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Computer-Aided Optimization of Phosphinic Inhibitors of Bacterial Ureases

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Urease inhibitors can be considered as a tool to control the damaging effect of ureolytic bacteria infections in humans which occur commonly in the developed countries. Computer-aided optimization of the aminomethylphosphinate structures by modifying both their N- and P-termini led to the invention of a novel group of inhibitors of bacterial ureases. Introduction of *P*-hydroxymethyl group into the molecule resulted in considerable increase of the inhibitory activity against enzymes purified from *Bacillus pasteurii* and *Proteus vulgaris* as compared with their *P*-methyl counterparts described previously. The designed compounds represent a competitive reversible class of urease inhibitors. The most potent, *N*-methyl-aminomethyl-*P*-hydroxymethylphosphinic acid, displayed K_i = 360 nM against *P. vulgaris* enzyme.

Introduction

The enzymatic hydrolysis of urea is a process involved in the development of various pathological conditions in humans and animals particularly associated with the urinary tract and the intestinal system.[1−3] Bacterial urease [EC 3.5.1.5] is a nickel-dependent amidohydrolase which catalyzes urea decomposition into ammonia and carbon dioxide with carbamate as the intermediate.[4,5] The infection with ureolytic bacteria causes a local rise in pH in areas physiologically neutral or acidic and results in numerous damaging consequences depending on the colonization site. In the kidney, the most severe effect is acute pyelonephritis.[6] Stone formation promoted by imbalanced urine pH is a more common condition leading to chronic inflammatory disorders in the urinary tract.[7,8] Precipitates build up, and insoluble material encrustation is a common hospital complication in patients requiring catheter treatment.[9−11] Bacterial strains infecting the urinary tract most commonly represent *Ureaplasma urealyticum* and *Proteus mirabilis* species, *Staphylococcus saprophyticus*, *Pseudomonas aeruginosa*, or *Klebsiella pneumoniae* may also be encountered.[12] Gastric disorders including gastritis, ulceration, and the most severe gastric carcinoma and primary gastric lymphoma are etiologically connected with *Helicobacter* species infections.[13,14] The ureolytic activity allows these pathogens to overcome acidic pH (part of the natural defense system at the stomach mucosal surfaces) and enables them to colonize the mucus layer due to acid resistance.[15,16] As the control of urease activity with effective inhibitors is a very promising tool for therapy of the diseases caused by urea hydrolyzing microorganisms, compounds able to interact with urease have been intensively studied.[17−21] Among many active structures described (e.g., urea analogues,[22,23] thiols,[24,25] quinones,[26,27] and heavy metal ions[28,29]), inhibitors derived from hydroxamic acid and phosphoramide are the best characterized.[4,30−39] Moreover, the large set of available bacterial urease structures (from *Klebsiella aerogenes*, *Bacillus pasteurii*, and *Helicobacter pylori*) has given the possibility to elucidate the enzymatic reaction mechanism as well as the mode of binding of chosen inhibitors.[25,40−47] In particular, the high efficiency of phosphoramidates is well justified by the crystal structures of *B. pasteurii* urease complexed with phosphordiamidate (DAP[a]).[44] The analysis of the obtained structures revealed that DAP is a classical transition state analogue interacting with both nickel ions present in the active site as well as forming several hydrogen bonds with the enzyme residues. An unfavorable feature of this class of compounds is their relatively low stability in water. Recently, using structure-based inhibitor design approach, we have elaborated the structure of aminophosphinates as potential inhibitors of bacterial ureases, with *P*-methylphosphinic acids being the most effective.[48] Among the studied phosphinates, *N*-methyl-aminomethyl-*P*-methylphosphinic acid and *N*-glycyl-aminomethyl-*P*-methylphosphinic acid exhibited the highest in vitro activity against *Proteus vulgaris* enzyme (K_i = 27 μM and K_i = 30 μM, respectively). Although the sulfur analogues of phosphinates designed by us, phosphinotioates, were significantly more active than their oxygen counterparts with the best inhibitory constant K_i = 0.45 μM against *P. vulgaris* enzyme, they were unfortunately characterized by very low stability in aqueous environment. Hydrolytic instability of phosphinotioates together with the release of toxic hydrogen sulfide has been already described for some model compounds.[49] The moderate activity of the previously described aminophosphinates, and

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[a]Abbreviations: $(Ac)_2O$, acetic anhydride; AcOH, acetic acid; Boc, *tert*-butoxycarbonyl; *t*-Bu, *tert*-butyl; Cbz, benzyloxycarbonyl; CH_2Cl_2, dichloromethane; DAP, phosphordiamidate; DMAP, 4-di(methylamino)-pyridine; EDC*HCl, *N*-[3-(dimethylamino)propyl]-*N*′-ethylcarbodiimide hydrochloride; EDTA, ethylenediaminetetraacetic acid; Et_2O, diethyl ether; EtOAc, ethyl acetate; EtOH, ethanol; HOBt, 1-hydroxybenzotriazole; MeOH, methanol; PhMe, toluene; TFA, trifluoroacetic acid.

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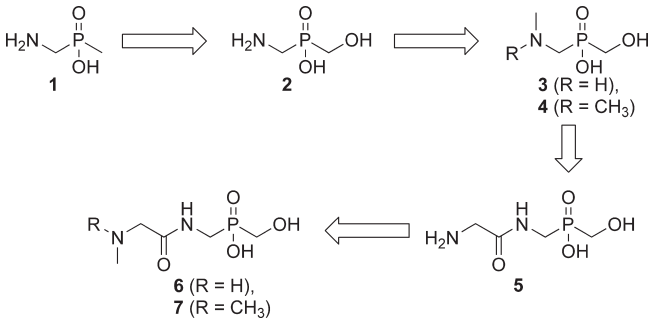
Scheme 1



the susceptibility to hydrolysis of their potent sulfur analogues inspired us to undertake further optimization of their structure. The present work describes the exploration of aminophosphinic acids as urease inhibitors using the computer-aided design relaying on the modification of both P- and N-termini. This approach led to the invention of several highly active and hydrolytically stable compounds.

Results and Discussion

Computer-Aided Design. Recently published results indicate that aminophosphinates represent a promising class of lead structures with particularly high potential for further optimization.[48] Therefore, we aimed at obtaining compounds that could offer therapeutic utility by improving activity and stability. On the basis of the lead structure of aminomethyl-*P*-methylphosphinate (**1**), a completely novel path of modification was proposed. For that purpose, the LUDI module of Insight 2000 program package was used, which allows to design structures de novo or to modify known inhibitors.[50,51] The previously described modeled complex of inhibitor **1** and urease was the starting point for these calculations. The modification of specified aminomethyl-*P*-methylphosphinate (**1**), relaying on the introduction of an OH group thus leading to aminomethyl-*P*-hydroxymethylphosphinate (**2**), was found favorable by the applied program (Scheme 1). The additional hydroxyl group was expected to form a hydrogen bond with Ala170 and increase the inhibitory activity in comparison to compound **1**.

