A Novel Class of Zinc-Binding Inhibitors for the Phosphatidylcholine-Preferring Phospholipase C from *Bacillus cereus*

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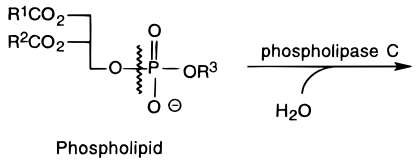
The phospholipase C (PLC) isozymes catalyze the hydrolysis of phospholipids to provide diacylglycerol (DAG) and a phosphorylated headgroup. Because DAG has been implicated in cellular signal transduction cascades in mammalian systems, there has been considerable interest in the development of inhibitors of these enzymes. Toward this end, we have discovered that the cyclic *N*,*N*′-dihydroxyureas **6**−**10** inhibit the phosphatidylcholine preferring PLC from *Bacillus cereus* (PLC_{Bc}). This class of inhibitors is believed to function by the bidentate chelation of the *N*,*N*′-dihydroxyurea array to one or more of the zinc ions at the active site of the enzyme. Because the affinities of these compounds correlate with the pK_as of the N−OH hydroxyl groups, it is apparent that one or both of the hydroxyl groups must be ionized for effective coordination to the zinc ions. It is also apparent that there may be rather strict steric requirements for these inhibitors.

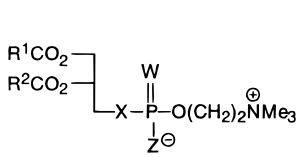
Introduction

Phospholipase C isozymes catalyze the hydrolysis of phospholipids to provide diacylglycerol (DAG) and a phosphorylated headgroup (Scheme 1). The products from the action of mammalian PLCs have been implicated in the cellular signal transduction cascade.[1] In particular, DAG and inositol triphosphate are important for their respective activation of protein kinase C and release of intracellular calcium.[2] The phosphatidylcholine-preferring phospholipase C from *Bacillus cereus* (PLC_{Bc}) is a bacterial enzyme with a known antigenic similarity to its mammalian counterpart.[3] This similarity has made studies of the PLC_{Bc} mechanism of action[4−6] and its selective inhibition[7] important biological pursuits.

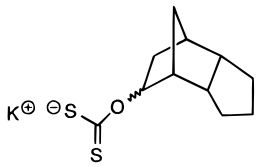
Scheme 1



In the context of developing inhibitors of PLC_{Bc}, it is noteworthy that most have been substrate analogues in which either the bridging or the nonbridging oxygens have been replaced.[7] For example, modest inhibitors of PLC_{Bc} activity have been identified wherein the phosphate oxygen of the scissile P−O bond was replaced with CH_2, CF_2, or NR groups to provide nonhydrolyzable phosphonate or phosphoramidate analogues as generally shown in **1**. Replacement of the nonbridging phosphate oxygens with sulfur also leads to inhibitors. Although a number of several monovalent anions and Tris have been found to be weak inhibitors of PLC_{Bc},[8] the only potent inhibitor of PLC_{Bc} that is not a substrate analogue is the xanthate derivative D609 (**2**).[9,10]



1: X = O, NR, S, CH_2, CF_2
W, Z = O, S combinations



2

To design novel, nonsubstrate analogues as inhibitors of PLC_{Bc}, it is helpful to examine the X-ray crystal structures of PLC_{Bc} in its native state[11] as well as in its complexes with various ions[12,13] and a phosphonate inhibitor.[14] For example, the structure of the PLC_{Bc}−inhibitor complex reveals PLC_{Bc} to be a monomeric, highly α-helical enzyme that contains three zinc ions in its active site (Figure 1). The nonbridging phosphate

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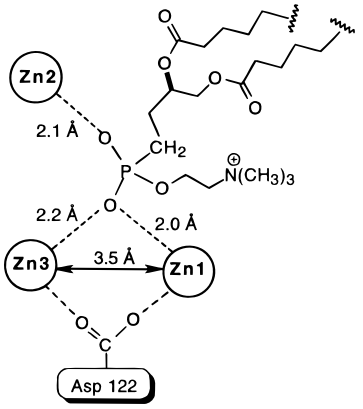
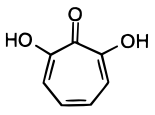


Figure 1. Interaction of phosphonate inhibitor with three zinc ions in the active site of PLC_{Bc} (adapted from ref 14).

oxygens of the substrate analogue are tightly coordinated with the three metal ions, suggesting a catalytic role for all three zincs. Moreover, Zn1 and Zn3 of PLC_{Bc} appear to comprise a 'bimetallic center' because the internuclear distance is only 3.5 Å, and they are bridged by a carboxylate residue.[15]

It occurred to us that a promising strategy for the discovery of inhibitors would be to identify bidentate ligands that would bind to this bimetallic site. For example, alkaline phosphatase, dopamine β-monooxygenase, and inositol monophosphatase, each of which contain similar bimetallic sites, are strongly inhibited by polyhydroxy tropolones containing three contiguous oxygen groups on the seven membered ring.[16,17] In these cases, the polyhydroxy tropolone presumably chelates to both metal ions at the active site.[16] When we tested the most active inhibitor, 2,7-dihydroxytropolone (**3**) against PLC_{Bc}, we found that it exhibited an inhibition constant (K_i) of 16 μM at pH 7.3, a potency comparable to the best of the previously described substrate analogues.[7] Although this was an interesting discovery, we recognized the problems associated with optimizing enzyme affinity by derivatizing the tropolone ring, which is known to have limited stability under certain conditions.[17−20] A stable zinc binding entity that could be easily derivatized into a variety of structures for optimal inhibition was needed.



