling time and 7 nm spectral resolution. The reverse phase column Microsorb MV-C18 (4.6 × 250 mm, 300 Å pore size) (Rainin) was eluted with a solvent system consisting of 0.1% TFA in acetontrile/water (3:1, component A) and 0.1% TFA in water (component B). The linear gradient of 10% to 40% of component A during 30 min was applied at the 1 mL/min flow rate. The reported chromatograms are for the 280 nm wavelength, unless indicated otherwise. Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F254 aluminum plates (Merck). Plates were visualized under UV light (254 nm) or with a 10% ethanol solution of phosphomolybdic acid. Dowex 1X8-200 and 50WX8-200 ion-exchange resins were used for preparative anion and cation exchange chromatography, respectively.

B. cereus PI-PLC was from Boehringer, and PI-PLC from *B. thuringiensis* was expressed in *E. coli* as described earlier.[56] Except for some experiments shown in Figure 1 all reactions were performed with the enzyme from *B. thuringiensis*. All compounds synthesized have the 1D-configuration of the inositol moiety. Synthesis of biotinylated pentaethylene glycol will be described elsewhere (Kubiak and Bruzik, unpublished). All other chemicals and solvents were from Aldrich or Sigma, and were used without additional purification.

Inositol 1,2-Cyclic Phosphate. (A) PI fraction of soybean phospholipid obtained as described earlier[29] (1 g, ca. 1.2 mmol) was dispersed in the solution of sodium deoxycholate (20 mM, 100 mL), containing EDTA (1 mM), and the pH was adjusted to 7.5 with 1 N HCl. PI-PLC (20 μg) was added, and the reaction was stirred gently at room temperature. The progress of the reaction was examined by TLC (chloroform−methanol−NH_4OH, 7:3:0.2) monitoring disappearance of the PI spot, and by HPAEC monitoring formation of IcP and IP. After all PI had been used up, but little IP had been formed, the pH of the reaction mixture was adjusted to 5.0 with HCl to precipitate SDC as a free acid. The mixture was extracted three times with chloroform−methanol (5:2, 100 mL). Phases were separated by centrifugation, and the final aqueous phase was freeze-dried. The residue was chromatographed on the formate form-Dowex 1X8-200 anion-exchange resin using a step-gradient of ammonium formate. The residual unextracted sodium deoxycholate was eluted off with water, and the IcP product was eluted with 50 mM ammonium formate. This fraction was freeze-dried to the give the final IcP product (27% assuming 100% purity of the starting material). ^{1}H NMR δ 4.51 (tr, H-2, J = 4.8 Hz, 1H), 4.12 (ddd, H-1, J = 4.8, 7.86, 20.2 Hz, 1H), 3.60 (tr, H-6, J = 8.4 Hz, 1H), 3.54 (ddd, H-3, J = 2.0, 3.8, 10.0 Hz, 1H), 3.44 (tr, H-4, J = 9.6, 1H), 3.09 (tr, H-5, J = 9.8, 1H). In a larger scale preparation the cleavage of 20 g of phosphoinositide fraction performed analogously gave 1.2 g of purified IcP.

(B) The soybean lecithin (12 g, Sigma) was solubilized in ether−water mixture (1:1, 800 mL). To this heterogeneous mixture was added with PI-PLC (30 μg), and the mixture was stirred at room temperature. The progress of the cleavage was followed by TLC, monitoring the formation of diacylglycerol (hexane−acetone, 8.5:1.5; R_f 0.3). The increase in diacylglycerol stopped after ca. 12 h. The mixture was extracted three times with chloroform−methanol (1:1 v/v, 500 mL), and the aqueous phase was concentrated to dryness under vacuum. The residue was chromatographed on the anion exchange column (Bio-Rad AG 1X4, 25 × 200 mm, formate form) collecting the 250 mM ammonium formate fraction (300 mL). This fraction was concentrated and re-evaporated several times with water to remove ammonium formate to give the essentially pure IcP (1.1 g), containing ca. 16% of ammonium formate. This product was used for further reactions without additional purification.