Subsequently, **2**−urease complex structure was optimized (Figure 1a). It was found that compound **2** is bound in a slightly different way than its methyl analogue **1**. Each phosphinyl oxygen atom interacts with an appropriate nickel ion, while the hydroxyl moiety forms two hydrogen bonds with His222 and Ala170 residues. A bifurcated intramolecular hydrogen bond between OH and NH_3^+ was also detected. Two hydrogen atoms of the inhibitor amine group are involved in the formation of hydrogen bonds with His323 and Cys322. One amine hydrogen atom is not a donor of a hydrogen bond to any protein residue. This causes energy loss for breakage of one hydrogen bond during desolvatation, which is not restored upon the complex formation. Thus *N*-methyl substituted analogue of **2**, compound **3**, was designed to overcome this disadvantage. The modeled structure of **3**−urease complex (Figure 1b) demonstrates that every inhibitor−enzyme interaction found in **2**−urease complex is preserved. To test the predicted formation of hydrogen bonds network, the dimethyl analogue **4** was also designed.

Subsequently, some dipeptide inhibitors were also designed (Scheme 1). The modeled structure of urease complex with *N*-glycyl derivative of parent compound **2** (inhibitor **5**)

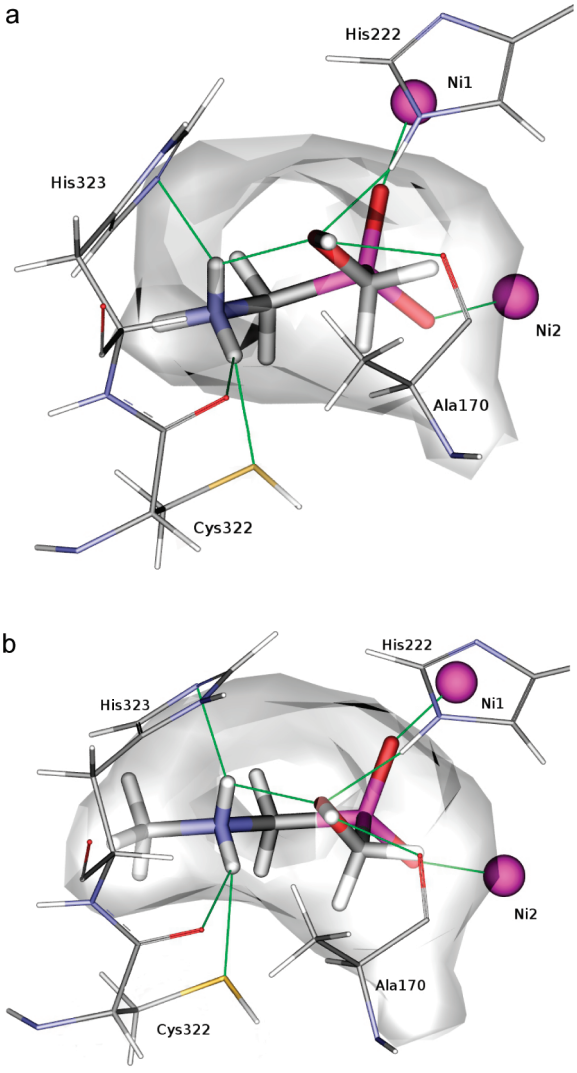


Figure 1. Modeled structures of complexes of designed inhibitors **2** (a) and **3** (b) with urease from *B. pasteurii*. Hydrogen bonds and interactions with nickel ions are presented as green solid lines.

is shown in Figure 2. The hydroxymethylphosphinyl portion of the inhibitor interacts with the enzyme in a similar manner as it was suggested for unsubstituted inhibitor **2**. Slightly changed geometry of the hydroxymethyl substituent is attributed to the lack of an intramolecular hydrogen bond between this fragment and N-terminus. The amide function of the inhibitor creates two hydrogen bonds with Cys322 and Arg339, while the terminal amine group interacts with His323 and Leu319. In this case also one amine hydrogen atom is not involved in the formation of hydrogen bond, thus *N*-methyl and *N*,*N*-dimethyl analogues (compounds **6** and **7**, respectively) were designed to test the energetically most favorable hydrogen bonds network.

Chemistry. Because the formation of phosphorus−carbon bonds via addition of phosphinic acid derivatives to aldehydes is well acknowledged,[52−56] this strategy was applied for the synthesis of the central building block **9** which contains a hydroxymethyl group. Indeed, by condensation of the Cbz-protected methyl phosphinate analogue of glycine **8**[48] with paraformaldehyde in the presence of triethylamine, the hydroxymethylphosphinate **9** was obtained in moderate yield (Scheme 2). Extending the reaction time or rising the temperature above the optimum (as described in the Experimental

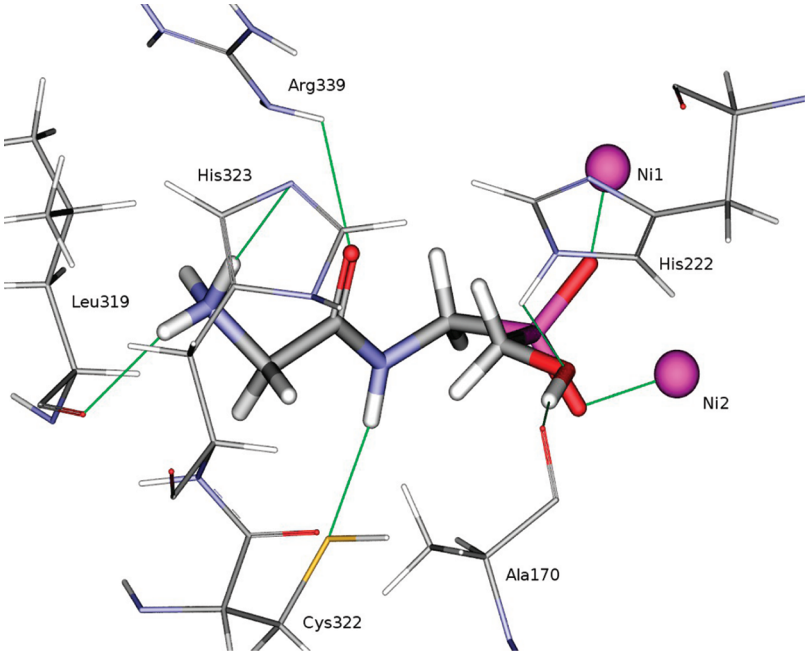
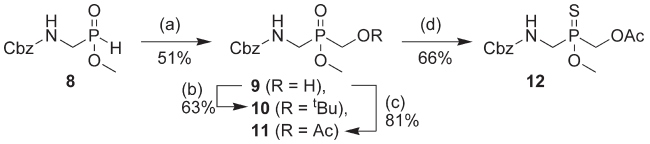


Figure 2. Modeled structure of complex of inhibitor **5** with *B. pasteurii* urease. Hydrogen bonds and interactions with nickel ions are presented as green solid lines.

Scheme 2[a]

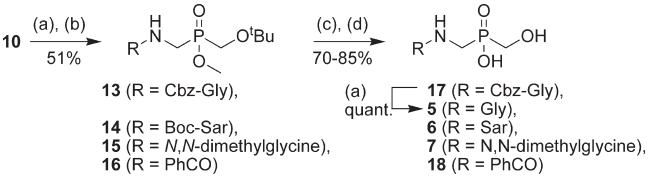


[a] Reagents and conditions: (a) $(CH_2O)_n$, PhMe, Et_3N, 75 °C, 45 min; (b) $CH_2{=}C(CH_3)_2$, CH_2Cl_2, conc H_2SO_4, rt, 24 h; (c) $(Ac)_2O$, pyridine, rt, 12 h; (d) Lawesson's reagent, PhMe, 90 °C, 90 min.