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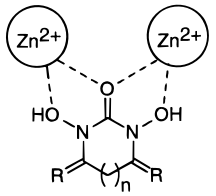
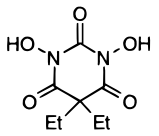


4: R = H_2 or O; n = 0,1,2

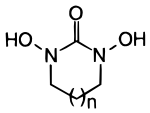
Because hydroxamic acids are well-known zinc chelators,[21] we reasoned that heterocyclic analogues of **3** having the general structure of **4** might be interesting candidates as possible inhibitors of PLC_{Bc}. These compounds contain an *N*,*N*′-dihydroxyurea functional group in place of the polyhydroxylated framework of the tropolone ring. While the dihydroxyurea moiety of **4** is technically not a hydroxamic acid, it seemed likely that it would retain the known zinc-chelating ability of hydroxamic acids. In a fashion similar to that proposed for **3**, the carbonyl group and the two *N*-hydroxyl groups on **4** would serve as Lewis basic sites for coordination to two of the zinc ions in the active site of PLC_{Bc}; this possibility was supported by preliminary docking studies using SYBYL. The synthesis and evaluation of a series of compounds for the general structure **4** as inhibitors of PLC_{Bc} is detailed herein.

Results and Discussion

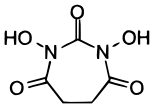
Design and Synthesis of Cyclic *N*,*N*′-Dihydroxyureas. In deciding what analogues of **4** should be evaluated as possible inhibitors of PLC_{Bc}, we wanted especially to probe two factors that might affect binding: (1) What role do the pK_as of the two hydroxyl groups play in determining inhibitor potency? and (2) Are there possible steric interactions that might interfere with binding? Toward addressing these questions, we focused upon the series of *N*,*N*′-dihydroxyurea derivatives **5**−**10**. Upon the basis of electronic effects, we predicted that the pK_as of **5** and **9** would be comparable to **3**, but **10** and **6**−**8** should be less acidic. The *N*,*N*′-dihydroxybarbituric acid **5** held the greatest potential for incurring unfavorable steric interactions on binding, whereas **9**, **10**, and **6**−**8** presenting correspondingly less likelihood for such interactions at the active site of PLC_{Bc}.



5



6: n = 0
7: n = 1
8: n = 2



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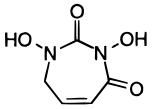

10

The barbituric acid **5** was known,[22] but it was necessary to devise tactics for synthesizing **6**−**10** (Schemes 2−4). Compounds **6** and **7** were prepared in a straightforward fashion by a sequence that commenced with the alkylation of *O*-benzylhydroxylurea with 1,2-dibromoethane or 1,3-dibromopropane in the presence of 2 equiv of KH in tetrahydrofuran (THF) at 0 °C to produce the cyclized products **12** and **13** (Scheme 2).[23] Initial attempts to remove the *O*-benzyl groups by hydrogenolysis using Pearlman's catalyst, Pd/C, and PtO_2 were unselective, and cleavage of the N−O bond was a significant side

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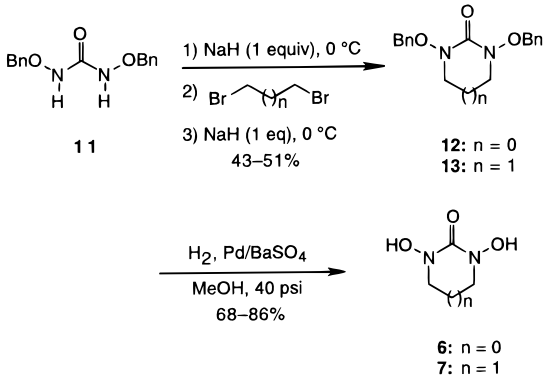
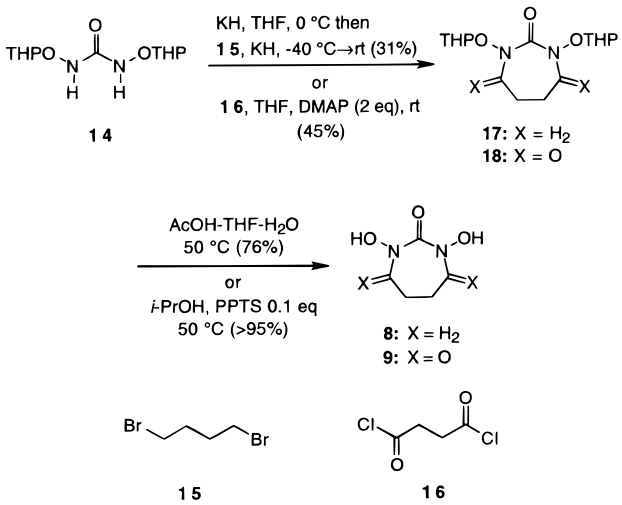
Scheme 2