Enzymatic Synthesis of *O*-Alkyl Inositol Phosphates: General Procedure. IcP (10−20 mg) was dissolved in deionized water (0.5 mL) containing a desired primary alcohol (80 mM−6.0 M). The pH of the reaction solvent was adjusted to 6.5−7.5 with a dilute HCl and NH_4OH. PI-PLC (50 μL, containing 10 μg of the protein) was added, and the progress of the reaction was followed by HPAEC, HPLC, or ^{31}P NMR. All reactions were run at ambient temperature. The reactions were stopped by adding 1 N HCl to pH 3, when the concentration of the phosphodiester product reached maximum. The mixture was loaded onto the Dowex 50WX8-200 cation-exchange column (6 × 60 mm), previously equilibrated with ammonium formate, and 10 mL of solution was collected and freeze-dried. The residue was redissolved in water (0.5 mL) and chromatographed on Dowex 1X8-200 anion exchange column (5 × 100 mm), formerly equilibrated with ammonium formate solution. The first fraction (15 mL) eluted off with water contained only an alcohol (in the case of nonionic alcohols). The product was eluted off typically with 30−50 mM ammonium formate (10−15 mL). This fraction was evaporated to dryness and analyzed by HPAEC, ^{31}P and ^{1}H NMR, and ES-MS. The products were frequently found to contain significant amount of ammonium formate, in which case they were redissolved in water and reconcentrated again.

With products of transesterification of certain alcohols (such as PEG-biotin) no suitable separation/detection method could be found to monitor the progress of transesterification. In this case, the reaction was continued until the concentration of IcP dropped to ca. 10% of its original value (as observed by HPAEC). The mixture was then acidified to pH 3, left for 2−3 h, and the IP byproduct was separated on the Dowex anion exchange column as described above. The eluates were monitored by HPAEC, and fractions containing IP were discarded. All fractions eluted prior to IP were pooled and concentrated. The residue was redissolved in small amounts of water, and the product was precipitated with acetone.

Control Experiments. (A) IcP (5 mM) in Tris-HCl buffer (0.1 M, pH 7.4) was stored at room temperature over the period of 4 days. (B) The analogous sample containing additionally 1.0 M glycerol was stored during 6 days. Under both conditions IcP was stable as determined by ^{31}P NMR (within 5% detection limit). (C) The PI-PLC preparation (ca. 1 mg of protein per 1 mL) was dialyzed against doubly distilled water, and the resulting enzyme solution (ca. 3.5 μg of protein) was used to hydrolyze IcP (10 mM) in 70 mM MOPS-Na buffer (pH 7.0)

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containing 10 mM EDTA-Na. ^{31}P NMR spectra revealed formation of only inositol 1-phosphate (within 2% detection limit).

***sn*-Glycero-3-phospho-(1′-*myo*-inositol) (4).** The solution of 1,2-dipalmitoyl-*sn*-glycero-3-phospho-1′-*myo*-inositol (17 mg, synthesized as described earlier[8] in butanol−methanol−water, 3:5:1) was added with methylamine/toluene (30%, 0.4 mL) and kept at 55 °C during 3 h. The mixture was diluted with water and extracted with chloroform, and the aqueous phase was evaporated to dryness. The residue was redissolved in water and passed through H-form Dowex 50X8-200, and the eluate was freeze-dried to give the pure diester **4**.

4: ^{1}H NMR (500 MHz, signal assignment assisted by ^{1}H−^{1}H COSY) δ 4.45 (tr, H-2, J = 2.8 Hz, 1H), 4.2−4.17 (H-1, H-2′, H-3″, m, 4H), 3.94 (tr, H-6, J = 9.7 Hz, 1H), 3.8 (dd, H-1′$_A$, J = 4.5, 11.9 Hz, 1H), 3.84 (tr, H-4, J = 9.8 Hz, 1H), 3.79 (dd, H-1′$_B$, J = 6.0, 11.8 Hz, 1H), 3.74 (dd, H-3, J = 2.8, 10.0 Hz, 1H), 3.52 (tr, H-5, J = 9.4 Hz, 1H); ^{13}C NMR δ 76.42 (d, J = 6.1 Hz), 73.93, 72.2, 71.32 (d, J = 6.5 Hz), 71.15, 70.73, 70.67 (d, J = 6.7 Hz), 66.52 (d, J = 5.5 Hz), 62.05; ^{31}P NMR δ 0.14 ppm; ESMS *m*/*z* 333.8.

***O*-1-(2-Amino-2-hydroxymethylene-3-hydroxypropyl) 1-*myo*-inositol phosphate (5):** ^{1}H NMR (500 MHz) δ 4.12 (tr, H-2, J = 2.8 Hz, 1H), 3.91 (m, H-1′A,B, 2H), 3.85 (ddd, H-1, J = 2.8, 8.1, 10.9 Hz, 1H, 3.62 (tr, H-6, J = 9.6 Hz, 1H), 3.6 (m, H-3′, H-4′, 4H), 3.49 (dd, H-4, J = 9.0, 9.9 Hz, 1H); 3.42 (dd, H-3, J = 2.8, 9.9 Hz, 1H), 3.20 (tr, H-5, J = 9.3 Hz, 1H); ^{31}P NMR δ −1.56 ppm.

