Design, Synthesis, and Evaluation of Novel Organophosphorus Inhibitors of Bacterial Ureases

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A new group of organophosphorus inhibitors of urease, *P*-methyl phosphinic acids was discovered by using the structure based inhibitor design approach. Several derivatives of the lead compound, aminomethyl(*P*-methyl)phosphinic acid, were synthesized successfully. Their potency was evaluated *in vitro* against urease from *Bacillus pasteurii* and *Proteus vulgaris*. The studied compounds constitute a group of competitive, reversible inhibitors of bacterial ureases. Obtained thiophosphinic analogues of the most effective structures exhibited kinetic characteristics of potent, slow binding urease inhibitors, with $K_i = 170$ nM (against *B. pasteurii* enzyme) for the most active *N*-(*N*′-benzyloxycarbonylglycyl)aminomethyl(*P*-methyl)phosphinothioic acid.

Introduction

Urease (urea amidohydrolase, E.C. 3.5.1.5) is an enzyme that catalyzes hydrolysis of urea to ammonia and carbamate, which is the final step of nitrogen metabolism in living organisms.[1,2] Carbamate decomposes rapidly and spontaneously, yielding a second molecule of ammonia. These reactions cause significant increase of solution pH.

Bacterial ureases are large heteropolymeric metalloproteins with nickel(II) ions present in their active sites.[3−6] A significant amino acid sequence similarity was observed between all ureases of a bacterial origin.[7,8] The mechanism of enzymatic reaction has been studied extensively by several research groups for many years.[2,5,9−11] On the basis of several crystal structures of complexes of *Bacillus pasteurii* urease, Ciurli and co-workers proposed the most reliable enzymatic reaction mechanism.[10] The active site of the native enzyme binds three water molecules and a hydroxide ion bridged between two nickel ions. Urea replaces these three water molecules and is bound by a network of hydrogen bonds as well as by the nickel ions.[12] An activated carbon atom of urea is attacked by the Ni-bridging hydroxide ion, forming a tetrahedral transition state. Subsequently, ammonia is released from the active site followed by the negatively charged carbamate.

Several classes of compounds are known to show considerable inhibitory activity against this enzyme with phosphoramidates being the most active.[13−17] It was shown that phenyl phosphordiamidate (PPD[a]) and its 4-substituted derivatives exhibit inhibitory properties in nanomolar range (PPD was a competitive slow-binding inhibitor with $K_i^* = 0.6$ nM against *B. pasteurii* enzyme).[16,18] Its mode of action relays on hydrolysis in the active site and release of phosphorodiamidic acid (**1**), which is the actual enzyme inhibitor and represents enzymatic reaction transition state analogue.[10,15] However, hydroxamic acids are the best recognized urease inhibitors.[13,19−22] The simplest analogue in this group is acetohydroxamic acid, which exhibited a competitive slow-binding inhibition with $K_i = 2.6$ μM against *Klebsiella aerogenes* enzyme. Various hydroxamate analogues, several aminoalkanehydroxamic acids, their *N*-substituted derivatives, as well as dipeptides, were examined (*N*-glycylglycinehydroxamic acid exhibited $IC_{50} = 0.79$ μM against *Helicobacter pylori* urease).[23,24] Interestingly, simple organosulfur compounds represented, for example, by β-mercaptoethanol, also exhibited considerable inhibitory activity.[25] This phenomenon was explained by the analysis of the crystal structure of β-mercaptoethanol−urease complex, in which the sulfur atom was bridged between the two nickel ions present in the active site.[6]

The studies on novel urease inhibitors are essential not only for the basic research on urease biochemistry but also for the possible development of a highly needed therapy for urease mediated bacterial infections.[1,8,26,27] Ammonia released in the urease catalyzed reaction is either a direct cause of clinical conditions or a crucial factor to the pathogen survival and a host colonization. Ureolytic activity of several microorganisms, i.e., *Proteus mirabilis*, *Proteus vulgaris*, and *Ureaplasma urealyticum*, is involved in the formation of urinary tract stones, which may lead to the chronic inflammation of kidney and its pelvis.[28−31] Additionally, urinary catheter obstruction in patients is caused by its colonization by urease-producing microorganisms, mainly *P. mirabilis*.[32,33] Moreover, the overproduction of ammonia by infectious microorganisms may contribute to ammonia encephalopathy or hepatic coma.[34−36]Acetohydroxamic acid, a potent urease inhibitor, was shown to be an efficient drug against diseases caused by ureolytic bacteria.[37−39] Another mechanism of urease involvement in pathogenic bacteria infection relies on the creation of a microenvironment suitable for the pathogen existence. *Helicobacter pylori* infection of stomach is possible only after local neutralization of gastric acid by released ammonia.[27,40,41] Furthermore, high concentration of ammonia disturbs mucosal permeability, in particular hydrogen ions passage through mucosal surface, and causes formation of peptic ulcers.[42,43] The immense significance of this medical problem was emphasized by the 2005 Nobel Prize in

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[a] Abbreviations: PPD, phenyl phosphordiamidate; Cbz, benzyloxycarbonyl, TMS, trimethylsilyl; EDC 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; HOBt, Hydroxybenzotriazol; DIPEA, diisopropylethylamine.

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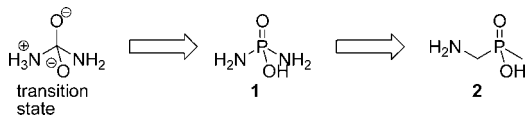
Scheme 1



Physiology or Medicine, awarded to Warren and Marshall for the research on the role of *H. pylori* in gastritis and peptic ulcer disease.

In this paper, we present the design, synthesis, and evaluation of novel ogranophosphorus inhibitors of bacterial urease. On the basis of the crystal structure of *Bacillus pasteurii* urease, several *P*-methyl phosphinic peptidic structures were designed by using the computer aided techniques. Successful synthesis of the designed compounds in enantiomerically pure forms allowed the kinetic evaluation of their potency against ureases purified from *B. pasteurii* and *P. vulgaris*. Obtained results showed high activity of the novel structures, with inhibitory constants in nanomolar range for the most active compounds, which proves the correctness of the applied strategy.

