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Synthesis and In Vitro Evaluation of Aspartate Transcarbamoylase Inhibitors

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Abstract

The design, synthesis, and evaluation of a series of novel inhibitors of aspartate transcarbamoylase (ATCase) are reported. Several submicromolar phosphorus-containing inhibitors are described, but all-carboxylate compounds are inactive. Compounds were synthesized to probe the postulated cyclic transition-state of the enzyme-catalyzed reaction. In addition, the associated role of the protonation state at the phosphorus acid moiety was evaluated using phosphinic and carboxylic acids. Although none of the synthesized inhibitors is more potent than N-phosphonacetyl-L-aspartate (PALA), the compounds provide useful mechanistic information, as well as the basis for the design of future inhibitors and/or prodrugs.

1. Introduction

Aspartate transcarbamoylase (ATCase) catalyzes the second step of the *de novo* pyrimidine biosynthesis, during which L-aspartic acid (Asp) is condensed with carbamyl phosphate (CP) to produce carbamoyl-L-aspartate (C-Asp) and inorganic phosphate (Equation 1).[1]

(Eq. 1)

Inhibitors of the pyrimidine biosynthesis[1] have been investigated as anticancer agents, and particularly, the potent ATCase inhibitor N-phosphonacetyl-L-aspartate **1** (PALA, K_i = 16 nM) has been evaluated in cancer chemotherapy.[2] In several studies, PALA was found to be a valuable agent, even though its current clinical utility appears limited to combination therapies. This inhibitor eradicated solid tumors in mice, but murine leukemias were resistant.[3] A combination therapy consisting of PALA and dipyridamole showed some effectiveness on various resistant cancer cells.[4] A comprehensive summary of preclinical and clinical studies

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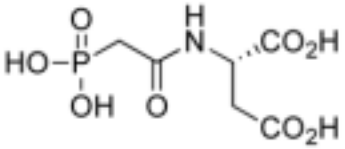
Supplementary Data

Additional experimental details (including the preparations of **2**, **3**, **9**, **10**, **11**, **14**, **22**, **24**, **28**, **29**, **38**, **40**, and **42**), spectral data, and copies of NMR spectra are provided as supplementary data. Supplementary data associated with this article can be found, in the online version, at doi:

with PALA has appeared.[5] PALA displays broad spectrum antitumor activity against solid tumors in a dose-dependent manner. Resistance was due to increased ATCase activity and expression, and PALA did not inhibit completely the *de novo* pyrimidine biosynthesis, because of its low bioavailability (with the presence of a possible total of four negative charges, PALA does not pass through membranes easily). Because PALA is unique in its mode of action and because it came close to passing all clinical trials for use as a single anticancer agent, much work has been devoted in order to find an even more potent and selective inhibitors of ATCase.[6] Unfortunately, in spite of more than 35 years of research, PALA remains the most potent ATCase inhibitor known to date.



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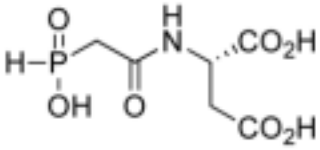

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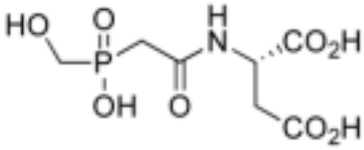

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In a communication,[7] we recently reported the synthesis and inhibitory activity of the novel phosphinates **2** and **3**, which were 26- and 12-fold less potent than PALA, respectively (K_i(**2**) = 417 nM, K_i(**3**) = 193 nM), but with a reduced overall charge. These compounds were the first submicromolar-level inhibitors of ATCase that did not have PALA's phosphonic acid moiety.[7]

Herein, we present the full study for a series of compounds possessing various replacements for PALA's phosphonacetyl moiety, especially phosphinic acids which have a reduced charge around phosphorus.

2. Results and Discussion

2.1 Synthesis

Synthesis of Phosphinates—Since our initial report,[7] we have developed and improved upon the synthesis of *H*-phosphinate **2** starting from isoprenyl-*H*-phosphinic acid **4**. This is shown in Scheme 1. The key building blocks **5** and **6** are also employed for the synthesis of other inhibitors described in the present work. Isoprenyl-*H*-phosphinic acid **4** was prepared through our own palladium-catalyzed hydrophosphinylation of isoprene.[8] Two methods were specifically developed for this step: either the homogeneous reaction[9] using a low palladium loading (0.25 mol%), or the heterogeneous reaction using our reusable polystyrene-nixantphos catalyst[10] (1 mol%). The latter method is ideal for the multigram preparation of **4**, since multiple runs can be conducted by simply filtering the catalyst at the end of one run and adding it to another. No loss of catalytic activity was observed after five runs, and the overall yield of **4** was quantitative.

Compound **4** was protected with triethyl orthoacetate/BF_3•OEt_2 to provide the key intermediate **5**, as we described previously.[11] Ozonolysis of **5** followed by oxidation with $NaClO_2$ delivers carboxylic acid **6**, which was used without purification to prepare amide **7**. Deprotection of **7**, through debenzylation then acid hydrolysis of the acetal protecting group, produces *H*-phosphinate inhibitor **2**. In our original synthesis of **2**,[7] carboxylic acid **6** was prepared from protected cinnamyl-*H*-phosphinate instead of protected isoprenyl-*H*-phosphinate **5**. During the ozonolysis/oxidation sequence of the cinnamyl group, benzoic acid is formed thus requiring the use of larger quantities of reagents, since it also reacts to form *N*-benzoyl-L-aspartic acid dibenzyl ester. With isoprenyl **5**, acetone is formed during ozonolysis and this cannot lead to

a carboxylic acid, not to mention the fact that acetone is easily evaporated before the $NaClO_2$ step.

Our interest in *H*-phosphinate **2** was two-fold: 1) the P-H analog has a reduced charge (it is less hydrophilic than PALA), so higher concentrations in cells could potentially be attained, improving upon PALA's bioavailability; and 2) this compound may be oxidized *in vivo* to PALA thus generating an even more potent inhibitor. There are indications that *H*-phosphinic acids can be oxidized *in vivo* to the corresponding phosphonic acids.[12] However, this would require oxidative mechanisms such as P450, which are not operating during assays on cell lines. Since **2** is itself a relatively good ATCase inhibitor, if it were activated *in vivo* to PALA, significant improvements in the concentration that might be achieved at the tumor site might be observed. As a result of the special potential of **2**, we decided to prepare a series of partial esters **9–11** (Scheme 2). Again, isoprenyl **5** was converted into the corresponding carboxylic acid and coupled as in Scheme 1, but this time with L-aspartic acid diethyl ester to afford **8**. Acid hydrolysis of **8** was conducted under controlled conditions to obtain an easily separated mixture of the two compounds **9** and **10**.

Debenzylation of **7** followed by acid hydrolysis under controlled conditions (1 h at 80 °C) afforded ethyl ester **11**. The syntheses of compounds **9–11** illustrate approaches to novel PALA prodrugs, especially since *H*-phosphinates can be easily converted into phosphonate esters (and other functionalities) through oxidative functionalization (for example: chlorination with oxalyl chloride or CCl_4 and reaction of the resulting phosphonochloridate with nucleophiles such as amines and alcohols).[13] Exploiting intermediates **7** and **8** for the purpose of PALA prodrug synthesis will be the object of future studies.

Since the proposed ordered cyclic transition-state for the proton transfer from nitrogen to phosphate oxygen in the reaction between Asp and CP catalyzed by ATCase (Figure 1, X-ray structures are generally drawn as Transition-State O-axial)[14] is reasonable and consistent with the inhibition observed with **2** and **3**, some heterocyclic analogs were synthesized next.

The simplest heterocycle **14** was synthesized from compound **12** as shown in Scheme 3. We have previously described the preparation of **12** (although the bis-isoprenyl analog would be better, we have not investigated this possibility).[15] Double ozonolysis of **12** followed by treatment with dimethylsulfide provided the corresponding dialdehyde[15] (along with benzaldehyde) which was immediately reacted under reductive amination conditions to produce compound **13**. Debenzylation then afforded heterocycle **14**.

The more elaborate "cyclo-PALA" heterocycle **19** was synthesized according to Scheme 4. The known hydroxymethyl-*H*-phosphinic acid **15**[16] undergoes a sila-Arbuzov reaction[17] with bromide **16**, and the crude mixture is esterified with diphenyldiazomethane[7,18] to afford **17** (hydrogenolysis of **17** gives hydroxymethyl inhibitor **3** in quantitative yield). Intermediate **17** was cyclized using Mitsunobu conditions[19] to produce crude **18**, which was hydrogenolyzed directly to the 5-membered amide **19**.

The 6-membered "cyclo-PALA" **27** was then synthesized as shown on Scheme 5. Isoprenyl-*H*-phosphinic acid **4** reacted with bromide **16** under sila-Arbuzov conditions,[17] and the resulting crude phosphinic acid was esterified to **20**, using $BnBr/Ag_2O$.[20] Ozonolysis and reduction with sodium borohydride afforded intermediate **21**. Since hydroxymethyl **3** was a potent inhibitor of ATCase, **21** was hydrogenolyzed to the homologous hydroxyethyl **22** for evaluation.

Compound **20** was converted into **23** using an ozonolysis/oxidation/esterification sequence in 63% overall yield. The esterification of the intermediate carboxylic acid was conducted with Ph_2CN_2[18] to facilitate purification. Hydrogenolysis of **23** afforded compound **24**.

To prepare **27**, compound **21** was cyclized using Mitsunobu conditions[19] as for the 5-membered amide, except this time, some vinyl phosphinate **25** also formed. In order to separate **26** from the impurities and **25**, the crude mixture was ozonolyzed before chromatography. Finally, compound **26** was hydrogenolyzed to deliver heterocyclic amide **27**.

Synthesis of Bi-substrate/Reactive Intermediate Analogs—PALA behaves as a bi-substrate analog. In spite of the many compounds that have been synthesized over the years, [6] several relatively simple bi-substrate analogs have never been investigated. In this section, we describe three of these analogs. The first two targets were carbamate **32** and urea **34** (Scheme 6).

