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Design, Synthesis, and Biological Evaluation of Phosphoramide Derivatives as Urease Inhibitors

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The design, synthesis, and biological evaluation of phosphoramide derivatives as urease inhibitors to reduce the loss of ammonia has been carried out. Forty phosphorus derivatives were synthesized and their inhibitory activities evaluated against that of jack bean urease. In addition, in vivo assays have been carried out. All of the compounds were characterized by IR, ^{1}H NMR, MS, and elemental microanalysis. In some cases, detailed molecular modeling studies were carried out, and these highlighted the interaction between the enzyme active center and the compounds and also the characteristics related to their activity as urease inhibitors. According to the IC_{50} values for in vitro inhibitory activity, 12 compounds showed values below 1 μM and 8 of them represent improvements of activity in comparison to the commercial urease inhibitor *N-n*-butylthiophosphorictriamide (NBPT) (100 nM) (AGROTAIN). On the basis of the activity results and the conclusions of the molecular modeling study, a structural model for new potential inhibitors has been defined.

KEYWORDS: Urease inhibitors; phosphoramides; fertilizers; urea

INTRODUCTION

Urea is a widely used nitrogen fertilizer in world agriculture. In soil, urea is hydrolyzed by urease (urea amidohydrolase; EC 3.5.1.5), a nickel-dependent enzyme that catalyzes the hydrolysis of urea to form ammonia and carbon dioxide (*1*). This in turn causes a sharp increase in pH and an accumulation of NH_4^+, which has negative side effects in agriculture and health. Thus, in surface applications, losses of gaseous NH_3 can occur and can constitute up to 50% of the fertilizer nitrogen applied, especially in soils with low buffer capacity, in calcareous soils, and in soils with a high organic C content. The other negative effect of urea hydrolysis is the accumulation of NO_2^-, which can damage germinating seeds, seedlings, and young plants. One approach to overcome the problems associated with the use of urea fertilizers is to find compounds that would inhibit the urease activity and thereby retard urea hydrolysis when applied to soils together with the fertilizer. The possibility of controlling the rate of the enzymatic urea hydrolysis using urease inhibitors is an important goal to pursue.

Ureases have been isolated from a wide variety of organisms, including plants, fungi, and bacteria (*2–4*). At present, crystallographic structures are available for only some of the bacterial ureases (*5–8*). However, the highly conserved amino acid sequences of all known ureases and the constant presence of two Ni ions, which are bridged by the carboxylate group of the carbamylated lysine ($Lys^{\alpha 220}$ in native *Bacillus pasteurii* urease, BPU) and coordinated by some surrounding histidine and aspartic residues [$His^{\alpha 249}$ and $His^{\alpha 275}$ for Ni(I) and $His^{\alpha 137}$, $His^{\alpha 139}$, and $Asp^{\alpha 363}$ for Ni(II) in native BPU], imply a common catalytic pathway (*6, 7, 9*). Jack bean (*Canavalia ensiformis*) urease (JBU) consists of six subunits each made of 840 amino acids, whereas *Klebsiella aerogenes* urease (KAU) has three subunits (α, β, and γ) with 101, 106, and 506 residues, respectively (*10*). The $\alpha\beta\gamma$ fragment of KAU is highly homologous to the single subunit of JBU. The structure of BPU reveals an analogous $\alpha\beta\gamma$ quaternary structure and a very similar active site geometry (*11*).

Among the known inhibitors of urease, the most efficient are phosphorodiamide and phosphorotriamide derivatives (*12–21*). This group includes the following: *N-n*-butylthiophosphoric-triamide (NBPT, **Figure 1**), which has been shown to form stable complexes with urease and is among the most efficient inhibitors of the enzyme (*22–24*); phenylphosphorodiamidate (PPD, **Figure 1**) (*25–27*), *N-n*-butylphosphoric-triamide (NBPTO, **Figure 1**) (*28, 29*), and *N*-diaminophosphoryl-4-fluorobenzamide (Flurofamide, **Figure 1**) (*30, 31*).

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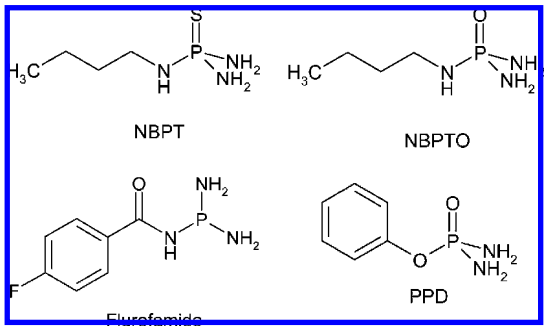


Figure 1. Some urease inhibitors, taken as references for the design of the new compounds.

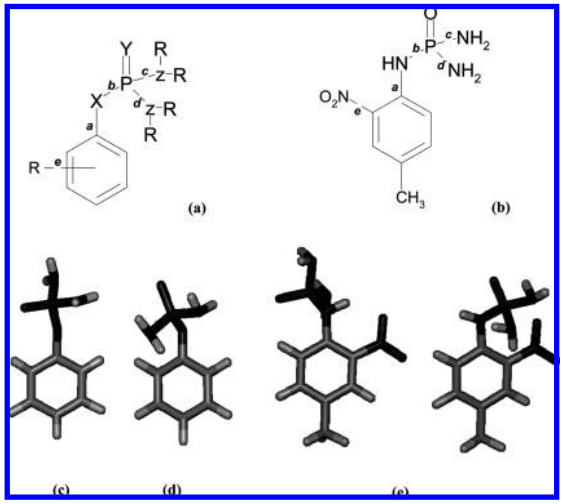
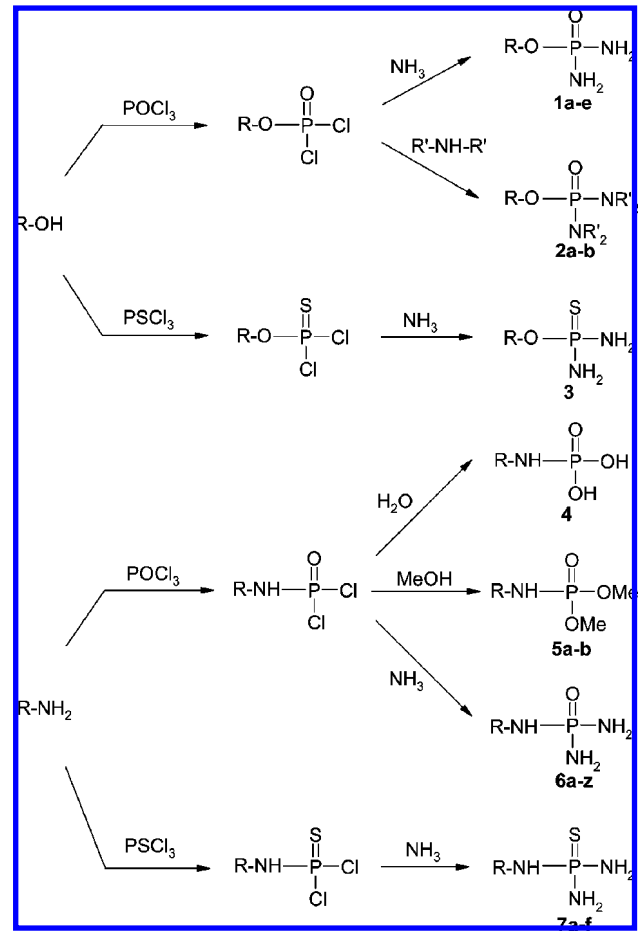


Figure 2. (**a**) General structure of the new compounds. **a**–**e**: bonds selected for the conformational analysis. (**b**) Bonds selected (**a**−**e**) for conformational analysis carried out for compound **6f**. (**c**) Crystallographic structure for PPD taken as a reference in the conformational analysis (CSD ref code: PPOSAM). (**d**) Representative lowest energy conformation for PPD. (**e**) Representative lowest energy conformations for compound **6f**.

The urease inhibition mechanism proposed for these compounds suggests that the oxygen atom bonded to the P can bridge the Ni ions, whereas the surrounding nitrogen and/or oxygen atoms bind to Ni(I) or Ni(II). Thus, in studies carried out on crystals of BPU obtained from PPD solutions (PDB code 3UBP) it has been established that a diamidophosphoric acid (DAP) molecule, the PPD enzymatic hydrolysis product, is present in the active site. DAP is coordinated to Ni(I) and Ni(II) by the P−O^- moiety, whereas one oxygen and one nitrogen bind to Ni(I) and Ni(II), respectively. The second nitrogen atom of DAP points away toward the cavity opening (*32, 33*). In the absence of crystallographic data for an NBPT−urease complex, the marked ability of NBPT to act as an inhibitor can be explained on the basis of its tridentate nature. In this system the lateral alkyl chain can point toward the cavity opening. As a consequence, the probability of urea reaching the Ni atom is greatly reduced when the active site is locked by the NBPT molecule. The design of new urease inhibitors has been carried out by taking as a starting point the reference urease inhibitors and the data published on BPU structures complexed with several inhibitors. The general structure of the new compounds is shown in **Figure 2a**.

In the proposed structures the P═Y moiety (Y = O, S) adopts the function of DAP's P−O^- moiety, whereas the surrounding nitrogen and/or oxygen atoms (X = O, NH; Z = O, N) can bind to Ni(I) and/or Ni(II) in a way similar to that observed for

Scheme 1. Synthetic Route for Compounds

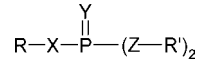


DAP. The lateral chain [R = alkyl, haloalkyl, aryl (substituted), alkyl-aryl, heteroalkyl, heteroaryl, alkyl-heteroalkyl, alkyl-heteroaryl] can be placed in the cavity opening. The introduction of alkylic chains on the terminal nitrogens and/or oxygens (R′ = ethyl, *n*-butyl) was proposed to modify the connection pattern of these atoms with the Ni atoms as well as with the residues involved in the active site.