Results and Discussion

Computer-Aided Design. Among the several known urease inhibitors, phosphordiamidates are the most efficient.[15,16,44] A very high activity of phosphorodiamidic acid (**1**), the simplest structure in this group, results from its close similarity to the transition state of the enzymatic reaction (Scheme 1). The main disadvantage to the widespread application of this class of inhibitors is their susceptibility to hydrolysis, particularly at low pH. To overcome such limitation, we considered the replacement of unstable P−N bonds with highly inert P−C linkages. Thus, aminomethyl(*P*-methyl)phosphinic acid (**2**) was chosen to be a lead compound, structurally analogous to phosphorodiamidic acid (**1**). It is well established that phosphinic acids act as very potent inhibitors of metal mediated enzymatic amide bond hydrolysis.[45−47] Moreover, it was shown that this class of compounds exhibit similar inhibitory properties to phosphoramides, as the computed free energy of binding is very similar.[48,49] Additionally, the positively charged aminomethyl moiety, although extended, is very similar to the transition state due to the fact that it retains the same charge.[50] On the basis of the crystal structure of **1**−*B. pasteurii* urease complex,[10] the binding mode of **2** in the enzyme active site was modeled (Figure 1).

As assumed, the binding pattern of **2** is comparable to that observed for **1**. Both oxygen atoms of **2** form hydrogen bonds with Asp$^{\alpha 363}$ and His$^{\alpha 222}$ and thus reproduce the same pattern that has been evidenced for **1**. The methyl group of **2** docks in the place of Ni(2)-bound phosphorodiamidic acid (**1**) amide group. Analogously to the distal amide moiety of **1**, the aminomethyl substituent of **2** projects toward the entrance of the active site and forms hydrogen bonds with His$^{\alpha 323}$ and Ala$^{\alpha 366}$.

Structure **2** offers highly interesting possibilities for further modifications in order to enhance significantly the affinity toward the enzyme by derivatization of its amino moiety. Taking into consideration both the kinetic characteristics of dipeptide hydroxamic acids[23] and the synthetic feasibility, a dipeptidic analogue of **2** was planned. Thus, the mode of interaction of the extended, nonsubstituted analogue, *N*-glycyl-aminomethyl(*P*-methyl)phosphinic acid (**3**) with urease active site, was modeled (Figure 2). Two novel interactions, in comparison to **2**−urease complex, occurred in the model, namely two hydrogen bonds formed by the carbonyl oxygen atom and the NH of the inhibitor

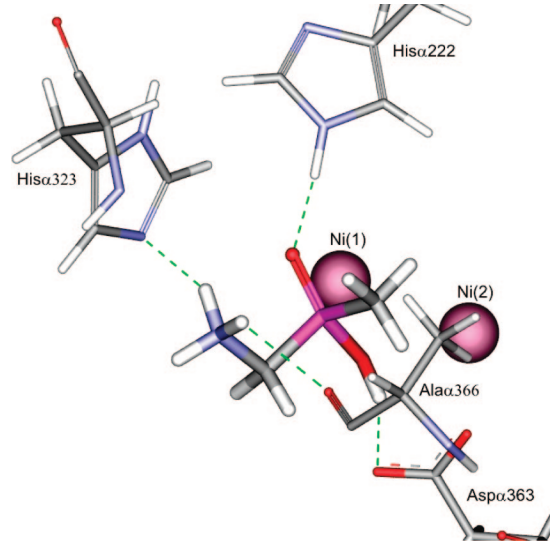


Figure 1. Computed structure of aminomethyl(*P*-methyl)phosphinic acid (**2**)−urease (*B. pasteurii*) complex. Hydrogen bonds are indicated as green dashed lines.

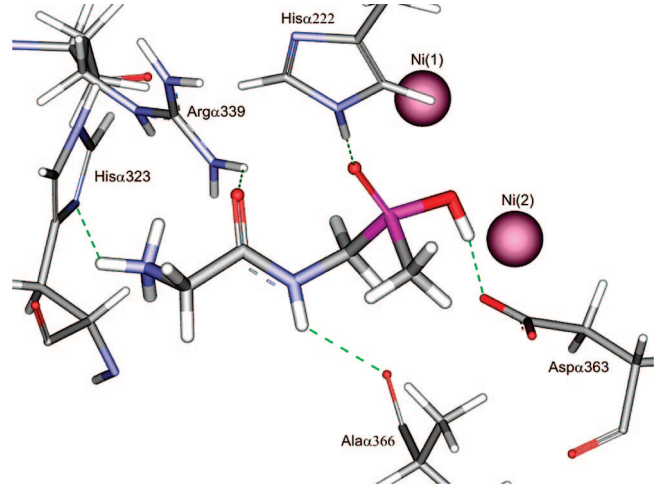
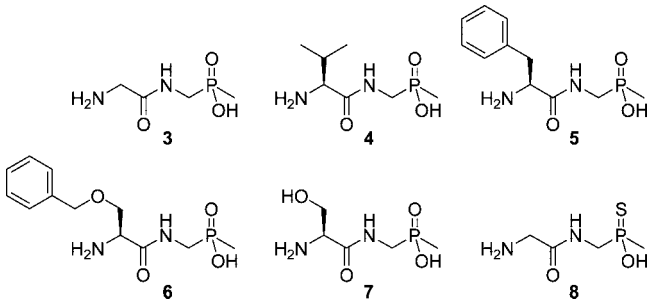


Figure 2. Modeled mode of binding of *N*-glycyl-aminomethyl(*P*-methyl)phosphinic acid (**3**) to bacterial urease active site. Hydrogen bonds are indicated as green dashed lines.

amide group with Arg$^{\alpha 339}$ and Ala$^{\alpha 366}$, respectively. These results strongly suggested that compound **3** should exert higher inhibitory effect toward bacterial urease comparing to **2**, which was further proved by calculation of LUDI scores, which are the measure of their potential enzyme affinity. The LUDI score obtained for **3** (SCORE = 462) was substantially higher than that calculated for **2** (SCORE = 338).

The next possible improvement of the inhibitor potency was the *N*-terminal amino acid residue structural extension. To enhance hydrophobic interactions with the enzyme, bulky substituents were introduced (compounds **4**−**6**). Optimization of the structure of these inhibitors' complexes with urease confirmed the correctness of proposed modifications. All hydrogen bonds found for **3**−urease complex were preserved, while hydrophobic bulky substituents docked well in the enzyme cavity. Computed LUDI scores (SCORE = 575, 593, and 504 for **4**, **5**, and **6**, respectively) also suggested higher possible efficacy of these inhibitors. Additionally, serine derivative **7** was chosen for further synthesis, as the molecular modeling showed that it could exhibit potency similar to **3**.



