Nonhydrolyzable analogs of phosphatidylinositol as ligands of phospholipases C†‡

Cornelia Mihai,[ac] Xiangjun Yue,[a] Li Zhao,[bd] Alex Kravchuk,[b] Ming-Daw Tsai[be] and Karol S. Bruzik*[a]

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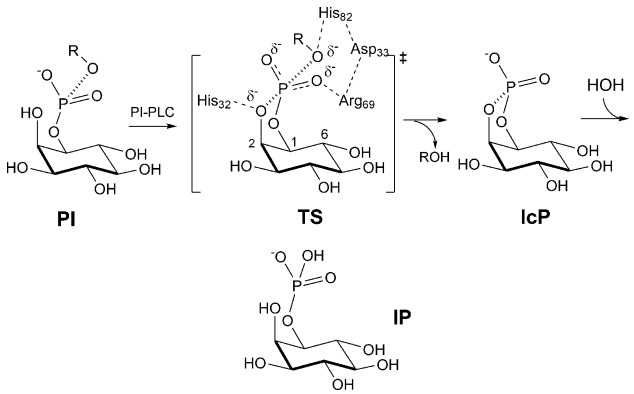
Phosphatidylinositol-specific phospholipases C (PI-PLCs) are important enzymes participating in transmembrane signal transduction. The structures of the two major species of these enzymes: bacterial Ca^{2+}-nondependent enzyme from *B. cereus* and mammalian Ca^{2+}-dependent PLCδ_1 from rat brain in the complexes with the polar head groups of their substrates have been previously solved. Although these structures show few differences as compared to free enzymes, there is a compelling evidence that full catalytic activity of PI-PLC necessitates interaction of the enzyme with the entire substrate, including the hydrophobic fatty acid chains. In this work we develop new tightly binding and cleavage-resistant analogs of phosphatidylinositol, using relatively minor modifications of the structure. Two synthesized analogs, 2-amino-2-deoxy-PI (**8**) and the conformationally constrained analog (**10**) had binding affinities (K_i) in 10 μM range. ^{15}N-^{1}H HSQC NMR spectra of uniformly ^{15}N-labeled bacterial Ca^{2+}-nondependent and Ca^{2+}-dependent phospholipases C, *bt*PLC and saPLC1, respectively, displayed changes upon ligand binding that suggest an occurrence of a conformational change.

Introduction

Phosphatidylinositol-specific phospholipases C (PI-PLCs) are key enzymes of signaling cascades that hydrolyze a minor membrane constituent, phosphatidylinositol 4,5-bisphosphate (PIP_2), and generate two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacyl glycerol (DAG).[1–3] Activation of PI-PLC occurs in response to numerous extracellular signals including growth factors, neurotransmitters and hormones, and involves a broad range of cell surface receptors. Binding of IP_3 to its receptor on the endoplasmic reticulum results in an increase of intracellular Ca^{2+} concentration, whereas DAG activates protein kinase C. Together, these changes control numerous cellular processes, such as DNA synthesis, cell proliferation, and neuronal activity.[1–3]

The catalytic mechanism of bacterial Ca^{2+}-nondependent PLC from *Bacillus thuringiensis* (*bt*PLC) involves (i) activation of the 2-hydroxy group of inositol by a composite general base (GB) dyad, Asp274-His32, and (ii) protonation of the leaving group by the general acid (GA) His82, networked with (iii) the negative charge stabilizing residue, Arg69, *via* an Asp33 intermediary (Scheme 1).[4,5] In the case of Ca^{2+}-dependent PLCδ_1, the negative charge stabilization function of Arg69 is replaced by Ca^{2+} cation, that of His82 GA by His356, whereas the identity of the GB remains unclear.[6] The available X-ray data for *B. cereus* PI-PLC and for mammalian PI-PLCδ_1 indicate that very small changes (if any) occur to the enzyme conformation upon binding of the polar head group of the phospholipid substrate.[7–9] However, since it is also known that activity of PI-PLC is reduced by a factor of 10^5 (k_{cat}/K_m) by the removal of hydrophobic side chains from the substrate,[4,10–12] it is quite possible that the reported X-ray structures of enzyme-head group complexes[8,9] do not represent the fully active enzyme conformations. For example, we have found that for *bt*PLC, formation of the catalytic triad



Scheme 1 General mechanism of bacterial Ca^{2+}-nondependent PI-PLC. Amino acid residue numbering is that of *Bacillus thuringiensis* enzyme.

[a] *Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, Chicago, IL 60612. E-mail: kbruzik@uic.edu*
[b] *Department of Chemistry, The Ohio State University, Columbus, Ohio 43210*
[c] *Department of Natural Sciences, Northwestern Oklahoma State University, Alva, OK 73717*
[d] *Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037*
[e] *Institute of Biological Chemistry, Academia Sinica, Taipei 115, Taiwan*
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Arg69-Asp33-His82 occurs only in the presence of the hydrophobic leaving group, and that enzyme interactions with substrate hydrophobic chains strongly affect both the general acid catalysis and negative charge neutralization functions of the enzyme.[4,11] The goal of this work was to synthesize and test analogs of phosphatidylinositol that while preserving as many of the active site interactions as possible, would still remain nonhydrolyzable to the PI-PLC isozymes.

The design of the ligands for the structural work is different from the design of tight-binding inhibitors, since in the former case preservation of the substrate's basic structural features is important. Ideally, a relatively minor modification should render the substrate analogs resistant to cleavage, while at the same time enhancing its binding affinity. For PI-PLC ligands, the available information indicates that the presence of the hydrophobic residues is important not only for catalysis, but also for substrate binding. The affinity of PLC toward the substrates with short carboxylic acid esters is lower, and is further drastically reduced when these chains are completely removed.[12,13] Thus, btPLC and PLCδ_1 bind the deacylated PI (Gro-PI) and IcP with very low affinity.[10,12] We conclude, that a successful ligand for investigation of the structure of the PLC-substrate complex should bear parts of diacyl glycerol residue. An additional parameter that must be taken into account during ligand design is its critical micellar concentration (cmc). In order to prevent ligand aggregation and the resulting complications (*e.g.* signal broadening in NMR spectra), most of the ligands synthesized in this work feature short chains, resulting in the cmc values above the binding constants.[13]

Two types of modifications have been previously explored to confer resistance of PI analogs to PLC cleavage: in the first case a nucleophilic 2-OH group of inositol was inverted (**1**),[14] deoxygenated (**2**),[15,16] fluorinated (**3**)[16] or methylated (**4**)[16,17] (Fig. 1). In general, these compounds were reported to be resistant to cleavage by PLC, but unfortunately showed only weak inhibitory properties (in the millimolar range). Alternatively, the scissile P–O bond was replaced with a C–P bond as in the phosphonate **5**.[18,19*a*] The phosphonate analogs were potent inhibitors with K_i in the 10 μM range, but we have deliberately excluded such ligands from our considerations since our goal was to obtain substrate analogs that would provide information about enzyme interactions with the leaving group. Zhang *et al.* have synthesized α-fluoroalkylphosphonate analogs of PIP_2 as a replacement for PLC-hydrolyzed PIP_2 that were able to restore properties of TRPM