Structural modifications are proposed to elucidate the chemical structure−biological activity relationship. Starting from an initial design, the chemical synthesis was modified and targeted according to biological activity data and molecular modeling calculations. For some of the compounds, a preliminary docking study was carried out to evaluate the possible interaction between the enzyme active center and the compounds as well as to highlight the characteristics related to their activity as urease inhibitors. The syntheses and structures of the phosphorated derivatives described here are presented in **Scheme 1** and **Table 1**. The compounds were identified and their purities established by the techniques commonly used in organic chemistry (**Table 2**). The molecular modeling calculations were performed on *Silicon Graphics* Octane2 workstations by applying the software package InsightII (*34*).

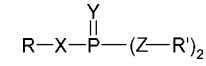
The urease inhibitory activity was determined by measurement of ammonium ions liberated (ionic HPLC) by urea hydrolysis (by urease enzyme action) for a fixed time, and the results are expressed as inhibitory concentration 50 (IC_{50}; **Table 3**) and percent inhibition (**Table 4**). Bearing in mind the aforementioned analogy between different ureases, and considering its commercial availability, we selected JBU for our biological tests.

Table 1. Experimental Data for the Synthesized Compounds



ref	R	X	Y	Z	R′	yield (%)	mp (°C)	C, H, N	% calcd/found
1a	4-nitrophenyl	O	O	N	H_2	23.1	115−116	$C_6H_8N_3O_4P$	C, 33.18; H, 3.69; N, 19.35
									C, 32.81; H, 3.71; N, 18.99
1b	phenyl	O	O	N	H_2	17.4	139−140	$C_6H_9N_2O_2P$	C, 41.86; H, 5.23; N, 16.28
									C, 41.72; H, 5.12; N, 16.20
1c	3,5-dimethylphenyl	O	O	N	H_2	29.3	98−99	$C_8H_{13}N_2O_2P$	C, 48.00; H, 6.50; N, 14.00
									C, 47.78; H, 6.28; N, 13.80
1d	4-benzyloxyphenyl	O	O	N	H_2	54.4	162−163	$C_{13}H_{15}N_2O_3P \cdot {}^{2}/_{3}H_2O$	C, 53.79; H, 5.63; N, 9.65
									C, 53.52; H, 5.30; N, 9.39
1e	4-methyl-2-nitrophenyl	O	O	N	H_2	8.4	122−123	$C_7H_{10}N_3O_4P \cdot {}^{4}/_{5}HCl$	C, 32.28; H, 4.15; N, 16.14
									C, 31.96; H, 4.45; N, 16.28
2a	4-nitrophenyl	O	O	N	C_2H_5	53.8	oil	$C_{14}H_{24}N_3O_4P$	C, 51.06; H, 7.29; N, 12.77
									C, 50.84; H, 7.40; N, 12.96
2b	4-nitrophenyl	O	O	N	C_4H_9	42.7	65−66	$C_{22}H_{40}N_3O_4P \cdot {}^{1}/_{2}HCl$	C, 57.48; H, 8.82; N, 9.14
									C, 57.30; H, 9.14; N, 9.11
3	4-benzyloxyphenyl	O	S	N	H_2	14.1	145−146	$C_{13}H_{15}N_2O_2PS$	C, 53.06; H, 5.10; N, 9.52
									C, 53.11; H, 5.28; N, 9.34
4	2-methylpyridyl	NH	O	O	H	2.4	174−175	$C_6H_9N_2O_3P$	C, 38.29; H, 4.79; N, 14.89
									C, 38.41; H, 5.15; N, 14.52
5a	3-methyl-2-pyridyl	NH	O	O	CH_3	14.5	146−147	$C_8H_{13}N_2O_3P \cdot {}^{1}/_{2}HCl$	C, 40.98; H, 5.76; N, 11.95
									C, 40.67; H, 6.06; N, 12.08
5b	2-benzothiazolyl	NH	O	O	CH_3	22.5	127−128	$C_9H_{11}N_2O_3PS$	C, 41.86; H, 4.26; N, 10.85
									C, 42.13; H, 4.32; N, 10.71
6a	bis(2-chloroethyl)	N	O	N	H_2	23.3	115−116	$C_4H_{12}Cl_2N_3OP$	C, 21.82; H, 5.45; N, 19.09
									C, 21.99; H, 5.39; N, 19.08
6b	1-adamantyl	NH	O	N	H_2	20.9	>300	$C_{10}H_{20}N_3OP$	C, 52.40; H, 8.73; N, 18.34
									C, 52.75; H, 8.74; N, 17.94
6c	1-naphthyl	NH	O	N	H_2	19.3	152−153	$C_{10}H_{20}N_3OP$	C, 52.40; H, 8.73; N, 18.34
									C, 52.75; H, 8.74; N, 17.94
6d	3-methoxyphenyl	NH	O	N	H_2	5.9	119−120	$C_7H_{12}N_3O_2P \cdot {}^{1}/_{4}H_2O$	C, 40.88; H, 5.96; N, 20.44
									C, 40.55; H, 5.63; N, 20.71
6e	4-cyclohexylphenyl	NH	O	N	H_2	2.5	224−225	$C_{12}H_{20}N_3OP$	C, 56.92; H, 7.91; N, 16.60
									C, 57.02; H, 7.91; N, 16.71
6f	4-methyl-2-nitrophenyl	NH	O	N	H_2	1.2	129−130	$C_7H_{11}N_4O_3P$	C, 36.52; H, 4.78; N, 24.35
									C, 36.56; H, 4.80; N, 24.74
6g	4-benzyloxyphenyl	NH	O	N	H_2	31.2	224−225	$C_{13}H_{16}N_3O_2P \cdot {}^{3}/_{4}H_2O$	C, 53.70; H, 6.02; N, 14.46
									C, 53.70; H, 5.85; N, 14.50
6h	2,5-dimethoxyphenyl	NH	O	N	H_2	0.9	122−123	$C_8H_{14}N_3O_3P \cdot {}^{2}/_{3}H_2O$	C, 39.50; H, 6.31; N, 17.28
									C, 39.20; H, 5.85; N, 17.38
6i	4-phenoxyphenyl	NH	O	N	H_2	16.8	136−137	$C_{12}H_{14}N_3O_2P \cdot {}^{1}/_{4}H_2O$	C, 53.83; H, 5.42; N, 15.70
									C, 53.47; H, 5.14; N, 15.67
6j	2-(4-fluorophenyl)ethyl	NH	O	N	H_2	2.1	238−239	$C_8H_{13}FN_3OP \cdot {}^{1}/_{7}HCl$	C, 43.20; H, 5.91; N, 18.90
									C, 43.37; H, 5.80; N, 18.80
6k	2-benzothiazolyl	NH	O	N	H_2	9.6	145−146	$C_7H_9N_4OPS$	C, 36.84; H, 3.95; N, 24.56
									C, 36.45; H, 3.91; N, 24.49
6l	3-morpholinylpropyl	NH	O	N	H_2	1.2	110−111	$C_7H_{19}N_4O_2P \cdot {}^{1}/_{2}HCl$	C, 34.96; H, 8.12; N, 23.31
									C, 35.15; H, 7.98; N, 23.66
6m	3-quinolyl	NH	O	N	H_2	0.7	141−142	$C_9H_{11}N_4OP$	C, 48.65; H, 4.95; N, 25.22
									C, 49.02; H, 4.87; N, 25.08
6n	phenyl	NH	O	N	H_2	4.9	119−120	$C_6H_{10}N_3OP \cdot {}^{1}/_{3}HCl$	C, 39.30; H, 5.64; N, 22.93
									C, 39.13; H, 5.46; N, 22.60
6o	4-nitrophenyl	NH	O	N	H_2	7.2	160−161	$C_6H_9N_4O_3P$	C, 33.33; H, 4.17; N, 25.92
									C, 33.05; H, 4.03; N, 26.00
6p	2-nitrophenyl	NH	O	N	H_2	21.4	168−169	$C_6H_9N_4O_3P$	C, 33.33; H, 4.17; N, 25.92
									C, 32.98; H, 4.10; N, 25.82
6q	2-phenylethyl	NH	O	N	H_2	2.7	118−119	$C_8H_{14}N_3OP \cdot {}^{1}/_{3}HCl$	C, 45.46; H, 6.79; N, 19.89
									C, 45.96; H, 6.74; N, 20.28
6r	2-trifluoromethoxyphenyl	NH	O	N	H_2	17.1	145−146	$C_7H_9N_4OPS$	C, 32.94; H, 3.53; N, 16.47
									C, 33.15; H, 3.49; N, 16.54
6s	2-ethoxycarbonyl-3-thienyl	NH	O	N	H_2	3.6	134−135	$C_6H_{10}N_3O_3PS \cdot {}^{1}/_{2}HCl$	C, 28.43; H, 4.15; N, 16.58
									C, 28.08; H, 4.64; N, 16.19
6t	6-ethoxy-2-benzothiazolyl	NH	O	N	H_2	4.9	171−172	$C_9H_{13}N_4O_2PS \cdot {}^{1}/_{4}HCl$	C, 38.42; H, 4.71; N, 19.92
									C, 38.37; H, 4.71; N, 19.64
6u	6-fluoro-2-benzothiazolyl	NH	O	N	H_2	34.2	179−180	$C_7H_8FN_4OPS \cdot {}^{1}/_{8}H_2O$	C, 33.84; H, 3.32; N, 22.56
									C, 33.96; H, 3.25; N, 22.27
6v	4-methoxy-2-benzothiazolyl	NH	O	N	H_2	27.4	184−185	$C_8H_{11}N_4PO_2S$	C, 37.21; H, 4.26; N, 21.70
									C, 37.43; H, 4.40; N, 21.49
6w	4-methyl-2-benzothiazolyl	NH	O	N	H_2	12.4	179−180	$C_8H_{11}N_4OPS \cdot {}^{1}/_{6}HCl$	C, 38.70; H, 4.50; N, 22.57
									C, 38.90; H, 4.56; N, 22.63