Scheme 3

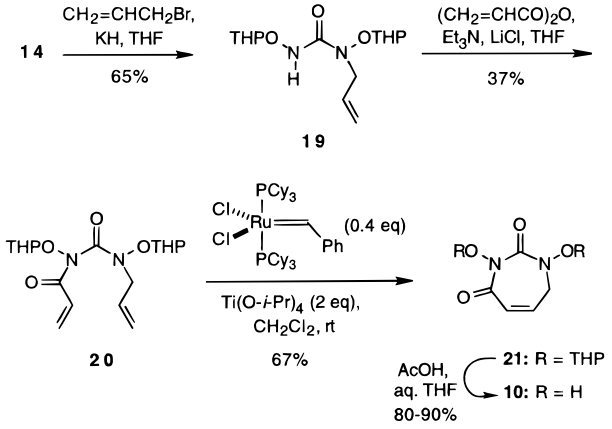


reaction. However, the *O*-benzyl groups were cleanly removed using $Pd/BaSO_4$ as the catalyst in MeOH (40 psi H_2) to afford the desired *N*,*N*′-dihydroxyureas **6** and **7** in good yields.

In initial experiments, we found that the reaction of **11** with 1,4-dibromobutane (**15**) in the presence of KH gave only low yields (<10%) of the seven-membered ring. However, when the THP-protected urea **14** was used as the reactant, **17** was obtained in 31% yield (Scheme 3). Removal of the THP groups under acidic conditions using AcOH−THF−H_2O provided **8** (76%). The tricarbonyl analogue **9** was also most efficiently prepared from **14**. Thus, when **14** was treated with succinyl chloride **16** and 2 equiv of 4-(*N*,*N*-dimethylamino)pyridine (DMAP) in THF, **18** was obtained in 45% yield. When bases such as Et_3N and *i*-Pr_2EtN were employed instead of DMAP, **16** appeared to decompose, perhaps by polymerization, and unreacted **14** was recovered. It was therefore crucial to use DMAP to activate the acid chloride toward acylation of **14**. Removal of the THP groups from **18** using standard conditions (AcOH−THF−H_2O) gave an inseparable mixture of the desired **9** together with a side product that was tentatively identified by ^1H NMR as being acyclic. However, when the deprotection was conducted in the less nucleophilic solvent isopropyl alcohol in the presence of catalytic pyridinium *p*-toluenesulfonate (PPTS), **9** was obtained in nearly quantitative yield.

The unsaturated dicarbonyl compound **10** was prepared by a sequence that featured a ring closing metathesis (RCM) reaction (Scheme 4).[24−27] The RCM substrate was readily prepared in two steps from **14**. In the event,

Scheme 4



14 was first monoalkylated with allyl bromide in the presence of KH to give **19** in 65% yield. Acylation of **19** was best achieved by reaction with acrylic anhydride, which was generated in situ, in the presence of LiCl to give **20** (37%).[28] The RCM of **20** to provide **21** using Grubbs's catalyst required the addition of Ti(O-*i*-Pr)$_4$ (2 equiv);[29,30] when the RCM reaction was performed in the absence of Ti(O-*i*-Pr)$_4$, only starting material was recovered. Removal of the THP groups of **21** under acidic conditions using AcOH in aqueous THF provided **10** in 80−90% yield.

***N*,*N*′-Dihydroxyureas 5−10 as Inhibitors of PLC$_{Bc}$.** With the *N*,*N*′-dihydroxyureas **5**−**10** in hand, the stage was set to evaluate their potencies as inhibitors of PLC$_{Bc}$ using an assay we had previously developed for determining the kinetic parameters of PLC$_{Bc}$ and mutants thereof. Briefly, the assay involves quantitation of the inorganic phosphate that is produced by the reaction of alkaline phosphatase with the phosphorylcholine that is liberated by the PLC$_{Bc}$-catalyzed hydrolysis of 1,2-di-*n*-hexanoyl-*sn*-glycero-3-phosphocholine (see Experimental Section).[31] The K_is of **3**, **5**−**10** were determined at pHs 7.3 and 9.5, and the results are summarized in Table 1. The pK_as of compounds **3**[16] and **5**[32] had been previously determined, so the pK_as of **8** and **10** were determined experimentally by alkaline titration.[33] Because of its rather low solubility, it was not possible to obtain reliable values for the pK_as of **9**, but they should be similar to those reported for **5**.

The *N*,*N*′-dihydroxybarbituric acid derivative **5** showed no detectable inhibition of PLC$_{Bc}$ at concentrations up to 1 mM and a pH of either 7.3 or 9.5. Although the simple, monocarbonyl derivatives **6**−**8** did not exhibit significant inhibition of PLC$_{Bc}$ at pH 7.3, they were modest inhibitors at pH 9.5. Interestingly, the potency varied with ring size

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Table 1. K_is of 3 and the Cyclic *N,N′*-Dihydroxyureas 5−10

compound	pK_{a1}, pK_{a2}	K_i (μM) at pH 7.3	K_i (μM) at pH 9.5
3	5.6, 7.0	16	23
5	5.6, 7.1	no inhibition	no inhibition
6	not determined	no inhibition	303
7	not determined	no inhibition	146
8	9.4, 11.2	no inhibition	71
9	not determined	no inhibition	no inhibition
10	5.0, 8.7	388	53

with the seven-membered analogue **8** being about twice as potent as the six-membered ring compound **7** and four times more effective than the five-membered ring derivative **6**. Although the tricarbonyl compound **9** did not inhibit the enzyme, its unsaturated, dicarbonyl analogue **10** exhibited modest activity at pH 7.3 and good inhibition at pH 9.5.