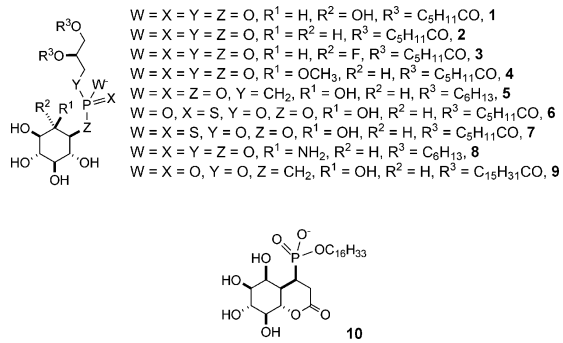


Fig. 1 Structures of PI analogs considered in this work.

transient-voltage-gated ion channels, however, the inhibitory power of these compounds was not determined.[19*b*] The goal of this work was to develop tight-binding and nonhydrolyzable ligands of phospholipase C using as few modifications of the native phosphatidylinositol structure as possible. These ligands would be useful in determination of the structures of PLC isozymes in complexes with their substrates by NMR and crystallographic methods, and also as research tools for investigation of inositol signaling pathways.

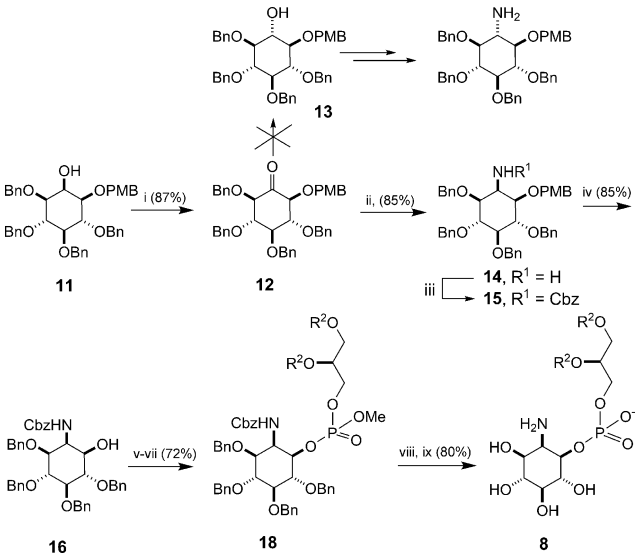
Results and discussion

Our earlier studies on the mechanism of bacterial PI-PLC indicated that interactions of enzyme with the inositol 2-OH group, the nonbridging phosphoryl oxygen atoms and the bridging oxygen atom of the leaving group provide the bulk of catalysis. Hence in our ligand design we strove to retain all three types of interactions. We have also found that the replacement of the *pro-S* oxygen atom in the phosphoryl group by sulfur reduces the rate of the cleavage reaction by 6 orders of magnitude, while preserving the negative charge of the phosphoryl residue. Thus, the phosphorothioate analog **6** could prove sufficiently resistant to PI-PLC to permit acquisition of X-ray diffraction or NMR data. The corresponding dithio analog **7** had relatively weak inhibitory properties.[16] In contrast to compounds **1–4** altered at the 2-position of inositol, the analog **8** featuring the axial amino group in this place should retain some form of interactions between the protonated 2-amino group and *e.g.* the general base residue (His32) of the enzyme (Scheme 1). We have also examined an analog in which the nonscissile C1–O bond was replaced by a C–C bond (**9**). Finally, we hypothesized that the conformationally constrained PI analog, such as the compound **10**, should have stronger binding affinity than the natural substrate, since due to the proximity of the 2-OH group and the phosphorus atom in this structure, it should mimic a near-attack-conformation (NAC)[20] directly preceding formation of the transition state. The structures of the previously synthesized and prospective substrate analogs are shown in Fig. 1.

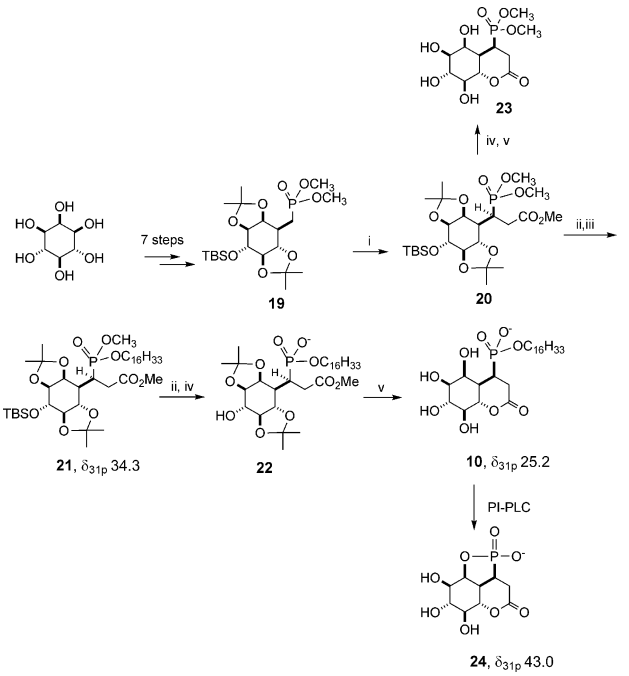
Synthesis of phosphatidylinositol analogs

The compounds **2** and **4** were prepared previously by others,[16,17] and therefore their syntheses are not described here. We used these compounds for comparison with the new ligands **6**, **8** and **10**. The analog **6** was prepared by the enzymatic separation of the mixture of R_p- and S_p-isomers of dihexanoyl phosphorothioate analog of PI, (R_p + S_p)-DHPsI. This mixture was treated with *bt*PLC, and after hydrolysis of the R_p-isomer, the remaining S_p-isomer was separated chromatographically from the products of the reaction.

The main challenge in the synthesis of the amino analog **8** was to retain the axial orientation of the amino group at the 2-position. In order to achieve this goal, we have designed two synthetic pathways (Scheme 2), both involving *scyllo*-inosose **12** as a key intermediate obtained in good yield from alcohol **11** by Swern oxidation. Unfortunately, reduction of **12** with several reagents regenerated the alcohol **11** as a major product, with very little *scyllo*-inositol derivative **13** (having an equatorial 2-OH). Although better yields of an equatorial



Scheme 2 Synthesis of 2-deoxy-2-amino-DHPI, **8**. (i): Ac_2O, DMSO; (ii): NH_4OAc, $NaBH_3CN$, MeOH; (iii): K_2CO_3, Cbz-Cl (R^1 = Cbz); (iv): CAN, acetonitrile–water; (v): Cl–P(OMe)($NiPr_2$), DIPEA; (vi): **17**, tetrazole; (vii): *tert*-BuOOH; (viii): Me_3N; (ix): H_2, Pd/C.