The phosphonate moiety for each was prepared through the reaction of dibenzylphosphite with paraformaldehyde to form **28**,[21] or with 1,3,5-tribenzylhexahydro-1,3,5-triazine to form **29**. [22] Amine salt **30** was converted to the corresponding isocyanate using triphosgene, as reported in the literature.[23] Addition of **28** gave carbamate **31**, whereas addition of **29** gave urea **33**. In both cases, the yield was moderate because isocyanate formation also leads to the symmetrical urea derived from **30**.[23]

The third bi-substrate analog is phosphate **36** (Scheme 7) in which the position of the carbon and oxygen has been inverted compared to carbamate **32**. Bromide **16** was phosphorylated using silver oxide[20] and dibenzylphosphate to afford intermediate **35**. Hydrogenolysis then provided phosphate **36** in quantitative yield. The simple two-step sequence thus produced **36** in outstanding yield from **16**.

Synthesis of carboxylic analogs—Some compounds where the phosphonate moiety of PALA is replaced with carboxylates were synthesized as shown in Scheme 8. Di-*tert*-butyl malonate was alkylated with bromide **16** under standard conditions to afford **37**. Hydrolysis of the *tert*-butyl ester groups, followed by hydrogenolysis produced malonate **38**. Esterification of malonic acid monobenzyl ester using EDC afforded perbenzylated intermediate **39**. Again hydrogenolysis provided carboxylate **40**.[7] Finally, homocarboxylate **42** was synthesized from succinic anhydride through reaction with **30**, followed by esterification with Ph_2CN_2.[18] This was done so that intermediate **41** can be purified easily. Hydrogenolysis then afforded homocarboxylate **42**.

2.2 Biological Evaluation

With a large number of potential inhibitors now at hand, evaluation against ATCase could be determined. The compounds were evaluated against the catalytic subunit of *E. coli* ATCase, which was purified from the strain/plasmid combination pEK17/EK1104 as previously described by Kantrowitz et al.[24] The colorimetric assay also developed by Kantrowitz,[25] was used to detect the formation of *N*-carbamoyl-L-aspartate, and inhibition was determined relative to carbamyl phosphate (K_m = 28 μM). Under these conditions, PALA **1** has an inhibition constant of K_i = 16 nM.[7] The results for the phosphorus-containing compounds are collected in Table 1. Compounds **9**, **10**, and **11** (Scheme 2) were not tested against ATCase since they are expected to be inactive on the purified enzyme. The partial esters of *H*-phosphinate **2** were prepared as possible prodrugs of *H*-phosphinate **2**, which itself is a potential *in vivo*, oxidatively-activated prodrug of PALA **1**.

Phosphorus-free carboxylate compounds **38**, **40**, and **42** were synthesized because similar carboxylate-containing compounds significantly inhibited the enzyme 3-dehydroquinate synthase (DHQS),[26] and replacing a phosphonate with carboxylate(s) has significant implications for the design of prodrugs.[27] DHQS was established as an example of an enzyme which exploits a phosphate residue in its substrate to achieve catalytic efficiency.[28] Indeed, the same is postulated for ATCase (Figure 1). The enzyme DHQS is a prime example of the

elegant and efficient way nature has evolved catalysis. Scheme 9 shows the mechanistic analogy between ATCase and DHQS.

With ATCase, nucleophilic attack of carbamyl phosphate by aspartate forms the tetrahedral intermediate **A**. Since a dianionic phosphate is one of the strongest base in nature, one of the phosphate oxygen can intramolecularly abstract the proton on the nitrogen atom (transition state **B**). Full proton transfer leads to reactive intermediate **C**, wherein the nitrogen is now neutral and the phosphate moiety is monoprotonated. Since a phosphate monoanion is now a much better leaving group, collapse of **C** would lead to inorganic phosphate dianion **D** and carbamoyl aspartate **C-Asp**. In the established sequence for DHQS, the molecular events are strikingly similar: the bound phosphate dianion in the substrate is oxidized to **E** with a tightly bound NAD cofactor.[28] One of the strongly basic phosphate oxygens then attacks the now acidic proton in of the ketone (transition-state **F**). Once protonated, phosphate **G** becomes a good leaving group, which collapses to produce α,β-unsaturated ketone **H** and inorganic phosphate dianion **D**. Thus, the two mechanisms would share striking similarities, particularly as far as the role of the phosphate group is concerned. In the case of DHQS, the transformation **E** to **H** is well supported experimentally.[28,29] For ATCase, computational studies and X-ray crystallography also support of the proposed cyclic transition-state (Figure 1).[14] In general, PALA's amide carbonyl oxygen is represented as being axial (Figure 1, Transition-State O-axial), gas-phase modeling places PALA's amide oxygen in the equatorial position so the phosphate oxygen is hydrogen-bonded to the amide NH. It is also interesting to note that in the enzyme active site, there does not seem to be residues that are binding to NH_2 of carbamyl phosphate **CP** other than lysine 267.

In the inhibition of DHQS, some dicarboxylates were very potent (nanomolar-level) inhibitors and therefore good mimics of the phosphate moiety.[26] Even the simplest monocarboxylate analog displayed an inhibition constant comparable to the Michaelis constant (K_m) of the substrate (DAHP). However, none of the carboxylates **38**, **40**, and **42** displayed any significant inhibition against ATCase. Since compound **40** is inactive, whereas *H*-phosphinate **2** is a good inhibitor, this clearly points to a geometrical effect. In the case of DHQS, the monoanion needs to be aligned with the hydrogen to be abstracted, and this remains possible because it is an in-plane reaction. On the other hand, with ATCase, the deprotonation requires a tetrahedral anionic moiety because the amide group in **38**, **40**, and **42** prevents the carboxylate from adopting the required "perpendicular" geometry. The potency of species (**2** and **3**) constrained to the phosphorus monoanion, also provides support for this mechanism. ATCase would bind to the monoanion, which is then deprotonated by an arginine residue[14] (Arg-54 in the *E. coli* enzyme). At the end of the sequence, inorganic phosphate can regenerate the basic residue (Arg) and leave the active site as monobasic phosphate. DHQS also has a single arginine residue[29] (Arg-130), which could act as a general base to deprotonate the phosphate monoanion once it is initially bound from solution.

None of the newly synthesized inhibitors were more potent than PALA **1** (Table 1). As discussed in our communication,[7] reduced-charge compounds **2** and **3** (entries 2 and 3, respectively) are interesting, at least mechanistically, and their activity is consistent with the proposed intramolecular proton transfer[14] depicted in Figure 1. On the other hand the simple heterocycle **14** (entry 4) is totally inactive, and this is not surprising since the carbonyl amide of PALA is expected to be very important for binding affinity and to mimic the developing negative charge in the transition-state (Figure 1). The 5-membered ring cyclo-PALA **19** (entry 5) is also inactive. This might, seemingly, be more surprising. But interestingly, the 6-membered homolog **27** (entry 8) shows significant inhibition [K_m(CP)/K_i(**27**) = 28] especially when compared to **19**. Again, electron density at the amide carbonyl should be critical in the mimicry of the transition-state: amide resonance (or dipolar interaction) in a 5-membered lactam is conformationally disfavored, thus decreasing electron-density at the carbonyl

oxygen. In 6-membered **27**, the carbonyl is more electron-rich than in **19**, because resonance better contributes to the negative charge on oxygen to mimic the postulated transition-state (Figure 1). Thus the drastic difference between **27** and **19** points to clear stereoelectronic effects. We surmise that the transition-state looks more like O-equatorial (Figure 1), which would be bound to Threonine 55 and Histidine 134 in the *E. coli* enzyme.

Hydroxyethyl **22** (entry 6) shows significant inhibitory activity, but it is still 13-times less potent than hydroxymethyl **3**, and it is no longer a competitive inhibitor, but instead it is uncompetitive. The inhibition displayed by the phosphinate-acetate **24** is interesting when compared to its reduced analog **22**. Although the inhibition constants are comparable, carboxylate **24** is once again a competitive inhibitor. The 6-membered cycloPALA **27** (entry 8) is more active than either **22** or **24** even if only slightly (a micromolar-level inhibitor). The 6-membered cyclic amide **27** also supports the sequence of events **A** to **C-Asp** and a cyclic transition-state (Figure 1), and is more tightly bound than the substrate **CP** (Scheme 9).

The last three entries in Table 1 (entries 9–11) are particularly revealing. Compounds **32**, **34**, and **38** were designed as bi-substrate analogs, related to PALA **1**. Carbamate **32** (entry 9) is mimicking the departure of phosphate in the transition-state. However, this compound does not inhibit ATCase at all. Carbamate **32** is likely a failure because of the reduced electron density on the carbonyl oxygen (carbamate versus amide). In fact, the activity of urea **34** (entry 10) is confirming this possibility: an electron-rich carbonyl oxygen must be present (ie. ureas are better hydrogen bond acceptors, and their carbonyl oxygen is more basic than the analogous carbamates). Most revealing perhaps, is the potent inhibition observed with phosphate **36** (entry 11). The difference between entry 9 and entry 11 is a switch between the carbon and oxygen in the phosphorus-containing moiety. While **36** is not as potent as PALA (13-fold weaker), its extreme ease of synthesis makes it a candidate for the preparation of esters in order to optimize its bioavailability and its potency further supports the above discussion of carbamate **32** and urea **34**.

For transition-state mimicry in the case of ATCase, the question remains as to which protonation state of the phosphate analog is optimum. Transition-state **B** might look like the starting material **A** (an early transition-state) and thus have a charge around the phosphorus closer to 2, or it could be more like the monoanionic product **D** (a late transition-state) with a charge closer to 1 (Scheme 10). Yet, if the transition-state were nearly symmetrical, the ideal state for the phosphate mimic would then be 1.5 negative charges. Although not as potent as PALA **1** by one order of magnitude, inhibitors **2** and **3** clearly support a mechanism in which the phosphate charge is reduced from two to one in the transition-state.