PLC_{Bc} must retain all three active site zinc ions for full activity,[4,5] so one must consider the possibility that **3**, **6**−**8**, and **10** are inhibitors of PLC_{Bc} because they sequester zinc ions from the enzyme active site; however, this interpretation seems unlikely. Namely, the K_i for the dihydroxytropolone **3** was 16 μM at pH 7.3 and 23 μM at pH 9.5. Inasmuch as both of these values are below the concentration of zinc ion (33 μM) in the assay, **3** cannot be inhibiting the enzyme by sequestering zinc ions. The structural similarity of **3** and the *N,N′*-dihydroxyureas **6**−**8** and **10** suggests that these compounds are also unlikely to inhibit the enzyme by sequestering zinc ions. In support of this hypothesis, we found that a sample of PLC_{Bc} (0.5 μM) in 1.0 mM dimethyl glutarate buffer (DMG) (pH = 7.3) containing 0.1 mM $ZnSO_4$ and **10** (6 mM) retained approximately three zinc ions (as determined by flame atomic absorption) after dialysis against 1.0 mM DMG buffer (three times, 1:1000, 3 h, 4 °C). The buffer from the last dialysis was used as a blank and contained no zinc ion. Analyses were performed in triplicate.

As might be expected, the pK_as of the two hydroxyl groups play a major role because the ionized *N*-hydroxyl function would be expected to bind better than a neutral hydroxyl group to an active site zinc ion. The first pK_a of **8** was determined to be 9.4, so that at pH 7.3 the *N,N′*-dihydroxyurea array in compounds **6**−**8** should be fully protonated. Because **6**−**8** were inactive at pH 7.3 but active at pH 9.5, it is apparent that at least one of the *N*-hydroxyl groups must be ionized in order for the *N,N′*-dihydroxyurea array to serve effectively as a ligand for the zinc ions at the active site. Assuming that ring size does not influence the pK_a, a comparison of the pK_as of **8**, **10**, and **5** reveals that placement of a carbonyl group adjacent to the *N*-hydroxyl group lowers its pK_a. Thus, the increased potency of **10** relative to **8** may be easily rationalized on the basis of its lower pK_as. That **10** is an order of magnitude more potent at pH 9.5 than at 7.3 suggests that both hydroxyl groups must be ionized for optimal binding. In this context, it is noteworthy that the dihydroxytropolone **3** would exist at least partially in its dianionic form at pH 7.3 and is thus approximately equipotent at pH 7.3 and 9.5.

It is at first surprising that **5** and **9**, which have pK_as approximately the same as **3**, are inactive as inhibitors of PLC_{Bc}. However, it is perhaps significant that both **5** and **9** have *two* carbonyl groups on the ring connecting the dihydroxyurea moiety. Because **10** has only one such carbonyl group, it occurred to us that one of the carbonyl groups in **5** and **9** might interfere with binding because of an unfavorable steric interaction with the enzyme. This hypothesis was supported by preliminary modeling and docking studies (SYBYL) of a complex of **5** with PLC_{Bc}. In this complex, which is shown in part in Figure 2, it appears that one of these carbonyl groups is forced to tie within 3 Å of the aromatic ring of the Phe 66 residue; such an interaction would clearly be detrimental to binding. Whether in fact this is true must be tested by further experimentation.

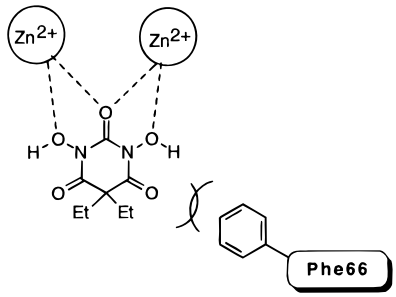

Figure 2. Interaction of ring carbonyl group of **5** with Phe66 of PLC_{Bc}.

In conclusion, a novel class of inhibitors for PLC_{Bc} has been designed and synthesized. This class of inhibitors is believed to function by the bidentate chelation of the *N,N′*-dihydroxyurea array to one or more of the zinc ions at the active site of the enzyme. Because the affinities of these compounds correlate with the pK_as of the N−OH hydroxyl groups, it is apparent that one or both of the hydroxyl groups must be ionized for effective coordination to the zinc ions. It is also apparent that there may be rather strict steric requirements for these inhibitors. Finally, it is possible that the *N,N′*-dihydroxyurea array might be more generally useful as a zinc binding group in the design of inhibitors of other metalloenzymes. These various questions are under current investigation on several fronts.