***O*-1-(6-Hydroxyhexyl) 1-*myo*-inositol phosphate (6):** ^{1}H NMR (D_2O) δ 4.23 (tr, H-2, J = 2.5 Hz, 1H), 3.91 (tr, H-1′, partially overlapped with H-1, 2H), 3.90 (ddd, H-2, partially overlapped with H-1′, 1H), 3.73 (tr, H-6, J = 10.0 Hz, 1H), 3.64 (tr, H-4, J = 9.5 Hz, 1H), 3.59 (tr, H-6′, J = 6.5 Hz, 2H), 3.55 (dd, H-3, J = 3.0, 10.0 Hz, 1H), 3.31 (tr, H-5, J = 9.5 Hz, 1H), 1.63 (p, H-2′, J = 7.0 Hz, 2H), 1.55 (p, H-5′, J = 7.0 Hz, 2H), 1.38−1.35 (m, H-3′, H-4′, overlapped, 4H); ^{31}P NMR δ 0.84; ESMS *m*/*z* 359.8.

***O*-Methyl 1-*myo*-inositol phosphate (7):** ^{1}H NMR δ 4.15 (tr, H-2, J = 2.8 Hz, 1H), 3.84 (ddd, H-1, J = 2.8, 8.2, 9.9 Hz, 1H), 3.65 (tr, H-6, J = 9.7 Hz, 1H), 3.56 (dd, H-4, J = 9.3 Hz, 10 Hz, 1H), 3.52 (dd, Me, J = 10.8 Hz, 3H), 3.46 (dd, H-3, J = 2.8, 10.0 Hz, 1H), 3.23 (tr, H-5, J = 9.2 Hz, 1H); ^{31}P NMR δ 1.1; ESMS 273.6.

***O*-*n*-Propyl 1-*myo*-inositol phosphate (8):** ^{1}H NMR δ 4.16 (tr, H-2, J = 2.6 Hz, 1H), 3.85 (ddd, H-1, J = 2.8, 8.3, 10.0 Hz, 1H), 3.76 (q, H-1′, J = 6.4 Hz, 2H), 3.66 (tr, H-6, J = 9.6 Hz, 1H), 3.57 (tr, H-4, J = 9.8 Hz, 1H), 3.47 (dd, H-3, J = 2.7, 9.8 Hz, 1H), 3.24 (tr, H-5, J = 9.3 Hz, 1H), 1.55 (q, H-2′, J = 7.3 Hz, 2 H), 0.83 (tr, Me, J = 7.4 Hz, 3H); ^{31}P NMR δ 0.29; ESMS *m*/*z* 301.0 (M − H$^+$).

***O*-*n*-Butyl 1-*myo*-inositol phosphate (9):** ^{1}H NMR δ 4.20 (tr, H-2, J = 2.6 Hz, 1H), 3.88 (trd, H-1′, partially overlapped with H-1, 2H), 3.89 (ddd, H-1, partially overlapped with H-1′, 1H), 3.71 (tr, H-6, J = 9.6 Hz, 1H), 3.61 (tr, H-4, J = 9.8 Hz, 1H), 3.50 (dd, H-3, J = 2.8, 10.0 Hz, 1H), 3.28 (tr, H-5, J = 9.4 Hz, 1H), 1.57 (p, H-2′, J = 6.7 Hz, 2H), 1.33 (s, H-3′, J = 7.8 Hz, 2H), 0.87 (tr, H-4′, J = 7.2 Hz, 3H); ^{31}P NMR δ 0.86 ppm; ESMS *m*/*z* 315.5.

***O*-Allyl 1-*myo*-inositol phosphate (10):** ^{1}H NMR δ 5.95 (m, H-2′, J = 5.28 Hz, 1H), 5.33 (dd, H-3′A, J = 1.1, 17.1 Hz, 1H), 5.19 (dd, H-3′B, J = 1.1, 10.5 Hz, 1H), 4.40 (m, 2H), 4.20 (tr, H-2, J = 2.8 Hz, 1H), 3.90 (ddd, H-1, J = 2.8, 8.4, 11.1 Hz, 1H), 3.70 (tr, H-6, J = 9.6 Hz, 1H), 3.61 (tr, H-4, J = 9.8 Hz, 1H), 3.50 (dd, H-3, J = 2.8, 10.0 Hz, 1H), 3.28 (tr, H-5, J = 9.4 Hz, 1H); ^{31}P NMR δ 0.63 ppm; ESMS *m*/*z* 299.4.