Section), yielded an unidentifiable mixture, composed mainly of oxidized phosphinic residues, as judged by the presence of ^{31}P NMR peaks in the area between 5 and 15 ppm. An alternative route is the broadly described 1,2-addition of phosphinates to aldehydes in the presence of trimethylsilyl chloride and triethylamine.[57] In our case, however, this method provided only tiny amounts of the desired compound **9**. The protection of the hydroxyl group both with an acid-sensitive (*tert*-butyl) and a base-sensitive (acetyl) group furnished the fully protected derivatives **10** and **11**, respectively. On the basis of our previous results, we also planned to synthesize some thiophosphinic analogues.[48] By carefully maintaining the temperature exactly at 95 °C in order to avoid introduction of sulfur into the carbamate or the ester functionalities, the reaction of **11** with Lawesson's reagent resulted in thiophosphinic compound **12**, which was purified by column chromatography (Scheme 2).

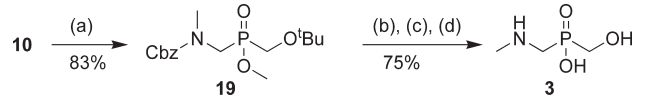
Further elongation of **10** was achieved by conventional carbodiimide/benzotriazole procedure. Thus, Cbz-group was removed by catalytic hydrogenolysis, and the free amine was acylated using various agents like natural and unnatural amino acids (Cbz-glycine for **13**, Boc-sarcosine for **14**, *N*,*N*-dimethyl glycine[58] for **15**) and benzoic acid (to obtain compound **16**). Hydrolysis of the methyl phosphinate with aqueous LiOH esters proceeded smoothly. Subsequent removal of the acid-sensitive protecting groups (Boc, ^{t}Bu) provided compounds **6**, **7**, **17**, and **18**, which were purified by reverse

Scheme 3[a]



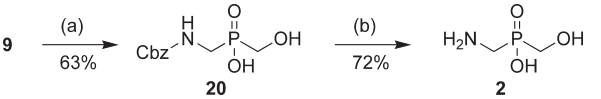
[a] Reagents and conditions: (a) H_2, Pd/C, CH_3OH, 1 atm, rt, 2.5 h; (b) ROH, EDC*HCl, HOBt, CH_2Cl_2, rt, 12 h; (c) (i) aq LiOH, dioxane, 48 h, rt; (ii) aq HCl; (d) 95% TFA, 14 h.

Scheme 4[a]



[a] Reagents and conditions: (a) NaH, CH_3I, THF, −10 °C to rt, 30 min; (b) H_2, Pd/C, CH_3OH, 1 atm, rt, 2.5 h; (c) (i) aq LiOH, dioxane, 48 h, rt; (ii) aq HCl; (d) (i) 95% TFA, 14 h; (ii) Dowex AG50 × 4 (H^+).

Scheme 5[a]



[a] Reagents and conditions: (a) (i) aq LiOH, dioxane, 48 h, rt; (ii) aq HCl; (b) (i) H_2, Pd/C, CH_3OH, 1 atm, rt, 2.5 h; (ii) Dowex AG50 × 4 (H^+).

phase silica gel chromatography. Compound **5** was derived from **17** after catalytic hydrogenation and Dowex purification (Scheme 3). *N*-Methylation of **10** followed by total deprotection furnished **3** (Scheme 4). Similarly, total deprotection of **9** provided compound **2** and, by removal of the methyl group alone, compound **20** (Scheme 5).

Simultaneous demethylation and deacetylation of **12** using aqueous LiOH provided **21**, the thiophosphinic analogue of **20** (Scheme 6).

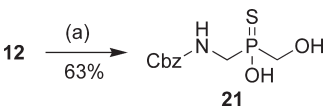
A relatively different approach was undertaken in order to prepare **4**. Initial attempts to transform **19** (after removal of Cbz-group) to its *N*,*N*-dimethyl analogue by alkylation with NaH/CH_3I were unsuccessful due to both incomplete reaction and the formation of a quaternized amine as a side-product, leading to a mixture difficult to separate. Reductive dimethylation of **10** using 10% Pd/C, H_2, and aqueous formaldehyde did not provide the desired dimethyl analogue either. At that point, the Kabachnik−Fields reaction, a classical three-component reaction applied to the synthesis of organophosphorus compounds, appeared to be quite promising.[59] Therefore we designed a three-component condensation of hydroxymethylphosphinic acid **22**, dimethyl amine, and formaldehyde as a mean to obtain **4**. Hydroxymethylphosphinic acid was prepared by reacting hypophosphorus acid, formaldehyde, and HCl in EtOH. This reaction provided the desired compound **22** but also the diaddition product bis(hydroxymethyl)-phosphinic acid **23** and unreacted H_3PO_2. Optimization of the reaction conditions by modifying the ratio of reagents and the reaction time, and monitoring its course with ^{31}P NMR, led to a mixture composed mainly of the monoadduct **22** (86% yield as judged by ^{31}P NMR) with only starting H_3PO_2 as a side product. After removal of volatile materials, the crude reaction mixture was used in the next step. Indeed, heating this crude reaction mixture, having added *p*-formaldehyde and dimethylamine using acidic activation, resulted in the production of **4** in 52% yield from H_3PO_2 after ion exchange purification. The reaction progress and the purification procedure were monitored by the pale positive ninhydrine color of the product and by the appearance of corresponding ^{31}P NMR signals (Scheme 7).

Biological Activity. Activity of the designed compounds was evaluated against enzymes purified from *P. vulgaris* CCM1956 and *B. pasteurii* CCM2056^T. All tested compounds exhibited inhibitory effect against both bacterial ureases. Progress curves of urease reaction and kinetic analysis of substrate dependence allowed classifying the designed molecules as competitive reversible inhibitors. Inhibition parameters (IC_{50} and K_i) for the compound of the most simple structure in the presented group, aminomethyl-*P*-hydroxymethylphosphinic acid (**2**), were significantly reduced in comparison to previously reported *P*-methyl lead compound **1** (K_i = 27.5 μM versus K_i = 425 μM for *P. vulgaris* enzyme). Results of the in vitro assays confirmed that the introduction of a hydroxyl group into lead molecule resulted in an increase of the inhibitor affinity to the enzyme. Then, the consequences of introduction of N-substitution (compounds **3**−**4**) were studied, taking into consideration our previously mentioned computational work. Interestingly, single substitution with methyl group leading to compound **3** resulted in an increase of activity by 2 orders of magnitude (K_i = 0.36 μM for *P. vulgaris* enzyme). This result is in good accordance with molecular modeling, which suggested such modification as favorable (Figure 1b). The incorporation of the next methyl group into the inhibitor structure, to yield compound **4**, resulted in 10-fold lowering of the inhibitory activity to K_i = 3.1 μM for *P. vulgaris* enzyme. This is the consequence of lack of one inhibitor−enzyme hydrogen bond in comparison to structure **3**, which is blocked by this additional methyl group. Thus the pattern of hydrogen bond network predicted by molecular modeling is in good agreement with obtained experimental affinities. Interestingly, inhibitors **18** and **20**, also possessing one NH hydrogen bond donor, exhibited inhibitory constants in medium micromolar range against *P. vulgaris* enzyme (K_i = 41.9 μM and 9.2 μM, respectively). The thioanalogue of compound **20**, structure **21**, showed relatively poor activity, with K_i equal to 143 μM (*P. vulgaris* enzyme). Moreover, in contrast to the *P*-methyl-thiophosphinates described previously, which were slow-binding inhibitors, it showed reversible, competitive inhibition. This could be attributed to the slightly different mode of binding of phosphinate group to nickel ions in case of *P*-methyl compounds in comparison to *P*-hydroxymethyl ones. For the *P*-methyl group, one phosphinyl oxygen/sulfur atom was bridged between two nickel ions, while for the *P*-hydroxymethyl set, each phosphinyl oxygen/sulfur atom interacts with a single nickel ion.