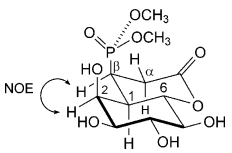
Scheme 3 Synthesis of the analog **10** from the phosphonate **19**. (i): *t*-BuLi, methyl chloroacetate; (ii): Me_3N; (iii): 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT), $C_{16}H_{33}OH$; (iv): TBAF; (v): TsOH, MeOH.

alcohol could be obtained starting from another substrate with a benzoyl group at the 1-position,[14] the separation of axial and equatorial products proved difficult. Hence, a strategy relying on a nuclophilic displacement of an activated equatorial alcohol **13** with an azide was not be pursued. Since the reducing agents preferentially attacked the carbonyl group in 2-inosose from the less hindered equatorial direction generating a product which features an axial group at the 2-position, we reasoned that this finding may be exploited for a direct conversion of the carbonyl into the axial amino group.

This assumption was validated by converting 2-inosose **12** into 2-deoxy-2-amino-inositol **14** *via* reductive amination of the ketone with ammonium acetate and sodium cyanoborohydride in methanol with very good yield. The amine **14** was not isolated, but instead directly protected with benzyloxycarbonyl group (Cbz) to generate compound **15**. The axial orientation of the 2-amino group was evident from the coupling pattern of inositol protons assigned by ^{1}H COSY NMR spectra of **15**. Thus, proton H-2 of **15** displayed 1.2 Hz coupling constant to H-3 and 4.5 Hz to H-1, consistent with equatoral—axial interaction and indicating equatorial orientation of H-2. The removal of the PMB group from **15** with ceric ammonium nitrate (CAN) in acetonitrile–water (4 : 1) produced the amino alcohol **16** in good yield. The subsequent phosphitylation with the with *P*-chloro-*N*,*N*-diisopropyl-*O*-methyl-phosphoramidite, followed by coupling with 1,2-dihexyl-*sn*-3-glycerol (**17**) and oxidation with *tert*-butyl hydroperoxide afforded the fully protected phosphate **18**. The deprotection of **18** by consecutive reactions with anhydrous trimethylamine and catalytic hydrogenolysis afforded the analog **8**.

The conformationally constrained analog **10** was synthesized in 6 steps from the phosphonate analog of inositol 1-phosphate **19**[21] (13 steps overall from *myo*-inositol) as shown in Scheme 3. In brief, alkylation of the α-carbon in the phosphonate **19** with methyl chloroacetate afforded the derivative **20**. The subsequent introduction of the hydrophobic ester group was achieved by demethylation of the phosphonate **20** with trimethylamine followed by the coupling with hexadecanol using the Narang's triester approach[22] to give the triester **21**. The demethylation of the phosphonate **21** and desilylation of the inositol 4-OH group afforded the alcohol **22**.

The cyclization of **22** was achieved in one step *via* acid-catalyzed removal of the isopropylidene groups followed by lactonization with the 6-OH group. It is noteworthy that our other numerous approaches to similar cyclizations including 6-OH alkylation, or formation of a hemiacetal with the 6-OH group, have proved unsuccessful. Since the analysis of the final product **10** was difficult due to its poor solubility and aggregation in aqueous and organic solvents, its stereochemical structure was established indirectly by the cyclization of the intermediate **20** followed by analysis of the neutral lactone **23**. ^{1}H NMR of **23** showed large vicinal coupling constants between ^{31}P and the axial protons at the 1- and α-positions ($^3J_{HCCP}$ = 19.0 Hz and 26.7 Hz, respectively), typical of the *trans*-orientation, whereas the NOESY spectrum showed a cross peak between H-2 and H-β in accordance with their diequatorial orientations (for full characterization of compound **23** see Supporting Information).



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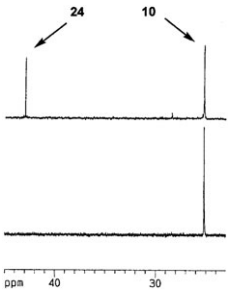

Fig. 2 ^{31}P NMR of the cleavage of the phosphonate **10** by *bt*PLC. Conditions: The solution of **10** (8 mM) in MOPS buffer (50 mM, pH 7.0, 0.5 mL) containing sodium deoxycholate (32 mM) and EDTA (2 mM) was treated with *bt*PLC (0.33 mg). The bottom spectrum was collected immediately after enzyme addition; the top spectrum was registered after 45 h at 23 °C.

In addition, the correct stereochemical structure of the final analog **10** was established by its PI-PLC-catalyzed cyclization to afford the cyclic phosphonate **24**, albeit with 10^6-fold lower activity than that of PI-PLC with natural substrate (V_{max} = 0.004 μmol mg^{-1} min^{-1}). The cleavage of the analog **10** by PI-PLC resulted in the formation of the product **24** displaying *ca.* 18 ppm downfield shift in ^{31}P NMR from that of the substrate **10**, consistent with formation of the five-membered ring phosphonate. Due to the racemic nature of the analog **10** synthesized and strict stereochemical specificity of PI-PLC, the cyclization reaction stopped at the 50% conversion (Fig. 2).

The analog **9** was prepared starting from compound **19**, using demethylation with trimethylamine followed by MSNT-catalyzed coupling with dipalmitoyl glycerol (see below) followed by acid-catalyzed deprotection of inositol ring. Not unexpectedly, the phosphonate analog **9** turned out to be a good substrate of *bt*PLC with V_{max} = 670 μmol mg^{-1} min^{-1}.

Inhibition of PI-PLC by PI analogs

Two of the ligands synthesized in this work, 2-deoxy-DHPI and 2-MeO-DHPI, have been previously tested by Martin and Wagman,[16] using *p*-nitrophenyl inositol phosphate as an assay substrate. The problem with their measurements was that due to a high K_m of the substrate (between 5 and 15 mM depending upon the buffer), the assays were performed under non-saturating substrate concentrations (0.2 mM). Hence, the K_i values reported for **2** (2.6 mM) and **4** (6.6 mM) are of uncertain reliability.