Experimental Section

General. Unless otherwise noted, all starting materials were obtained from commercial suppliers and were used without further purification. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Tetrahydrofuran (THF) and toluene were distilled from potassium/benzophenone ketyl immediately prior to use. Triethylamine and methylene chloride were distilled from calcium hydride and stored over 4 Å molecular sieves under nitrogen. All reactions involving organometallic reagents or other air-sensitive reagents were executed under an atmosphere of dry nitrogen or argon, using oven- or flame-dried glassware. Spectra were recorded on compounds that were ≥95% pure by HPLC or ^{1}H NMR spectroscopy. IR spectra of oils were recorded as thin films (NaCl plates), whereas the IR spectra of solids were determined as solutions in $CHCl_3$. The ^{1}H and ^{13}C NMR spectra were determined unless otherwise indicated as solutions in $CDCl_3$ at the indicated field; chemical shifts are expressed in parts per million (δ units) downfield from tetramethylsilane. Splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quartet; p, pentuplet; m, multiplet; comp, complex multiplet; br, broad.

1,3-*N,N′*-Dibenzyloxyimidazolid-2-one (12). A slurry of NaH (5.4 mg, 0.14 mmol) in DMF (0.2 mL) was added to **11** (37 mg, 0.14 mmol) in DMF (0.2 mL). The reaction was stirred for 10 min before slowly adding dibromoethane (11.7 μL, 0.14 mmol). After 30 min, NaH (5.4 mg, 0.14 mmol) in DMF (0.4

mL) was added, and the mixture was stirred for 12 h. The reaction was poured into sat. aqueous $NaHCO_3$ (10 mL). The mixture was extracted with CH_2Cl_2 (3 × 10 mL). The organic layers were combined. The organic phase was washed with H_2O (10 mL) and dried ($MgSO_4$). The solvents were removed under reduced pressure. The crude material was purified by flash chromatography eluting with 5% $Et_2O-CH_2Cl_2$ to provide 10 mg of **12** (43%) as a viscous oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44−7.33 (comp, 10 H), 4.99 (s, 4 H), 3.07 (s, 4 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 136.0, 129.3, 128.5, 128.4, 78.0, 44.5; IR 2883, 1752, 1454, 1369, 1240, 1013, 749 cm^{-1}; mass spectrum (CI) *m/z* 299.1393 [$C_{17}H_{19}N_2O_3$ (M + 1) requires 299.1396].

1,3-*N,N′*-Dibenzyloxytetrahydropyrimid-2-one (13). A slurry of NaH (8 mg, 0.19 mmol) in DMF (0.2 mL) was added to **11** (51 mg, 0.19 mmol) in DMF (0.8 mL). The reaction was cooled to 0 °C. After 10 min, dibromopropane (19 μL, 0.19 mmol) was added dropwise. After 30 min, NaH (5.4 mg, 0.14 mmol) in DMF (0.2 mL) was added to the reaction. The mixture was stirred for 12 h. The reaction was poured into sat. aqueous $NaHCO_3$ (10 mL). The aqueous phase was extracted with EtOAc (3 × 10 mL). The organic layers were combined and dried ($MgSO_4$). The solvents were removed under reduced pressure. The residue was purified by flash chromatography eluting with 50% EtOAc−hexanes to provide 30 mg of **13** (51%) as an off white solid; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.49−7.34 (comp, 10 H), 4.99 (s, 4 H), 3.26 (t, J = 6.2 Hz, 4 H), 1.89 (p, J = 5.9 Hz, 2 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 159.5, 135.9, 129.5, 128.4, 128.3, 77.1, 49.1, 20.6; IR 1690, 1454, 1294, 1183 cm^{-1}; mass spectrum (CI) *m/z* 313.1546 [$C_{18}H_{21}N_2O_3$ (M + 1) requires 313.5522].

1,3-*N,N′*-Dihydroxyimidazolid-2-one (6). A solution containing **12** (10 mg, 0.06 mmol) and 5 wt % Pd/$BaSO_4$ (2.4 mg) in MeOH (4 mL) was evacuated under aspirator pressure and then charged with H_2 at 40 psi (repeated three times). The reaction was shaken on a Parr shaker for 12 h. The reaction was filtered through a plug of glass wool to remove the 5 wt % Pd/$BaSO_4$ catalyst. The solvents were removed under reduced pressure. The residue was purified by flash chromatography eluting with 10% MeOH−CH_2Cl_2 to provide 4.5 mg of **6** (68%) as light brown oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.31 (s, 4 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 106.9, 46.0; mass spectrum (CI) *m/z* 119.0457 [$C_3H_7N_2O_3$ (M + 1) requires 119.0457].

1,3-*N,N′*-Dihydroxytetrahydropyrimid-2-one (7). A solution containing **13** (124 mg, 0.40 mmol) and 5 wt % Pd/$BaSO_4$ (21 mg) in MeOH−THF (12 mL, 7:5) was evacuated under aspirator pressure and then charged with H_2 at 40 psi (repeated three times). The reaction was shaken on a Parr shaker for 12 h. The Pd/$BaSO_4$ was removed by filtration through glass wool to remove the Pd/$BaSO_4$. The solvents were removed under reduced pressure. The residue was purified by flash chromatography eluting with 20% MeOH−CH_2Cl_2 to provide 45 mg of **7** (86%) as a light brown solid; mp = 122−124 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.37 (t, J = 5.9 Hz, 4 H), 2.01 (p, J = 5.9 Hz, 2 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.5, 49.9, 19.9; IR 3303, 2870, 1650, 1534, 1310 cm^{-1}; mass spectrum (CI) *m/z* 133.0611 [$C_4H_9N_2O_3$ (M + 1) requires 133.0613].