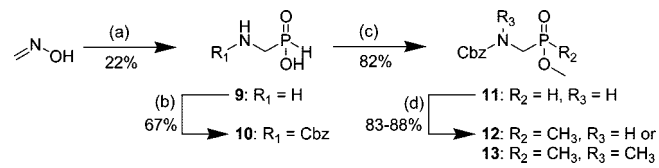
Finally, on the basis of the well-established high potency of organosulfur compounds against urease,[6,51] we decided to make an additional modification by introducing a sulfur atom into the structure of phosphinic dipeptides. Thus, the oxygen atom of phosphinic group that bridges nickel ions could be replaced by a sulfur atom, yielding thiophosphinic dipeptide (**8**) in order to obtain a similar pattern of interactions with that observed in the crystal structure of β-mercaptoethanol−urease complex.

Chemistry. Although the synthesis of 1-aminomethylphosphinic acid **9** (the analogue of glycine) has been intensively studied,[52−54] none of these methods were successful in our hands either because of multistep character and thus very low total yield of the process or because of toxic and expensive reagents required. Therefore, a recently described[55] method was attempted and proved to be successful. In this procedure, aqueous solution of NaH_2PO_2 and formaldoxime was added to hot methanolic solution of HCl, and the product was separated by ion-exchange chromatography (Scheme 2). The main byproduct of this reaction is the diaddition product, namely bis(aminomethyl)phosphinic acid, which is easily observed in ^{31}P NMR spectra (14.2 ppm for the monoadduct, 29.2 ppm for the diadduct in D_2O). Benzyloxycarbonyl (Cbz) protection of **9** proceeded smoothly, and the product **10** was obtained in pure form. NMR characterization of this compound is possible in D_2O/NaOD solution, otherwise only broad signals are observed, suggesting that aggregation or micelle formation occurs, most likely as a result of hydrogen bonding.[56] Subsequent *O*-methylation with trimethylsilyl diazomethane provided **11** in high yield. Although no other special precautions were taken, the P−H compound was immediately used after purification to avoid possibility of oxidation to the corresponding phosphonate. *P*-methylation was achieved by direct alkylation with CH_3I using NaH as a base. By using one equivalent of the base, compound **12** was obtained while two equivalents of the reagents resulted in both *P*- and *N*-methylation, providing **13** in very good yield after chromatographic purification (Scheme 2).

Further elongation of **12** was achieved by conventional carbodiimide/benzotriazole procedure. Thus, Cbz-group was removed by catalytic hydrogenolysis and the free amine was acylated using several natural amino acids in the presence of EDC·HCl and HOBt. After column chromatography, compounds **14**−**17** (Scheme 3) were obtained as mixture of two enantiomers for the first pseudodipeptide and two diastereoisomers in the three other cases. The purified compounds were finally deprotected in two subsequent synthetic steps. The methyl ester was removed by using TMSBr and then methanol. The benzyloxycarbonyl group was removed using HBr in acetic acid. Compounds **3**−**5** were purified by means of cation exchange resin column chromatography by water elution, and the purity of the final products was confirmed by HPLC using a reverse phase MZ-Analytical column, Kromasil, C18.

By careful planning of the deprotection sequence, two additional inhibitors were obtained. Indeed, by subjecting **17** to catalytic hydrogenolysis followed by treatment with TMSBr, the serine analogue **7** was obtained (Scheme 4), while reacting **17** only with TMSBr provided the benzyl protected serine analogue **6**. By a similar reaction sequence compound **13** was transformed to potential inhibitor **18**.

Scheme 2[a]



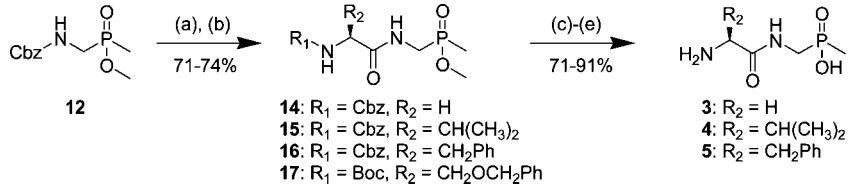
[a] Reagents and conditions: (a) NaH_2PO_2, CH_3OH, HCl, reflux, 30 min.; (b) Cbz-Cl, K_2CO_3, rt; (c) $TMSCHN_2$; (d) NaH, CH_3I, THF, −15°C to rt.

To obtain Cbz-protected inhibitors starting from **12** and **14**, these compounds were demethylated with excess of aqueous LiOH in dioxane followed by acidification with diluted HCl and column chromatography purification to provide pure **19** and **20** (Scheme 5) as judged by ^1H NMR.

After evaluating the previously synthesized phosphinic inhibitors, we turned our attention to preparation of the thio-analogues of the most potent ones. The replacement of amide bonds in physiologically active peptides with thioamide bonds is one of several backbone modifications used frequently in the search for more potent and/or selective compounds than the parent structures. Despite numerous literature examples[57] for the replacement of the oxo group of phosphorus (P═O) with the thio (P═S), to the best of our knowledge, there is no reference for thiophosphinic pseudopeptides. Very recently,[58] Acher et al. described the synthesis and agonist activity of a thiophosphonate toward group III metabotropic glutamate receptors. To synthesize the thiophosphinic analogue of **20**, Lawesson's reagent was chosen as thionating agent because phosphorus pentasulfide P_2S_5, commonly used for such transformations in the past, is associated with low and variable yields, long reaction times, and large excess of reagent are required.[59] Because Lawesson's reagent is the reagent of choice for transformations of functional groups like amides and urethanes, we thought that compound **12** would be the best substrate for the thionation because there is no amide bond present. Indeed, taking into consideration the reactivity of Lawesson's reagent toward the carbonyl group[60] (Figure 3) and the literature data for the (P═O) to (P═S) transformation,[61] we anticipated that by careful control of the temperature we could selectively thionate phosphorus atom without affecting the carbamate function.