In this work, the evaluation of inhibitors was performed by a continuous UV assay using phosphorothiolate dioctanoyl analog of PI, DOsPI, as a substrate that has a small K_m (75 μM).[13,23] The assays were performed at substrate concentrations ranging from 25 μM to 400 μM, while inhibitor concentrations were kept constant. For each inhibitor, the type of inhibition was assessed, and the K_i value for each inhibitor calculated using the following expressions:[24]

$$\text{Competitive inhibition: } K_i = K_m\,[I]/(K_p - K_m)$$

$$\text{Uncompetitive inhibition: } K_i = K_p\,[I]/(K_m - K_p)$$

$$\text{Non-competitive inhibition: } K_i = V_p\,[I]/(V_m - V_p)$$

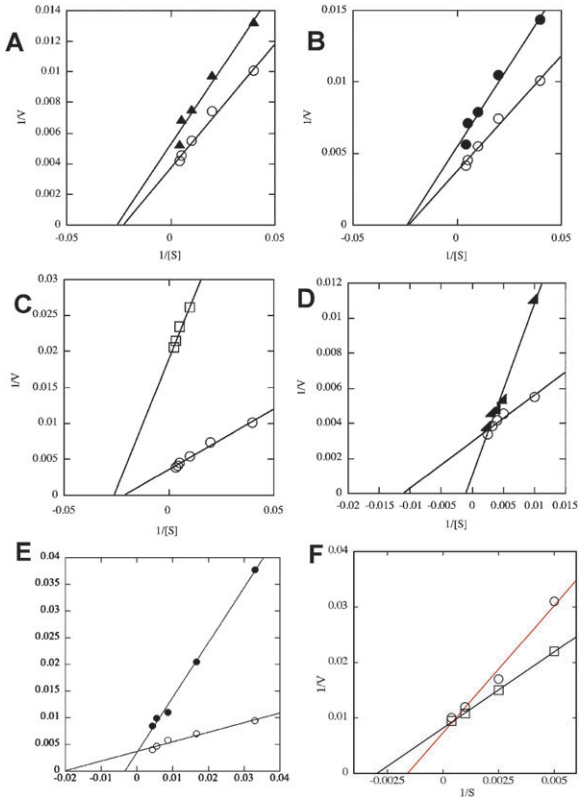

Fig. 3 Lineweaver–Burk plots for *bt*PLC (A–E) and saPLC1 inhibition (F): (A) 2-deoxy-DHPI (**2**): (○) DOsPI, (▲) DOsPI + 2 mM **2**; (B) 2-*O*-methyl-DHPI (**4**): (○) DOsPI; (●) DOsPI + 5 mM **4**; (C) S_p-DHPsI (**6**): (○) DOsPI, (□) DOsPI + 100 μM **6**; (D) 2-deoxy-2-amino-dhPI (**8**): (○) DOsPI, (▲) DOsPI + 250 μM **8**; (E) (**10**): (○) DOsPI, (●) DOsPI + 100 μM **10**; F: (□) DOsPI, (○) DOsPI + 250 μM **8**.

where K_m is the Michaelis constant of the substrate, K_p is the apparent Michaelis constant of the substrate in the presence of inhibitor, K_i is the inhibition constant, [I] is the concentration of inhibitor, V_m is the maximum velocity, and V_p is the apparent maximum velocity (with inhibitor).

Unexpectedly, 2-deoxy-PI, **2**, was found to be a poor substrate with a cleavage rate *ca.* 10^6-fold slower than that of PI. In view of the absence of the nucleophilic 2-OH group, the instability of compound **2** is interesting since it indicates that PLC can substitute a water molecule for the missing hydroxyl group to effect a direct hydrolysis, albeit at a very low rate. Our experiments also showed that this compound is a weak uncompetitive inhibitor (Fig. 3A) with similar effects on both K_m and V_{max} and the calculated K_i of 5 mM. 2-*O*-Methyl-DHPI (**4**) was found to be one of the most stable of all ligands compared. It was completely resistant to 1 mg of WT PI-PLC for more than one week, hence the upper limit of the cleavage rate is 7.7 × 10^{-5} μmol min^{-1} mg^{-1}). However, it was a rather weak non-competitive inhibitor with K_i value of 11 mM (Fig. 3B). S_p-DHPsI (**6**) was cleaved by *bt*PLC more than 10^6 times slower than the natural substrate (V_{max} = 9 × 10^{-4} μmol min^{-1} mg^{-1}). This compound was a partially non-competitive inhibitor with a significant effect on maximum velocity of substrate and lower effect on K_m (Fig. 3C). Using the expression for a non-competitive inhibition, the K_i was

calculated at 23 μM. A possible explanation of these inhibitory behaviors of ligands **2**, **4** and **6** is that phenomena such as allosteric activation by the added inhibitors (increasing V_{max}) and surface dilution in the micelles (reducing V_{max}) are additional parts of the overall interactions. If the active site-binding of an inhibitor (competitive inhibition) is not significantly stronger than that of the substrate, these phenomena can lead to apparent modes of inhibition other than competitive. For compound **8**, in addition to the replacement of the 2-OH group with the amino group, which prevents the nucleophilic attack, this analog was also equipped with ether-linked instead of ester-linked chains. This modification of DAG moiety should provide stronger interactions with PLC.[19] Compound **8** was found to be resistant to cleavage by *bt*PLC for more than one week using 5 mM concentration of **8** and 1 mg of btPLC. The estimated upper limit of the rate of the cleavage is hence 8.5×10^{-5} μmol min^{-1} mg^{-1}). Fig. 3D indicates that this compound is a strong competitive inhibitor with a K_i of 15 μM. Hence, this compound appears to be one of the most promising ligands synthesized to date, offering a conservative structure modification, tight binding and complete resistance to enzymatic cleavage. Compound **8** was also a weaker inhibitor of saPLC1 with K_i = 0.23 mM (Fig. 3F). The bicyclic compound **10** was cleaved by *bt*PLC at low rate, and was also a clean competitive inhibitor with K_i = 21.3 μM (Fig. 3E). Given that btPLC is strictly stereospecific and that the synthesized compound was racemic, the K_i for compound **10** is just 10.6 μM. Additional modifications must be performed to improve stability of this compound to PI-PLC–catalyzed cleavage.

Conformational changes of PI-PLC upon ligand binding

Our goal was to examine binding properties of the synthetic substrate analogs and detect structural changes in PLC upon ligand binding. We have used ^{15}N-^{1}H HSQC NMR of uniformly ^{15}N-labeled enzymes as the means to generate signature data, and to identify the best ligands for further structural studies, either by multidimensional NMR or X-ray crystallography. The partial spectra of the arginine side chains of *bt*PLC are shown in Fig. 4. Note, that the rates of cleavage of compounds **2**, **6** and **10** by the WT enzyme were somewhat too high for these ligands to be used in NMR experiments, therefore an active site mutant, Arg69Asp, was used. Fig. 4A clearly shows signals from all nine arginine side chains ($^{\varepsilon}$NH region) of *bt*PLC. The assignment of the resonaces of Arg69, Arg71 and Arg163 was achieved using the approriate mutants. The comparison between the ^{1}H-^{15}N HSQC spectra of WT and Arg69Asp (Fig. 4A–B) shows that in the mutant, the signals of not only Arg69 but also Arg71 disappeared, while the signal of Arg163 was slightly shifted upfield. Upon addition of the analog **6** (Fig. 4C) to this mutant, the signal of Arg71 reappeared in a somewhat shifted position, and the signal of Arg163 moved downfield by almost 3 ppm (^{1}H). The small residual signal at the original position of Arg163 indicates that the ligand-bound and unbound protein species remain in a slow conformational exchange on the NMR time scale. Similar results were observed for analogs **4** and **8** with the same mutant (spectra not shown). In contrast, addition of 10 mM **4** to WT (Fig. 4D) produced much less dramatic