Dipeptides **5**−**7** and **17** also exhibited satisfactory activity with K_i values in the range of 0.43−16.2 μM. As expected, activity of compound **5** (K_i = 7.3 μM for *P. vulgaris* enzyme) compared with its *P*-methyl analogue (K_i = 30 μM) was considerably improved. Interestingly, the trend of inhibitory activity upon subsequent *N*-methylation is different from that observed for series of compounds **2**−**4**. Nonsubstituted and *N*-methylated inhibitors (**5** and **6**) exhibited similar potency (K_i = 7.3 μM and K_i = 16.2 μM, respectively), while the *N*,*N*-dimethylated one (**7**) showed considerably improved affinity to toward the enzyme (K_i = 0.43 μM against *P. vulgaris* enzyme). Although the modeled structure of **5**−urease complex (Figure 2) disclosed that one terminal amine hydrogen atom is not a hydrogen bond donor (two other hydrogen atoms are bound by His323 and Leu319), *N*-methylation did not improve enzyme affinity. It could be explained by molecular modeling of **6**−urease complex, where one hydrogen bond with Leu319 carbonyl function is broken and again one amine hydrogen atom does not interact with the enzyme. Finally, the *N*,*N*-dimethylated analogue **7** creates the appropriate hydrogen bond network. The amine hydrogen atom as well as other hydrogen bond donors of this inhibitor interact with the enzyme residues, thus the inhibitory activity is increased.

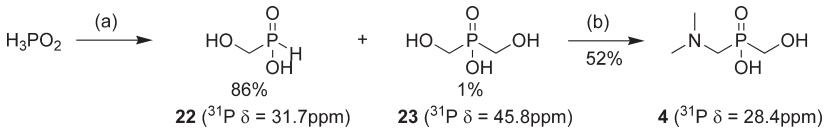
B. pasteurii urease was generally less susceptible, although still significantly inhibited, by the studied compounds and exhibited

Scheme 6[a]



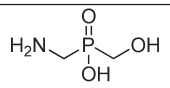
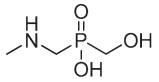
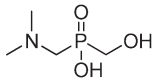
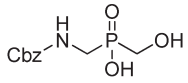
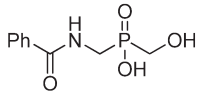
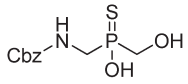
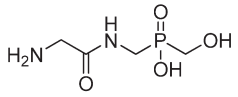
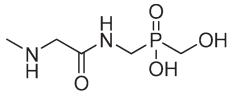
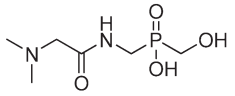
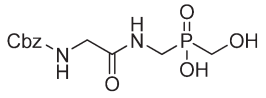
[a] Reagents and conditions: (a) (i) aq LiOH, dioxane, 48 h, rt; (ii) aq HCl.

Scheme 7[a]



[a] Reagents and conditions: (a) $(CH_2O)_n$, aq HCl, EtOH, 100 °C, 48 h; (b) (i) 40% aq Me_2NH, aq HCl, $(CH_2O)_n$, 90 °C, 5 h; (ii) Dowex AG50 × 4 (H^+).

Table 1. Inhibitory Activities (K_i and IC_{50} Values) of *P*-Hydroxymethyl Phosphinates against *B. pasteurii* and *P. vulgaris* Ureases

		P. vulgaris urease		*B. pasteurii* urease	
no	Compound	IC_{50} [μM]	K_i [μM]	IC_{50} [μM]	K_i [μM]
2		47.7 ±8.3	27.5 ±8.6	423 ±33	95 ±10
3		0.96 ±0.23	0.36 ±0.10	1.59 ±0.65	0.43 ±0.03
4		17.2 ±3.6	3.10 ±0.56	67.6 ±2.7	5.0 ±1.0
20		377 ±25	41.9 ±5.5	889 ±60	376 ±93
18		28 ±6.6	9.2 ±2.1	69.0 ±1.1	31.9 ±4.2
21		976 ±15	143 ±27	1420 ±112	428 ±35
5		14.9 ±1.2	7.3 ±3.1	85 ±2.7	30.5 ±5.8
6		20.6 ±1.0	16.2 ±3.2	25.4 ±1.1	10.6 ±1.3
7		0.86 ±0.14	0.43 ±0.10	2.15 ±0.66	1.15 ±0.12
17		3.6 ±1.0	1.96 ±0.19	5.0 ±1.2	1.35 ±0.19

nearly identical pattern of the structure−activity relationship. This correlation between *P. vulgaris* and *B. pasteurii* enzymes proves the effectiveness of computer model based on crystal structure of the latter enzyme.

To summarize, within the presented class of urease inhibitors two highly active compounds (**3** and **7**) were found with K_i values of 360 nM and 430 nM against *P. vulgaris* enzyme, respectively (Table 1). Thus, considerable improvement of phosphinic acids activity was made in comparison to previously reported compounds, where the most active compound, *N*-methylaminomethyl-*P*-methylphosphinic acid, exhibited $K_i = 27\ \mu M$ against *P. vulgaris* urease. The potency of the new series is higher than that of acetohydroxamic acid, a slow-binding inhibitor of urease approved by FDA in 1983 for treatment of chronic urinary tract infections ($K_i^* = 2.6\ \mu M$ against bacterial urease from *K. aerogenes*).[60] In clinical trials, acetohydroxamic acid markedly reduced blood ammonia levels in patients with cirrhosis and with high *H. pylori* density in gastric mucosa.[61] The administration of this inhibitor abolished *H. pylori* cytoplasmic and external urease activity and significantly reduced its chemotactic swarming ability.[62] Inhibition of *H. pylori* urease activity was shown to decrease microorganism viability in acidic environment.[63] In the urinary tract and in the stomach, the proposed (though still widely discussed) role of urease in infection development is to alkalinize the environment to allow bacterial cells to survive and propagate.[64] The *Helicobacter* urease is present both in cytoplasmic and surface associated (or extracellular) form. Though the role of the surface-bound urease is still argued, it was proved that inhibition of this enzyme alone by a poorly diffusible inhibitor, fluorofamide (*N*-(diaminophosphinyl)-4-fluorobenzamide), was sufficient to reduce *H. pylori* resistance to acid despite the presence of 5 mM urea.[65,66] We presume that the aminophosphinates effect upon ureolytic microorganisms growth would also rely on interfering with urea decomposition crucial for survival in acidic environment rather than direct bacteriocidal activity. We expect that the active doses of aminophosphinates necessary to exert effect in vivo comparable with acetohydroxamates might be lower due to the remarkable stability of C−P bond containing compounds. Hydrolytical stability of the new inhibitors was confirmed by ^{31}P and ^{1}H NMR. Neither 6 months storage as frozen solutions nor one week incubation at room temperature had detectable effect upon 10 mM samples' spectra. In view of emerging problems concerning the currently available therapy (growing resistance rates to

antibiotics, severe teratogenic, psychoneurological, and musculointegumenatary side effects of acetohydroxamate) the inhibitory activity and the stability of presented compounds in water solution suggest the possibility of their application.