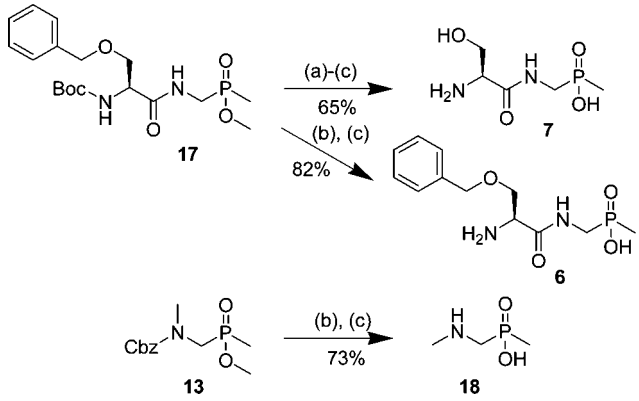
Therefore, after detailed experimentation, we found that the optimal reaction conditions were: heating at 95 °C, for 2 h in dry toluene. Under these conditions, compound **21** was obtained in 82% yield after purification (Scheme 6). It can be noted that the increase of the ^{31}P NMR chemical shift, 54.4 ppm for **12** compared to 96.1 ppm for **21**, which is very diagnostic. In the further step, Cbz group was removed using HBr/AcOH without affecting the thiophosphinic function. Catalytic hydrogenolysis was excluded because of the well-known poisoning effect of sulfur. After liberating the amine from its hydrobromic salt using DIPEA, conventional carbodiimide method provided protected thiophosphinic dipeptide **22**, using Cbz-Gly-OH as acylating agent. When we tried to apply the previously described deprotection sequence (TMSBr then HBr/AcOH), we found out that after treating **22** with TMSBr, the obtained product was **20**, where sulfur−oxygen exchange had occurred quantitatively as judged by mass spectroscopy (Scheme 7). This prompted us to search the literature for analogous results. The only similar example is a study published in 1985,[62] describing the high

Scheme 3[a]



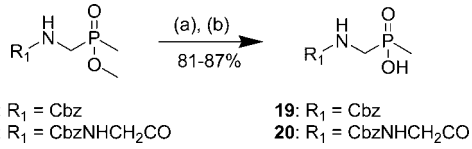
[a] Reagents and conditions: (a) H_2, Pd/C, CH_3OH, 1 atm, rt; (b) R_1-NH-CH(R_2)-COOH, EDC·HCl, HOBt, CH_2Cl_2, rt, 12 h; (c) TMSBr, CH_2Cl_2, rt, 2 h, then CH_3OH; (d) 33% HBr/AcOH, rt, 1 h; (e) Dowex AG50 × 4 (H^+).

Scheme 4[a]



[a] Reagents and conditions: (a) H_2, Pd/C, CH_3OH, 1 atm, rt; (b) TMSBr, CH_2Cl_2, rt, 2 h, then CH_2OH; (c) Dowex AG50 × 4 (H^+).

Scheme 5[a]



[a] Reagents and conditions: (a) aq LiOH, dioxane, 48 h, rt; (b) aq HCl.

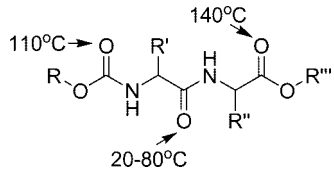


Figure 3. Reactivity of Lawesson's reagent toward the carbonyl groups of protected dipeptides.

acidity catalysis of the sulfur exchange reaction for a very simple thiophosphinate, namely *O*-methyl dimethylthiophosphinate. Furthermore, desulfurization under acidic conditions was reported very recently[58] for a thiophosphinate.

Taking this information into consideration, we decided to perform alkaline hydrolysis of the methyl ester. Indeed, by treating **22** with LiOH for 48 h, Cbz-protected thiophosphinic acid **24** (Scheme 6) was obtained after typical workup followed by column chromatography purification, which was confirmed by mass spectrometry and ^{31}P NMR comparison with **20** (48.7 ppm for **20** and 88.4 ppm for **24**). Analogously, compound **25** was obtained from **21**. For the thiophosphinic dipeptide **8**, a similar strategy was pursued, but Fmoc-Gly-OH was used as a substrate in coupling step in order to receive simultaneous cleavage of the Fmoc group and the methyl ester upon alkaline hydrolysis. Dowex purification furnished pure compound **8**.

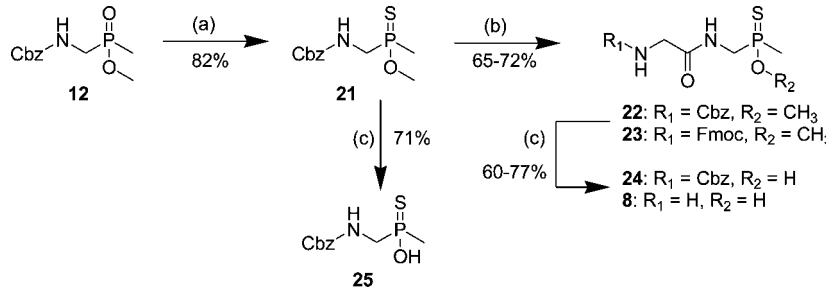
Biological Activity. The inhibitory potential of the synthesized *P*-methyl phosphinic and tiophosphinic acids was assessed against urease from *Bacillus pasteurii* (*Sporosarcina pasteurii*) CCM 2056^T and *Proteus vulgaris* CCM 1956. Affinity purification using cellufine sulfate combined with hydroxylapatite ion-exchange chromatography yielded urease preparations of 3800 nkat mg $^{-1}$ specific activity (40% recovery) and 2550 nkat mg $^{-1}$ (27% recovery) for *Bacillus* and *Proteus* strains, respectively. *B. pasteurii* enzyme was kinetically characterized by K_m of 28 mM and V_{max} of 0.063 mM s^{-1}, for *P. vulgaris* urease K_m was 12.4 mM and V_{max} 0.035 mM s^{-1}. Urease activity was evaluated using Berthelot color reaction procedure, as other methods of ammonia quantification based on pH changes (e.g., phenol red assay) lead to artificial results due to strong buffering properties of the studied inhibitors.[63] All analyzed compounds exhibited interesting inhibitory activity against bacterial ureases, however, their potency varied substantially from K_i 340 μM to 170 nM for **2** and **24**, respectively, against *B. pasteurii* enzyme (Table 1). There were no significant differences in susceptibility of the two purified microbial ureases toward presented inhibitors, which is most probably the result of very similar structure of their active sites.[8]

As expected, originally designed lead compound **2** exhibited moderate activity of the competitive, reversible mode (K_i = 340 μM, *B. pasteurii* enzyme). Further modification of this structure caused considerable affinity improvement for all designed compounds toward chosen enzymes. Noteworthy, a very small alternation of inhibitor structure by substituting an amine hydrogen with a methyl group yielded over 1 order of magnitude decrease of inhibitory constant (K_i = 18 μM for compound **18**). According to molecular modeling studies, in **2**−urease complex, one of the amine hydrogen atoms of the inhibitor is not engaged in the hydrogen bonding. Thus, the negative enthaphlic effect of desolvatation of **2** upon binding to the enzyme is eliminated by *N*-methylation.