***O*-(2-Hydroxyethyl) inositol phosphate (11):** ^{1}H NMR δ 4.18 (tr, H-2, J = 2.6 Hz, 1H), 3.88 (ddd, H-1 partially overlapped with H-1′, 1H), 3.68 (m, H-2′, 2H), 3.66 (tr, H-6 partially overlapped with H-2′, J = 9.6 Hz, 1H), 3.56 (dd, H-4, J = 9.4, 9.9 Hz, 1H), 3.47 (dd, H-3, J = 2.7, 9.9 Hz, 1H), 3.24 (tr, H-5, J = 9.3 Hz, 1H); ^{31}P NMR (D_2O) δ 0.14 ppm; ESMS *m*/*z* 303.4.

***O*-1-[(2*S*)-Hydroxypropyl)] 1-*myo*-inositol phosphate (12):** ^{1}H NMR δ 4.17 (tr, H-2, J = 2.9 Hz, 1H), 3.92 (qdd, H-2′ partially overlapped with H-1, 1H), 3.88 (ddd, H-1, J = 3.0, 8.5, 10.2 Hz, 1H), 3.81 (ddd, H-1′A, J = 3.4, 5.7, 10.6 Hz, 1H), 3.66 (tr, H-6 partially overlapped with H-1′B, J = 9.3 Hz, 1H), 3.65 (ddd, H-1′B, J = 6.3, 7.0, 10.6 Hz, 1H), 3.56 (tr, H-4, J = 9.5 Hz, 1H), 3.46 (dd, H-3, J = 2.8, 10.0 Hz, 1H), 3.24 (tr, H-5, J = 9.3 Hz, 1H), 1.08 (d, H-3′, J = 6.4 Hz, 3H); ^{31}P NMR δ 0.63 ppm; ESMS *m*/*z* 317.7.

D-Mannito-1-phospho-(1-*myo*-inositol) (13): ^{1}H NMR δ 4.07 (tr, H-2, J = 2.7 Hz, 1H), 4.0 (m, H′-1, 1H), 3.89 (m, H′-1, 1H), 3.78 (dtr, H-1, J = 10.8, 2.8 Hz, 1H), 3.6 (m, 3H), 3.58−3.54 (m, 3H), 3.51−3.41 (m, 3H), 3.35 (dd, H-3, J = 10, 2.7 Hz, 1H), 3.12 (tr, H-5, J = 9.3 Hz, 1H); ^{31}P NMR δ 0.99 ppm; ESMS *m*/*z* 423.0.

L-Idito-1-phospho-(1-*myo*-inositol) (14): ^{1}H NMR δ 4.23 (tr, H-2, J = 2.6 Hz, 1H), 4.02−3.9 (m, H-1′, H-2′, H-1, 4H), 3.84 (m, H-3′, 1H), 3.78−3.6 (m, 6H), 3.53 (dd, H-3, J = 10.0 Hz, 1H), 3.31 (tr, H-5, J = 9.5 Hz, 1H); ^{31}P NMR δ 0.63 ppm; ESMS *m*/*z* 423.0.

***O*-1-(3-Aminopropyl) 1-*myo*-inositol phosphate (15):** ^{1}H NMR δ 4.04 (tr, H-2, J = 2.4 Hz, 1H), 3.85 (trd, H-1′, overlapped, 2H), 3.75 (ddd, H-2, J = 2.9, 9.7, 12.4 Hz, 1H), 3.55 (tr, H-6, J = 9.6 Hz, 1H), 3.46 (tr, H-4, J = 9.3 Hz, 1H), 3.35 (dd, H-3, J = 2.5, 10.1 Hz, 1H), 3.13 (tr, H-5, J = 9.2 Hz, 1H), 2,96 (tr, H-3′, J = 7.3 Hz, 2H), 1.82 (p, H-2′, J = 6.6 Hz, 2H); ^{31}P NMR δ 0.68 ppm; ESMS *m*/*z* 317.0.

Cholinephospho-(1-*myo*-inositol) (16): ^{1}H NMR δ 4.28 (m, H-1′A, H-1′B, 2H), 4.15 (tr, H-2, J = 2.8 Hz, 1H), 3.87 (ddd, H-1, J = 2.8, 8.4, 9.9 Hz, 1H), 3.67 (tr, H-6, J = 9.7 Hz, 1H), 3.58 (m, H-2′, 2H), 3.56 (tr, H-4, 9.3 Hz, 1H), 3.47 (dd, H-3, J = 2.7, 10 Hz, 1H), 3.24 (tr, H-5, J = 9.3 Hz, 1H), 3.13 (s, Me, 9H); ^{31}P NMR δ −1.06; ESMS *m*/*z* 329.9.