Table 1. Continued



ref	R	X	Y	Z	R′	yield (%)	mp (°C)	C, H, N	% calcd/found
6x	3-nitro-2-pyridyl	NH	O	N	H_2	1.4	212−213	$C_5H_8N_5O_3P$	C, 27.65; H, 3.69; N, 32.26 C, 27.73; H, 3.70; N, 32.58
6y	2-difluoromethoxyphenyl	NH	O	N	H_2	12.5	111−112	$C_7H_{10}F_2N_3O_2P$	C, 32.91; H, 4.11; N, 16.45 C, 32.58; H, 4.30; N, 16.47
6z	2-methoxy-4-nitrophenyl	NH	O	N	H_2	28.2	153−154	$C_7H_{11}N_4O_4P$	C, 34.15; H, 4.47; N, 22.76 C, 34.32; H, 4.42; N, 22.32
7a	2-benzothiazolyl	NH	S	N	H_2	37.3	111−112	$C_7H_9N_4PS_2$	C, 34.42; H, 3.69; N, 22.95 C, 34.13; H, 3.86; N, 22.67
7b	4-benzyloxyphenyl	NH	S	N	H_2	8.9	156−157	$C_{13}H_{16}N_3OPS \cdot {}^1/_2HCl$	C, 50.12; H, 5.30; N, 13.49 C, 49.98; H, 5.41; N, 13.35
7c	3-morpholinylpropyl	NH	S	N	H_2	52.2	170−171	$C_7H_{19}N_4OPS \cdot {}^1/_8HCl$	C, 34.63; H, 7.88; N, 23.09 C, 34.53; H, 7.76; N, 23.12
7d	4-phenoxyphenyl	NH	S	N	H_2	9.6	122−123	$C_{12}H_{14}N_3OPS \cdot {}^1/_4HCl$	C, 49.98; H, 4.94; N, 14.58 C, 49.90; H, 5.18; N, 14.31
7e	2-phenylethyl	NH	S	N	H_2	35.8	92−93	$C_8H_{14}N_3PS$	C, 44.65; H, 6.51; N, 19.53 C, 44.73; H, 6.48; N, 19.83
7f	3-methylpyridyl	NH	S	N	H_2	11.9	142−143	$C_6H_{11}N_4PS$	C, 35.64; H, 5.44; N, 27.72 C, 35.29; H, 5.38; N, 27.48

MATERIALS AND METHODS

Chemistry and Synthetic Methods. Melting points were determined with a Mettler FP82+FP80 apparatus (Greifense, Switzerland) and are uncorrected. The ^{1}H NMR spectra were recorded on a Bruker 400 Ultrashield (Rheinstetten, Germany) spectrometer, using tetramethylsilane (TMS) as the internal standard. Infrared spectra (IR) were obtained using a Thermo Nicolet FT-IR Nexus spectrophotometer with samples as KBr pellets. Elemental microanalyses were carried out on vacuum-dried samples using an elemental analyzer (LECO, CHN-900 elemental analyzer). Mass spectra were recorded using a Hewlett-Packard MSD 5973N spectrometer (GC 6890plus/direct insertion probe, DIP). Silica gel 60 (0.040−0.063 mm) 1.09385.2500 (Merck KGaA, Darmstadt, Germany) was used for column chromatography and Alugram SIL G/UV$_{254}$ (layer = 0.2 mm) (Macherey-Nagel GmbH & Co. KG, Düren, Germany) was used for thin layer chromatography. All starting materials were commercially available research grade chemicals and were used without further purification. Chemicals were purchased from E. Merck (Darmstadt, Germany), Scharlau (FEROSA, Barcelona, Spain), Panreac Química S.A. (Montcada i Reixac, Barcelona, Spain), Sigma-Aldrich Química, S.A. (Alcobendas, Madrid, Spain), Acros Organics (Janssen Pharmaceuticalaan 3a, Geel, Belgium), or Lancaster (Bischheim-Strasbourg, France). The experimental data for the synthesized compounds are shown in **Table 1**, and the spectroscopic data are given in **Table 2**.

Preparation of O-Substituted Diamidophosphates. *General Procedure for Compounds **1a,b.*** Ammonia gas was bubbled rapidly for 10 min through a solution of the appropriate dichloride intermediate (10 mmol) in dichloromethane (CH_2Cl_2) (50 mL). The resulting precipitate was collected and was extracted into boiling ethyl acetate (100 mL) for 2 h. The mixture was filtered while hot. The product, which crystallized from the filtrate when stored in the cold overnight, was isolated and purified by recrystallization from ethyl acetate.

*4-Nitrophenyldiamidophosphate (**1a**):* from 4-nitrophenyldichloridophosphate.

*Phenyldiamidophosphate (**1b**):* from phenyldichloridophosphate.

*General Procedure for O-Substituted Diamidophosphates **1c−e.*** A solution of the appropriate alcohol (10 mmol) in dry dioxane (50 mL) was added to a solution of phosphoryl chloride (80 mmol) and potassium carbonate (20 mmol) in dry dioxane (50 mL). The mixture was heated under reflux for 24 h. The solution was filtered, and the solvents were removed under reduced pressure. The residue was suspended in CH_2Cl_2, and ammonia gas was bubbled through for 10 min. The resulting precipitate was collected and was extracted into boiling ethyl acetate (100 mL) for 2 h. The mixture was filtered while hot. The product, which crystallized from the filtrate when stored in the cold overnight, was isolated and purified by recrystallization from ethyl acetate.

*3,5-Dimethylphenyldiamidophosphate (**1c**):* from 3,5-dimethylphenol.

*4-Benzyloxyphenyldiamidophosphate (**1d**):* from 4-benzyloxyphenol.

*4-Methyl-2-nitrophenyldiamidophosphate (**1e**):* from 4-methyl-2-nitrophenol.

Preparation of *N*-Alkyl-Substituted Diamidophosphates. *General Procedure for Compounds **2a−**.* The appropriate amine (20 mmol) was added dropwise to a solution of the appropriate dichloride intermediate (5 mmol) in diethyl ether (100 mL). The mixture was stirred for 10 h. The solution was filtered, and the diethyl ether was removed under reduced pressure. The residue was suspended in dry *n*-hexane (50 mL) and filtered off. The resulting solid was recrystallized from *n*-hexane/methanol.

*4-Nitrophenyl-N,N,N′,N′-tetraethyldiamidophosphate (**2a**):* from diethylamine and 4-nitrophenyldichloridophosphate.

*4-Nitrophenyl-N,N,N′,N′-tetrabutyldiamidophosphate (**2b**):* from dibutylamine and 4-nitrophenyldichloridophosphate.

***O*-[4-(Benzyloxy)phenyl]diamidothiophosphate (3).** A solution of 4-benzyloxyphenol (10 mmol, 2.00 g) in dry dioxane (50 mL) was added to a solution of thiophosphoryl chloride (80 mmol, 13.5 g, 8.5 mL) and potassium carbonate (20 mmol, 2.12 g) in dry dioxane (50 mL). The mixture was heated under reflux for 24 h. The solution was filtered, and the solvents were removed from the filtrate under reduced pressure. The residue was suspended in CH_2Cl_2, and ammonia gas was bubbled through the mixture for 10 min. The resulting precipitate was collected and was extracted into boiling ethyl acetate (100 mL) for 2 h. The mixture was filtered while hot. The product, which crystallized from the filtrate when stored in the cold overnight, was isolated and purified by recrystallization from ethyl acetate: yield = 14.1%; mp 145−146 °C; IR (KBr), υ_{max} 3389, 3246, 3150−3000, 2900, 1504, 1197, 1014, 927, 825 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 4.75 (s, 4H, 2 NH_2, exchangeable with D_2O), 5.06 (s, 2H, CH_2), 6.96 (d, 2H, H_3 + H_5, J_{3-2} = 9.1 Hz), 7.11 (dd, 2H, H_2 + H_6, J_{2-3} = 9.0 Hz), 7.34 (dt, 1H, $H_{4'}$, $J_{4'-3'}$ = 7.2 Hz), 7.40 (t, 2H, $H_{3'}$ + $H_{5'}$), 7.45 (d, 2H, $H_{2'}$ + $H_{6'}$, $J_{2'-3'}$ = 6.8 Hz); MS, *m*/*z* (abundance) 294 ($M^{\bullet+}$, 61), 200 (6), 95 (31), 91 (100). Anal. Calcd for $C_{13}H_{15}N_2O_2PS$ (%): C, 53.06; H, 5.10; N, 9.52. Found: C, 53.11; H, 5.28; N, 9.34.

Preparation of *N*-(Pyridin-2-ylmethyl)phosphoramidic acid (4). A solution of 2-aminomethylpyridine (2.03 mL, 30 mmmol) and triethylamine (4.18 mL, 30 mol) in diethyl ether (10 mL) was added dropwise to a cooled (ice bath) solution of phosphoryl chloride (2.79 mL, 30 mmol) in diethyl ether (50 mL) under nitrogen. The mixture was stirred for 24 h at room temperature. The solution was filtered,