On the basis of results obtained by Kabashi and co-workers for hydroxamic acids,[23] as well as our molecular modeling studies, dipeptidyl structures were synthesized and evaluated. Such extension of the lead compound, represented by structures **3**−**7**, proved successful, with inhibitory constants being in low micromolar range. Interestingly, the simplest structure in this group, namely compound **3**, appeared to be the most effective (K_i = 21 μM for *B. pasteurii* enzyme).

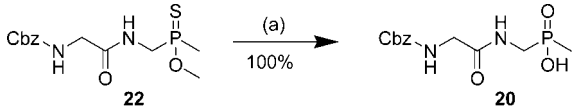
Combination of sulfur containing compounds with dipeptidyl structures leading to compound **24** caused exceptional improvement of inhibitory activity, and K_i* = 170 nM was obtained for this structure. This value is about 3 orders of magnitude better than the one of the analogous oxygen compound **20**. Moreover, it was found that kinetic characteristic of **24** was completely different, showing slow binding mode of action (Figure 4). Initial rates of the progress curves recorded in the presence of **24** were hyperbolically dependent on the inhibitor concentration, suggesting mechanism B of inhibitor binding.[64] Thus, the inhibitor most likely undergoes the conformational change after binding to the enzyme and a slow-dissociating enzyme−inhibitor complex is formed.

Scheme 6[a]



[a] Reagents and conditions: (a) Lawesson's reagent, 95°C, 2 h; (b) (i) 33% HBr/AcOH; (ii) Cbz-Gly-OH or Fmoc-Gly-OH, DIPEA, EDC·HCl, HOBt, CH_2Cl_2, rt, 12 h; (c) (i) aq LiOH, dioxane, 48 h, rt; (ii) aq HCl 0.5M.

Scheme 7[a]



[a] Reagents and conditions: (a) TMSBr, CH_2Cl_2, rt, 2 h, then CH_3OH.

A comparison of protected and nonprotected dipeptides **20** and **3** strongly suggests that structure **8** should be more effective than **24**. Although compound **8** was synthesized in pure form, it was impossible to obtain its reliable kinetic data due to its high instability in the assay mixture. Nevertheless, incomplete measurements suggest very promising inhibitory properties (data not shown). High inhibitory efficiency of sulfur analogues in comparison to their oxygen counterparts most probably results from stronger interaction of sulfur atom with nickel ions present in the urease active site. Similar phenomenon was observed in the crystal structure of urease complex with mercaptoethanol.[6]

Conclusions

This paper is the first report on the highly active bacterial urease inhibitors belonging to the organophosphinate class of compounds. The computer aided design using crystal structures of *Bacillus pasteurii* urease allowed the development of the novel inhibitors and proved very successful. Combination of structural features of urease inactivators described so far, namely phosphoramidates, hydroxamic dipeptides, and sulfur compounds, and computational and experimental verification of this approach led to structure **24** exhibiting $K_i = 170$ nM against *B. pasteurii* and 450 nM against *P. vulgaris* enzymes. Thus, it ranks among the most potent small molecular weight inhibitors of bacterial ureases. Moreover, studied compounds are characterized by the presence of a chemically inert C−P bond, assuring their stability in physiological conditions. Several well-known examples of successful applications of biologically active phosphonates and phosphinates (glyphosate, phosphinothricin, aledronate, phosphomycin) indicate high potential of this group of compounds. Moreover, proposed scaffold of urease inhibitor offers the possibility of convenient further modifications and extensions that could give rise to structures with improved inhibitory activity or/and selectivity toward enzymes of chosen origin.

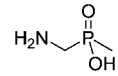
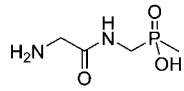
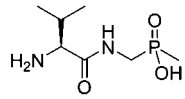
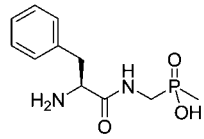
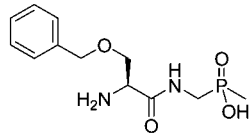
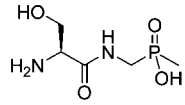
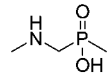
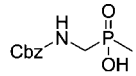
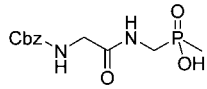
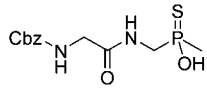
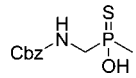
Experimental Section

General. All materials were purchased from commercial suppliers (Aldrich, Sigma, Fluka, Meck) at the highest commercial quality and used without purification unless otherwise stated. Dry tetrahydrofuran (THF) and methylene chloride were obtained by distillation of commercially available predried solvents from NaH. All of the compounds, for which analytical and spectroscopic data are quoted, were chromatographically homogeneous. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm silica gel plates (60F-254) using UV light as visualizing agent and an aqueous solution of cerium molybdate/H_2SO_4 ("Blue Stain") or 2% (w/v) ninhydrin in ethanol and heat in both cases as developing agent. Purification of compounds by column chromatography was carried out on silica gel (70−230 mesh). RPHPLC analyses were carried out on an analytical instrument using a MZ-Analytical column 250 mm × 4 mm, Kromasil 100 (C18, 5 μm), at a flow rate of 0.5 mL/min. Solvent A: 10% CH_3CN, 90% H_2O, 0.1% TFA. Solvent B: 90% CH_3CN, 10% H_2O, 0.09% TFA. The following gradients were used: (A) $t = 0$ min (0% B), $t = 20$ min (35% B), $t = 25$ min (60% B), $t = 32$ min (100% B), $t = 34$ min (100% B), $t = 38$ min (40% B); (B) $t = 0$ min (0% B), $t = 20$ min (10% B), $t = 25$ min (60% B), $t = 32$ min (100% B), $t = 34$ min (100% B), $t = 38$ min (40% B); (C) $t = 0$ min (0% B), $t = 20$ min (25% B), $t = 25$ min (60% B), $t = 32$ min (100% B), $t = 34$ min (100% B), $t = 38$ min (40% B); (D) $t = 0$ min (0% B), $t = 10$ min (25% B), $t = 45$ min (75% B), $t = 50$ min (100% B), $t = 55$ min (100% B), $t = 60$ min (40% B). Eluted peaks were detected by a UV detector at 254 or 210 nm. Reported retention times are counted in minutes. In NMR measurements, $CDCl_3$, D_2O and CD_3OD were used as solvents. ^1H, ^{31}P, and ^{13}C NMR spectra were recorded on a 200 MHz spectrometer. Proton and carbon chemical shifts are referenced to residual solvent. ^{31}P chemical shifts are reported on δ scale (in ppm) downfield from 85% H_3PO_4. The following abbreviations were used to explain the NMR multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. Before microanalysis, samples were dried under high vacuum at 25 °C for 24 h in a dry pistol. All spectroscopy and analytical data were obtained in the Laboratory of Organic Chemistry of the University of Athens.