L-Serine-3-phospho-(1-*myo*-inositol) (17): ^{1}H NMR δ 4.09 (tr, H-1′, J = 5.4 Hz, 2H), 4.04 (tr, H-2, J = 2.7 Hz, 1H), 3.81 (tr, H-2′, J = 4.29 Hz, 1H), 3.75 (ddd, H-1, J = 12.00, 9.00, 3.00 Hz, 1H), 3.53 (tr, H-6, J = 9.8 Hz, 1H), 3.41 (dd, H-4, J = 18.6, 9.3 Hz, 1H), 3.35 (dd, H-3, J = 10.02, 1.8 Hz, 1H), 3.12 (tr, H-5, J = 9.3 Hz, 1H); ^{31}P NMR δ −0.74 ppm; ESMS *m*/*z* 346.

(Pentaethyleneglyco)phospho-1-*myo*-inositol (18): ^{1}H NMR δ 4.24 (tr, H-2, 1H), 4.05 (m, CH_2OP, 2H), 3.94 (ddd, H-1, 1H), 3.68−3.77 (m, OCH_2CH_2O, 16H), 3.63 (m, H-4, H-6, C*H*$_2$OH, 4H), 3.53 (dd, H-3, 1H), 3.31 (tr, H-5, 1H); ^{31}P NMR δ 0.49 ppm; ESMS *m*/*z* 479.0 (M − H$^+$).

(Biotinylpentaethyleneglyco)phospho-1-*myo*-inositol (19): ^{1}H NMR δ 4.60 (dd, 1H), 4.42 (dd, 1H), 4.25 (m, CH_2OCO, H-2, 3H), 4.05 (m, CH_2OP, 2H), 3.94 (ddd, H-1, 1H), 3.60−3.80 (m, OCH_2CH_2O, 16H), 3.52 (dd, H-3, 1H), 3.78 (m, 2H), 2.96 (dd, 1H), 2.75 (d, 1H), 2.42 (tr, 2H), 1.32−1.80 (m, 6H); ^{31}P NMR δ 0.44 ppm; ESMS *m*/*z* 706.

Glucose-6-phospho-(1-*myo*-inositol) (20): 5.20 (d, J = 4.8 Hz), 4.62 (d, J = 8.0 Hz), 4,23 (tr, J = 2.7 Hz, H-2, 1H), 4.16 (ddd, J = 5.3, 9.6, 1 Hz), 4.11 (tr, J = 4.9 Hz), 4.06 (q, J = 5.3 Hz), 3.93 (m, H-1, 1H), 3.71 (tr, J = 9.7 Hz, 1H), 3.61 (tr, J = 9.7 Hz, 1H), 3.53−3.43 (m, 3H), 3.29 (tr, J = 9.4 Hz, 1H), 3.25 (m); ^{31}P NMR δ 0.65 ppm; ESMS *m*/*z* 421.4.

Uridine-5′-phospho-(1-*myo*-inositol) (22): ^{1}H NMR δ 7.89 (d, J = 8.0 Hz, 1H), 5.92 (d, H-1′, J = 4.7 Hz, 1H), 5.90 (d, J = 8.0 Hz, 1H), 4.31 (m, H-5′, 2H), 4.2 (m, H-2 overlapped with H-4′ and H-2′, 3H), 4.09 (m, H-3′, 1H), 3.92 (m, H-1, 1H), 3.71 (tr, H-6, J = 9.6 Hz, 1H), 3.61 (tr, H-4, J = 9.8 Hz, 1H), 3.50 (dd, H-3, J = 2.8, 6.0 Hz, 1H), 3.27 (tr, H-5, J = 9.4 Hz, 1H); ^{31}P NMR δ −0.47 ppm; ESMS *m*/*z* 485.

Transesterification of Ser-Tyr-Ser-Met with IcP. The solution (50 μL) of tetrapeptide (1 mg) in water was treated with PI-PLC solution (4 μL, 1 mg/mL). The progress of the reaction was monitored by RP-HPLC on C18 column using gradient elution as specified earlier. The reaction was stopped after 48 h and the mixture subjected to LC-ESMS, using chromatographic conditions as described earlier.

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Supporting Information Available: ^{1}H and ^{31}P NMR spectra and ESMS data for compounds **2**−**23** synthesized in this work (27 pages). See any current masthead page for ordering and Internet access instructions.

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