Table 2. Spectroscopic Data (IR, ^{1}H NMR, and MS) for Synthesized Compounds

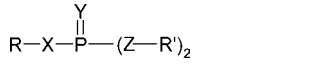
ref	IR (KBr, cm^{-1})	^{1}H NMR (DMSO-$_{d6}$, 400 MHz) δ (J, Hz)	MS (m/z % abundance)
1a	3420−3223, 1200	4.55 (bs, 4H, 2 NH_2[a]), 7.42 (d, 2H, H_2, H_6, J_{2-3} = 5.8), 8.24 (d, 2H, H_3, H_5, J_{3-2} = 6.5)	217 ($M^{\bullet+}$, 5), 95 (100)
1b	3366−3229, 1182	4.33 (d, 4H, 2 NH_2[a]), 7.09 (dt, 1H, H_4), 7.18 (d, 2H, H_2, H_6, J_{2-3} = 8.2), 7.30 (t, 2H, H_3, H_5)	172 ($M^{\bullet+}$, 87), 94 (100)
1c	3435−3322, 1190	2.23 (s, 6H, 2 CH_3), 4.28 (s, 4H, NH_2[a]), 6.72 (s, 1H, H_4); 6.80 (s, 2H, H_2, H_6)	200 ($M^{\bullet+}$, 40), 122 (100)
1d	3428−3367, 1225	4.25 (bs, 4H, 2 NH_2[a]), 5.04 (s, 2H, CH_2), 6.92 (d, 2H, H_3, H_5, J_{3-2} = 9.0), 7.08 (d, 2H, H_2, H_6, J_{2-3} = 8.9), 7.32 (d, 2H, $H_{2'}$ $H_{6'}$), 7.38 (t, 1H, H_4), 7.42 (t, 2H, $H_{3'}$, $H_{5'}$)	278 ($M^{\bullet+}$, 7), 91 (100)
1e	3446−3298	2.28 (s, 3H, CH_3), 7.30 (dd, 1H, H_5, J_{5-6} = 8.5), 7.33 (bs, 4H, 2 NH_2[a]), 7.53 (s, 1H, H_3), 7.62 (d, 1H, H_6, J_{6-5} = 8.5)	231 ($M^{\bullet+}$, 2), 77 (100)
2a	(NaCl) 3178−3109, 1190	1.12 (t, 12H, $CH_2\underline{CH_3}$, J = 7.1), 3.16 (m, 8H, $\underline{CH_2}CH_3$), 7.41 (d, 2H, H_2, H_6, J_{2-3} = 9.3), 8.22 (dd, 2H, H_3, H_5, J_{3-2} = 9.2)	
2b	3095, 3083, 1222	0.87 (dt, 12H, $H_{4'}$, $H_{4''}$, $J_{4'-3'}$ = 7.4), 1.28 (m, 8H, $H_{3'}$, $H_{3''}$), 1.46 (m, 4H, $H_{2'}$), 1.76 (m, 4H, $H_{2''}$), 2.77 (m, 4H, $H_{1'}$), 3.11 (m, 4H, $H_{1''}$), 7.33 (d, 2H, H_2, H_6, J_{2-3} = 9.3), 8.15 (d, 2H, H_3, H_5, J_{3-2} = 9.2), 10.02 (bs, HCl[a])	441 ($M^{\bullet+}$, 5), 86 (100)
3	3389−3246, 825	4.75 (s, 4H, 2 NH_2[a]), 5.06 (s, 2H, CH_2), 6.96 (d, 2H, H_3, H_5, J_{3-2} = 9.1), 7.11 (dd, 2H, H_2, H_6, J_{2-3} = 9.0), 7.34 (dt, 1H, $H_{4'}$, $J_{4'-3'}$ = 7.2), 7.40 (t, 2H, $H_{3'}$, $H_{5'}$), 7.45 (d, 2H, $H_{2'}$, $H_{6'}$, $J_{2'-3'}$ = 6.8)	294 ($M^{\bullet+}$, 61), 91 (100)
4	3423, 1232	4.24 (bs, 2H, CH_2), 7.99 (s, 1H, H_5), 8.62 (s, 1H, H_4), 8.88 (bs, 4H, H_6, NH, 2 OH[a]), 9.04 (s, 1H, H_2)	188 ($M^{\bullet+}$, 5), 69 (100)
5a	3377, 1216	2.12 (s, 3H, Ar−CH_3), 3.45 (dd, 6H, 2 OCH_3), 6.68 (t, 1H, H_5), 7.62 (d, 1H, H_4, J_{4-5} = 7.1), 7.75 (d, 1H, H_6, J_{6-5} = 6.0), 7.77 (bs, 1H, NH[a])	216 ($M^{\bullet+}$, 5), 96 (100)
5b	3404, 1185	3.64 (d, 6H, 2 OCH_3), 7.18 (t, 1H, H_6), 7.32 (d, 2H, H_5, H_4), 7.70 (d, 1H, H_7, J_{7-6} = 7.9), 12.2 (bs, 1H, NH[a])	258 ($M^{\bullet+}$, 100)
6a	3416−3254, 1259	3.24 (m, 4H, CH_2N), 3.66 (t, 4H, CH_2Cl, J_{2-1} = 7.6), 3.77 (bs, 4H, NH_2[a])	220 ($M^{\bullet+}$, 10), 92 (100)
6b	3420−3100, 1261	1.55 (s, 6H, 2 H_4, 2 H_7, 2 H_9), 1.64 (s, 2H, H_{10}), 1.82 (s, 4H, 2H_2, 2H_6), 1.96 (s, 2H, H_3, H_5), 2.09 (s, 1H, H_8), 3.09 (bs, 1H, NH[a]), 3.35 (s, 4H, 2NH_2[a])	229 ($M^{\bullet+}$, 15), 94 (100)
6c	3433−3225, 1199	4.06 (s, 4H, 2 NH_2), 6.71 (d, 1H, NH), 7.36 (m, 2H, H_3, H_4), 7.46 (s, 2H, H_6, H_7), 7.56 (d, 1H, H_2, J_{2-3} = 6.9), 7.82 (s, 1H, H_8), 8.22 (s, 1H, H_5)	221 ($M^{\bullet+}$, 15), 191 (100)
6d	3423−3335, 1202	3.67 (s, 3H, CH_3), 3.93 (bs, 4H, 2 NH_2[a]), 6.31 (dd, 1H, H_4, J_{4-5} = 8.1), 6.68 (d, 1H, H_6, J_{6-5} = 8.1), 6.75 (t, 1H, H_2), 6.86 (d, 1H, NH[a]), 7.00 (t, 1H, H_5)	201 ($M^{\bullet+}$, 48), 79 (100)
6e	3445−3293, 1202	1.22 (t, 1H, $H_{4'a}$), 1.33 (m, 4H, $H_{3'e}$, $H_{2'a}$, $H_{5'e}$, $H_{6'a}$), 1.71 (m, 5H, $H_{4'e}$, $H_{3'a}$, $H_{5'a}$, $H_{2'e}$, $H_{6'e}$), 2.34 ($H_{1'a}$), 3.88 (bs, 4H, 2 NH_2[a]), 6.71 (d, 1H, NH[a]), 6.94 (d, 2H, H_3, H_5, J_{3-2} = 8.5), 7.01 (d, 2H, H_2, H_6, J_{2-3} = 8.5)	253 ($M^{\bullet+}$, 93),132 (100)
6f	3378−3261, 1229	2.28 (s, 3H, CH_3), 4.48 (d, 4H, 2 NH_2[a]), 7.44 (dd, 1H, H_5, J_{5-6} = 8.8, J_{5-3} = 1.7), 7.83 (d, 1H, H_6, J_{6-5} = 8.7), 7.92 (s, 1H, H_3), 8.24 (d, 1H, NH[a])	230 ($M^{\bullet+}$, 27), 184 (100)
6g	3393−3245, 1145	3.86 (bs, 4H, 2 NH_2[a]), 4.99 (s, 2H, CH_2), 6.63 (d, 1H, NH[a]), 6.80 (d, 2H, H_3, H_5, J_{3-2} = 8.8), 7.04 (d, 2H, H_2, H_6, J_{2-3} = 8.8), 7.32 (d, 2H, $H_{2'}$, $H_{6'}$, $J_{2'-3'}$ = 7.0), 7.38 (t, 1H, $H_{4'}$, $J_{4'-5'}$ = $J_{4'-3'}$ = 7.4), 7.41 (t, 2H, $H_{3'}$, $H_{5'}$)	277 ($M^{\bullet+}$, 5), 108 (100)
6h	3407−3249, 1221	3.66−3.74 (s, 6H, 2 CH_3), 4.10 (s, 4H, 2 NH_2[a]), 5.45 (d, 1H, NH[a]), 6.29 (dd, 1H, H_4, J_{4-3} = 8.7, J_{4-6} = 3.2), 6.79 (d, 1H, H_3, J_{3-4} = 8.8), 7.09 (d, 1H, H_6, J_{6-4} = 2.8)	231 ($M^{\bullet+}$, 58), 138 (100)
6i	3374−3233, 1148	3.96 (bs, 4H, 2 NH_2[a]), 6.86 (t, 4H, H_3, H_5, $H_{2'}$, $H_{6'}$), 6.93 (d, 1H, NH[a]), 7.02 (t, 1H, $H_{4'}$, $J_{4'-3'}$ = 6.7), 7.15 (d, 2H, H_2, H_6, J_{2-3} = 7.9), 7.31 (t, 2H, $H_{3'}$, $H_{5'}$)	263 ($M^{\bullet+}$, 36), 185 (100)
6j	3394−3251, 1244	2.70 (t, 2H, CH_2Ph, J_{2-1} = 7.5), 2.94 (m, 2H, CH_2NH), 3.36−3.43 (bs, 5H, 2H_2, NH[a]), 7.10 (t, 2H, $H_{3'}$, $H_{5'}$), 7.24 (m, 2H, $H_{2'}$, $H_{6'}$)	217 ($M^{\bullet+}$, 5), 109 (100)
6k	3401−3262, 1207	4.30 (bs, 4H, 2 NH_2[a]), 7.11 (t, 1H, H_6), 7.27 (t, 1H, H_5), 7.50 (d, 1H, H_4, J_{4-5} = 7.8), 7.74 (d, 1H, H_7, J_{7-6} = 7.4), 9.13 (bs, 1H, NH[a])	228 ($M^{\bullet+}$, 25), 150 (100)
6l	3415, 1175	1.55 (q, 2H, 2 H_2), 2.30 (m, 6H, 2 H_1, 2 $H_{3'}$, 2 $H_{5'}$), 2.77 (q, 2H, 2 H_3), 3.38 (bs, 5H, NH, 2 NH_2[a]), 3.56 (t, 4H, 2 $H_{2'}$, 2 $H_{6'}$)	222 ($M^{\bullet+}$, 5), 100 (100)
6m	3427−3226, 1184	4.20 (bs, 4H, 2 NH_2[a]), 7.46 (m, 3H, H_6, H_7, NH), 7.70 (m, 1H, H_5), 7.84 (m, 1H, H_8), 7.87 (d, 1H, H_4), 8.74 (d, 1H, H_2)	222 ($M^{\bullet+}$, 10), 144 (100)
6n	3391−3248, 1147	3.94 (bs, 4H, 2 NH_2[a]), 6.72 (dt, 1H, H_4, J_{4-3} = 8.5, J_{4-2} = 4.2), 6.87 (d, 1H, NH[a]), 7.10 (d, 4H, H_2, H_3, H_5, H_6, J = 4.1)	171 ($M^{\bullet+}$, 63), 93 (100)
6o	3480−3363, 1182	4.28 (bs, 4H, 2 NH_2[a]), 7.27 (d, 2H, H_2, H_6, J_{2-3} = 9.2), 8.04 (d, 2H, H_3, H_5, J_{3-2} = 9.2), 8.08 (d, 1H, NH[a])	216 ($M^{\bullet+}$, 63), 108 (100)
6p	3430−3232, 1241	4.53 (s, 4H, 2NH_2[a]), 6.96 (t, 1H, H_4), 7.60 (t, 1H, H_5), 7.92 (d, 1H, H_6, J_{6-5} = 8.5), 8.11 (d, 1H, H_3, J_{3-4} = 8.5), 8.33 (d, 1H, NH[a])	216 ($M^{\bullet+}$, 25), 170 (100)
6q	3388−3252, 1145	2.72 (t, 2H, CH_2Ph), 2.96 (dd, 2H, CH_2NH, J_{1-2} = 16.3, 8.9), 3.40 (bs, 5H, 2H_2, NH[a]), 7.19 (dd, 3H, $H_{2'}$, $H_{6'}$, $H_{5'}$, J = 12.4, 7.1), 7.29 (t, 2H, $H_{3'}$, $H_{5'}$)	199 ($M^{\bullet+}$, 8), 108 (100)
6r	3441−3252, 1220	4.20 (bs, 4H, 2 NH_2[a]), 5.86 (d, 1H, NH, J = 7.1), 6.85 (dt, 1H, H_5, J = 8.2), 7.19 (t, 1H, H_4, J = 7.3), 7.23 (dd, 1H, H_3, J_{3-4} = 8.2, J_{3-5} = 1.4), 7.69 (dd, 1H, H_6, J_{6-5} = 8.3, J_{6-4} = 1.3)	255 ($M^{\bullet+}$, 100), 177 (100)
6s	3404−3329, 1248	3.20−3.53 (bs, 4H, 2 NH_2), 3.79 (s, 3H, OCH_3), 7.34 (d, 1H, H_4, J_{4-5} = 5.5), 7.38−7.43 (bs, 1H, NH·HCl[a]), 7.55 (d, 1H, H5, J_{5-4} = 5.5)	235 ($M^{\bullet+}$, 5), 125 (100)
6t	3380−3265, 1228	1.33 (t, 3H, CH_3, J = 6.9), 4.02 (q, 2H, CH_2, J = 6.9), 4.24 (bs, 4H, 2 NH_2[a]), 6.86 (dd, 1H, H_5, J_{5-4} = 8.7, J_{5-7} = 2.5), 7.36 (ds, 1H, H_7, J_{7-5} = 2.4), 7.39 (d, 1H, H_4, J_{4-5} = 8.7), 8.90 (bs, 1H, NH[a])	272 ($M^{\bullet+}$, 30), 165 (100)
6u	3398−3224, 1194	4.30 (bs, 4H, 2 NH_2[a]), 7.11 (dt, 1H, H_7, J = 9.2), 7.49 (dd, 1H, H_5, J_{5-4} = 8.7), 7.68 (dd, 1H, H_4, J_{4-5} = 8.8), 9.12 (bs, 1H, NH[a])	246 ($M^{\bullet+}$, 75), 168 (100)
6v	3382−3360, 1155	3.86 (s, 3H, CH_3), 4.28 (bs, 4H, 2 NH_2[a]), 6.87 (d, 1H, H_5, J_{5-6} = 7.9), 7.07 (t, 1H, H_6, J_{6-5} = J_{6-7} = 8.0), 7.33 (d, 1H, H_7, J_{7-6} = 7.8), 8.90 (bs, 1H, NH[a])	258 ($M^{\bullet+}$, 84), 79 (100)
6w	3378−3246, 1184	2.50 (s, 3H, CH_3), 4.27 (bs, 4H, 2 NH_2[a]), 7.01 (t, 1H, H_6, J_{6-5} = J_{6-7} = 7.6), 7.10 (d, 1H, H_5, J_{5-6} = 7.2), 7.57 (d, 1H, H_7, J_{7-6} = 7.7), 9.08 (bs, 1H, NH[a])	242 ($M^{\bullet+}$, 70), 79 (100)