Molecular Modeling. Crystal structure of phosphorodiamidic acid (**1**)−*Bacillus pasteurii* urease complex[10] was obtained from the Protein Data Bank[65] (refcode 3UBP) and used as starting point for all computations. Hydrogen atoms were added using Insight 2000 program from Accelrys, assuming pH 7.0. All molecular mechanics calculations were done using Discover program with cff97 force field and conjugate gradient minimizer. Minimizations were done up to energy change of 0.02 kcal/mol. To obtain designed inhibitor−enzyme complex, phosphorodiamidic acid were appropriately modified using the Builder module of Insight 2000 program. Then the structures of the obtained complexes were minimized in two steps. First, positions of all hydrogen atoms were optimized; second, positions of all atoms of enzyme active site and inhibitor were minimized. Finally, optimized structures were scored using LUDI scoring function from Insight 2000 package.[66]

Chemistry. Methyl *N*-benzyloxycarbonylaminomethyl(*P*-methyl)phosphinate (12). NaH, 60% dispersion in mineral oil (117 mg, 2.93 mmol), was added to a cooled (−10 °C) solution of **11** (670 mg, 2.93 mmol) in THF (27 mL) under argon. The reaction mixture was treated with CH_3I (0.37 mL, 6.00 mmol) and stirred at −10 °C for 1 h and at rt for 0.5 h. The reaction was treated with ethyl acetate, washed with water, dried (Na_2SO_4), and evaporated. The crude material was purified by column chromatography, eluting

Table 1. Inhibition of *Bacillus pasteurii* and *Proteus vulgaris* Ureases by Studied Compounds[a]

Compound	Structure	*Bacillus pasteurii* CCM 2056 ^{T}		*Proteus vulgaris* CCM 1956	
		IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)
2		1100±10.5	340±22	2530±42	425±16
3		60±0.6	21±5	86±5.1	30±0.8
4		485±9	120±18	617±17	208±7
5		600±7.3	215±12	754±31	176±7
6		80±0.1	25±0.9	183±11	32.6±1.2
7		150±1.4	37±3	342±23	41±2
18		60±0.3	18±0.7	153±4	27±0.3
19		123±1.3	43±3	319±8.6	65±0.5
20		450±5.4	135±6	640±26	178±15
24		1.8±0.1*	0.17±0.02*	3.1±0.3*	0.45±0.06*
25		112±0.6*	17.5±0.2*	158±1.3*	14±0.4*

[a] *Steady state kinetics studies.

with $CH_2Cl_2-CH_3OH$ (9.5:0.5), to give 624 mg of **12** as colorless oil; (83% yield). ^{1}H NMR ($CDCl_3$): δ 1.40 (d, J = 13.9 Hz, 3H), 3.49 (m. 2H), 3.63 (d, J = 10.3 Hz, 3H), 5.00 (s, 2H), 6.09 (bt, 1H), 7.29 (s, 5H). ^{13}C NMR ($CDCl_3$): δ 11.3 (d, J_{PC} = 93.3 Hz), 38.1 (d, J_{PC} = 104.9 Hz), 50.5 (d, J_{PC} = 7.1 Hz), 67.0, 128.1, 128.2, 128.5, 136.4, 157.0. ^{31}P NMR ($CDCl_3$) δ 54.4. MS (ESI^+) m/z 258.1 (M + 1).

Methyl *N*-(*N*′-Benzyloxycarbonylglycyl)aminomethyl(*P*-methyl)phosphinate (14). A mixture of 10% Pd/C (30 mg) and **12** (300 mg, 1.16 mmol) in methanol (30 mL) was stirred at room temperature under an atmosphere of hydrogen until all the starting material was consumed as observed by TLC (2.5 h) The catalyst was filtered off through celite, and the mixture was washed with methanol and water/methanol (1:9). Solvents were combined and evaporated in vacuo to give a solid (142 mg) that was dried over P_2O_5. Dichloromethane (5 mL) was then added, followed by addition of Cbz-Gly-OH (206 mg, 1 mmol), EDC·HCl (190 mg, 1 mmol), HOBt (152 mg, 1 mmol), and the reaction mixture was stirred at room temperature for 12 h. The organic solvent was then removed in vacuo, the reaction mixture was partitioned between ethyl acetate and water, the organic phase was successively washed with 5% $NaHCO_3$, 1% HCl, and water, dried over Na_2SO_4, evaporated, and the crude product was purified by column chromatography using $CH_2Cl_2-CH_3OH$ (9.5:0.5) as eluent. Compound **14** was obtained in 72% yield (238 mg). ^{1}H NMR ($CDCl_3$): δ 1.46 (d, J = 13.9 Hz, 3H), 3.48−3.70 (m, 4H), 3.69 (d, J = 10.3 Hz, 3H), 5.11 (s, 2H), 5.65 (bs, 1H), 7.10 (bs, 1H), 7.33 (s, 5H). ^{13}C NMR ($CDCl_3$): δ 12.5 (d, J_{PC} = 93.5 Hz), 38.0 (d, J_{PC} = 93.5 Hz), 44.7, 51.9 (d, J_{PC} = 8.0 Hz), 67.4, 128.1, 128.2, 128.5, 137.4, 156.9, 170.0. ^{31}P NMR ($CDCl_3$): δ 53.1. MS (ESI^+) m/z 415.1 (M + 1).