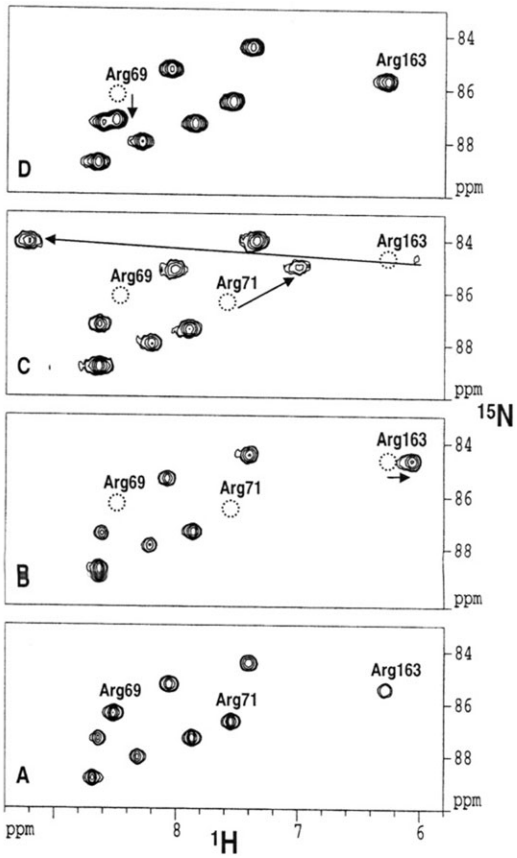

Fig. 4 Arginine side chain ($^{\varepsilon}$NH) region of ^{1}H-^{15}N HSQC spectra of uniformly ^{15}N-labeled btPLC. (A) WT, (B) R69D, (C) R69D + 8 mM **6**, (D) WT + 10 mM **4**. All samples were in 10% D_2O-90% H_2O, 50 mM HEPES, pH 7.5. The protein concentration was *ca.* 0.4 mM. The open circles with dashed line represent the original locations of the arginine cross peaks in the spectrum of WT. Spectra were acquired on a Bruker DRX-800 spectrometer at 37 °C.

changes in the spectra than those observed for Arg69Asp. In the arginine region, only Arg69 was shifted downfield. These results suggest that Arg69Asp mutant undergoes a conformational change upon ligand binding. It is unclear at the moment whether the same is true for the WT enzyme, since the differences observed (with a different ligand) are smaller. Whether this is related to weak binding of the ligand **4** or greater conformational stability of WT *bt*PLC is also uncertain.

Another PLC that we are interested in is a Ca^{2+}-dependent PI-PLC from *Streptomyces antibioticus* (saPLC1).[25] This enzyme employs a unique mechanism in which the 6-hydroxyl group, rather than 2-OH, is an attacking nucleophile.[26] Ligand **8** inhibited saPLC1 in a competitive manner with K_i = 230 μM, and was completely resistant to this enzyme.

The effect the ligand **8** binding on the conformation of saPLC1 was qualitatively assessed using ^{15}N-^{1}H HSQC spectra (Fig. 5). In the absence of Ca^{2+}, no obvious spectroscopic changes could be observed in the ^{15}N-^{1}H HSQC spectrum upon addition of 0.2 mM **8** (data not shown). After adding Ca^{2+} to this system, about 30 signals in the amide backbone region moved to different positions (Fig. 5). These results indicate that compound **8** binds to the enzyme in a Ca^{2+}-dependent manner.

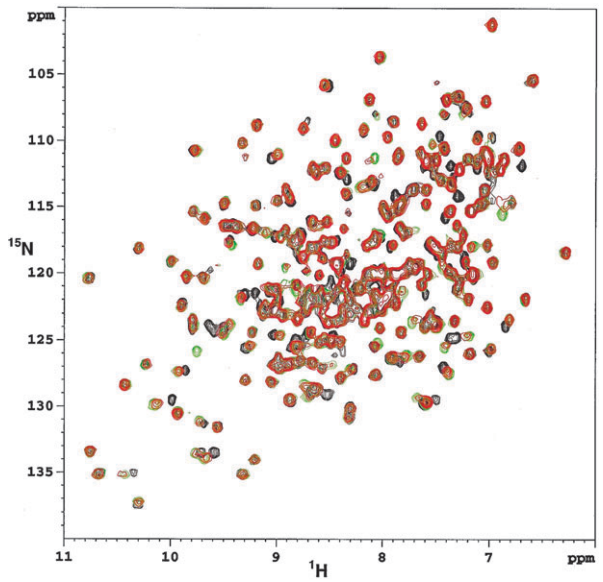

Fig. 5 The backbone amide region of ^{15}N-^{1}H HSQC spectra of uniformly ^{15}N-labeled saPLC1. Overlaid are spectra for saPLC1 (black), saPLC1 + 1 mM free Ca^{2+} (green) and saPLC1 + 1 mM free Ca^{2+} + 0.2 mM **8** (red). All samples were in 10% D_2O–90% H_2O, 50 mM HEPES, 1 mM EDTA, pH 7.0. The protein concentration was 0.2 mM. Spectra were acquired on a Bruker DRX-800 spectrometer at 37 °C.

As a control, we also examined the changes in the ^{15}N-^{1}H HSQC spectrum of saPLC1 upon Ca^{2+} titration. As shown in Fig. 5, about 50 signals in the amide backbone region have shifted upon Ca^{2+} addition. Interestingly, overlaying three spectra (free enzyme, enzyme + Ca^{2+}, enzyme + Ca^{2+} + **8**) demonstrates that most of the signals, which were subject to changes upon inhibitor binding, also experienced shifts upon calcium binding. This ultimately suggests that Ca^{2+} and **8** binds to saPLC1 in a closely related region, possibly the active site.

Conclusions

We have synthesized several analogs of phosphatidylinositol with the aim of achieving strong binding and complete resistance to PI-PLC with relatively minor conservative modifications of the PI structure. Among six compounds prepared, 2-deoxy-2-amino analog **8**, and the conformationally constrained analog **10** were the strongest competitive inhibitors. In addition, compound **8** was also completely resistant to cleavage by the enzymes, while compound **10** underwent slow cleavage. The latter analog will require additional modification to achieve complete hydrolytic resistance. Using these conservative analogs of PI and ^{15}N-^{1}H HSQC NMR spectra we have shown that both *bt*PLC and saPLC1 seem to undergo conformational transition upon ligand binding. Further studies are needed to solve the structures of these enzymes in complexes with the ligands.