Table 2. Continued

ref	IR (KBr, cm^{-1})	^1H NMR (DMSO-$_{d6}$, 400 MHz) δ (J, Hz)	MS (m/z % abundance)
6x	3417−3202, 1195	4.35 (s, 4H, 2 NH_2[a]), 7.06 (dd, 1H, H_6, J_{6-5} = 8.4, J_{6-4} = 4.6), 8.55 (m, 3H, H_4, H_5, NH)	217 ($M^{\bullet+}$, 6), 171 (100)
6y	3347−3206, 1190	4.17 (bs, 4H, 2 NH_2[a]), 5.58 (dd, 1H, CH), 6.76−6.83 (m, 1H, H_6), 7.10−7.19 (m, 3H, H_4, H_5, NH), 7.60 (dd, 1H, H_3)	237 ($M^{\bullet+}$, 58), 159 (100)
6z	3425−3224, 1185	3.95 (s, 3H, OCH_3), 4.37−4.38 (bs, 4H, 2 NH_2[a]), 6.26−6.28 (d, 1H, NH[a]), 7.65 (d, 1H, H_5, J_{5-6} = 9.0), 7.70 (s, 1H, H_3), 7.83 (d, 1H, H_6, J_{6-5} = 8.9)	246 ($M^{\bullet+}$, 38), 57 (100)
7a	3372−3277, 883	3.37 (bs, 1H, NH[a]), 4.58 (s, 4H, 2 NH_2[a]), 7.14 (dt, 1H, H_3), 7.30 (dt, 1H, H_4), 7.52 (d, 1H, H_5, J_{5-4} = 8.0), 7.78 (d, 1H, H_2, J_{2-3} = 7.9)	244 ($M^{\bullet+}$, 10), 150 (100)
7b	3425−3300, 734	4.23 (bs, 4H, 2 NH_2[a]), 4.97 (s, 2H, CH_2), 6.78 (d, 1H, NH[a]), 6.82 (d, 2H, H_3, H_5, J_{3-2} = 8.9), 7.12 (d, 2H, H_2, H_6, J_{2-3} = 8.8), 7.32 (m, 1H, $H_{4'}$), 7.38 (m, 4H, $H_{2'}$, $H_{3'}$, $H_{5'}$, $H_{6'}$)	293 ($M^{\bullet+}$, 23), 108 (100)
7c	3415−3258, 863	1.59 (q, 2H, $2H_2$), 2.30 (m, 6H, 2 H_3, 2 $H_{3'}$, 2 $H_{5'}$), 2.82 (q, 2H, $2H_1$), 3.37 (bs, 4H, 2 NH_2[a]), 3.56 (t, 4H, 2 $H_{2'}$, 2 $H_{6'}$), 3.80 (bs, 1H, NH[a])	238 ($M^{\bullet+}$, 7), 57 (100)
7d	3403−3224, 868	4.34 (bs, 4H, 2 NH_2[a]), 6.88 (t, 4H, H_3, H_5, $H_{2'}$, $H_{6'}$), 7.04 (t, 1H, $H_{4'}$, $J_{4'-3'}$ = 7.4), 7.12 (d, 1H, NH[a]), 7.24 (d, 2H, H_2, H_6, J_{2-3} = 8.8), 7.32 (t, 2H, $H_{3'}$, $H_{5'}$)	279 ($M^{\bullet+}$, 82), 185 (100)
7e	3343−3255, 895	2.75 (t, 2H, CH_2 (C2), J_{1-2} = 7.7), 3.01 (m, 2H, CH_2 (C1)), 3.80 (bs, 5H, 2 NH_2, NH[a]), 7.19 (t, 3H, $H_{2'}$, $H_{4'}$, $H_{6'}$), 7.29 (t, 2H, $H_{3'}$, $H_{4'}$)	215 ($M^{\bullet+}$, 50), 124 (100)
7f	3367−3280, 892	3.95 (bs, 4H, 2 NH_2[a]), 4.05 (m, 2H, CH_2), 4.53 (d, 1H, NH[a]), 7.31 (dt, 1H, H_5, J_{5-4} = 7.7, J_{5-6} = 4.8), 7.80 (d, 1H, H_4, J_{4-5} = 7.8), 8.41 (d, 1H, H_6, J_{6-5} = 4.6), 8.56 (s, 1H, H_2)	202 ($M^{\bullet+}$, 26), 107 (100)

[a] Exchangeable with D_2O.

Table 3. Urease Inhibitory Activity of More Active Compounds



ref	R	X	Y	Z	R′	IC_{50}(nM) jack bean
1a	4-nitrophenyl	O	O	N	H_2	63
1c	3,5-dimethylphenyl	O	O	N	H_2	26
1d	4-benzyloxyphenyl	O	O	N	H_2	16
6f	4-methyl-2-nitrophenyl	NH	O	N	H_2	3.5
6j	2-(4-fluorophenyl)ethyl	NH	O	N	H_2	49
6k	2-benzothiazolyl	NH	O	N	H_2	2
6o	4-nitrophenyl	NH	O	N	H_2	300
6p	2-nitrophenyl	NH	O	N	H_2	3
6q	2-phenylethyl	NH	O	N	H_2	14
6t	6-ethoxy-2-benzothiazolyl	NH	O	N	H_2	5
6u	6-fluoro-2-benzothiazolyl	NH	O	N	H_2	5
6v	4-methoxy-2-benzothiazolyl	NH	O	N	H_2	10
6w	4-methyl-2-benzothiazolyl	NH	O	N	H_2	10
6x	3-nitro-2-pyridyl	NH	O	N	H_2	10
6z	2-methoxy-4-nitrophenyl	NH	O	N	H_2	20
PPD[a]						3
NBPT[a]						100

[a] As reference.

and the solvents were removed under reduced pressure. The residue was suspended in methanol, and ammonia gas was bubbled through for 10 min. The solvent was removed, and the resulting solid was suspended in water (100 mL), filtered off, washed with water, and recrystallized from methanol/water: yield = 2.4%; mp 174−175 °C; IR (KBr), υ_{max} 3423, 3100−3000, 3000−2887, 1605, 1549, 1465, 1232, 1127, 910 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 4.24 (bs, 2H, CH_2), 7.99 (s, 1H, H_5), 8.62 (s, 1H, H_4), 8.88 (bs, 4H, H_6 + NH + 2OH, exchangeable with D_2O), 9.04 (s, 1H, H_2); MS, m/z (abundance) 188 ($M^{\bullet+}$, 5), 124 (55), 69 (100). Anal. Calcd for $C_6H_9N_2O_3P$ (%): C, 38.29; H, 4.79; N, 14.89. Found: C, 38.41; H, 5.15; N, 14.52.