Overall, the results observed with our compounds support the need for: 1) a tetrahedral, at least monoanionic, phosphate mimic (**1**, **2**, **3**, **22**, **27**); and 2) significant electronic density on the amide carbonyl oxygen along with proper spatial orientation (**19** vs **27**, and **32** vs **36**, for example). In the case of PALA, both the monoanionic and dianionic phosphonate group are accessible, and the amide oxygen has high electron density. This suggests that *N*-methyl-PALA might be an improved inhibitor, and this will be explored in the near future (compounds like *N*-methyl-PALA have not been described in the literature). The preparation of cyclic transition-state analogs is challenging because although phosphorus monoanions can be good inhibitors, the transition-state has a higher negative charge (anywhere between 2 and 1), and in addition, an additional functional group mimicking the amino group in the tetrahedral intermediate needs to be perpendicular to the plane of the ring.

3. Conclusion

The present work provides a number of new compounds for the inhibition of ATCase, and a better understanding of PALA's activity. Potential prodrugs of (or related to) PALA could be accessible through either in vivo metabolic oxidation of *H*-phosphinate **2**, better translocation of reduced-charge compounds such as **2** and **3**, or oxidative esterification of intermediates related to **9**–**11**. While none of the described compounds showed an inhibition of ATCase superior to that of PALA, the results provide guidelines for the design of new inhibitors. Compounds **2**, **3**, and **36** are all submicromolar inhibitors with functionalities significantly differing from the phosphonate moiety in PALA. The described compounds also shed some light on the mechanism of the ATCase-catalyzed transformation. PALA itself, while appearing more like a bi-substrate analog, may indeed behave like a transition analog as Stark originally suggested. Although our work (like all other prior works) has failed to identify an inhibitor that is more potent than PALA *in vitro*, several research avenues remain to be explored. Two directions are identified as: 1) the development of PALA-related prodrugs through the masking of charges especially around the phosphorus atom in order to improve PALA's bioavailability; and 2) the design of cyclic or conformationally restricted transition-state analogs. In spite of the 38 years that have elapsed since Stark's discovery of PALA, much remains to be investigated for the design of potent *in vitro* and *in vivo* inhibitors of ATCase, and ultimately to improve upon PALA's anticancer activity. The potential *in vivo* oxidation of *H*-phosphinic acid **2** to produce PALA remains to be established.

4. Experimental Section

4.1 General information

Concentrated hypophosphorous acid was obtained by rotary evaporation (0.5 mmHg) of the 50 wt. % aqueous solution at room temperature for 20–30 min before reaction. *Caution: overdrying H_3PO_2 may result in the formation of a yellow solid of high phosphorus content that could be pyrophoric.* Tetrahedrofuran (THF) was freshly distilled from sodium benzophenone ketyl under N_2. Toluene, *t*-amyl alcohol, acetonitrile (ACN) and dichloromethane (CH_2Cl_2) were freshly distilled from CaH_2 under N_2. Dimethylformamide (DMF) over 4Å sieves and pyridine over 3Å were used. Triethylamine (Et_3N) was distilled from CaH_2 and stored over 4Å sieves. Absolute ethanol, methanol, *t*-butanol (*t*BuOH) and chloroform were used as received. ^{1}H NMR spectra were recorded on a 300-MHz spectrometer. Chemical shifts for ^{1}H NMR spectra are reported (in parts per million) relative to internal tetramethylsilane (Me_4Si, $\delta = 0.00$ ppm) with $CDCl_3$ or H_2O ($\delta = 4.75$ ppm) in D_2O. ^{13}C NMR spectra were recorded at 75 MHz and in some cases at 100 MHz. Chemical shifts for ^{13}C NMR spectra are reported (in parts per million) relative to $CDCl_3$ ($\delta = 77.0$ ppm). ^{31}P NMR spectra were recorded at 121 MHz, and chemical shifts reported (in parts per million) relative to external 85% phosphoric acid ($\delta = 0.0$ ppm).

4.2 General procedures

Procedure A—Neat *H*-phosphinic acid **15** or **4** (4.6 mmol, 1.0 equiv.) was placed under vacuum in a dry two-neck flask for 10 min then placed under N_2 and dissolved in distilled toluene (0.3 M, 16 mL). *N*-(bromoacetyl)-L-aspartic acid dibenzyl ester **16** (2.0 g, 4.6 mmol, 1.0 equiv.), HMDS (2.4 mL, 11.5 mmol, 2.5 equiv.) and TMSCl (1.5 mL, 11.5 mmol, 2.5 equiv.) were added at room temperature under N_2. The reaction mixture was refluxed for 14 h under N_2. The solution was cooled down, quenched by adding methanol (5 mL) and concentrated in vacuo.

Procedure B—To a solution of hydroxyl **17** or **21** (1.0 equiv.) in THF (0.05 M) was added triphenylphosphine (1.0 equiv.) and diisopropyl azodicarboxylate (1.0 equiv.) at room

temperature, under N_2. After 30 min at room temperature, the crude mixture was quenched with water and concentrated in vacuo.

Procedure C—To a solution of ester (0.6 g) in a mixture THF/H_2O (2/1, v/v, 4.5 mL) was added 10 wt% Pd/C (0.30 g). The reaction was placed in a hydrogenator under 48 psi H_2 at room temperature. After 24 h, the reaction mixture was filtered through Celite® and concentrated in vacuo. The resulting oil was dissolved in water (20 mL) and washed with ethyl acetate (3 × 10 mL). The aqueous layer was concentrated to afford the acid.

Procedure D—To triphosgene (0.36 g, 1.2 mmol, 0.6 equiv.) in CH_2Cl_2 (9.0 mL) at −15 °C was added over 2 h a solution of L-aspartic acid dibenzyl ester *p*-toluenesulfonate **30** (1.16 g, 2.4 mmol, 1.2 equiv.) and triethylamine (1.3 mL, 9.6 mmol, 4.8 equiv.) in CH_2Cl_2 (8.0 mL) under N_2. After the addition, the reaction mixture was allowed to warm to room temperature over 1 h. Then a solution of ester **28** or **29** (2.0 mmol, 1.0 equiv.) in CH_2Cl_2 (10.0 mL) was added dropwise at room temperature. After 17h at room temperature, the reaction mixture was concentrated in vacuo. The resulting oil was diluted in ethyl acetate (10 mL) and filtered through a cotton plug. The filtrate was concentrated in vacuo and the resulting oil was purified by chromatography over silica gel.

4.3 Synthesis of the "cyclo-PALA" heterocycle 19 (Scheme 4)

4.3.1 (*N*-acetyl-L-aspartic acid dibenzyl ester)-hydroxymethyl-phosphinic acid benzhydryl ester (17)—Procedure A. The resulting oil was dissolved in distilled ACN (10 mL) and treated with Ph_2CN_2[18] (1.0 M in toluene) until complete esterification (persistence of the pink color and ^{31}P NMR monitoring, 2 singlets at 48 ppm) at room temperature under N_2. After removal of the solvent in vacuo, the resulting oil was dissolved in ethyl acetate (50 mL) and washed with 10% aqueous tartaric acid (1 × 10 mL) and brine (2 × 10 mL). The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The resulting oil was purified by chromatography over silica gel (hexanes/ethyl acetate, 3/7, v/v) to afford the ester **17** (1.4 g, 50 % yield). ^1H NMR (300 MHz, $CDCl_3$) δ: 7.65 (d, J = 7.0 Hz, 0.5 H, N*H*), 7.50 (d, J = 7.0 Hz, 0.5 H, N*H*), 7.26–7.36 (m, 20 H, C$H_{arom.}$), 6.56 (d, J = 9.0 Hz, 1 H, Ph_2CH-), 5.01–5.15 (m, 4 H, *Bn* -CH_2-), 4.85 (m, 1 H, N-C*H*-), 3.70–3.84 (m, 2 H, P-CH_2-OH), 2.74–3.05 (m, 4 H, P-CH_2-CO & CH-CH_2-CO_2Bn); ^{13}C NMR (75.45 MHz, $CDCl_3$) δ: 170.8 & 170.6, 170.4 & 170.2, 165.7 (2 d, J_{PCC} = 3.0 Hz), 140.6 (2 C), 135.5, 135.2, 128.9, 128.8, 128.7, 128.7, 128.6, 128.5, 127.3, 127.2, 78.8 (d, J_{POC} = 6.0 Hz), 67.9, 67.2 & 67.1, 60.0 (d, J_{PC} = 108.5 Hz), 49.5 & 49.3, 37.0 (2 d, J_{PC} = 77.5 Hz), 36.3; ^{31}P NMR (121.47 MHz, $CDCl_3$) δ: 48.03 (s, 50 %), 48.00 (s, 50 %); $^+$) 616.2100, found 616.2103. HRMS (ESI+) calcd. for $C_{34}H_{35}NO_8P$, ([M+H]

4.1.2 2-(3-Benzhydryloxy-3,5-dioxo-3λ5-[1,3]azaphospholidin-1-yl)-succinic acid dibenzyl ester (18)—Procedure B. The resulting oil was purified by chromatography over silica gel (hexanes/ethyl acetate, 6/4 to 4/6, v/v) to afford the mixture of the ester **18** and triphenylphosphine oxide (0.614 g). ^1H NMR (300 MHz, $CDCl_3$) δ: 7.26–7.40 (m, 20 H, *aro* CH), 6.58 (d, J = 9.0 Hz, 1 H, Ph_2CH-), 5.12 (s, 2 H, *Bn* -CH_2-), 5.06 (s, 2 H, *Bn* -CH_2-), 4.45–3.56 (m, 1 H, N-CH-), 2.04–3.15 (m, 6 H, P-CH_2-N & P-CH_2-CO & CH-CH_2-CO_2Bn); ^{31}P NMR (121.47 MHz, $CDCl_3$) δ: 46.25 (s, 22 %, 1 diastereoisomer), 46.16 (s, 62 %, 1 diastereoisomer), 30.51 (s, 16 %, Ph_3PO).