***N,N′*-Bis(tetrahydropyranyloxy)urea (14).** To a solution of $THPONH_2$[34] (2.97 g, 25.4 mmol) in THF (60 mL) was added a solution of phosgene (35.6 mmol) in toluene (5 mL) at −5 °C over 15 min. The reaction was then allowed to warm slowly to room temperature over 1 h while continuously stirring vigorously via mechanical stirring and then the reaction was stirred for an additional 6 h. The reaction was poured into sat. aqueous NH_4Cl (50 mL). The aqueous phase was extracted with EtOAc (3 × 50 mL). The organic phase was dried ($MgSO_4$) and the solvents were removed under reduced pressure. The crude material was purified by flash chromatography eluting with 75% EtOAc−hexanes to provide 3.09 g of **14** (94%) as a white foam; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.14 (s, 2 H), 4.89−4.86 (comp, 2 H), 4.03−3.91 (comp, 2 H), 3.64−3.56 (comp, 2 H), 1.81−1.56 (comp, 12 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 160.3, 160.0, 103.6, 103.3, 63.5, 28.3, 24.9, 19.5, 19.4; IR 3478, 3250, 2945, 2870, 1682, 1487, 1114, 1037 cm^{-1}; mass spectrum (CI) *m/z* 261.1456 [$C_{11}H_{21}N_2O_5$ (M + 1) requires 261.1450].

1,3-*N,N′*-Bis(tetrahydropyranyloxy)pentahydro-1,3-diazepin-2-one (17). To a solution of **14** (138 mg, 0.53 mmol) in THF (10 mL) at 0 °C was added KH (21 mg, 0.53 mmol). The reaction was stirred rapidly for 15 min before adding 1,4-dibromobutane (63 μL, 0.53 mmol) via syringe. The reaction was allowed to warm to 23 °C in 1 h before adding KH (21 mg, 0.53 mmol). After 24 h of rapid stirring, the reaction was diluted with EtOAc (10 mL) and then poured into sat. aqueous NH_4Cl (10 mL). The organic layer was separated. The aqueous phase was extracted with EtOAc (3 × 10 mL). The organic layers were combined and dried ($MgSO_4$). The solvents were removed under reduced pressure. The crude material was purified by flash chromatography to provide 45 mg of **17** (27%) as a clear oil and starting material (8 mg, 0.03 mmol); ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.95−4.92 (m, 1 H), 4.89−4.87 (m, 1 H), 4.16−3.93 (comp, 2 H), 3.63−3.42 (comp, 3 H), 1.90−1.48 (comp, 16 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.6, 102.6, 101.9, 62.8, 62.6, 55.6, 55.5, 28.5, 28.4, 27.0, 26.7, 25.2, 19.0, 18.8; IR 2940, 1710, 1441, 1204, 1113, 1039 cm^{-1}; mass spectrum (CI) *m/z* 315.1912 [$C_{15}H_{27}N_2O_5$ (M + 1) requires 315.1912].

1,3-*N,N′*-Dihydroxypentahydro-1,3-diazepin-2-one (8). A solution of **17** (22 mg, 0.07 mmol) in AcOH−THF−H_2O (3.5 mL, 4:2:1) was stirred for 12 h at room temperature. The solvents were removed under reduced pressure. The crude material was purified by flash chromatography eluting with 10% MeOH−CH_2Cl_2 to provide 7.8 mg of **8** (76%) as a white solid; mp = 100−102 °C; ^{1}H NMR (300 MHz, D_2O) δ 3.39−3.36 (comp, 4 H), 1.64−1.60 (comp, 4 H); ^{13}C NMR (75 MHz, D_2O) δ 166.4, 56.1, 25.7; IR 3249, 2934, 1663, 1468, 1260, 1021 cm^{-1}; mass spectrum (CI) *m/z* 147.0769 [$C_5H_{11}N_2O_3$ (M + 1) requires 147.0770].

***N,N′*-Bis(tetrahydropyranyloxy)dihydro-1,3-diazepin-2,4,7-trione (18).** To a solution of **14** (188 mg, 0.72 mmol) and succinyl dichloride (80 μL, 0.72 mmol) in THF (8 mL) at room temperature was added DMAP (176 mg, 1.5 mmol). The reaction immediately turned light purple in color, and a dark purple precipitate was observed in the solution. After 48 h, the reaction was poured into sat. aqueous NH_4Cl (15 mL). The aqueous phase was extracted with EtOAc (3 × 15 mL). An insoluble dark purple precipitate was present in both phases. The organic layers were dried ($MgSO_4$), and the solvents were removed under reduced pressure. The crude material was purified by flash chromatography eluting with 75% EtOAc−hexanes to provide 111 mg of **18** (45%) as a viscous oil and 81 mg of starting material; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.00−4.93 (comp, 2 H), 4.13−4.01 (comp, 2 H), 3.60−3.51 (comp, 2 H), 3.06−2.84 (comp, 4 H), 2.06−1.48 (comp, 12 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.9, 147.5, 102.4, 102.3, 62.7, 31.1, 28.3, 27.7, 24.8, 17.7; IR 2945, 1731, 1711, 1303, 1205, 1141, 1036 cm^{-1}; mass spectrum (CI) *m/z* 343.1505 [$C_{15}H_{23}N_2O_7$ (M + 1) requires 343.1505].