Preparation of N-Substituted Amidophosphates. *General Procedure for Compounds **5a,b**.* A solution of the appropriate amine (40 mmol) in CH_2Cl_2 (20 mL) was added dropwise to a solution of phosphoryl chloride (7.40 mL, 80 mmol) in CH_2Cl_2 (50 mL) under nitrogen. The mixture was stirred for 24 h at room temperature. The solution was filtered, and the solvents were removed under reduced pressure. The residue was suspended in methanol, and ammonia gas was bubbled through for 10 min. The solvent was removed, and the resulting solid was extracted into boiling ethyl acetate (100 mL) for 2 h. The mixture was filtered while hot. The product, which crystallized from the filtrate when stored in the cold overnight, was isolated and purified by recrystallization from ethyl acetate.

*Dimethyl N-[(3-methyl)pyridin-2-yl]amidophosphate (**5a**):* from 3-methyl-2-aminopyridine.

*Dimethyl N-[(1,3-benzothiazol)-2-yl]amidophosphate (**5b**):* from 2-aminobenzothiazole.

Preparation of Phosphorictriamides. *General Procedure for Compounds **6a−z**.* A solution of the appropriate amine (20 mmol) in dry CH_2Cl_2 (20 mL) was added dropwise to a cooled (ice bath) solution of phosphoryl chloride (80 mmol) in dry CH_2Cl_2 (50 mL) under nitrogen. The mixture was stirred for 10 h at room temperature. The solution was filtered, and the solvents were removed under reduced pressure. The residue was suspended in CH_2Cl_2, and ammonia gas was bubbled through for 10 min. The resulting solid was collected and extracted into boiling ethyl acetate (100 mL) for 2 h. The mixture was filtered while hot. The product, which crystallized from the filtrate when stored in the cold overnight, was isolated and purified by recrystallization from ethyl acetate.

*N,N-Bis(2-chloroethyl)phosphorictriamide (**6a**):* from bis(2-chloroethyl)amine.

*N-(1-Adamantyl)phosphorictriamide (**6b**):* from 1-adamantylamine.

*N-(α-Naphthyl)phosphorictriamide (**6c**):* from 1-naphthylamine.

*N-(3-Methoxyphenyl)phosphorictriamide (**6d**):* from 3-methoxyaniline.

*N-(4-Cyclohexylphenyl)phosphorictriamide (**6e**):* from 4-cyclohexylaniline.

*N-(4-Methyl-2-nitrophenyl)phosphorictriamide (**6f**):* from 4-methyl-2-nitroaniline.

*N-[4-(Benzyloxy)phenyl]phosphorictriamide (**6g**):* from 4-(benzyloxy)aniline.

*N-(2,5-Dimethoxyphenyl)phosphorictriamide (**6h**):* from 2,5-dimethoxyaniline.

*N-(4-Phenoxyphenyl)phosphorictriamide (**6i**):* from 4-phenoxyaniline.

*N-[2-(4-Fluorophenyl)ethyl]phosphorictriamide (**6j**):* from 2-(4-fluorophenyl) ethylamine.

*N-(1,3-Benzothiazol-2-yl)phosphorictriamide (**6k**):* from 1,3-benzothiazol-2-amine.

*N-(3-Morpholin-4-ylpropyl)phosphorictriamide (**6l**):* from 3-morpholin-4-ylpropan-1-amine.

*N-Quinolin-3-ylphosphorictriamide (**6m**):* from quinolin-3-amine.

*N-Phenylphosphorictriamide (**6n**):* from aniline.

*N-(4-Nitrophenyl)phosphorictriamide (**6o**):* from 4-nitroaniline.

*N-(2-Nitrophenyl)phosphorictriamide (**6p**):* from 2-nitroaniline.

*N-(2-Phenylethyl)phosphorictriamide (**6q**):* from 2-phenylethylamine.

*N-(2-Trifluoromethoxyphenyl)phosphorictriamide (**6r**):* from 2-trifluoromethoxyaniline.

*N-(2-Methoxycarbonylthien-3-yl)phosphorictriamide(**6s**):* from methyl 3-amino-2-thiophenecarboxylate.

*N-(6-Ethoxy-1,3-benzothiazol-2-yl)phosphorictriamide (**6t**):* from 2-amino-6-ethoxybenzothiazole.

*N-(6-Fluoro-1,3-benzothiazol-2-yl)phosphorictriamide (**6u**):* from 2-amino-6-fluorobenzothiazole.

Table 4. Data for in Vivo Urease Assays over Ranges of Incubation Time and pH

	% inhibition (pH 4.5)			% inhibition (pH 5.9)			% inhibition (pH 8.4)		
compd	5 days	15 days	30 days	5 days	15 days	30 days	5 days	15 days	30 days
control	0	0	0	0	0	0	0	0	0
6f	92	63	6	82	22	0	94	87	73
6k	0	7	0	0	10	0	67	3	1
6p	88	74	12	78	14	0	86	90	81
NBPT[a]	79	9	0	75	44	8	95	88	43

[a] As reference.

*N-(4-Methoxy-1,3-benzothiazol-2-yl)phosphorictriamide (**6v**):* from 2-amino-4-methoxybenzothiazole.

*N-(4-Methyl-1,3-benzothiazol-2-yl)phosphorictriamide (**6w**):* from 2-amino-4-methylbenzothiazole.

*N-[(3-Nitro)pyridin-2-yl]phosphorictriamide (**6x**):* from 2-amino-3-nitropyridine.

*N-(2-Difluoromethoxyphenyl)phosphorictriamide (**6y**):* from 2-difluoromethoxyaniline.

*N-(2-Methoxy-4-nitrophenyl)phosphorictriamide (**6z**):* from 2-methoxy-4-nitroaniline.

Preparation of Phosphorothioictriamides. *General Procedure for Compounds **7a**−**f**.* A solution of the appropriate amine (20 mmol) in dry CH_2Cl_2 (20 mL) was added dropwise to a cooled (ice bath) solution of thiophosphoryl chloride (80 mmol) in dry CH_2Cl_2 (50 mL). The mixture was stirred for 2 h at room temperature. The solution was filtered, and the solvents were removed under reduced pressure. The residue was suspended in CH_2Cl_2, and ammonia gas was bubbled through for 10 min. The resulting solid was collected and was extracted into boiling ethyl acetate (100 mL) for 2 h. The mixture was filtered while hot. The product, which crystallized from the filtrate when stored in the cold overnight, was isolated and purified by recrystallization from ethyl acetate.

*N-(1,3-Benzothiazol-2-yl)phosphorothioictriamide (**7a**):* from 1,3-benzothiazol-2-amine.

*N-[4-(Benzyloxy)phenyl]phosphorothioictriamide (**7b**):* from 4-(benzyloxy)aniline.

*N-(3-Morpholin-4-ylpropyl)phoshorothioictriamide (**7c**):* from 3-morpholin-4-ylpropan-1-amine.

*N-(4-Phenoxyphenyl)phosphorothioictriamide (**7d**):* from 4-phenoxyaniline.

*N-(2-Phenylethyl)phosphorothioictriamide (**7e**):* from 2-phenylethylamine.

*N-(Pyridin-3-ylmethyl)phosphorothioictriamide (**7f**):* from 1-pyridin-3-ylmethanamine.

Preparation of Phenylphosphonic Acid. A solution of phenylphosphonic dichloride (5.85 g, 30 mmol) in water was stirred for 2 h. A solution of 20% NaOH (30 mL) was added to give a pH 6−7. The solid was filtered off and recrystallized from methanol/water: yield = 72.0%; mp 163−164 °C; IR (KBr), υ_{max} 3200−2850, 1459, 1144, 1083, 1019, 938 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 7.26 (bs, 2H, OH, exchangeable with D_2O), 7.47 (m, 3H, $2H_3$, H_4), 7.68 (m, 2H, $2H_2$); MS, *m/z* (abundance) 158 ($M^{\bullet+}$, 5), 124 (48), 95 (51), 77 (40), 69 (28). Anal. Calcd for $C_6H_7O_3P$ (%): C, 45.57; H, 4.43; N, 0.00. Found: C, 45.36; H, 4.38; N, 0.03.

Molecular Modeling Methods. All of the computational work was performed on *SiliconGraphics* Octane2 workstations by applying the software package InsightII. The starting atomic coordinates were obtained from the structurally related CSD (*35*) structure PPOSAM shown in **Figure 2c**,**d** (CSD System version 5.26: search and information retrieval with ConQuest (*36*) version 1.7; structure visualization with Mercury (*37*) version 1.3). The three-dimensional models of the studied compounds were constructed using atoms and structural fragments from the *Builder* module. The protocol can be summed up as follows: (a) Initial construction of the model. (b) Hierarchized systematic conformational analysis: determination of the rotation-sensitive bonds; election of a 30° window to check each selected dihedron. First filtration: elimination of the conformations that are nondistinguishable by symmetry. Second filtration: elimination of conformations that present steric impediments. Third filtration: calculation of the energy of conformations and elimination of those conformations having a relative energy >10 kcal/mol at a global minimum. Fourth filtration: optimization of the geometry of the conformations and elimination of those having a relative energy >10 kcal/mol at a global minimum. All of the molecular mechanics calculations were carried out using the consistent valence force field ESFF (*38*) (*Search and Compare* module, InsightII). (c) Analysis of conformational trajectory (*Analysis* module InsightII) and selection of representative lowest energy conformation. Root mean square (rms) deviations of the structures were monitored. (d) Mechano-quantics optimization of the conformations obtained in the previous step, with the molecular orbital calculations package *Mopac* (AM1 (*39*) semiempirical approach, *AMPAC*/*MOPAC* InsightII module). The first approach to the study for the proposal of a union mode for compound **6f** in the catalytic site in the enzyme was constructed by taking the complex DAP−BPU as template; the data were obtained for PDB (reference 3UBP). The lowest energy conformations obtained for **6f** were superimposed through a manual docking process, taking as a superposition pattern the P−O moiety on the DAP for P═O.