4.1.3 2-(3-Hydroxy-3,5-dioxo-3l5-[1,3]azaphospholidin-1-yl)-succinic acid (19)—Procedure C. Yellow oil (0.183 g, 44 % yield from hydroxyl **17**). ^1H NMR (300 MHz, D_2O) δ: 5.00 (t, J = 7.0 Hz, 1 H, N-C*H*-), 3.31 (d, J_{HCP} = 11.5 Hz, 2 H, P-CH_2-N), 2.93 (dd, J = 17.0 Hz, J = 6.5 Hz, 1 H, N-CH-CH_2-), 2.72 (dd, J = 17.0 Hz, J = 6.5 Hz, 1 H, N-CH-CH_2-), 2.43 (d, J_{HCP} = 13.0 Hz, 2 H, P-CH_2-CO); ^{13}C NMR (75.45 MHz, D_2O) δ: 174.3, 172.6 (d, J_{PCC}

= 12.0 Hz), 172.3, 53.1 & 53.0, 48.3 (d, J_{PC} = 88.5 Hz), 33.2, 32.8 (d, J_{PC} = 88.5 Hz); ^{31}P NMR (121.47 MHz, D_2O) δ: 33.84 (s); HRMS (ESI^+) calcd. for $C_7H_{11}O_7NP$, ($[M+H]^+$) 252.0273, found 252.0266.

4.2 Synthesis of the 6-membered "cyclo-PALA" 27 (Scheme 5)

4.2.1 (*N*-acetyl-L-aspartic acid dibenzyl ester)-(3-methyl-but-2-enyl)-phosphinic acid benzyl ester (20)—Procedure A. The resulting oil was dissolved in ethyl acetate (50 mL) and washed with brine (1 × 30 mL), the aqueous layer was extracted with ethyl acetate (2 × 50 mL). The combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. To the resulting oil in chloroform (75 mL) was added benzyl bromide (4.3 mL, 35.9 mmol, 1.2 equiv.). The mixture was refluxed and silver oxide (I) was added in portions every 30 minutes (4.0 g, 17.0 mmol, 0.6 equiv., in 4 portions) under N_2. After cooling down, the suspension was filtered through Celite® and the the solvent was removed in vacuo. The resulting oil was purified by chromatography over silica gel (hexanes/ethyl acetate, 6/4, v/v to ethyl acetate 100 %) to afford the ester **20** (10.2 g, 59 % yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.59 (d, J = 8.0 Hz, 1 H, N*H*), 7.25–7.35 (m, 15 H, C$H_{arom.}$), 4.98–5.14 (m, 7 H, *Bn* -CH_2- & -C*H*=), 4.89–4.95 (m, 1 H, N-C*H*), 2.78 (d, J_{HCP} = 14.5 Hz, 2 H, P-CH_2-CH=), 2.58–3.08 (m, 4 H, N-CH-CH_2- & P-CH_2-CO), 1.72 (d, J = 5.0 Hz, 3 H, CH_3-C=), 1.59 (m, 3 H, CH_3-C=); ^{13}C NMR (75.45 MHz, $CDCl_3$) δ: 170.5 (2 s), 170.3, 164.9, 138.4 (d, J_{PCCC} = 14.0 Hz), 136.5, 135.6, 135.3, 128.9 (2 C), 128.8 (2 C), 128.7 (4 C), 128.6 (2 C), 128.3, 128.2, 111.8 (d, J_{PCC} = 10.0 Hz), 67.8 (2 s), 67.1, 67.0 & 66.8 (2 d, J_{POC} = 7.0 Hz), 49.2, 37.0 (2 d, J_{PC} = 75.5 Hz), 36.5 & 36.4, 30.2 & 30.0 (2 d, J_{PC} = 94.0 Hz), 26.1 (d, J_{PCCCC} = 3.0 Hz), 18.3; ^{31}P NMR (121.47 MHz, $CDCl_3$) δ: 50.26 (s, 52 %), 49.78 (s, 48 %); HRMS (EI) calcd. for $C_{32}H_{36}NO_7P$, ($[M]^+$) 577.2229, found 577.2235.

4.2.2 (*N*-acetyl-L-aspartic acid dibenzyl ester)-hydroxyethyl-phosphinic acid benzyl ester (21)—After saturation of a solution of the ester **20** (2.0 g, 3.47 mmol, 1.0 equiv.) in CH_2Cl_2 (0.2 M, 17.0 mL) at −78 °C by ozone, Me_2S (1.8 mL, 24.3 mmol, 7.0 equiv.) was added under N_2 at −78 °C. The reaction mixture was allowed to warm up to room temperature overnight then the solution was concentrated in vacuo. The resulting oil was dissolved in methanol (0.2 M, 17 mL) and treated by sodium borohydride in portions (0.20 g, 5.2 mmol, 1.5 equiv.) at 0°C. The reaction was followed by TLC, after completion the reaction mixture was diluted with ethyl acetate (50 mL) and washed with brine (1 × 20 mL), the aqueous layer was extracted with ethyl acetate (2 × 50 mL). The combined organic layer was dried over $MgSO_4$ and concentrated in vacuo. The resulting oil was purified by chromatography over silica gel (hexanes/ethyl acetate, 1/9, v/v to ethyl acetate/methanol 98/2, v/v) to afford the ester **21** (1.09 g, 57 % yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.03 (d, J = 8.5 Hz, 0.5 H, N*H*), 7.59 (d, J = 8.0 Hz, 0.5 H, N*H*), 7.26–7.36 (m, 15 H, C$H_{arom.}$), 5.03–5.15 (m, 6 H, *Bn* -CH_2-), 4.86–4.98 (m, 1 H, N-C*H*-), 3.77–3.99 (m, 2 H, -CH_2-OH), 2.80–3.18 (m, 4 H, N-CH-CH_2 & P-CH_2-CO), 1.94–2.38 (m, 2 H, P-CH_2-CH_2OH); ^{13}C NMR (75.45 MHz, $CDCl_3$) δ: 170.8 & 170.4, 170.7, 165.4, 136.4 (d, J_{POCC} = 6.0 Hz), 135.6, 135.6, 135.3 (2 s), 128.9 (2 C), 128.8 (2 C), 128.7 (2 C), 128.6 (4 C), 128.3, 128.2, 67.9 & 67.8, 67.1, 66.7 & 66.6 (d, J_{POC} = 9.5 Hz), 56.4, 49.4 & 49.1, 39.2 (d, J_{PC} = 75.5 Hz), 36.6 & 36.4, 31.5 (d, J_{PC} = 95.0 Hz) & 31.2 (d, J_{PC} = 95.5 Hz); ^{31}P NMR (121.47 MHz, $CDCl_3$) δ: 52.05 (s, 51 %), 51.69 (s, 49 %);HRMS (EI) calcd. for $C_{29}H_{32}NO_8P$, ($[M]^+$) 553.1866, found 553.1875.

4.2.3 2-(4-Benzyloxy-2,4-dioxo-4λ^5-[1,4]azaphosphinan-1-yl)-succinic acid dibenzyl ester (26)—Procedure B. The resulting oil was dissolved in dicholoromethane (25 mL). After saturation of the solution at −78 °C by ozone, Me_2S (1.7 mL, 23.2 mmol, 7.0 equiv.) was added under N_2 at −78 °C. The reaction mixture was allowed to warm up to room temperature overnight then the solution was concentrated in vacuo. The resulting oil was purified by chromatography over silica gel (hexanes/ethyl acetate, 3/7, v/v) to afford the ester

26 as a lightly yellow oil (1.2 g, 33 % yield). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.25–7.35 (m, 15 H, C$H_{arom.}$), 4.93–5.17 (m, 7 H, *Bn* -CH_2- & N-C*H*-), 3.42–3.74 (m, 2 H, P-CH_2-CH_2-N), 2.83–3.14 (m, 4 H, P-CH_2-CO & N-CH-CH_2-), 1.93–2.21 (m, 2 H, P-CH_2-CH_2-N); ^{13}C NMR ($CDCl_3$, 75.45 MHz) δ: 170.7, 169.3 & 169.2, 165.9 (d, J_{PCC} = 5.0 Hz) & 165.8 (d, J_{PCC} = 5.0 Hz), 135.8, 135.5, 135.2, 129.0 (2 C), 128.9 (3 C), 128.7 (3 C), 128.6 (2 C), 128.4 (2 C), 67.9 & 67.8, 67.2, 67.0 (d, J_{POC} = 7.5 Hz) & 66.9 (d, J_{POC} = 6.5 Hz), 57.6 & 57.1, 43.9 (d, J_{PCC} = 26.0 Hz), 36.9 (d, J_{PC} = 74.5 Hz) & 36.8 (d, J_{PC} = 73.5 Hz), 35.0 (2 s), 27.5 (d, J_{PC} = 94.5 Hz) & 27.3 (d, J_{PC} = 95.5 Hz); ^{31}P NMR ($CDCl_3$, 121.47 MHz) δ: 49.23 (s, 55 %), 48.77 (s, 45 %); HRMS (CI, NH_3) calcd. for $C_{29}H_{31}NO_7P$, ([M+H]$^+$) 536.1838, found 536.1829.

4.2.4 2-(4-Hydroxy-2,4-dioxo-4λ^5-[1,4]azaphosphinan-1-yl)-succinic acid (27)— Procedure C. Orange oil (0.30 g, 64 % yield). ^{1}H NMR (D_2O, 300 MHz) δ: 4.88 (t, J = 8.0 Hz, 1 H, N-C*H*-), 3.65 (t, J = 6.5 Hz, 1 H, P-CH_2-CH_2-N), 3.57 (t, J = 6.5 Hz, 1 H, P-CH_2-CH_2-N), 2.99 (dd, J_{HCP} = 17.0 Hz, J = 6.5 Hz, 1 H, N-CH-CH_2-), 2.69–2.83 (m, 3 H, N-CH-CH_2- & P-CH_2-CO), 1.87–1.96 (m, 2 H, P-CH_2-CH_2-N); ^{13}C NMR (D_2O, 100.6 MHz) δ: 174.7, 173.0, 170.4, 57.0, 44.3 (d, J_{PCC} = 5.0 Hz), 38.1 (d, J_{PC} = 74.0 Hz), 33.9, 27.5 (d, J_{PC} = 95.5 Hz); ^{31}P NMR (D_2O, 121.47 MHz) δ: 40.56 (s); HRMS (ESI+) calcd. for $C_8H_{13}NO_7P$, ([M+H]$^+$) 266.0429, found 266.0427.