Experimental

General

All reagents were obtained from Aldrich Chemical Company and Fisher Scientific. All solvents used for moisture sensitive reactions were anhydrous, and were purchased from Aldrich. These solvents were stored in vacuum-tight ampoules over appropriate drying agents and transferred to the reaction vessel by molecular distillation under vacuum. Chloroform and methylene chloride were passed through a column of basic alumina to remove traces of water and acid, and stored over calcium hydride or phosphorus pentoxide. Acetonitrile and toluene were stored over calcium hydride and diisopropylethyl amine (DIPEA) was stored over sodium hydride. THF was stored over Na–K alloy or lithium aluminium hydride. Anhydrous methanol was dried by magnesium turnings, in the presence of catalytic amounts of iodine, distilled and stored over 3 Å molecular sieves. ^{1}H NMR spectra were obtained with NMR spectrometers operating at 300 and 500 MHz frequencies, while ^{13}C NMR spectra were obtained at 75 and 125 MHz. ^{31}P NMR spectra were obtained at 121.5 MHz with Bruker DPX-300 NMR spectrometer and the chemical shifts are referenced to external 80% orthophoshoric acid.

PLC Assay. The assay used in this work is a modified procedure first reported by Hendrickson and co-workers[29] and expanded in our recent publication.[13] For *bt*PLC assay, the appropriate amount of a 10 mM stock solution of DOsPI in DI water to provide the final substrate concentration of 25–400 μM was loaded into a semi-micro UV cuvette. The buffer solution (40 μL, 50 mM MOPS, 1 mM EGTA, pH 7.0) was added into the cuvette followed by the solution of 4,4′-dithiopyridine (DTP) in ethanol (10 μL, 100 mM). An aliquot of the aqueous solution of an inhibitor (1.0–10 mM depending on a ligand) was added next to obtain a desired concentration of an inhibitor shown in Fig. 3. The mixture was diluted with distilled water to 390 μL, and the contents of the cuvette were stirred and equilibrated for 3 min at room temperature to achieve optical clarity. Background absorbance was recorded for 200 s at 324 nm (25 °C) against the MOPS buffer on a Hitachi U3010 spectrophotometer. The reaction was initiated by adding 10 μL of a freshly prepared enzyme solution, and the absorbance at 324 nm was recorded for 200–400 s. The molar extinction coefficient of 4-pyridyl thiol, ε = 19 800 M^{-1} cm^{-1}, was used to calculate the initial rates of reaction. The dependencies of these rates *vs.* the substrate concentrations were fitted to kinetic equation, described above, using either GraFit or KaleidaGraph software to obtain the kinetic parameters. The assay conditions for saPLC1 were the same except 3 mM $CaCl_2$ was included in the assay mixture.

Synthesis

Alcohol **11** was synthesized according to a published procedure.[27] Synthesis of the phosphonate **19** was performed as reported previously.[21] *bt*PLC and its mutants were expressed as described earlier.[4] Expression and purification of saPLC1 is described in the reference 28.[28] Uniformly ^{15}N-labeled WT saPLC1 was expressed in M9 minimal media using $^{15}NH_4Cl$ as a single nitrogen source and purified as described earlier.[28] Two-dimensional ^{15}N-^{1}H HSQC spectra of the uniformly ^{15}N-labeled protein at Ca^{2+} concentration ranging from 0 to 5 mM were obtained on a Bruker DRX-800 spectrometer at 37 °C.

(S_p)-(1,2-Di-*O*-hexanoyloxypropane-3-thiophospho)-1-D-*myo*-inositol (6). The mixture of (R_p + S_p) DHPsI diastereomers (0.285 g, 0.52 mmol), synthesized analogously as described earlier,[30] was dispersed by sonication in water (30–40 mL) and the pH of the solution obtained was adjusted to pH = 7.0 with 50 mM ammonium formate. To this mixture was added PI-PLC from *B. thuringiensis* (40 μg) in 50 mM MOPS buffer (pH 7.0, 50 μL). The mixture was stirred vigorously at room temperature for 5 h, monitoring the progress of the reaction by ^{31}P NMR. When the R_p-DHPsI was completely cleaved, the mixture was extracted twice with diethyl ether (2 × 20 mL) and the aqueous extract was loaded on a C_{18}-reverse phase column. Elution with 4 column volumes of water delivered all the compounds soluble in water, including IcPs, while the fractions obtained from elution with 4–5 column volumes of methanol contained S_p-DHPsI. The methanolic fractions were concentrated, the residue dispersed in water and the solution passed through a cation-exchange column (ammonium-form). The freeze-drying of the fraction eluted from the column afforded pure **6** as an ammonium salt (0.102 g, 90% recovered from the initial mixture) as a white solid. ^{1}H NMR (CD_3OD) δ δ 0.90 (t, J = 6.9 Hz, 6H), 1.33–1.40 (m, 8H), 1.59–1,66 (m, 4H), 2.31–2.39 (m, 4H), 3.18–3.25 (m, 1H), 3.45 (dd, 1H), 3.64 (t, J = 9.5 Hz 1H), 3.79 (t, J = 9.5 Hz, 1H), 4.11–4.22 (m, 5H), 4.4 (m, 1H), 5.25 (m, 1H); ^{31}P NMR (D_2O) δ57.0. 61; ES-MS (m/z) 545.

1-D-1-*O*-(4-Methoxybenzyl)-3,4,5,6-tetra-*O*-benzyl-*scyllo*-inosose (12). A solution of alcohol **11** (0.5 g, 0.75 mmol) in anhydrous DMSO (10 mL) was added dropwise with acetic anhydride (1 mL, 10.6 mmol) under nitrogen and stirred at room temperature for about 14 h. The reaction mixture was added stepwise to the stirred aqueous solution of sodium bicarbonate (25 g in 125 mL water) during 1 h and then stirred for 2 more hours. The precipitate formed was filtered off and washed with water. The recrystallization from hexane–ethyl acetate (1 : 1) afforded pure 2-inosose **12** (0.435 g, 87%). R_f 0.48 (hexane–ethyl acetate, 2 : 1). ^{1}H NMR ($CDCl_3$) δ 3.61–3.65 (m, 2H), 3.82 (s, 3H), 3.87 (t, J = 9 Hz, 1H), 4.12–4.17 (m, 2H), 4.48 (d, J = 12 Hz, 1H), 4.52 (d, J = 12 Hz, 1H), 4.75–4.94 (m, 8H), 6.86 (d, 2H), 7.28–7.39 (m, 22 H); ^{13}C NMR ($CDCl_3$) δ 53.16, 72.98, 73.29, 75.83, 75.87, 75.98, 82.16, 83.54, 83.85, 113.81, 127.47, 127.64, 127.68, 127.88, 127.93, 128.31, 128.43, 129.56, 129.86, 137.55, 138.32, 138.37, 138.42, 159.54, 202.34.