1,3-*N,N′*-Dihydroxy-5,6-dihydro-1,3-diazepin-2,4,7-trione (9). To a solution of **18** (22 mg, 0.064 mmol) in THF−*i*-PrOH (1:1) at 50 °C was added PPTS (5 mg, 0.02 mmol). After 2 h, PPTS (5 mg, 0.02 mmol) was added to the reaction. After 2 h, the reaction was complete by TLC, and the flask was cooled to −78 °C for 5 h. The product crystallized into tiny white crystals that were isolated by first transferring the reaction mixture to an epindorf tube. The tube was briefly centrifuged at high speed. The mother liquor was decanted. The crystals were washed with cold *i*-PrOH. The excess solvent was removed under reduced pressure to provide 3.5 mg of **9** in quantitative yield; ^{1}H NMR (300 MHz, CD_3OD) δ 2.96 (s, 4 H); ^{13}C NMR (75 MHz, CD_3OD) δ 170.7, 150.8, 31.4; mass spectrum (CI) *m/z* 175.0361 [$C_5H_7N_2O_5$ (M + 1) requires 175.0355].

***N*-(2-Propenyl)-*N,N′*-bis(tetrahydropyranyloxy)urea (19).** To a solution of **14** (740 mg, 2.9 mmol) and allyl bromide (295 μL, 3.4 mmol) in THF (18 mL) was added KH (111 mg, 2.9 mmol) at −60 °C. The reaction was warmed to room temperature over 1 h and then stirred at room temperature for an additional 12 h. The reaction was quenched by pouring it into sat. aqueous $NaHCO_3$ (50 mL). The layers were

separated, and the aqueous phase was extracted with CH_2Cl_2 (3 × 100 mL). The organic layers were dried ($MgSO_4$). The solvents were removed under reduced pressure. The crude material was purified by flash chromatography eluting with 75% EtOAc−hexanes to provide 556 mg of **19** (65%) as a white foam; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.12 (s, 0.5 H), 9.07 (s, 0.5 H), 5.89 (m, 1 H), 5.31−5.19 (comp, 2 H), 4.95 (dt, J = 11.8, 3.4 Hz, 1 H), 4.75 (m, 1 H), 4.36−4.27 (m, 1 H), 4.05−3.91 (comp, 3 H), 3.66−3.48 (comp, 2 H), 1.90−1.46 (comp, 12 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.7, 161.5, 132.0, 132.9, 119.7, 119.6, 106.3, 106.0, 103.2, 102.4, 66.3, 66.1, 63.2, 62.9, 54.2, 29.7, 29.6, 28.8, 28.7, 25.7, 25.3, 21.7, 21.6, 19.6, 19.2; IR 3308, 2944, 2868, 1701, 1458, 1037 cm^{-1}; mass spectrum (CI) m/z 301.1768 [$C_{14}H_{25}N_2O_5$ (M + 1) requires 301.1763].

***N*-(Acryloxycarbamoyl)-*N′*-(2-propenyl)-*N*,*N′*-bis(tetrahydropyranyloxy)urea (20).** To a solution of acrylic acid (252 μL, 3.68 mmol) and Et_3N (994 μL, 7.08 mmol) in THF (10 mL) at −20 °C was added acryloyl chloride (252 μL, 3.40 mmol) dropwise via syringe.[35] The mixture was stirred for a 1 h, and LiCl (132 mg, 3.11 mmol) was added followed by dropwise addition of a solution of **19** (849 mg, 2.83 mmol) in THF (9 mL). The reaction was allowed to warm to room temperature and stirred for an additional 3.5 h. The reaction was diluted with EtOAc (50 mL) and poured into sat. aqueous NH_4Cl (70 mL). The aqueous phase was extracted with EtOAc (3 × 150 mL). The organic layers were combined and dried ($MgSO_4$). The solvents were removed under reduced pressure. The crude material was purified by flash chromatography to provide 369 mg of **20** (37%) as a light brown oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.68 (dd, J = 17.1, 10.3 Hz, 0.5 H), 6.60 (dd, J = 17.1, 9.8 Hz, 0.5 H), 6.48 (dd, J = 16.9, 1.8 Hz, 1 H), 6.06−5.92 (m, 1 H), 5.81 (dd, J = 10.3, 1.6 Hz, 0.5 H), 5.80 (dd, J = 10.0, 2.1 Hz, 0.5 H), 5.36−5.14 (comp, 3 H), 5.09−5.03 (m, 1 H), 4.59−4.49 (comp, 0.5 H), 4.46−4.36 (comp, 0.5 H), 4.31−4.18 (comp, 1 H), 4.06−3.92 (comp, 2 H), 3.66−3.52 (comp, 2 H), 1.96−1.50 (comp, 12 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.3, 154.5, 132.0, 130.8, 126.80, 126.81, 126.75, 118.6, 118.4, 63.1, 62.7, 62.5, 54.4, 53.4, 28.6, 28.5, 28.2, 25.0, 24.8, 18.7, 18.6, 18.5, 18.3; IR 2946, 1698, 1403, 1206, 1115, 1038 cm^{-1}; mass spectrum (CI) m/z 355.1869 [$C_{17}H_{27}N_2O_6$ (M + 1) requires 355.1859].