4.3. Synthesis of the carbamate and urea analogs (32 and 34, Scheme 6)

4.3.1 2-(Bis-benzyloxy-phosphorylmethoxycarbonylamino)-succinic acid dibenzyl ester (31)—Procedure D. Purification by chromatography over silica gel (hexanes/ethyl acetate, 8/2 to 6/4,v/v) to afford the ester **31** as a light yellow oil (0.625 g, 50 % yield). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.24–7.39 (m, 20 H, C$H_{arom.}$), 5.88 (d, J = 8.0 Hz, 1 H, N*H*), 5.00–5.12 (m, 8 H, *Bn* -CH_2-), 4.57–4.62 (m, 1 H, N-C*H*-CH_2), 4.27–4.42 (m, 2 H, P-CH_2-O), 3.05 (dd, J = 17.0 Hz, J = 4.0 Hz, 1 H, -CH_2-CO_2Bn), 2.83 (dd, J = 17.0 Hz, J = 5.0 Hz, 1 H, -CH_2-CO_2Bn); ^{13}C NMR ($CDCl_3$, 75.45 MHz) δ: 170.7, 170.3, 155.1 (d, J_{PCOC} = 10.0 Hz), 136.0 (d, J_{POCC} = 5.5 Hz, 2 C), 135.5, 135.3, 128.9 (10 C), 128.8 (2 C), 128.7 (2 C), 128.6 (2 C), 128.3 (4 C), 68.5 (d, J_{POC} = 6.5 Hz,), 68.4 (d, J_{POC} = 6.0 Hz), 68.9, 67.2, 58.3 (d, J_{PC} = 170.0 Hz), 50.9, 36.8; ^{31}P NMR ($CDCl_3$, 121.47 MHz) δ: 21.49 (s); HRMS (EI) calcd. for $C_{34}H_{34}NO_9P$, ([M]$^+$) 631.1971, found 631.1947.

4.3.2 2-[3-Benzyl-3-(bis-benzyloxy-phosphorylmethyl)-ureido]-succinic acid dibenzyl ester (33)—Procedure D. Purification by chromatography over silica gel (hexanes/ethyl acetate, 7/3,v/v) to afford the ester **33** as a light yellow oil (0.620 g, 43 % yield). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.12–7.38 (m, 25 H, C$H_{arom.}$), 6.13 (bs, 1 H, N*H*), 4.92–5.10 (m, 8 H, O-CH_2-Ph), 4.77–4.82 (m, 1 H, N-C*H*-CH_2-), 4.52 (d, J = 16.0 Hz, 1 H, N-CH_2-Ph), 4.40 (d, J = 16.0 Hz, 1 H, N-CH_2-Ph), 3.59 (m, 2 H, P-CH_2-N), 2.99 (dd, J = 17.0 Hz, J = 5.0 Hz, 1 H, N-CH-CH_2-), 2.84 (dd, J = 17.0 Hz, J = 5.0 Hz, 1 H, N-CH-CH_2-); ^{13}C NMR ($CDCl_3$, 75.45 MHz) δ: 171.4, 170.8, 157.8, 136.6, 136.3, 136.2, 135.7 (d, J_{POCC} = 8.5 Hz, 2 C), 129.0 (2 C), 128.9 (4 C), 128.8 (5 C), 128.5 (10 C), 128.5 (2 C), 127.8 (4 C), 68.3 (d, J_{POC} = 7.5 Hz, 2 C), 67.5, 66.9, 51.0 (d, J_{PCNC} = 18.5 Hz), 50.9, 42.9 (d, J_{PCNC} = 160.0 Hz), 37.2; ^{31}P NMR ($CDCl_3$, 121.47 MHz) δ: 25.00 (s); HRMS (EI) calcd. for $C_{41}H_{41}N_2O_8P$, ([M]$^+$)720.2601, found 720.2616.

4.3.3 2-Phosphonomethyl-oxycarbonylamino-succinic acid (32)—Procedure C. Orange oil (0.26 g, 100 % yield). ^{1}H NMR (D_2O, 300 MHz) δ: 4.43 (t, J = 6.0 Hz, N-C*H*-CH_2), 4.05 (d, J_{HCP} = 8.5 Hz, 2 H, P-CH_2-O), 2.80 (d, J = 6.0 Hz, 2 H, -CH_2-CO_2H); ^{13}C NMR (D_2O, 75.45 MHz) δ: 170.7, 174.5, 157.6, 60.3 (d, J_{PC} = 159.0 Hz), 50.6, 36.0; ^{31}P NMR (D_2O, 121.47 MHz) δ: 17.54 (s); HRMS (ESI+) calcd. for $C_6H_{11}NO_9P$, ([M+H]$^+$) 272.0171, found 272.0176.

4.3.4 2-(3-Phosphonomethyl-ureido)-succinic acid (34)—Procedure C. Orange oil (0.133 g, 100 % yield). ^{1}H NMR (D_2O, 300 MHz) δ: 4.49 (t, J = 5.5 Hz, 1 H, N-C*H*-CH_2-), 3.27 (d, J_{HCP} = 11.5 Hz, 2 H, P-CH_2-N), 2.79 (d, J = 5.5 Hz, 2 H, N-CH-CH_2-); ^{13}C NMR (D_2O, 75.45 MHz) δ: 175.5, 174.9, 159.3, 49.8, 37.1 (d, J_{PCNC} = 150.0 Hz), 36.4; ^{31}P NMR (D_2O, 121.47 MHz) δ: 20.20 (s); HRMS (ESI+) calcd. for $C_6H_{12}N_2O_8P$, ($[M+H]^+$) 271.0331, found 271.0323.

4.4 Synthesis of the phosphate analog 36 (Scheme 7)

4.4.1 Dibenzyl-(*N*-(2-hydroxy-acetyl)-L-aspartic acid dibenzyl ester)-phosphate (35)—To a mixture of dibenzylphosphate (0.278 g, 1.0 mmol., 1.0 equiv.), *N*-(bromoacetyl)-L-aspartic acid dibenzyl ester **16** (0.434 g, 1.0 mmol, 1.0 equiv.) in acetonitrile (0.1 M, 10 mL) was added silver (I) oxide (0.231 g, 1.0 mmol, 1.0 equiv.). The reaction mixture was heated at 60 °C under N_2 for 16 h. After cooling down, the suspension was filtered thought Celite® and concentrated in vacuo. The resulting oil was diluted by CH_2Cl_2 (50 mL) and washed with water (3 × 10 mL). The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The resulting oil was purified by chromatography over silica gel (hexanes/ethyl acetate, 6/4, v/v) to afford the ester **35** as a lightly yellow oil (0.595 g, 94 % yield). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.26–7.34 (m, 20 H, C$H_{arom.}$), 5.13 (s, 2 H, *Bn* -CH_2-), 5.02–5.07 (m, 6 H, *Bn* -CH_2-), 4.91 & 4.88 (2 t, J = 4.5 Hz, 1 H, N-C*H*-CH_2), 4.39 (d, J_{HCOP} = 7.0 Hz, 2 H, P-O-CH_2-CO), 3.05 (dd, J = 17.0 Hz, J = 4.5 Hz, 1 H, N-CH-CH_2-), 2.85 (dd, J = 17.0 Hz, J = 4.5 Hz, 1 H, N-CH-CH_2-); ^{13}C NMR ($CDCl_3$, 75.45 MHz) δ: 170.6, 170.0, 167.0 (d, J_{POCC} = 9.0 Hz), 135.3 (d, J_{POCC} = 6.5 Hz, 2 C), 135.2, 135.0, 128.8, 128.7 (4 C), 128.6 (4 C), 128.5 (2 C), 128.4, 128.3, 128.2 (4 C), 69.9 (d, J_{POC} = 5.0 Hz, 2 C), 67.6, 66.9, 65.1 (d, J_{POC} = 6.0 Hz), 48.3, 36.1; ^{31}P NMR ($CDCl_3$, 121.47 MHz) δ: −0.685 (s); HRMS (EI) calcd. for $C_{34}H_{34}NO_9P$, ($[M]^+$) 631.1971, found 631.1979.

4.4.2 *N*-(2-hydroxy-acetyl)-L-aspartic acid-phosphate (36)—Procedure C. Orange oil (0.357 g, 100 % yield). ^{1}H NMR (D_2O, 300 MHz) δ: 4.69 (t, J = 6.0 Hz, 1 H, N-C*H*-CH_2), 4.25 (d, J_{HCOP} = 7.0 Hz, 2 H, P-O-CH_2-CO), 2.83 (m, *2* H, N-CH-CH_2-); ^{13}C NMR (D_2O, 75.45 MHz) δ: 174.5, 173.8, 171.4 (d, J_{POCC} = 9.0 Hz), 63.8 (d, J_{POC} = 5.0 Hz), 48.7, 35.5; ^{31}P NMR (D_2O, 121.47 MHz) δ: 0.40 (s); HRMS (ESI+) calcd. for $C_6H_{10}NO_9P$, ($[M+H]^+$) 272.0173, found 272.0171.

4.5 Biological assay

Before testing, each compound was neutralized to pH 7.0 with 1.0 M aqueous NaOH then concentrated to dryness. For the compounds containing a phosphorus atom, the phosphorus assay[30] was used to determine accurate concentrations, and dilute solutions were prepared from this standardized solution. All compounds tested were at least 97% pure as determined by ^{1}H- and ^{31}P-NMR. The potential oxidation of **2** into PALA could not be detected over a period of two weeks (^{31}P-NMR monitoring).