1-D-1-*O*-(4-Methoxybenzyl)-2-deoxy-2-benzyloxycarbonylamino-3,4,5,6-tetra-*O*-benzyl-*myo*-inositol (15). The inosose **12** (250 mg, 0.38 mmol) was dispersed in anhydrous THF (5 mL) and stirred until the ketone dissolved completely. Ammonium acetate (293 mg, 3.8 mmol) was added and the mixture stirred for a few minutes until solution was clear. A solution of sodium cyanoborohydride (27.6 mg, 0.42 mmol) was added dropwise at room temperature and the mixture stirred for 2 h. The progress of the reaction was monitored by TLC (hexane–ethyl acetate, 2 : 1). The reaction mixture was concentrated, the residue redissolved in ethyl acetate, and washed with sodium bicarbonate and brine. The organic phase was separated, dried over sodium sulfate and concentrated. Purification by column chromatography (hexane–ethyl acetate, 2 : 1) afforded pure amine (225 mg, 90%) as a white solid (R_f 0.24, hexane–ethyl acetate, 2 : 1). To the solution of the amine in dioxane-water (1.5 : 1, 15 mL) solid potassium carbonate (51.4 mg, 0.32 mmol) was added in portions and the mixture was stirred for a few minutes. Benzyl chloroformate (100 μL, 0.7 mmol) was added stepwise under nitrogen and the reaction was vigorously stirred for 1 h. The progress of the reaction was monitored by TLC (hexane–ethyl acetate, 2 : 1). When no more starting material was observed by TLC, the mixture was concentrated to dryness, the residue redissolved in methylene chloride and the solution washed with brine, dried and concentrated. Purification by column chromatography on silica gel (hexane–ethyl acetate, 2 : 1) gave pure **15** (207 mg, 90% from the amine) as a white solid. R_f 0.55 (hexane–ethyl acetate, 2 : 1); $[\alpha]_D^{20}$ = −5° (c 1.6, chloroform); ^{1}H NMR ($CDCl_3$:CD_3OD, 1 : 1) δ 3.55–3.71 (m, 5H), 3.87 (s, 3H), 4.56 (d, J = 10.7 Hz, 1H), 4.63 (d, J = 11 Hz, 1H), 4.83–5.00 (m, 10H), 5.26 (s, 2H), 6.91 (d, 2H), 7.28–7.45 (m, 27H); ^{13}C NMR ($CDCl_3$) δ 55.43, 71.82, 72.15, 75.85, 76.17, 77.91, 78.22, 81.61, 82.75, 113.96, 127.77, 127.89, 127.99, 128.17, 128.24, 128.28, 128.49, 128.53, 128.69, 129.36, 130.12, 136.58,138.02, 138.68, 138.74, 157.20, 159.46.

1-D-2-Deoxy-2-*N*-benzyloxycarbonylamino-3,4,5,6-tetra-*O*-benzyl-*myo*-inositol (16). Compound **15** (200 mg, 0.25 mmol) was dissolved in acetonitrile–water (4 : 1, v/v, 25 mL). To this solution, CAN (690 mg, 1.26 mmol) was added in portions at room temperature, and the mixture stirred for 30 min. When TLC showed complete consumption of the starting material, the mixture was concentrated, the residue redissolved in chloroform and the organic extract was washed with brine several times. Organic extract was separated, dried and concentrated. Purification by silica gel chromatography (hexane–ethyl acetate, 2 : 1 → 1 : 1) afforded pure compound **16** (144 mg, 85%) as a white solid. R_f 0.25 (hexane–ethyl acetate, 2 : 1). ^{1}H NMR ($CDCl_3$) δ 3.55–3.71 (m, 5H), 4.55 (m, 1H), 4.64–4.93 (m, 8H), 5.17 (s, 2H), 5.27 (d, 1H), 7.24–7.42 (m, 25H).

1,2-Di-*O*-hexyl-*sn*-*glycero*-3-phospho)-D-2-deoxy-2-benzyloxycarbonylamino-3,4,5,6-tetra-*O*-benzyl-*myo*-inositol (18). Into the solution of alcohol **16** (144 mg, 0.21 mmol) in chloroform (2 mL) was added *via* syringe *P*-chloro-*N*,*N*-diisopropyl-*O*-methyl-phosphoramidite (0.25 mmol) at room temperature, under nitrogen. The reaction was stirred for 24 h, solvents were removed under vacuum, and the residue dried in vacuo for a few more hours. Tetrazole (1 mmol) and 1,2-di-*O*-hexyl-*sn*-glycerol **17** (86 mg, 0.33 mmol) (see Supporting Information for description of synthesis of **17**‡) were added and a minimum volume of anhydrous acetonitrile was transferred into the reaction vessel *via* distillation under vacuum. The suspension obtained was stirred for 24 h at room temperature under nitrogen, monitoring the progress of the reaction by ^{31}P NMR. The solution of crude phosphite triester in dry chloroform, was cooled to −10 °C and *tert*-butyl hydroperoxide in decane (0.5 mmol) was added stepwise *via* a syringe. The reaction mixture was warmed up to room temperature and aqueous sodium bicarbonate solution was

added. The organic extract was separated, washed with brine and dried over sodium sulfate, filtered and concentrated. The crude product was purified on silica gel (hexane–ethyl acetate, 4 : 1 → 1 : 1) to give pure **18** as a mixture of two diastereomers (100 mg, 72%) in a form of a colorless oil. R_f 0.20 (hexane–ethyl acetate, 2 : 1); ^{1}H NMR ($CDCl_3$) δ 0.89 (m, 12H), 1.31 (m, 24H), 1.56 (m, 8H), 3.25–3.78 (m, 24H), 4.02–4.06 (m, 4H), 4.51 (4.55 (m, 2H), 4.74–4.92 (m, 16H), 5.17 (s, 2H), 7.21–7.37 (m, 50H); ^{31}P NMR ($CDCl_3$) δ 1.25, 1.45 (1 : 1).

1,2-Di-*O*-hexyl-*sn*-3-*glycero*-3-phospho-D-2-deoxy-2-amino-*myo*-inositol (8). The triester **18** (100 mg, 0.098 mmol) was demethylated with anhydrous trimethylamine during 48 h at 50 °C to afford pure product, which was then subjected to hydrogenolysis with palladium on charcoal in dry methanol. After 24 h the reaction was complete, the mixture was filtered through Celite and the solution concentrated to dryness. The residue was redissolved in water, and the solution was passed through a reverse phase column. The product was eluted off with methanol and concentrated. The residue was dissolved in water and converted into an ammonium salt using a cation exchange column. Freeze-drying gave the pure compound **8** (38 mg, 70%) as a white powder. ^{1}H NMR ($CDCl_3$:CD_3OD:D_2O, 3 : 3 : 1) δ 0.8 (m, 6H), 1.20–1.30 (m, 12H), 1.48 (m, 4H), 3.23 (m, 2H), 3.39–3.45 (m, 5H), 3.50–3.68 (m, 6H), 3.82 (t, J = 4.5, 1H), 3.90 (m, 2H), 4.09 (m, 1H); ^{31}P NMR (D_2O) δ 0.70; ES-MS (m/z) 501.