Biology. *In Vitro Urease Activity* (*40*). One milliliter of buffer solution (KH_2PO_4/K_2HPO_4, 70 mM, pH 7), 1 mL of inhibitor solution (at different concentrations), and 10 μL of jack bean urease solution (2.8 mg/mL, Sigma U-1500 35.500) were mixed in test tubes and incubated for 30 min at 37 °C (blank solution consisted of the same mixture without inhibitor). Afterward, 1 mL of urea solution (6 mg/mL) was added, with incubation again for 30 min at 37 °C. The reaction was stopped by the addition of 0.033 N HCl. Each inhibitor dose was tested in triplicate. The level of ammonium released was determined by ionic chromatography (Dionex DX-120), and the inhibition percentage [INH (%)] was calculated as

$$\text{INH}\ (\%) = 100 - ((A_{INH}/A_B) \times 100) \quad (1)$$

where A_{INH} and A_B are the ppm of ammonium in the test tubes with and without inhibitor, respectively.

Urease Activity in Soil. Sieved soil samples (5 g each) were placed in 60 mL glass bottles and moistened with 1.5 mL of water. After 1 h, a solution (1 mL) containing 10 mg/mL of urea and 50 μg/mL of inhibitor was added. The soil control bottle was prepared by adding 1 mL of water instead of the 5 g of soil, whereas in the blank bottle 1 mL of urea plus inhibitor solution was substituted for 1 mL of 10 mg/mL urea solution. Each treatment was tested in quintuplicate. The bottles were stoppered and incubated at room temperature for periods of 5, 10, and 15 days. After those times, incubated soil samples were extracted for 1 h with 50 mL of 2 M KCl containing 5 mg/L of phenylmercuric acetate (continuous mechanical shaking). Samples were then filtered through 8 μm filter papers, and the urea content in the extracts was determined using a modified version of the method described by Mulvaney and Bremner (*41*).

Urea Determination Procedure. A solution of 30 mL of an acidic reagent was prepared by mixing 1.5 mL of an aqueous diacetyl monoxime solution (25 mg/mL) and 0.90 mL of an aqueous thiosemicarbazide solution (2.5 mg/mL) and adding an acidic solution (10 mL of 96% H_2SO_4 + 250 mL of 85% H_3PO_4 + water up to 500 mL) until the final volume (30 mL) was obtained. Urea measurements were determined in a 96-well plate, placing in each well 0.1 mL of the extracts and 0.25 mL of acidic reagent. The plate was sealed with an adhesive film and incubated at 85 °C for 30 min, followed by cooling to 4 °C for 20 min. The film was then removed, and the absorption at

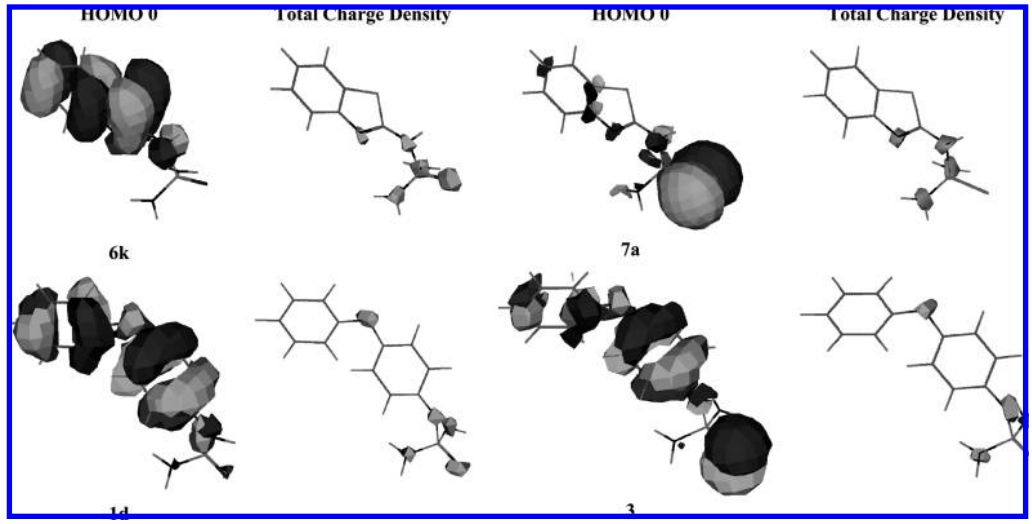


Figure 3. Influence of fragment Y: O versus S.

545 nm was measured using a UV−vis plate reader (*41*). All extracts were analyzed in triplicate.

The inhibition percentage [INH (%)] was calculated using the method proposed by Martens and Bremner (*42*)

$$\mathrm{INH}\ (\%) = 100 \times \left(\frac{\mathrm{UH_B} - \mathrm{UH_{INH}}}{\mathrm{UH_B}} \right) \qquad (2)$$

where UH_B and UH_{INH} are the amounts of urea hydrolyzed in the soil sample in the absence or presence of inhibitor, respectively.

Analyses of the Soils Employed in the Experiments. The analyses of the different soils used were as follows.

Acidic soil (Anaya de Alba, Spain): pH 4.5; electrical conductivity at 25 °C, 0.8 dS m^{-1}; organic matter content, 10.3 g kg^{-1}; 0.7 g kg^{-1} N; 33.3 mg kg^{-1} P (Olsen); 0.2 cmolc kg^{-1} K; 0.82 cmolc kg^{-1} Mg; 3.38 cmolc kg^{-1} Ca; 0.12 cmolc kg^{-1} Na; 29.14 mg kg^{-1} Fe; 0.53 mg kg^{-1} Cu; 0.05 mg kg^{-1} Zn; 75.68 mg kg^{-1} Mn; sand, 591.4 g kg^{-1}; silt, 335.7 g kg^{-1} and clay, 73 g kg^{-1}.

Moderated acidic soil (Las Planas, Spain): pH 5.9; electrical conductivity at 25 °C, 0.85 dS m^{-1}; organic matter content, 9.7 g kg^{-1}; 0.4 g kg^{-1} N; 78.6 mg kg^{-1} P (Olsen); 0.29 cmolc kg^{-1} K; 3.34 cmolc kg^{-1} Mg; 6.35 cmolc kg^{-1} Ca; 0.14 cmolc kg^{-1} Na; 13.97 mg kg^{-1} Fe; 0.35 mg kg^{-1} Cu; 0.02 mg kg^{-1} Zn; 22.88 mg kg^{-1} Mn; calcium carbonate, 13.1 g kg^{-1}; sand, 866.3 g kg^{-1}; silt, 57.1 g kg^{-1} and clay, 76.6 g kg^{-1}.

Calcareous soil (Mendigorría, Spain): pH 8.4; electrical conductivity at 25 °C, 0.89 dS m^{-1}; organic matter content, 29.9 g kg^{-1}; 4.1 g kg^{-1} N; 57 mg kg^{-1} P (Olsen); 0.25 cmolc kg^{-1} K; 2.15 cmolc kg^{-1} Mg; 19.66 cmolc kg^{-1} Ca; 1.11 cmolc kg^{-1} Na; 14.7 mg kg^{-1} Fe; 2.61 mg kg^{-1} Cu; 8.04 mg kg^{-1} Zn; 1.53 mg kg^{-1} Mn; calcium carbonate, 308.2 g kg^{-1}; sand, 459.3 g kg^{-1}; silt, 402.9 g kg^{-1} and clay, 137.8 g kg^{-1}. The methodology is described by Jackson et al. (*43*).

Reference Substances. AGROTAIN (CAS Registry No. 94317-64-3) was obtained from Agrow Australia Pty Ltd. and the composition is NBPT (25%), *N*-methylpyrrolidone (10%), and other unspecified components (60−65%). Phenylphosphorodiamidate (PPD) (phenyldiamidophosphate, **1b**) and phenylphosphonic acid were prepared as described earlier in this section.

RESULTS AND DISCUSSION

In this study a total of 40 phosphorus derivatives were synthesized. For all of the synthesized compounds in vitro inhibitory activity tests were carried out. The results for the most active compounds are given in **Table 3**. PPD and NBPT (the only commercial product currently available as an urease inhibitor) were used as the reference compounds for the assays, and their values are also included in **Table 3**.

A preliminary molecular modeling study was carried out on the synthesized compounds with the aim of obtaining data about the structure−activity relationships. In a previous phase a conformational analysis was carried out. The conformational freedom of the target compounds encouraged us to use the geometric data obtained for some structurally analogous molecules as a reference. The reference crystallographic structure for PPD (reference CSD: PPOSAM), obtained from the Cambridge Structural Database, was taken as template for building the compounds. The rotations selected for this analysis are summarized in **Figure 2a**, and the starting configuration is given in **Figure 2c**.

Once the models for the compounds had been constructed, the initial geometries were fully minimized to an energy gradient below 10^{-3} kcal mol^{-1} $Å^{-1}$. The minimum energy conformers were superimposed, with the P═Y moiety and the surrounding nitrogens or oxygens from the central structure taken as adjusting atoms. The effectiveness of the superimposed models was evaluated in terms of the root mean square (rms) values obtained. The energy differences between the different conformations analyzed for each trajectory were in the range of 2−5 kcal. As a representative example, the lowest energy conformations obtained for compound **6f** are given in **Figure 2e**.