The assay was performed with *E. Coli* His-tagged C3 ATCase provided by Professor Evan Kantrowitz (Boston College).[24] The assay was performed as described by Professor Evan Kantrowitz[25] at room temperature, in the presence of 0.1 M Tris-acetate pH 8.3, 75 ng/mL His-tagged C3 ATCase and 10 mM L-aspartate. Kinetic was measured with four carbamyl phosphate concentrations (20, 40, 60, 80 μM) and five inhibitors concentrations (0, 0.5, 1.0, 3.0, 6.0 μM) to determine the inhibition constant (K_i) and the mode of inhibition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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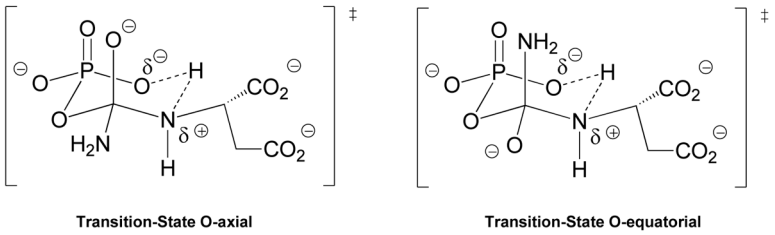
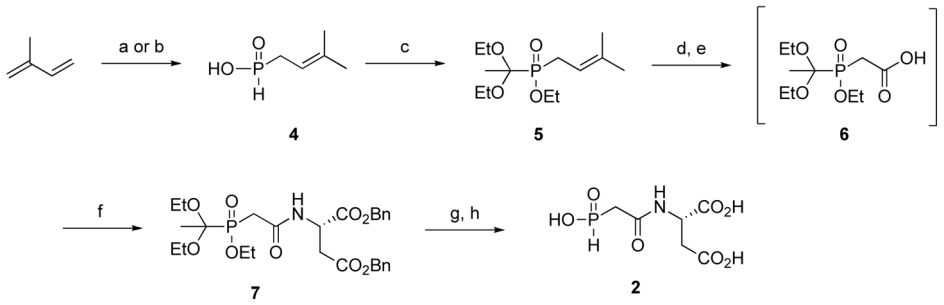
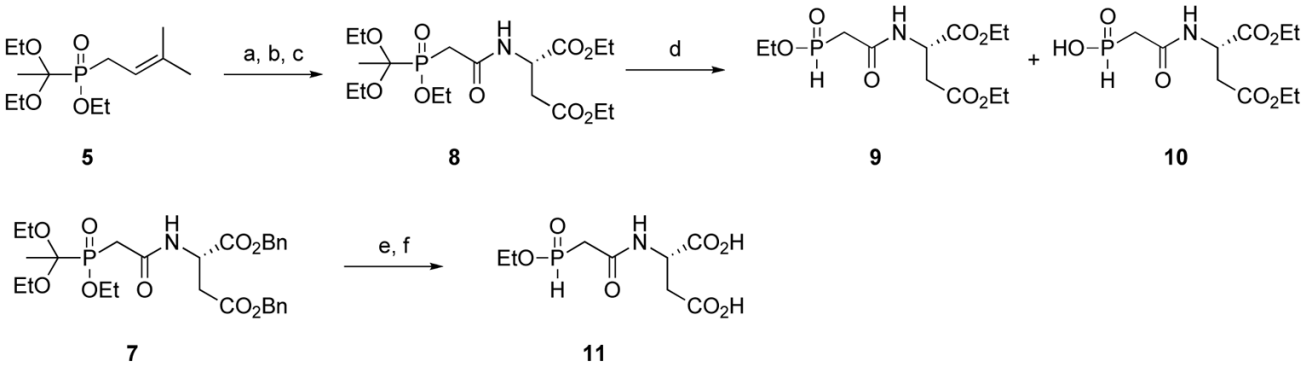


Figure 1.
Postulated transition-state



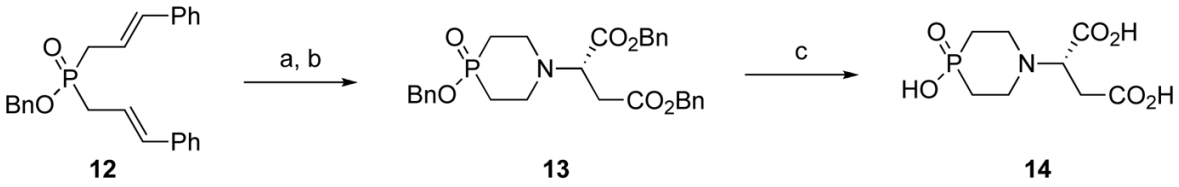
Scheme 1.

Reagents and conditions: (a) H_3PO_2 (1.0 equiv.), Pd_2dba_3 (0.125 mol %), xantphos (0.275 mol %), DMF, 85 °C, 13 h, sealed tube, 83 %; (b) H_3PO_2 (1.5 equiv.), nixantphos catalyst (1.2 mol %), ACN, 85 °C, 15 h, sealed tube, 5 runs, 100 %; (c) $CH_3C(OEt)_3$ (6.0 equiv.), BF_3•OEt_2 (0.2 equiv.), r.t., N_2, 14 h, 64 %; (d) O_3, CH_2Cl_2, −78 °C, then Me_2S (6.8 equiv.), −78 °C to r.t., N_2, 14 h; (e) $NaClO_2$ (1.5 equiv.), NaH_2PO_4•H_2O (1.5 equiv.), 2-methyl-2-butene (2.0 equiv.), *t*BuOH/H_2O, 0 °C then r.t., 1 h; (f) L-Aspartic acid dibenzyl ester *p*-toluenesulfonate (1.0 equiv.), DMAP (3.0 equiv.), EDC (3.0 equiv.), Et_3N (1.1 equiv.), r.t., 17 h, N_2,THF, 66 % from **5**; (g) H_2, Pd/C, THF/H_2O, 17 h; (h) Amberlite IR 120 plus, THF/H_2O, 80 °C, 15 h, 57% from **7** after ion exchange chromatography.



Scheme 2.

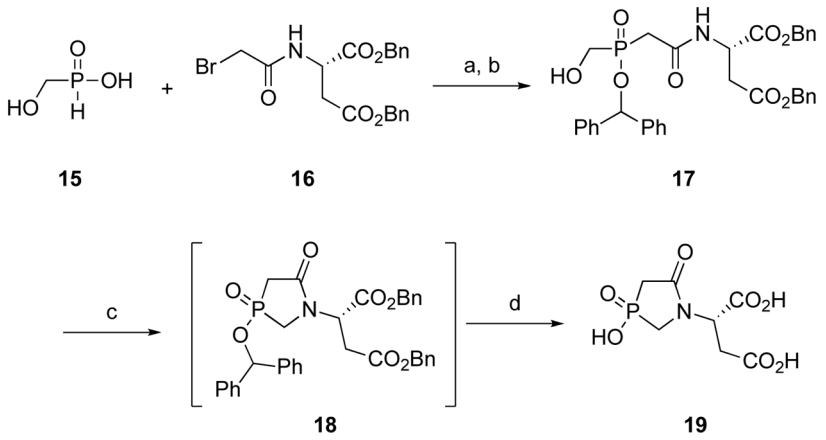
Reagents and conditions: (a) O_3, CH_2Cl_2, −78 °C then Me_2S (6.8 equiv.), −78 °C to r.t., N_2, 14 h; (b) $NaClO_2$ (1.5 equiv.), $NaH_2PO_4 \cdot H_2O$ (1.5 equiv.), 2-methyl-2-butene (2.0 equiv.), *t*BuOH/H_2O, 0 °C then r.t., 1 h; (c) L-aspartic acid diethyl ester hydrochloride (1.0 equiv.), DMAP (3.0 equiv.), EDC (3.0 equiv.), Et_3N (1.1 equiv.), r.t., 17 h, N_2,THF, 50 % from **5**; (d) Amberlite IR 120 plus, THF/H_2O, 80 °C, 5 h, 37 % for **9** and 60 % for **10**; (e) H_2, Pd/C, THF/H_2O, 23 h; (f) Amberlite IR 120 plus, THF/H_2O, 80 °C, 1 h, 59 % from **7**.



12 13 14

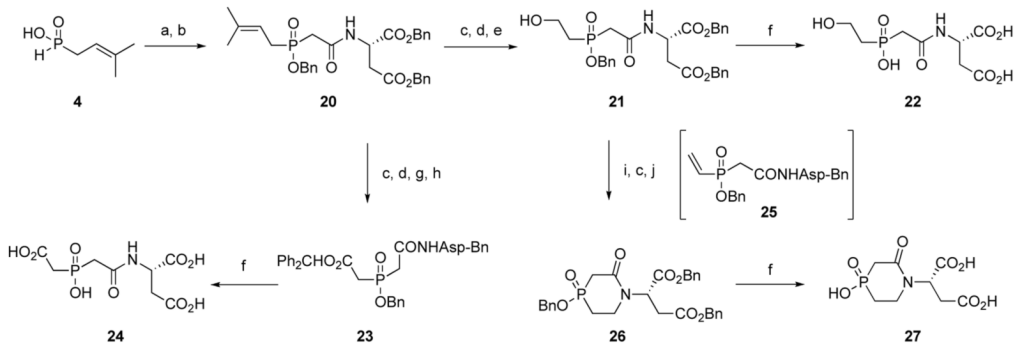
Scheme 3.

Reagents and conditions: (a) O_3, CH_2Cl_2, −78 °C then Me_2S (12.0 equiv.), −78 °C to r.t., N_2, 14 h; (b) L-aspartic acid dibenzyl ester p-toluenesulfonate (3.0 equiv.), $NaCNBH_3$ (7.7 equiv.), Et_3N (3.0 equiv.), AcOH to pH 4–6, r.t., 4 h, ACN, 54%; (c) H_2, Pd/C, THF/H_2O, 24 h, 85 %.