Conclusions

Taking all the results together, a newly developed class of potent low-molecular-weight bacterial urease inhibitors has been described. Although the small volume of urease active site and the flexibility of its large portion make computer-aided design difficult, it was possible to find interesting novel structures and predict their relative potency in silico. Successful synthesis of the designed compounds allowed measurement of their activities against chosen bacterial ureases and to prove the correctness of the applied computational methods. It was found that this class of compounds is significantly more active than their *P*-methyl analogues. Moreover, appropriate methylation of terminal amine group led to significant increase of inhibitory potency by creating a compatible number of hydrogen bond donors. The major advantage of the presented novel group of urease inhibitors lays in the combination of strong inhibitory activity and hydrolytic stability, which promote their possible application. Moreover, their structures are still susceptible to further modifications when searching for other, possibly more potent inhibitors of this enzyme.

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 either UV light as visualizing agent or 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 agents. Purification of compounds by column chromatography was carried out on silica gel (70−230 mesh), reverse phase silica gel (Kieselgel 60, RP-18, 40−63 μm), and Dowex AG50 × 4 cation exchange resin. RP-HPLC analyses were carried out 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), $t = 10$ min (35% B), $t = 35$ min (100% B), $t = 37$ min (100% B), $t = 40$ min (40% B). B: $t = 0$ min (0% B), $t = 10$ min (25% B), $t = 45$ min (75% B), $t = 50$ min (100% B), $t = 52$ min (100% B), $t = 60$ min (40% B). Eluted peaks were detected by a UV detector at 254 nm. Reported retention times are reported in minutes. In NMR measurements, $CDCl_3$, D_2O, and CD_3OD were used as solvents. ^{1}H, ^{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 tested compounds **2**−**7**, **17**−**18**, and **20**−**21**, gave satisfactory microanalysis (see Supporting Information) confirming ≥95% purity. All spectroscopy and analytical data were obtained in the Laboratory of Organic Chemistry of the University of Athens.

Molecular Modeling. Molecular modeling was done using Insight 2000 program package from Accelrys. The starting structure for novel inhibitor design was the optimized complex of aminomethyl-*P*-methylphosphinate with *B. pasteurii* urease described previously.[48] The optimization of known inhibitor substituents was performed using LUDI module of this package.[50,51] LUDI program parameters were as follows: Preselect, 2.00; MinSeparation, 2.0; DensL, 50; DensP, 50; MinSurf, 0; LinkWeight, 1.00; LipoWeight, 1.00; HbondWeight, 1.00; Aliphatic_Aromatic, No; Reject_Bifurcated, No; No_Unpaired_Polar, No; Electrostatic_Check, No; MinimumScore, 0; MaximumFits, 5000; MaximumHits, All; MaxUnfilledCavity, 0; ScoringFunction, Energy_Estimate_1; Invert, No; SearchType, Fast; Linkage, One; MaxAlignmentAngle, 30.00; MaxRMS, 0.6; RotableBonds, Two_At_A_Time. Minimizations were done with the program Discover from Insight 2000 package with the use of cff97 forcefield. Conjugate gradient minimizer was applied. Optimizations were done up to rms derivative equals to 0.01 A. Minimizations were done in two steps: first the structure of ligand was optimized, while the structure of protein was frozen, then, both ligand and enzyme residues that interact with the inhibitor were optimized. In the case of dipeptides, which were obtained by appropriate modification of parent structure **4**, the minimization protocol was different. First, the N-teminal inhibitor residue and surrounding enzyme residues were optimized, while the rest of the inhibitor and enzyme was frozen. In the second step, the structure of whole inhibitor and all active site residues were optimized.

Chemistry. *N*-Benzyloxycarbonylaminomethyl(hydroxymethyl)-(*O*-methyl) Phosphinate (9). Paraformaldehyde (84 mg, 2.89 mmol) and triethylamine (95 μL, 70 mmol) were added to a solution of **8** (320 mg, 1.40 mmol) in toluene (3 mL) and the reaction was heated at 75 °C for 45 min. The mixture was allowed to cool to room temperature and concentrated in vacuo. The residue was purified by column chromatography, eluting with CH_2Cl_2−MeOH (9.5−0.5) to give 194 mg (51%) of **9** as a colorless gum.

^{1}H NMR ($CDCl_3$) δ 3.43 (m, 2 H), 3.73 (d, $J = 10.3$, 3 H), 3.83 (s, 2H), 5.08 (s, 2 H), 6.21 (bt, 1 H), 7.32 (s, 5H). ^{13}C NMR ($CDCl_3$) δ 36.3 (d, $J_{PC} = 105.3$ Hz), 52.3 (d, $J_{PC} = 7.1$ Hz), 56.6 (d, $J_{PC} = 103.1$ Hz), 67.9, 128.4, 128.7, 128.8, 136.0, 157.7. ^{31}P NMR ($CDCl_3$) δ 50.6. MS(H^+) = 274.1

***O*-Methyl *N*-Benzyloxycarbonylaminomethyl(*tert*-butoxymethyl)-phosphinate (10).** To an ice-cooled solution of **2** (0.15 g, 0.55 mmol) in CH_2Cl_2 (5 mL), a catalytic amount of concentrated sulfuric acid was added. Then, a large excess of liquid isobutene (20 mL) was added and the mixture was stirred at room temperature for 24 h. After careful removal of isobutene, the reaction mixture was partitioned between CH_2Cl_2 and aqueous Na_2CO_3, and the organic layer was washed with H_2O and brine, dried over Na_2SO_4, and concentrated in vacuo. The resulting colorless oil was purified by column chromatography using CH_2Cl_2−MeOH (9.3−0.7) as eluent to afford the product as colorless oil. Yield: 63% (0.11 g).

^{1}H NMR ($CDCl_3$) δ 1.17 (s, 9H), 3.49−3.92 (m, 4H), 3.71 (d, $J = 10.4$ Hz, 3H), 5.11 (s, 2H), 5.38 (bs, 1H), 7.24−7.39 (m, 5H). ^{13}C NMR ($CDCl_3$) δ 27.0, 37.4 (d, $J_{PC} = 102.5$ Hz), 52.2 (d, $J_{PC} = 7.2$ Hz), 57.5 (d, $J_{PC} = 115.5$ Hz), 67.3, 75.7, 128.2, 128.4, 128.7, 136.5, 156.4. ^{31}P NMR ($CDCl_3$) δ 47.2. MS (ESI+) m/z 330.1 (M + 1).