1,3-*N*,*N′*-Bis(tetrahydropyranyloxy)-5,6-dihydro-1,3-diazepin-2,4-dione (21). A solution of **20** (84 mg, 0.24 mmol) was stirred rapidly in degassed, dry CH_2Cl_2 (24 mL) at 0 °C, and Ti(O-*i*-Pr)$_4$ (140 μL, 0.48 mmol) was added dropwise. After 10 min, a solution of Grubbs's catalyst[36] (78 mg, 0.095 mmol) in degassed, dry CH_2Cl_2 (6 mL) was added dropwise via syringe over 30 min. The reaction was rapidly stirred for 30 min at 0 °C. The ice bath was then removed, and the solution was stirred for 4 h at room temperature. The ruthenium catalyst was removed by filtration through a short column of SiO_2 using EtOAc to elute the product. The solvents were removed under reduced pressure, and the brown residue was purified by flash chromatography eluting with EtOAc to provide 58 mg of **21** (74%) as a very dark brown oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.91−6.82 (m, 1 H), 6.28−6.23 (m, 1 H), 5.13−5.01 (comp, 2 H), 4.58−3.97 (comp, 4 H), 3.70−3.57 (comp, 2 H), 2.12−1.51 (comp, 12 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.0, 140.9, 128.4, 128.0, 104.6, 103.9, 102.6, 102.1, 63.6, 62.4, 48.5, 28.3, 27.8, 27.7, 24.9, 19.1, 17.8; IR 2945, 1728, 1690, 1205, 1115, 1035, 938 cm^{-1}; mass spectrum (CI) m/z 327.1554 [$C_{15}H_{23}N_2O_6$ (M + 1) requires 327.1556].

1,3-*N*,*N′*-Dihydroxy-5,6-dihydro-1,3-diazepin-2,4-dione (10). A solution of **21** (31 mg, 0.095 mmol) in AcOH/THF/H_2O (3.5 mL, 4:2:1) was heated to 40 °C for 24 h. The solvents were removed under reduced pressure. The crude material was purified by flash chromatography eluting with CH_2Cl_2−MeOH (7.5:2) to provide 9 mg of **10** (60%) as a colorless glass; ^{1}H NMR (300 MHz, CD_3OD) δ 6.91 (dt, J = 10.8, 6.6 Hz, 1 H), 6.15 (d, J = 10.8 Hz, 1 H), 4.24 (d, J = 6.6 Hz, 2 H); ^{13}C NMR (125 MHz, D_2O) δ 165.1, 154.4, 143.4, 127.2, 49.5; IR (MeOH) 3409, 2874, 1711, 1680, 1632, 1242, 828, 670; mass spectrum (CI) m/z 159.0399 [$C_5H_7N_2O_4$ (M+1) requires 159.0406], 159 (base), 143, 102.

Determination of K_is for Compounds 5−10. The inhibition constants (K_i) for compounds **3** and **5**−**10** were determined with a PLC_{Bc} assay based on the quantitation of inorganic phosphate, performed in 96-well plates.[31] In this procedure, the PLC_{Bc} reaction was allowed to proceed for a defined period of time, at which point the activity was quenched with the biological buffer Tris, a known PLC_{Bc} inhibitor.[13] The phosphorylcholine product of the PLC_{Bc} reaction was then converted to inorganic phosphate (Pi) and choline through the action of alkaline phosphatase (APase). A blue-colored complex with Pi was then formed by the addition of solutions containing ammonium molybdate, ascorbic acid, trichloroacetic acid, and sodium metaarsenite; this "molybdenum blue" complex had a λ_{max} of 700 nm and was read with a microplate reader. To determine K_is, the assay was performed in an analogous fashion, except the synthesized organic compounds were added at the following concentrations: **3** at 10 μM; **5** at 1 mM**, 6** at 1 mM; **7** at 40 μM; **8** at 40 μM; **9** at 80 μM; **10** at 60 μM. The data obtained in the presence of the inhibitor was then compared to data gathered in its absence. The V_{int} vs concentration of phosphatidylcholine plots of these two data sets (from a minimum of eight substrate concentrations) were fit to the Michaelis−Menten curves using the program KaleidaGraph. A K_i was then deduced from the apparent V_{max} and K_m values in the presence of the inhibitor. Because 2,7-dihydroxytropolone (**3**) is a known inhibitor of alkaline phosphatase, a great excess of APase (>10-fold relative to a typical assay) was added to all the kinetic assays. Control experiments indicated that under these conditions of high APase concentration phosphorylcholine was rapidly and quantitatively converted to Pi and choline, even in the presence of **3**.

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Supporting Information Available: Copies of ^{1}H NMR spectra of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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