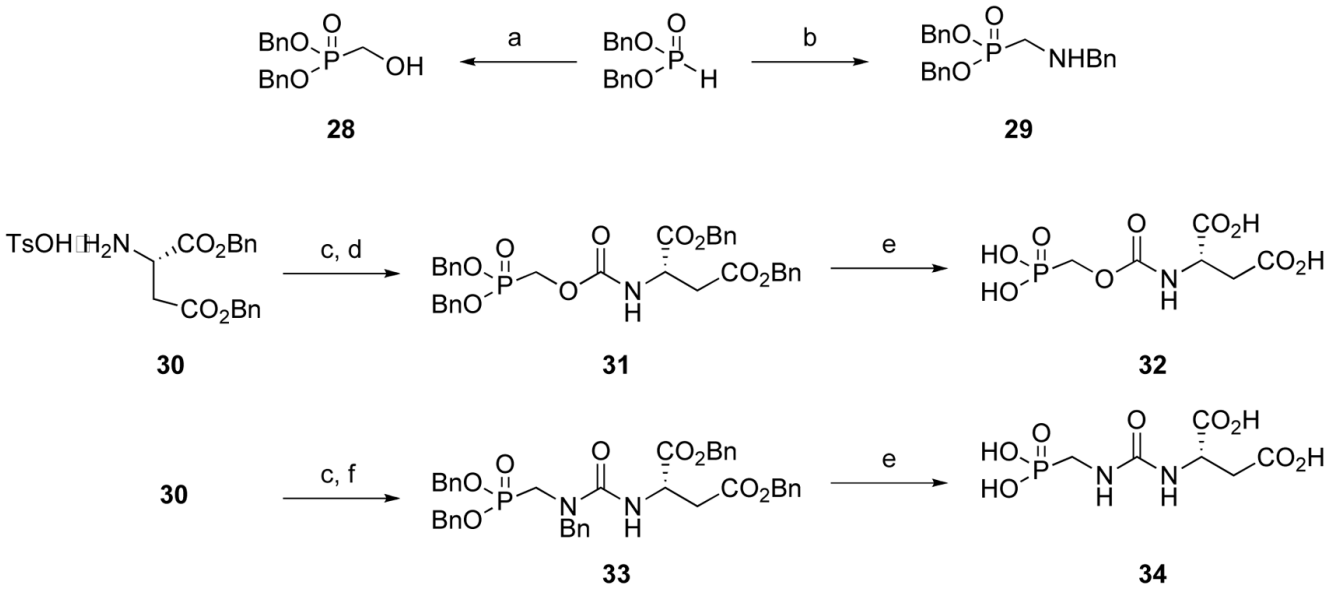
Scheme 4.

Reagents and conditions: (a) N-(bromoacetyl)-L-aspartic acid dibenzyl ester **16** (1.0 equiv.), HMDS (2.5 equiv.), TMSCl (2.5 equiv.), toluene, reflux, 14 h; (b) Ph_2CN_2, toluene, 50 % from **15**; (c) DIAD (1.0 equiv.), Ph_3P (1.0 equiv.), THF, 30 min, r.t.; (d) H_2, Pd/C, THF/H_2O, 24 h, 44 % from **17**.



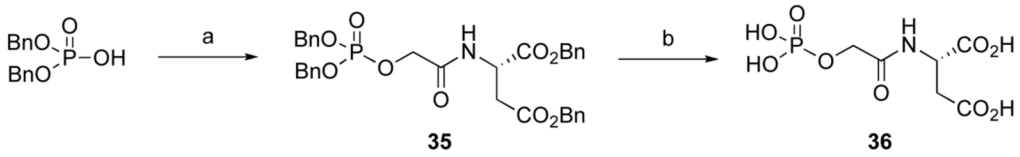
Scheme 5.

Reagents and conditions: (a) N-(bromoacetyl)-L-aspartic acid dibenzyl ester **16** (1.0 equiv.), HMDS (2.5 equiv.), TMSCl (2.5 equiv.), toluene, reflux, 14 h; (b) BnBr (1.2 equiv.), Ag_2O (0.6 equiv.), $CHCl_3$, reflux, N_2, 2 h, 59 % from **4**; (c) O_3, CH_2Cl_2, −78 °C; (d) Me_2S (7.0 equiv.), −78 °C to r.t., N_2, 14 h; (e) $NaBH_4$ (1.9 equiv.), MeOH, 0 °C, 1 h, 61 % from **20**; (f) H_2, Pd/C, THF/H_2O, 24 h, 100 % for **22**, 94 % for **24**, 100 % for **27**; (g) $NaClO_2$ (1.5 equiv.), $NaH_2PO_4.H_2O$ (1.5 equiv.), 2-methyl-2-butene (2.0 equiv.), *t*BuOH/H_2O, 0 °C then r.t., 1 h; (h) Ph_2CN_2, toluene, r.t., 63 % from **20**; (i) DIAD (1.0 equiv.), Ph_3P (1.0 equiv.), THF (0.05 M), 30 min, r.t.; (j) Me_2S (7.0 equiv.), −78 °C to r.t., N_2, 14 h, 33 % from **21**.



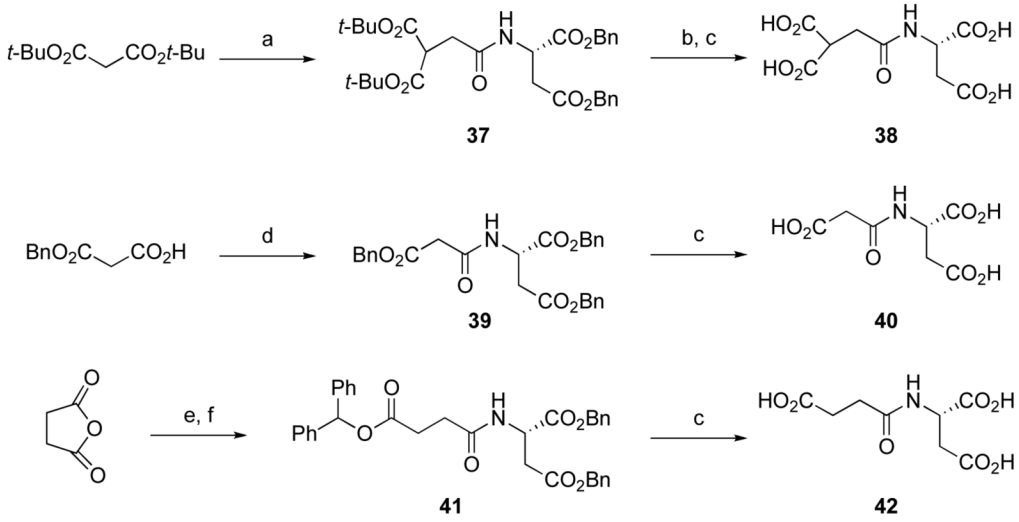
Scheme 6.

Reagents and conditions: (a) $(CH_2O)_n$ (1.0 equiv.), Et_3N (0.11 equiv.), 50°C, 45 min then 90 °C, 1.5 h, 61 %; (b) 1,3,5-tribenzylhexahydro-1,3,5-triazine (0.33 equiv.), toluene, reflux, 14 h, 73 %; (c) **30** (1.2 equiv.), Et_3N (4.8 equiv.), CH_2Cl_2, triphosgene (0.6 equiv.), − 15 °C, 2 h then r.t., 1 h; (d) hydroxymethyl-phosphonic acid dibenzyl ester **28** (1.0 equiv.), r.t., 17 h, 50 % from **30**; (e) H_2, Pd/C, THF/H_2O, 24 h, 100 % for both **32** and **34**; (f) **29** (1.0 equiv.), r.t., 17 h, 43 % from **30**.



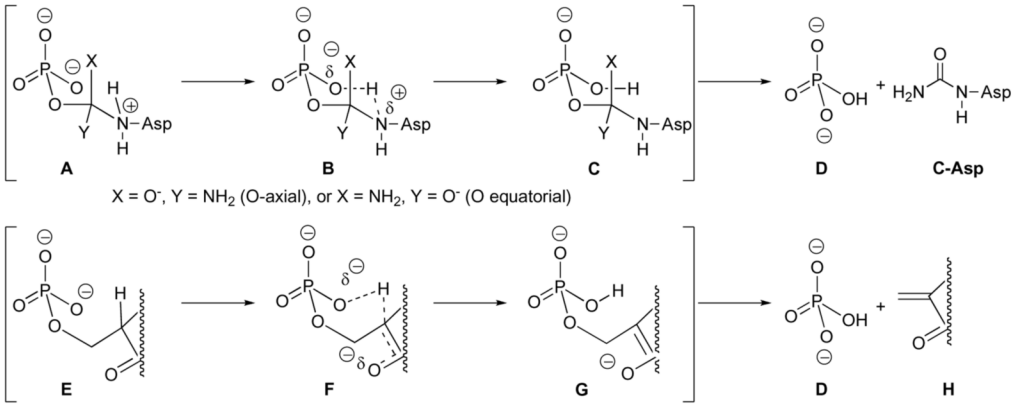
Scheme 7.

Reagents and conditions: (a) *N*-(bromoacetyl)-L-aspartic acid dibenzyl ester **16** (1.0 equiv.), Ag_2O (1.0 equiv.), CH_3CN, 60 °C, 19 h, 94 %; (b) H_2, Pd/C, THF/H_2O, 24 h, 100 %.



Scheme 8.