Alkylation of phosphonate 19. Into a solution of phosphonate **19** (3.36 g, 7 mmol) in anhydrous THF (30 mL) was added dropwise a solution of 1.7 M *t*-BuLi in pentane (4.94 mL, 8.4 mmol, 1.2 equiv.). The mixture was stirred at −78 °C for 1.5 h methyl chloroacetate (0.920 mL, 10.5 mmol, 1.5 equiv.) and the temperature was allowed to warm up to 20 °C. The reaction mixture was diluted with chloroform (300 mL), washed with brine, and dried over Na_2SO_4. Evaporation of all solvents followed by chromatography on silica gel using hexane-acetone (1 : 1, v/v) as an eluting solvent gave the product **20** (3.55 g, 92%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.09 (s, 6H, $SiMe_2$), 0.90 (s, 9H, $SiBu^t$), 1.31 (s, 3H, CH_3), 1.32 (s, 3H, CH_3), 1.34 (s, 3H, CH_3), 1.47 (s, 3H, CH_3), 2.31 (m, 1H, PCH), 2.50–3.10 (m, 3H, CH, CH_2CO_2Me), 3.21 (dd, 1H, J = 4.9 Hz, CH), 3.63 (s, 3H, OCH_3), 3.60–3.80 (m, 6H, $P(OMe)_2$), 3.90 (dd, 1H, J = 6.4 Hz, CH), 4.55 (dd, 1H, J = 4.3 Hz, CH). ^{31}P NMR (121 MHz, $CDCl_3$): δ 35.40 ppm.

Exchange of the ester grop in the phosphonate 20. The solution of the phosphonate **20** (1.1 g, 2 mmol) in trimethylamine (5 mL) was heated in a sealed ampoule at 80 °C for 4 days. Evaporation of Me_3N gave the demethylated product quantitatively as indicated by ^{31}P NMR, which was used directly in the next step. The mixture of the demethylated product from above, 1-(mesilytene-2-sulfonyl)-3-nitro-1,2,4-triazole (1.18 g, 4 mmol, 2 equiv.) and 1-hexadecanol (720 mg, 3 mmol, 1.5 equiv.) in anhydrous pyridine (5 mL) was stirred at room temperature for 24 h. The reaction mixture was diluted with chloroform (300 mL), washed with brine, and dried over Na_2SO_4. Evaporation of all solvents followed by chromatography on silica gel using hexanes-acetone (2 : 1, v/v) as an eluent gave the phosphonate diester **21** (550 mg, 36%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.10 (s, 6H, $SiMe_2$), 0.89 (m, 3H, CH_3), 0.90 (s, 9H, $SiBu^t$), 1.26 (s, 28H, CH_2), 1.34 (s, 3H, CH_3), 1.37 (s, 3H, CH_3), 1.40 (s, 3H, CH_3), 1.46 (s, 3H, CH_3), 2.33 (m, 1H, PCH), 2.50–3.20 (m, 3H, CH, CH_2CO_2Me), 3.26 (dd, 1H, J = 4.9 Hz, CH), 3.66 (s, 3H, OCH_3), 3.60–3.80 (m, 5H, P(OMe), $P(OCH_2R)$, 3.91 (dd, 1H, J = 6.4 Hz, CH), 4.03 (m, 2H, CH), 4.64 (m, 1H, CH). ^{31}P NMR (121 MHz, $CDCl_3$) δ 33.76 ppm.

Formation of the alcohol 22. A solution of the phosphonate **21** (530 mg, 0.7 mmol) was treated with trimethylamine (5 mL) analogously as described above. Evaporation of trimethylamine gave the demethylated product quantitatively, which was further dissolved in the solution of tetra-n-butylammonium fluoride in THF (1 M, 5 mL) and stored at room temperature for 2 h. Evaporation of the solvent followed by chromatography on silica gel using chloroform–methanol-ammonium hydroxide (40 : 10 : 1, v/v) as an eluent gave the phosphonate monoester **22** (300 mg, 65%) as a colorless oil. This compound was used for further steps without additional purification as described below.

Formation of the δ-lactone 10. A mixture of the demethylated phosphonate **22** (65 mg, 0.1 mmol) and *p*-toluenesulfonic acid monohydrate (5 mg) in methanol (2 mL) was heated at 60 °C for 2 days. Evaporation of all solvents followed by chromatography on silica gel eluting with chloroform–methanol–water-ammonium hydroxide (60 : 40 : 5 : 1, v/v) gave the bicyclic phosphonate **10** (27 mg, 50%) as an amorphous colorless solid. ^{1}H NMR (500 MHz, CD_3OD): δ 4.25 (dd, J = 9.3, 11.2 Hz, H-6, 1H), 4.19 (tr, J 3.0 Hz, H-2, 1H), 3.89 (m, OCH_2, 2H), 3.65 (tr, J = 10.6 Hz, H-4, 1H), 3.49 (tr, J = 9.2 Hz, H-5, 1H), 3.47 (dd, 3.0 Hz, 9.8 Hz, H-3, 1H), 3.22 (m, 1H), 2.76–2.87 (m, 2H), 2.52 (m, 1H), 1.97 (m, 1H), 1.62 (m, 2H), 1.31 (m, 26H), 0.89 (tr, 3H). ^{31}P NMR (121 MHz, D_2O): δ 25.2 ppm. HR ESMS: Calcd. for $C_{25}H_{46}O_9P$: 521.2879; Found: 521.2869.

Formation of lactone 23. Lactone **23** was obtained from compound **20** analogously as described for compound **21**. ^{1}H NMR (400 MHz, Py-d_5) δ 5.05 (d, J = 9.4, 11.0 Hz, H-6, 1H), 4.82 (brs, H-2, 1H), 4.65 (tr, J = 8.9 Hz, H-4, 1H), 4.23 (tr, J = 9.0 Hz, H-5, 1H), 4.14 (dd, J = 2.7, 9.5 Hz, H-3, 1H), 3.68 (d, J = 9.1 Hz, 3H), 3.65 (d, J = 8.5 Hz, 3H), 3.50 (dddd, J = 2.3, 8.8, 11.0, 17.0 Hz, H_b, 1H), 3.26 (ddd, J = 10.7 Hz, 16.6 Hz, 26.7 Hz, H_{aa}, 1H), 3.07 (dtr, J = 2.5 Hz, 16.1 Hz, H_{ae}, 1H), 2.61 (dddd, J = 2.2, 9.0, 11.2, 19.0 Hz, H-1, 1H). ^{13}C NMR (Py-d_5) δ 171.6, 171.5, 80.65, 80.53, 76.2, 75.8, 74.4, 71.3, 53.2, 53.1, 53.0., 39.4, 30.2, 29.7, 29.6, 28.2. ^{31}P NMR (Py-d_5) δ 36.0.

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