***N*-Benzyloxycarbonylaminomethyl(*P*-acetoxymethyl)(*O*-methyl) Phosphinate (11).** To a stirred solution of **9** (0.30 g, 1.10 mmol) and DMAP (10 mg, 0.082 mmol) in pyridine (5 mL), acetic anhydride (0.13 mL, 1.30 mmol) was added at room temperature. After stirring for 12 h at room temperature, water (5 mL) was added and the aqueous phase was extracted with ethyl acetate. The organic phase was washed with HCl 1 M and brine. After drying over Na_2SO_4, concentration of the organic phase afforded crude **11**, which was purified by column chromatography, eluting with CH_2Cl_2−MeOH (9.5−0.5) to give 280 mg (81%) of the desired product as colorless gum.

^{1}H NMR ($CDCl_3$) δ 2.10 (s, 3H), 3.44−3.92 (m, 2 H), 3.72 (d, J = 11.1 Hz, 3 H,), 4.30 (dd, J = 4.0 Hz, 1H), 4.55 (dd, J = 7.1 Hz 1H), 5.11(s, 2 H), 6.35 (bs, 1 H), 7.34 (s, 5H). ^{13}C NMR ($CDCl_3$) $\delta\delta$ 21.0, 38.6 (d, J_{PC} = 106.74 Hz), 52.8 (d, J_{PC} = 7.8 Hz), 57.0 (d, J_{PC} = 107.9 Hz), 67.5, 128.3, 128.4, 128.7, 136.3, 156.7, 170.5. ^{31}P NMR ($CDCl_3$) δ 46.0. MS (ESI+) m/z 316.1

***N*-Benzyloxycarbonylaminomethyl(*P*-acetoxymethyl) (*O*-Methyl)-phosphinothioate (12).** A mixture of **11** (95 mg, 0.30 mmol) and Lawesson's reagent (120 mg, 0.30 mmol) was heated at 95 °C in sodium dried toluene (1.5 mL) for 2 h. Evaporation in vacuo gave a pale-yellow oil, which was chromatographed with gradient of CH_2Cl_2−EtOAc (9.5−0.5) to give 61 mg of **12** as oil (60%).

^{1}H NMR ($CDCl_3$) δ 2.11 (s, 3H), 3.56−4.00 (m, 2 H), 3.67 (d, J = 12.1 Hz, 3 H), 4.32 (dd, J = 3.8 Hz, 1H), 4.67 (dd, J = 6.7 Hz, 1H), 5.12 (s, 2 H), 5.17 (bs, 1 H), 7.34 (s, 5H). ^{13}C NMR ($CDCl_3$) δ 20.8, 42.4 (d, J_{PC} = 85.7 Hz), 52.5 (d, J_{PC} = 6.9 Hz), 61.5 (d, J_{PC} = 82.6 Hz), 67.7, 128.4, 128.6, 128.8, 136.2, 156.2, 170.5. ^{31}P NMR ($CDCl_3$) δ 91.1. MS (ESI+) m/z 332.2 (M + 1).

***N*-(*N′*-Benzyloxycarbonylglycyl)aminomethyl *tert*-Butoxymethyl (*O*-Methyl) Phosphinate (13).** A mixture of 10% Pd/C (30 mg) and **10** (329 mg, 1 mmol) in methanol (20 mL) was stirred at room temperature under an atmosphere of hydrogen until 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 mixture (1:9). Solvents were combined and evaporated in vacuo to give a solid (190 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), and 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 **13** was obtained in 72% yield (280 mg).

^{1}H NMR ($CDCl_3$): δ 1.17 (s, 9H), 3.40−4.00 (m, 4H), 3.72 (d, J = 11.2 Hz,3H), 3.88 (d, J = 4.8 Hz, 2H), 5.10 (s, 2H), 5.80 (bs, 1H), 7.10 (bs, 1H), 7.33 (s, 5H). ^{13}C NMR ($CDCl_3$): δ 27.1, 35.5 (d, J_{PC} = 102.9 Hz), 44.6, 52.3 (d, J_{PC} = 7.2 Hz), 57.6 (d, J_{PC} = 116.3 Hz), 67.3, 75.9, 128.3, 128.4, 128.7, 136.4, 169.5, 169.6. ^{31}P NMR ($CDCl_3$): δ 47.6. MS (ESI+) m/z 387.3 (M + 1).

***N*-(*N′*-*tert*-Butoxycarbonylsarcosyl)aminomethyl *tert*-Butoxymethyl (*O*-Methyl) Phosphinate (14).** Following the same procedure as described for compound **13**, compound **14** was obtained in 77% yield.

^{1}H NMR ($CDCl_3$) δ 1.17(s, 9H), 1.42 (s, 9H), 2.89 (s, 3H), 3.40−4.00 (m, 6H), 3.73 (d, J = 10.0 Hz, 3H), 6.80 (bs, 1H). ^{13}CNMR ($CDCl_3$) δ 27.1, 28.5, 35.3 (d, J_{PC} = 102.1 Hz), 36.1, 52.3 (d, J_{PC} = 6.6 Hz), 53.1, 57.7 (d, J_{PC} = 120.0 Hz), 76.2, 81.0, 128.3, 128.4, 128.7, 136.4, 154.7, 169.5. ^{31}P NMR ($CDCl_3$) δ 47.6. MS (ESI+) m/z 367.3 (M + 1).

***N*-(*N′*,*N′*-Dimethylglycyl)aminomethyl *tert*-Butoxymethyl (*O*-Methyl) Phosphinate (15).** Following the same procedure as described for **13**, compound **15** was obtained in 76% yield.

^{1}H NMR ($CDCl_3$) δ 1.22 (s, 9H), 2.37 (s, 6H), 3.43−3.60 (m, 4H), 3.76 (d, J = 11.7 Hz, 3H), 3.95−4.17 (m, 2H), 7.80 (bs, 1H). ^{13}C NMR ($CDCl_3$) δ 27.1, 27.0, 35.4 (d, J_{PC} = 98.2 Hz), 45.8, 52.3 (d, J_{PC} = 6.7 Hz), 58.2 (d, J_{PC} = 106.7 Hz), 62.7, 76.4, 170.6. ^{31}P NMR ($CDCl_3$) δ 47.3. MS (ESI+) m/z 281.1 (M + 1).

Benzoylaminomethyl(*tert*-butoxymethyl)(*O*-methyl)phosphinate (16). Following the same procedure as described for **13**, compound **16** was obtained in 82% yield.

^{1}H NMR ($CDCl_3$) δ 1.20 (s, 9H), 3.45−3.84 (m, 4H), 3.71 (d, J = 10.4 Hz,3H), 7.05 (bs, 1H), 7.41 (m, 3H), 7.80 (d, J = 6.98 Hz, 2H). ^{13}CNMR ($CDCl_3$) δ 27.1, 36.6 (d, J_{PC} = 102.8 Hz) 51.3 (d, J_{PC} = 6.8 Hz), 58.5 (d, J_{PC} = 116.2 Hz), 75.9, 127.3, 128.6, 128.8, 130.3, 132.0, 133.6 136.7, 165.7. ^{31}P NMR ($CDCl_3$) δ 45.9. MS (ESI+) m/z 300.4 (M + 1).