***N*-Glycylaminomethyl(*P*-methyl)phosphinic acid (3).** A solution of phosphinate **14** (31.4 mg, 0.10 mmol) and TMSBr (23 mg, 0.15 mmol) in dry CH_2Cl_2 (1 mL) was stirred for 2 h at room temperature. Then the solvent was evaporated and the residue was treated with ethyl acetate (2 mL) and CH_3OH (2 mL). The solution was stirred for 30 min at room temperature and evaporated in vacuo. The crude product was triturated with dry diethyl ether to furnish

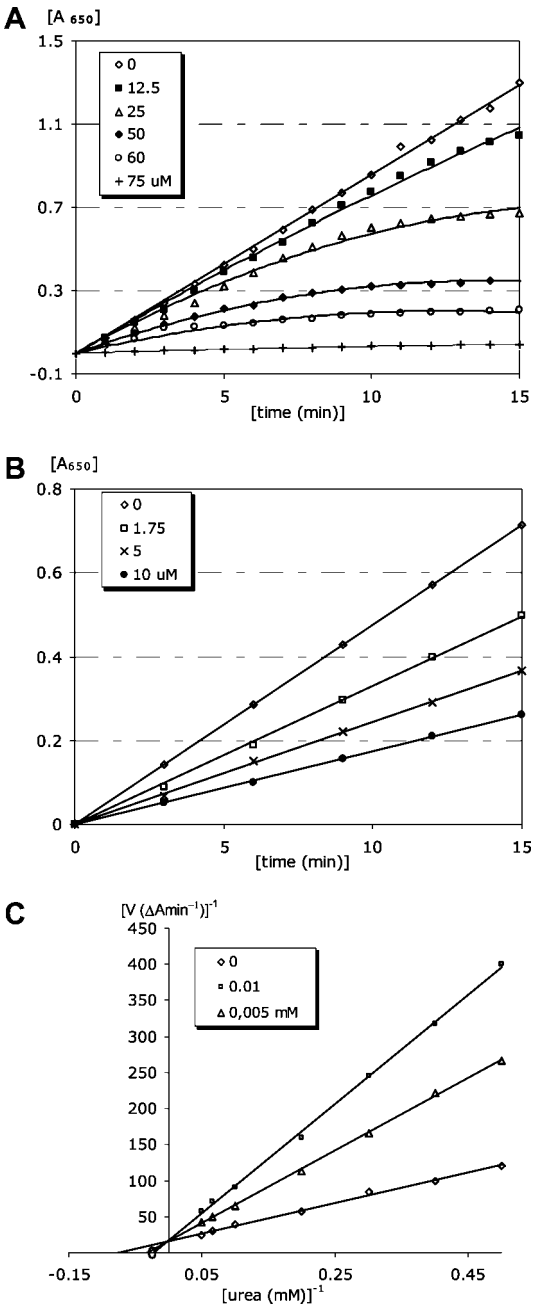


Figure 4. Time-dependent inhibition of *B. pasteurii* urease as a function of **24** concentration in nonincubated (A) and incubated **24**−urease systems (B). Steady-state kinetic analysis of the mode of **24** inhibition (C).

the free hydroxyphosphinyl product as solid. To this solid, 33% HBr/AcOH (1 mL) was added; the reaction mixture was stirred at room temperature for 1 h. Dry diethyl ether (7 mL) was then added to precipitate an orange solid. The supernatant was decanted, the procedure repeated twice, and the remained solid was purified using a Dowex AG50 × 4 cation exchange resin and water as eluent. The fractions that gave positive color reaction with ninhydrin were combined and evaporated under vacuum to give **3** (15 mg, 91% yield). ^{1}H NMR (D_2O): δ 1.20 (d, J = 13.9 Hz, 3H), 3.38 (d, J = 8.8 Hz, 2H), 3.71 (s, 2H). ^{13}C NMR (D_2O): δ 12.8 (d, J_{PC} = 91.7 Hz), 38.9 (d, J_{PC} = 104.2 Hz), 40.6, 167.0. ^{31}P NMR (D_2O): δ 42.2. MS (ESI^+) m/z 166.9 (M + 1). Analytical HPLC method B: t_R = 12.4 min. HPLC purity > 97%. Anal. ($C_4H_{11}N_2O_3P\cdot 0.5H_2O$) C, H, N.

***N*-Benzyloxycarbonylaminomethyl(*P*-methyl)phosphinic acid (19).** rotected phosphinate **12** (51 mg, 0.20 mmol) was dissolved in dioxane (0.25 mL) and water (0.20 mL). To this solution, aqueous 2 M LiOH (0.25 mL) was added and the mixture was vigorously stirred at room temperature for 48 h. Water was added, the aqueous solution was washed with ethyl acetate and acidified to pH 1 with 0.5 M HCl, and the aqueous phase was extracted with ethyl acetate twice. The organic extracts were combined, dried over Na_2SO_4, and evaporated in vacuo to give the free hydroxyphosphinyl compound. The crude product was purified by column chromatography using CH_2Cl_2/CH_3OH/AcOH (9:1:0.5) as eluent to give **19** (42 mg, 87% yield). ^{1}H NMR ($CDCl_3$): δ 1.43 (d, J = 13.9 Hz, 3H), 3.52 (bs, 2H), 5.40 (bs, 1H), 7.31 (s, 5H). ^{13}C NMR ($CDCl_3$): δ 13.1, (d, J_{PC} = 97.2 Hz), 38.9 (d, J_{PC} = 101.2 Hz), 67.6, 127.4, 128.4, 128.5, 128.8, 136.2, 156.3. ^{31}P NMR ($CDCl_3$): δ 52.0. MS (ESI^+) m/z 244 (M + 1). t_R = 16.3 min. HPLC purity > 98%, gradient D. Anal. ($C_{10}H_{14}NO_4P$) C, H, N.

***O*-Methyl *N*-Benzyloxycarbonylaminomethyl(*P*-methyl)phosphinothioate (21).** A mixture of **12** (43 mg, 0.17 mmol) and Lawesson's reagent (68 mg, 0.17 mmol) was heated at 95 °C in sodium dried toluene (1 mL) for 2 h. Evaporation in vacuo gave a pale-yellow oil, which was chromatographed (5% EtOAc-CH_2Cl_2) to give 38 mg of **21** as viscous oil (82%). ^{1}H NMR ($CDCl_3$): δ 1.77 (d, J = 13.9 Hz, 3H), 3.50 (m, 2H), 3.65 (d, J = 10.4 Hz, 3H), 3.73 (s, 2H), 5.12 (s, 2H), 5.22 (bs, 1H), 7.35 (s, 5H). ^{13}C NMR ($CDCl_3$): δ 19.7 (d, J_{PC} = 72.3 Hz), 45.0 (d, J_{PC} = 84.7 Hz), 51.8 (d, J_{PC} = 6.8 Hz), 67.6, 128.4, 128.6, 128.8, 136.2, 156.3. ^{31}P NMR ($CDCl_3$): δ 96.1. MS (ESI^+) m/z 274.2 (M + 1).