According to our initial proposal, which stated that the compounds must interact with the Ni atoms and surrounding residues in the active site of the enzyme through the Y (Y = O, S), X (X = N, O), and/or Z (Z = O, N) heteroatoms bonded to P, we selected the charge density distribution and the location and atomic contribution to the HOMO 0 orbital as the first descriptor parameters for the structure−activity relationships. Schematic views of the data obtained for some representative compounds are provided in **Figures 3**−**5**. The lack of inhibitory activity for compounds in which Y = S can be ascribed to the observed difference in the charge density distribution when this parameter is compared to that obtained for compounds with Y = O. It can be seen from **Figure 3** that a deep negative zone appears on O for the derivatives with P═O (for example, **1d** or **3**) and that this disappears for analogous compounds with P═S (for example, **6k** or **7a**). In a similar way, the distribution and atomic contribution for the orbital HOMO 0 is observed. In the P═S derivatives the S atom contributes to a greater extent to this orbital, whereas for P═O derivatives the contribution is much smaller for the O.

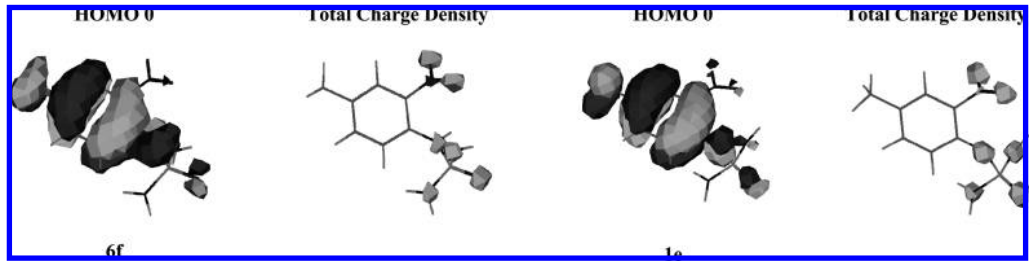


Figure 4. Influence of fragment X: NH versus O.

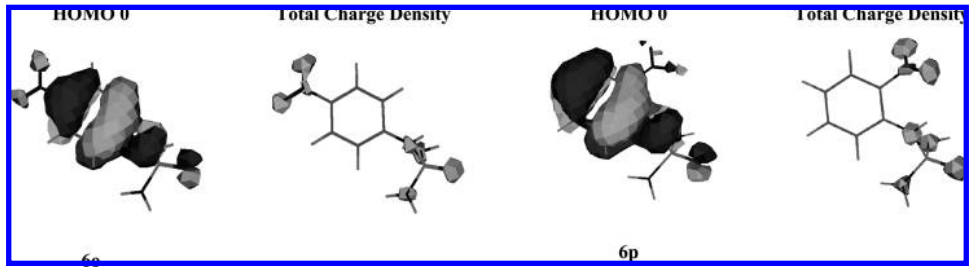


Figure 5. Influence of substituent location for aryl derivatives: **2** versus **4**.

With respect to fragment X (X = O, NH), the derivatives in which X is NH generally have greater inhibitory activity. In this case the presence of the N also produces a slight reinforcement of the electronic density on the oxygen of the P═O fragment as well as a greater contribution to the HOMO orbital in the zone that surrounds this fragment (see **Figure 4** for compounds **6f** and **1e**). This situation could be related to the better direct interaction with Ni atoms and the residues of the catalytic center. In addition, the presence of this X fragment, which acts as a bridge between R and P, introduces greater conformational freedom and a flexibility that can facilitate the interaction with the active site. Thus, it is observed that phenylphosphonic acid, which was synthesized and evaluated as a reference, is totally inactive in our tests. This observation can be explained by considering that the absence of the X group makes it impossible to adopt a valid conformation for an effective interaction with the active site. On the other hand, in the O-substituted diamidophosphate derivatives **1**, in which X is oxygen, there is a high probability of hydrolysis and degradation.

The presence of alkylic chains on the Z heteroatoms (compounds **2**, *N*-alkyl-substituted diamidophosphate derivatives) leads to the disappearance of the inhibitory activity, and this could be related to the steric impediment making the interaction with the Ni atoms impossible. With respect to the structural variations that affect the fragment R, the more active compounds are those that incorporate a flat aromatic or heteroaromatic ring, with great variability possible regarding the volume of such a ring. This situation is compatible with our starting hypothesis, according to which these fragments would arrange in a preferred conformation in which they were oriented toward the free cavity of the enzyme. With respect to the type and location of the substituents present in the ring, the best results are obtained for an atomic grouping that is able to participate in hydrogen bonds, particularly NO_2 (for example, **6f** or **6p**), located at position 2 for the aryl derivatives. This finding can be interpreted, in the first instance, as being a consequence of the potential of this group to participate in hydrogen bonds with the residues near the Ni atoms, thus reinforcing the union with the active site. For the heteroaryl derivatives such as the benzothiazoles (for example, **6k**, **6t**, **6u**, **6v**, and **6w**), the heteroatoms located in the ring can act in a similar way. A schematic representation of the differences in the charge density and HOMO 0 distribution obtained for compounds **6o** and **6p** is shown in **Figure 5**. The presence of a NO_2 group at position 2 leads to a zone of charge density that is favorable for the formation of hydrogen bonds in the vicinity of the active site.

For some of the compounds, a preliminary docking study was carried out to evaluate the possible interaction between the enzyme active center and the active compounds, thus exploring the previously proposed characteristics related to their activity as urease inhibitors. A manual superposition of the selected lowest energy conformations (obtained from the conformational analysis described previously) was carried out (see **Figure 2b** as an example), taking the DAP molecule in the complex DAP−BPU (PDB: 3ubp) as a superposition template (**Figure 6a**). Thus, superposition of the P═Y moiety of the selected derivatives was made on the P−O^- fragment of DAP, whereas group X was systematically superposed onto the rest of the O and/or N of this molecule in a search for the best orientation of the R group toward the opened cavity of the active center.

A schematic view of the preliminary results obtained for compound **6f** is shown in **Figure 6b**. It can be observed that the P═O fragment is located between the two Ni atoms, with the groups NH_2 and NH oriented toward the residues that surround the Ni atoms, whereas the aryl group orients itself toward the open enzyme cavity. A study into the stability (by means of molecular dynamics techniques) of these compounds is currently under way. The first data from this study allowed us to corroborate the applicability of our starting hypothesis and the validity of the proposed structural model.

The high in vitro activity levels that these compounds have as urease inhibitors justifies testing of these compounds in vivo. With this aim in mind, the activities in soils with different pH values were determined versus a control and using NBPT as a reference. Data obtained in these in vivo urease assays over a range of incubation pHs are given in **Table 4**.

In acidic soils, NBPT shows very low inhibition percentages—less than 40% after 10 days of incubation. However, compounds **6f** and **6p** have inhibition activities of about 65%. The behavior of compounds **6f** and **6p** is comparable to that of NBPT in neutral soils and in alkaline soils, with these compounds showing higher values than the reference compound, especially after 30 days of incubation. Despite the high level

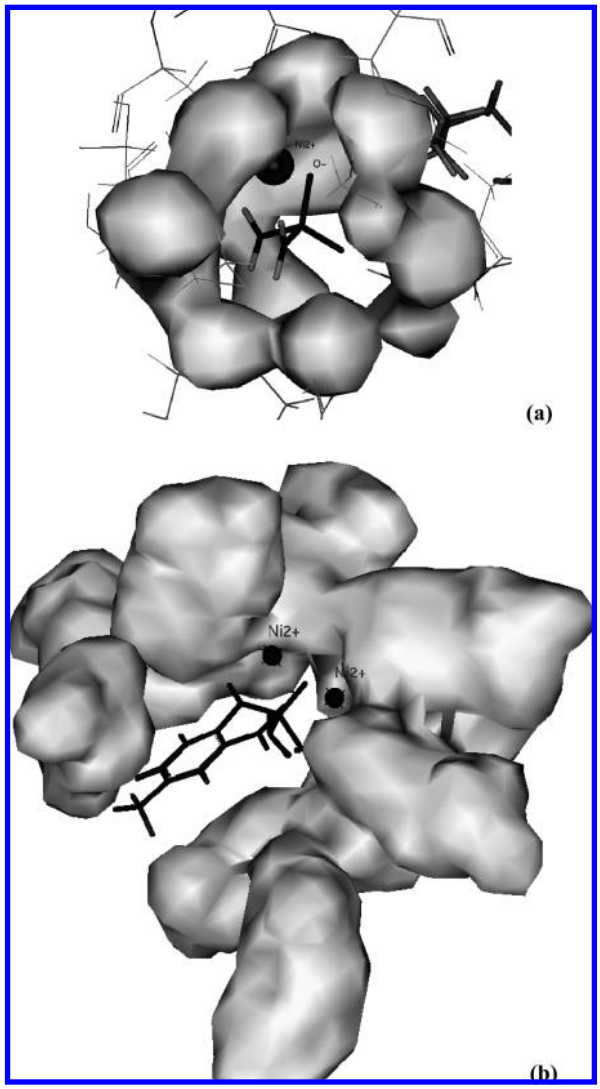


Figure 6. (**a**) Scheme for the active site of diaminophosphoric acid (DAP)-inhibited *Bacillus pasteurii* urease (BPU, ref code PDB: 3UBP). (**b**) Schematic proposal for the active site of BPU in the complex with compound **6f** (Ni^{2+} atoms are displayed as spheres).

of in vitro activity (IC_{50} = 2 nM) of compound **6k**, this inhibitor possibly undergoes more rapid degradation in soil.

These results could be related to the different stabilities of these compounds (**6f** and **6p**) and NBPT in the acidic soil used. In fact, Hendrickson and Douglass (*44*) observed that the efficiencies of NBPT and analogues such as urease inhibitors were clearly lower in acidic soils than in neutral soils. This result seemed to be related with NBPT degradation at low pH. Recently, Tao et al. (*45*) also observed lower NBPT efficiency as a urease inhibitor in acidic soils, and these authors related this result to both soil pH and microbial activity. Studies carried out in our laboratory using different acidic soils also confirmed the relationship between NBPT degradation and soil pH and microbial activity, although in our case soil pH seemed to be more important than microbial activity (data not shown). These results are of practical importance, because ammonium volatilization in urea-amended acidic soils with high organic matter contents is very significant (*1*).

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