Benzoylaminomethyl(hydroxymethyl)phosphinic Acid (18). Compound **16** (49 mg, 0.16 mmol) was dissolved in dioxane (0.45 mL) and aqueous 2 M LiOH (0.25 mL) was added, and the mixture was vigorously stirred at room temperature for 48 h. After addition of water, the aqueous solution was washed with ethyl acetate and acidified to pH 1 with 0.5 M HCl. Then the aqueous phase was extracted two times with ethyl acetate. The organic extracts were combined, dried over Na_2SO_4, and evaporated in vacuo. The crude product was purified by column chromatography using CH_2Cl_2−CH_3OH−AcOH (9:1:0.5) as eluent to give 29 mg of the free hydroxyphosphinyl compound. This compound was treated with 95% TFA for 14 h. The volatiles were removed by repeated addition/evaporation of CH_2Cl_2. Trituration with Et_2O followed by purification with reverse phase silica gel and water−acetonitrile (8.5−1.5) as eluent provided **18** as a hygroscopic solid (30 mg, 82%).

^{1}H NMR (CD_3OD) δ 3.79−3.84 (m, 4H), 7.46 (m, 3H), 7.84 (d, J = 6.97 Hz, 2H). ^{13}CNMR (CD_3OD): δ 33.7 (d, J_{PC} = 100.9 Hz), 63.6 (d, J_{PC} = 112.3 Hz), 127.3, 128.4, 131.8, 136.5, 169.2. ^{31}P NMR (CD_3OD) δ 42.4. MS (ESI−) m/z 228.1 (M− 1). Analytical HPLC method B: t_R = 9.0 min. HPLC purity >97%.

***N*-(*N′*-Benzyloxycarbonylglycyl)aminomethyl(hydroxymethyl)-phosphinic Acid (17).** Following the same procedure as described for **18**, compound **17** was obtained in 80% yield.

^{1}H NMR (CD_3OD) δ 3.70 (d, J = 5.2 Hz, 2H), 3.78−3.89 (m, 4H), 5.10 (s, 2H), 7.33 (s, 5H). ^{13}CNMR (CD_3OD) δ 35.1 (d, J_{PC} = 98.6 Hz), 43.6, 57.1 (d, J_{PC} = 113.0 Hz), 66.7, 127.7, 127.9, 128.3, 136.1, 158.0, 172.3. ^{31}P NMR (CD_3OD) δ 44.1. MS (ESI−) m/z 315.1 (M − 1). Analytical HPLC method B: t_R = 13.2 min. HPLC purity >97%.

***N*-Sarcosyl(*P*-aminomethyl) (*P*-hydroxymethyl) Phosphinic Acid (6).** Following the same procedure as described for **18**, compound **6** was obtained in 85% yield.

^{1}H NMR (CD_3OD) δ 2.75 (s, 3H), 3.73 (d, J = 9.0 Hz,2H), 3.84 (d, J = 5.6 Hz,2H), 3.89 (s, 2H). ^{13}CNMR (CD_3OD) δ 32.4, 36.1 (d J_{PC} = 96.7 Hz), 58.3 (d, J_{PC} = 108.5 Hz), 65.7, 165.7. ^{31}P NMR (CD_3OD): δ 41.2. MS (ESI−) m/z 195.2 (M − 1).

***N*-(*N′*,*N′*-Dimethylglycyl) (*P*-Aminomethyl)(*P*-hydroxymethyl) Phosphinic Acid (7).** Following the same procedure as described for **18**, compound **7** was obtained in 85% yield.

^{1}H NMR (D_2O) δ 2.79 (s, 6H), 3.43 (d, J = 8.7 Hz, 2H), 3.57 (d, J = 6.1 Hz, 2H), 3.90 (s, 2H). ^{13}C NMR (D_2O): δ 37.0 (d, J_{PC} = 98.5 Hz), 43.8, 58.7 (d, J_{PC} = 112.6, Hz), 58.3 165.8. ^{31}P NMR (D_2O) δ 36.7. MS (ESI+) m/z 211.1 (M + 1).

***N*-Glycyl(*P*-aminomethyl) (*P*-Hydroxymethyl) Phosphinic Acid (5).** Following the same procedure for catalytic hydrogenolysis as described for **13**, compound **5** was obtained in 73% yield after trituration with Et_2O and purification with Dowex AG50 × 4 cation exchange resin using water as eluent and collecting the fractions showing positive ninhydrin reaction.

^{1}H NMR (D_2O) δ 3.30 (d, J = 9.6 Hz, 2H), 3.43(d, J = 5.9 Hz, 2H), 3.63 (s, 2H). ^{13}C NMR (D_2O): δ 37.1 (d J_{PC} = 96.7 Hz), 40.4, 58.7 (d J_{PC} = 111.4 Hz), 166.8. ^{31}P NMR (D_2O) δ 34.3. MS (ESI−) m/z 181.3 (M − 1).

***N*-Benzyloxycarbonylmethylaminomethyl(*O*-methyl)(*tert*-butoxymethyl)phosphinate (19).** Sixty percent Dispersion of NaH in mineral oil (12 mg, 0.29 mmol) was and added to a cooled (−10 °C) solution of **10** (95 mg, 0.29 mmol) in THF (2.7 mL). The reaction mixture was treated with MeI (37 μL, 0.6 mmol) and stirred at −10 °C for 1 h and at rt for 0.5 h. Then it was partitioned between EtOAc and water, washed with water, dried (Na_2SO_4), and evaporated. The crude material was purified by column chromatography, eluting with CH_2Cl_2−MeOH (9.5−0.5), to give 82 mg (83%) of **16** as colorless oil.

^{1}H NMR ($CDCl_3$) δ 1.18 (s, 9H), 3.05 (s, 3H), 3.50−3.84 (m, 4H), 3.78 (d, J = 10.1 Hz,3H), 5.11 (s, 2H), 7.33 (s, 5H). ^{13}CNMR ($CDCl_3$) δ 27.1, 30.0, 36.1 (d, J_{PC} = 99.9 Hz), 45.1, 51.1 (d, J_{PC} = 6.8 Hz), 58.3 (d, J_{PC} = 106.3 Hz), 75.4, 128.1, 128.3, 128.7, 136.7, 156.0, 156.7. ^{31}P NMR ($CDCl_3$) δ 48.4. MS (ESI+) m/z 344.7 (M + 1).

Hydroxymethyl((methylamino)methyl) Phosphinic Acid (3). Compound **19** (70 mg, 0.20 mmol) was subjected to catalytic hydrogenolysis, as described for compound **13**. Then the reaction product 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. After addition of water, the aqueous solution was washed with ethyl acetate and acidified to pH 1 with 0.5 M HCl. Then the aqueous phase was extracted two times with ethyl acetate. The organic extracts were combined, dried over Na_2SO_4, and evaporated in vacuo. The crude product was purified by column chromatography using $CH_2Cl_2-CH_3OH-AcOH$ (9:1:0.5) as eluent to give 29 mg of the free hydroxyphosphinyl compound. This compound was treated with 95% TFA for 14 h. The volatiles were removed by repeated addition/evaporation of CH_2Cl_2, and the remaining solid was purified using 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** (21 mg, 75% yield).