***O*-Methyl *N*-(*N*-Benzyloxycarbonylglycyl)aminomethyl(*P*-methyl)phosphinothioate (22).** Cbz-protected compound **21** (270 mg, 1 mmol) was stirred for 1 h at 20 °C in 33% HBr/AcOH (3 mL). Dry diethyl ether (20 mL) was then added to precipitate a white solid. The supernatant liquid was decanted, the procedure repeated twice, and the remained solid was dried over P_2O_5. DIPEA (170 mL, 1 mmol) was added to a suspension of the resulting hydrobromide in CH_2Cl_2 (5 mL), followed by addition of Cbz-Gly-OH (206 mg, 1 mmol), EDC·HCl (190 mg, 1 mmol), and HOBt (152 mg, 1 mmol). The reaction mixture was stirred at room temperature for 12 h. Then organic solvent was removed in vacuo, the reaction mixture was partitioned between ethyl acetate and water, the organic phase was successively washed with 5% $NaHCO_3$, 1% HCl, and water, dried over Na_2SO_4, evaporated, and the crude product was purified by column chromatography using CH_2Cl_2−CH_3OH (9.5:0.5) as eluent. Compound **22** was obtained in 72% yield (238 mg). ^{1}H NMR ($CDCl_3$): δ 1.73 (d, J = 13.9 Hz, 3H), 3.65−3.87 (m, 4H), 3.60 (d, J = 10.3 Hz, 3H), 5.10 (s, 2H), 5.73 (bs, 1H), 6.90 (bs, 1H), 7.33 (s, 5H). ^{13}C NMR ($CDCl_3$): δ 20.0 (d, J_{PC} = 73.2 Hz), 42.8 (d, J_{PC} = 81.3 Hz), 44.9, 51.9 (d, J_{PC} = 6.9 Hz), 67.7, 128.4, 128.6, 128.8, 136.1, 156.8, 169.2. ^{31}P NMR ($CDCl_3$): δ 96.4. MS (ESI^+) m/z 331.2 (M + 1).

***N*-(*N'*-Benzyloxycarbonylglycyl)aminomethyl(*P*-methyl)phosphinothioic Acid (24).** Following the same procedure described above for **19**, compound **24** was obtained (77% yield). ^{1}H NMR (CD_3OD): δ 1.49 (d, J = 13.9 Hz, 3H), 3.40−3.69 (m, 4H), 5.10 (s, 2H), 7.33 (s, 5H). ^{13}C NMR (CD_3OD): δ 12.4 (d, J_{PC} = 92.9 Hz), 38.9 (d, J_{PC} = 105.1 Hz), 43.7, 66.7, 127.7, 127.8, 128.3, 137.8, 156.1, 171.1. ^{31}P NMR (CD_3OD): δ 88.4. MS (ESI^+) m/z 316.8 (M + 1). t_R = 30.7 min. HPLC purity > 98% gradient D. Anal. ($C_{12}H_{17}N_2O_4PS$) C, H, N.

Urease Purification. *Bacillus pasteurii* CCM 2056^T and *Proteus vulgaris* CCM 1956 were each cultured aerobically for 48 h at 37 °C in 1 L of medium, pH 9.0, containing (per liter) 20 g of yeast extract, 20 g of urea, and 1.0 mM $NiCl_2$.[67] Cell pellet was collected by centrifugation at 12000*g* for 15 min at 4 °C, washed twice with 20 mM sodium phosphate, pH 6.5, and disrupted ultrasonically in 20 mM phosphate/1 mM EDTA buffer pH 6.5. Insoluble cell debris was removed by centrifugation at 15000*g* for 30 min, and 5 mL of supernatant was applied to cellufine sulfate step A and B columns (14 mm × 150 mm) using urease affinity purification protocol described by Icatlo et al.[68] Fractions of 1.5 mL were collected and assayed for protein content by the method of Bradford[69] and urease activity by the phenol−hypochlorite colorimetric method.[63] Step B column void volume containing partially purified urease was combined with urease fractionated with 20 mM phosphate pH 7.4/

0.15 M NaCl and applied to hydroxyapatite column (14 mm × 100 mm) equilibrated with 20 mM phosphate buffer pH 7.4 and eluted with linear gradient from 20 to 100 mM phosphate (50 mL), collecting 1.5 mL fractions. Urease-containing fractions eluted at around 50 mM phosphate were pooled and stored at 4 °C.

Enzyme Assay. Bacterial urease was assayed at 30 °C in 3 mM phosphate buffer pH 7.0. Separate reactions (200 μL total volume) were terminated by introduction of 0.5 mL phenol−sodium nitroprusside solution immediately followed by addition of 0.5 mL NaOH−sodium hypochlorite mixture.[63] The amount of ammonia liberated was assayed by measuring indophenol blue formed in Berthelot reaction over 15 min incubation at 30 °C following the changes at 650 nm. Compounds studied as inhibitors did not affect indophenol readings when introduced to standard curve assays using ammonium sulfate.

Kinetic parameters K_m and V_{max} of noninhibited urease were determined by measuring initial rates of reactions carried out in the standard assay mixtures containing 2−100 mM urea.

Inhibition Studies. Progress curves were obtained by initiation of urease reaction with addition of 50 pkat enzyme into assay mixtures containing increasing concentrations of inhibitors and urea, which are in the range of 2 to 100 mM. Each assay was run in triplicate. K_i values were dertermined from Lineweaver−Burk plots after testing at least 5 inhibitor concentrations. IC_{50} values were obtained from measurements performed in the presence of saturating urea concentration 100 mM.

Steady-state kinetic measurements were performed in two ways. The enzyme was preicubated with assayed inhibitor concentration in separate aliquots of reaction buffer prior to reaction initiation by incorporation of urea. Amounts of enzyme and inhibitor in each reaction mixture were analogous to the corresponding progress curve experiment. In fast dilution assays, reactions were started by introducing portions of previously preincubated 30 times concentrated enzyme−inhibitor mixture into assay buffer samples containing urea. In each case, the enzyme was allowed to interact with inhibitor for 30 min before contacting substrate. Linear regression analysis was used to calculate steady-state inhibition constants K_i* using equation:[64]

$$v_s = \frac{v_{max} S_0}{K_M\left(1 + \frac{I}{K^*}\right) + S_0} \quad (1)$$

Concentration of inhibitor causing 50% enzyme activity loss (IC_{50}) was calculated from linear regression of urease activity versus the logarithm of inhibitor concentration.

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Supporting Information Available: elemental analysis and HPLC purity for all target compounds; details of synthesis and spectral data for compounds **4**−**8**, **10**, **11**, **13**, **15**−**18**, **20**, **23**, **25**. This material is available free of charge via the Internet at http://pubs.acs.org.

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