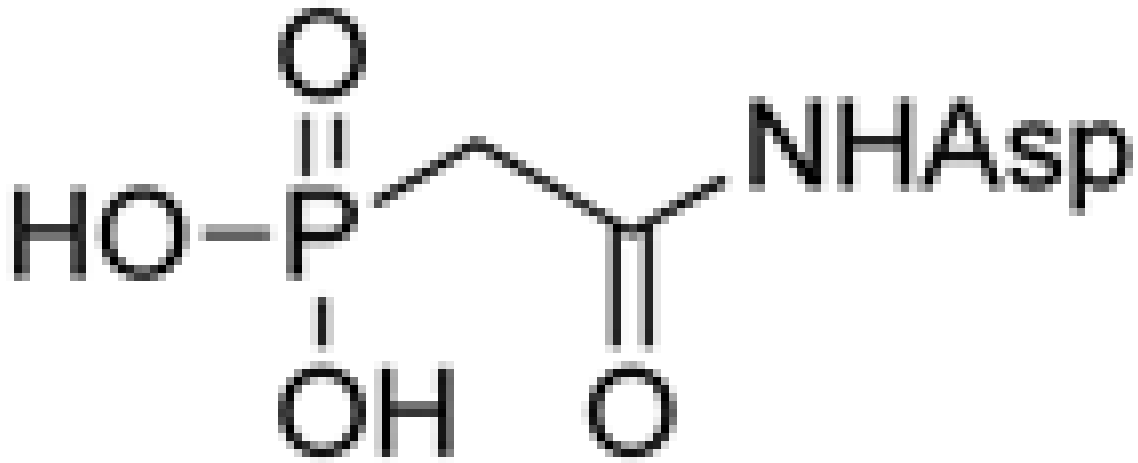
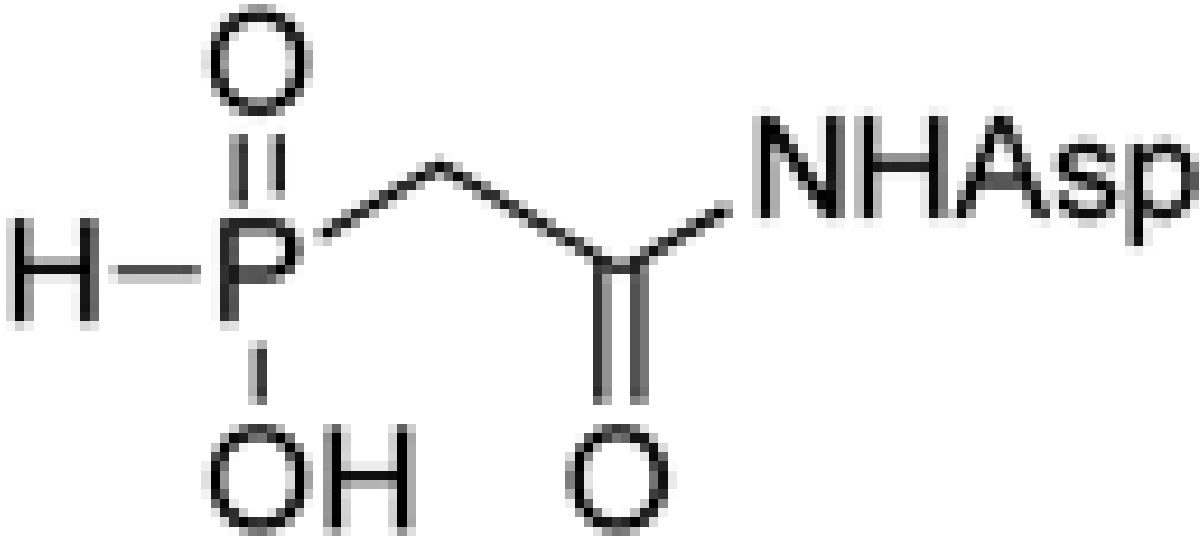
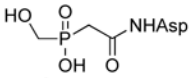
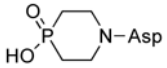
Reagents and conditions: (a) NaH (1.0 equiv.), THF, r.t., 5 min then N- (bromoacetyl)-L-aspartic acid dibenzyl ester **16** (0.77 equiv.), 89 %; (b) TFA, CH_2Cl_2, 0 °C, 15 h; (c) H_2, Pd/C, THF/H_2O, 24 h, 95 % from **37**; 83 % from **39**; 99 % from **41**; (d) L-aspartic acid dibenzyl ester p-toluenesulfonate **30** (1.0 equiv.), EDC•HCl (2.5 equiv.), DMAP (2.5 equiv.), Et_3N (1.1 equiv.), THF, r.t., 5 h, 78 %; (e) L-aspartic acid dibenzyl ester p-toluenesulfonate **30** (1.0 equiv.), Et_3N (6.0 equiv.), THF, r.t., 4 h; (f) Ph_2CN_2, THF, r.t., 33 % from succinic anhydride.

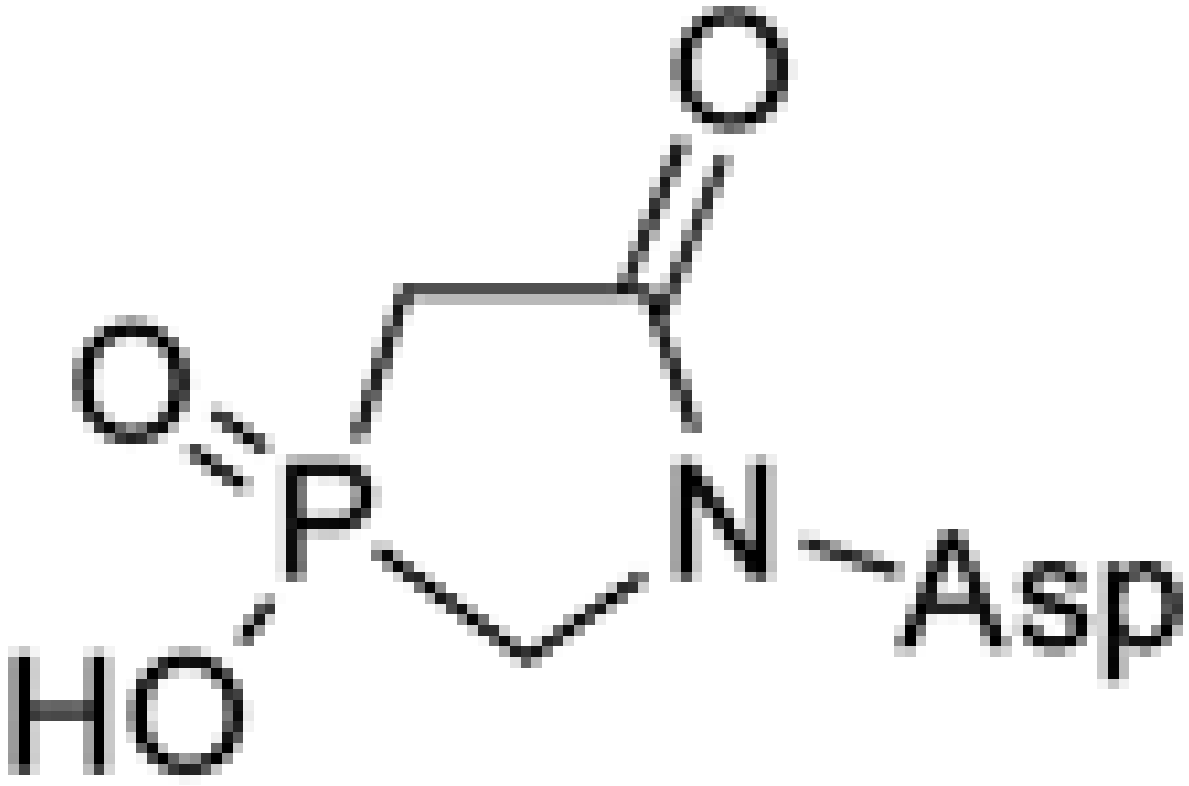
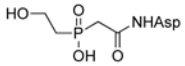
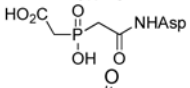
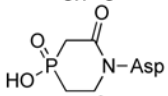
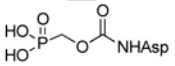
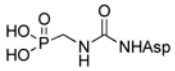


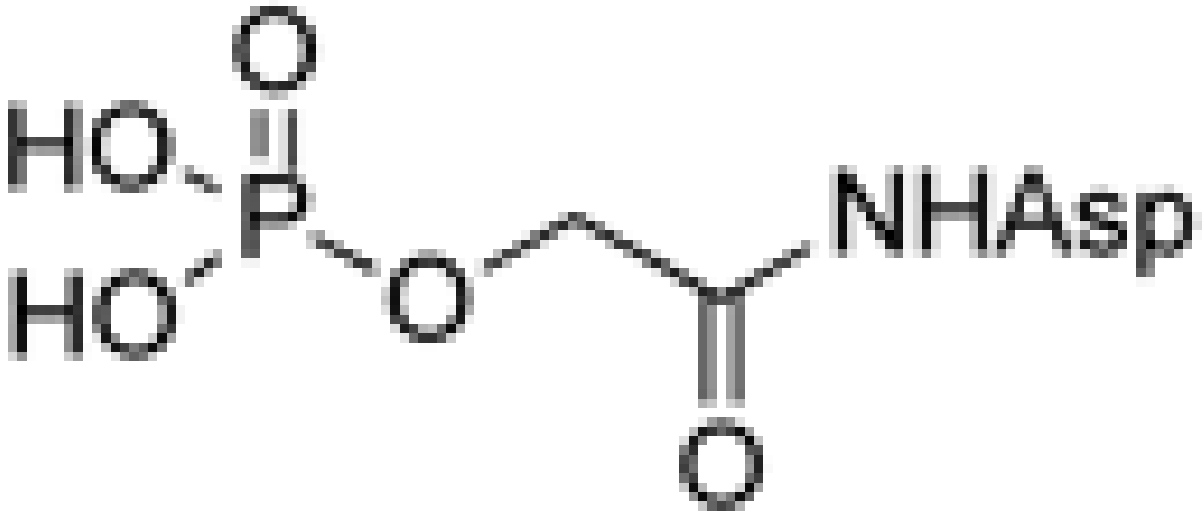
Scheme 9.
Mechanistic analogy between ATCase and 3-dehydroquinate synthase (DHQS)

Table 1

[a] Inhibition constants for the synthesized inhibitors

Entry	Inhibitor	Compound number[I]
1		**1**
2		**2**
3		**3**
4		**14**

Entry	Inhibitor	Compound number[I]
5		**19**
6		**22**
7		**24**
8		**27**
9		**32**
10		**34**

Entry	Inhibitor	Compound number[I]
11		**36**

[a] Colorimetric assay detecting the formation of *N*-carbamoyl-L-aspartate.[25] The *E. coli* catalytic subunit of ATCase was used.

[b] Inhibition relative to carbamyl phosphate K_m = 28 μM. Unless otherwise noted, all active compounds were competitive inhibitors.

[c] Compound **22** was an uncompetitive inhibitor.