^{1}H NMR (D_2O) δ 2.55 (d, J = 4.9 Hz, 3H), 2.97 (d, J = 4.4 Hz, 2H), 3.49 (bs, 2H). ^{13}C NMR (D_2O) δ 35.3, 46.0 (d, J_{PC} = 84.2 Hz), 59.7 (d, J_{PC} = 117.8 Hz). ^{31}P NMR (D_2O) δ 28.3. MS (ESI−) m/z 138.0 (M − 1).

***N*-Benzyloxycarbonylaminomethyl(hydroxymethyl)phosphinic Acid (20).** Compound **9** (17 mg, 0,06 mmol) was treated with LiOH as described for **3**. Following the same workup and column chromatography eluting with $CH_2Cl_2-MeOH-AcOH$ (9−1−0.5) compound **20** was obtained (10 mg, 63%).

^{1}H NMR (CD_3OD): δ 3.56 (d, J = 8.5 Hz, 2H), 3.78 (d, J = 5.6 Hz,2H), 5.10 (s, 2H), 7.33 (s, 5H). ^{13}CNMR (CD_3OD) δ 37.1 (d, J_{PC} = 102.5 Hz), 57.6 (d, J_{PC} = 110.0 Hz), 66.8, 127.7, 127.9, 128.3, 136.9, 159.5. ^{31}P NMR (CD_3OD) δ 44.2. MS (ESI−) m/z 258.1 (M − 1). Analytical HPLC method B: t_R = 13.4 min. HPLC purity >97%.

Aminomethyl(hydroxymethyl)phosphinic Acid (2). Compound **20** (10 mg, 0.037 mmol) was subjected to catalytic hydrogenolysis, as described for compound **13**. Purification using Dowex AG50 × 4 cation exchange resin, water as eluent, and collection of the fractions that gave positive color reaction with ninhydrin furnished **2** (3 mg 72% yield).

^{1}H NMR (D_2O) δ 3.15 (d, J = 10.1 Hz, 2H), 3.37 (d, J = 9.2 Hz, 2H). ^{13}C NMR (D_2O) δ 37.3 (d, J_{PC} = 98.5 Hz), 59.2 (d, J_{PC} = 113.5 Hz).^{31}P NMR (D_2O) δ 20.4. MS (ESI−) m/z 124.2 (M − 1).

***N*-Benzyloxycarbonylaminomethyl(hydroxymethyl)phosphinothioic Acid (21).** Compound **12** (22 mg, 0,08 mmol) was treated with LiOH as described for **3**. After chromatographic purification using $CH_2Cl_2-CH_3OH-AcOH$ (9:1:0.1) as eluent, compound **21** was obtained (14 mg, 63%).

^{1}H NMR (CD_3OD) δ 3.60 (d, J = 8.0 Hz, 2H), 3.74 (d, J = 5.3 Hz, 2H), 5.10 (s, 2H), 7.33 (s, 5H). ^{13}C NMR (CD_3OD) δ 38.2 (d, J_{PC} = 95.3 Hz), 58.6 (d, J_{PC} = 112.5 Hz), 66.5, 127.6, 127.9, 128.3, 136.8, 159.8. ^{31}P NMR (CD_3OD): δ 69.3. MS (ESI+) m/z 276.1 (M + 1). Analytical HPLC method A: t_R = 22.1 min. HPLC purity >97%.

***N*,*N*-Dimethyl-(*P*-aminomethyl)(*P*-hydroxymethyl) Phosphinic Acid (4).** A mixture of hypophosphorus acid (50%) (0.13 g, 1 mmol), *p*-formaldehyde (0.036 g, 1.2 mmol), and 6 M solution of HCl (0.2 mL, 1.2 mmol) in EtOH (4 mL) was heated at 100 °C for 48 h. The volatiles were then removed in vacuo, and the crude product was dissolved in water (2 mL), followed by the addition of dimethylamine 40% (0.11 g, 1 mmol) and 6N HCl (0.5 mL). The solution was warmed to 90 °C and *p*-formaldehyde (0.072 g, 2 mmol) was added, and the mixture was kept at 90 °C for additional 5 h. After removing the volatiles, the crude product was dissolved in water and purified using a Dowex AG50 × 4 cation exchange resin with water as eluent. Fractions that gave a slightly positive color reaction with ninhydrin and exhibited a peak at 28.4 ppm in ^{31}P NMR were combined and evaporated under vacuum to give **4** as hygroscopic solid (0.08 g, 52% yield).

^{1}H NMR (D_2O) δ 2.80 (s, 6H), 3.21 (d, J = 8.3 Hz,2H), 3.55 (d, J = 7.0 Hz, 2H). ^{13}C NMR (D_2O) δ 45.8, 54.7 (d, J_{PC} = 82.7 Hz), 60.1 (d, J_{PC} = 118.3 Hz). ^{31}P NMR (D_2O) δ 28.4. MS (ESI−) m/z 152.2 (M − 1).

Urease Purification. Ureases were partially purified from the soluble extract of *B. pasteurii* CCM 2056^T and *P. vulgaris* CCM 1956 cells using two chromatography steps procedure. Both strains were grown aerobically at 37 °C in a medium containing (per liter) 20 g of yeast extract, 20 g of urea, and 1 mM $NiCl_2$, pH 9.0. After 12 h, incubation cells were harvested by centrifugation, washed with lysis buffer containing 50 mM phosphate, pH 7.5/1 mM β-mercaptoetanol/1 mM EDTA, and sonicated. The soluble fraction was recovered after high-speed centrifugation and loaded onto BioGel column. Desalted crude extract was loaded onto the anion-exchange column Q Sepharose and linear gradient of 0−1 M NaCl was used. Enzymatically active fractions were precipitated with 1.5 M $(NH_4)_2SO_4$, applied onto the Phenyl Sepharose column, and developed using 50 mM phosphate buffer with a decreasing linear gradient of $(NH_4)_2SO_4$ from 1.5 to 0 M.[67] Fractions with urease activity were eluted with 350 mM $(NH_4)_2SO_4$ and dialyzed against 50 mM phosphate buffer, pH 7.5, with addition of 1 mM $NiCl_2$.

The partially purified urease from *B. pasteurii* CCM 2056^T exhibited a Michaelis−Menten saturation kinetics with K_m 19.3 ± 1.7 mM, and specific activity about 1234.8 U/mg. The same parameter for urease isolated from *P. vulgaris* CCM 1956 was about 1615.3U/mg with K_m 10.4 ± 0.9 mM. Unit was defined as μmol/min/mL of released ammonium ions. The yield of enzyme preparation was 36.5% and 42.2% for both above-mentioned strains, respectively.

Protein concentration during preparation was measured using Bradford method with a bovine serum albumin as a standard. Enzymatic activity of purified urease was quantified spectrophotometrically using indophenol color reaction.[68]

Inhibition Studies. To evaluate the inhibitory activity of the compounds, purified enzyme was incubated at 30 °C in 3 mM phosphate buffer pH 7.0 containing increasing concentrations of inhibitors and urea (from 10 to 100 mM). Urease activity was determined by means of modified Berthelot colorimetric reaction as previously reported. The concentrations of inhibitor causing 50% loss of the enzyme activity (IC_{50}) as well as K_i values for all analyzed compounds were calculated using appropriate equations of GraphPad Prism 5 software program.

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Supporting Information Available: Elemental analysis for all target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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