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Development of an Empirical Model for Activity Prediction of Ureases's Inhibitors using PM6 Semiempirical Method

Desenvolvimento de um Modelo Empírico para Predição de Atividade de Inibidores de Ureases Usando o Método Semiempírico PM6

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In order to design new urease inhibitors, it is important to better understand the reactions that occurs in its binuclear nickel active site. As a step toward this goal, we evaluated by theoretical methods the spin multiplicity and the state of protonation of the oxygen located between the Ni(II) ions. In a second stage, parameters such as the interaction enthalpy, the Gibbs free energy required for the inhibitor to go from the aqueous phase to the interior of the urease and the entropic losses associated to the freezing of bonds after the binding of the inhibitors to the urease were used to develop correlations with the measured experimental inhibitor constant values. The quantification of these parameters for some phosphinic acids derivatives from the literature allowed us to obtain a good empirical model for the correlation between experimental activity data and the theoretical parameters (r=0.92). The model was employed for the prediction of the relative activity of a series of new proposed compounds by the organophosphorous.

Keywords: Urease; organophosphorus compounds; free energy models; semiempirical method

1. Introduction

Urease (EC 3.5.1.5) is a metalloenzyme found in a wide variety of plants, fungi and bacteria. It catalyzes the hydrolysis reaction of urea to form ammonia and carbamate, which is the last step in the nitrogen metabolism of living organisms. The carbamate is decomposed rapidly and spontaneously to produce carbon dioxide and a second molecule of ammonia.[1]

This enzyme is of great importance for researches related to agriculture, environment and medicine. For this reason, methodologies have been developed for the determination of urease activity in different matrices, such as soil and biologic tissue.[2-4] Strategies based on the inhibition of urease have been considered for the treatment of infections caused by ammonia-producing bacteria, and to achieve greater efficiency in the use of urea as a nitrogen source in agriculture and for reducing environmental pollution.[5,6] Several classes of compounds are known to exhibit inhibitory activity for this enzyme, and the class of phosphoramidates is one of the most actives.[7]

Different approaches have been developed for the construction of correlation models useful for the prediction of the activities of synthetic or natural compounds, especially QSAR models. Although models with high quality statistics can be obtained with the QSAR approach, in many cases the interpretation of such models is made difficult by the huge amount of descriptors that can be used for their construction. The use of more generalized descriptors, such as thermodynamic descriptors, could make easier the interpretation of such correlations and also extend their applicability to the design of new bioactive compounds.

The process of enzyme-ligand molecular recognition is driven by a combination of enthalpic and entropic effects. These effects can be estimated by the binding free energy (ΔG_{bind}) between the enzyme and the ligands, which is directly related to the experimentally-determined inhibition constant, K_i. Different computational methods have been developed to estimate ΔG_{bind}, such as thermodynamic integration, the free energy perturbation and the linear interaction energy methods.[8-11] However, for molecular systems with a large number of atoms, such as proteins, the obtainment of accurate estimates of ΔG_{bind} with any of these methods has a high computational cost.

A simple way for calculating ΔG_{bind} is the development of models based on empirical equations in which the free energy is partitioned in a series of terms that can be evaluated separately. The influence of the most important free energy terms for the enzyme-ligand interaction can be calibrated in these empirical models by fitting to available experimental

values.[12] With the aim of investigating the application of this approach to urease inhibitors, in the first step of this work we made a study of the urease active site, evaluating the spin multiplicity of the two Ni (II) ions present in this site, the protonation state of an oxygen atom located between the Ni (II) ions and the effect of the dielectric constant of the surrounding medium. In a second step, a study was done to obtain the initial docking structures of complexes between urease and inhibitors known in the literature. Because of the approximations involved in the docking method, this step was complemented by a semiempirical quantum mechanical study applied to the best docking poses, to obtain more accurate results in relation to the structural and energetic points of view. Moreover, semiempirical quantum mechanical methods are fast enough to be applied to systems containing hundreds of atoms. Next, we sought the development of an empirical equation to predict the potential urease inhibitory activity of some proposed phosphorylhydrazones, based on data from compounds with experimentally observed inhibitory activity. The model was used to evaluate changes in the structures of proposed phosphorylhydrazones in order to optimize the interaction with the enzyme and increase the efficiency of these compounds.

2. Methodology

2.1. Study of the spin multiplicity of the Ni (II) ions and the protonation state of the oxygen atom located between the Ni ions (II)

A search was performed in the Protein Data Bank (PDB) in order to select crystallographic structures of ureases presenting an oxygen atom between the nickel atoms in the enzyme's active site. Two urease structures were selected with this feature (PDB codes 1FWJ and 1EJW).[1] Amino acid residues with at least one atom located within a radius of 6.0 Å sphere around the nickel atoms were selected with RasMol program[9] as input data for the semiempirical calculations. Prior to these calculations, the necessary hydrogen atoms were added with the Babel program.[14] The calculations were done with the Mopac 2009 program[15] with the semiempirical PM6 method.[16] PM6 contains parameters for transition metals, necessary for calculations of the nickel-containing urease enzyme. All atomic coordinates were fully optimized during energy minimization, with exception of the atoms of the polypeptide chain.

Analysis of the number of potential ligands near the Ni(II) ions indicated a probable octahedral environment, so the expected number of unpaired electrons in each Ni(II) ion would be two. We considered two possibilities: (i) two unpaired electrons in one of the Ni(II) ions with parallel spins, but anti-parallel to the spins of the electrons in the other, resulting in a singlet state, and (ii) all four unpaired electrons with parallel spins, resulting in a quintet state.[17] Both systems were modeled with semi-empirical molecular orbital calculations, using the Restricted Hartree-Fock (RHF) Hamiltonian for the first case and the Unrestricted Hartree-Fock (UHF) Hamiltonian for the second. The oxygen atom located between the nickel atoms was considered as a water molecule or as a hydroxide ion. The COSMO continuum solvation model was adopted to include the effect of the medium in the resolution of the Schrödinger equation, using a dielectric constant equal to 4.0 to mimic the enzyme's interior.[18] As a criterion to compare the results, after geometry optimization 14 atomic distances measured from the two Ni (II) ions in each structure were compared with the respective experimental values (Figure 1, Table 1S and 2S of the Supplementary Material).

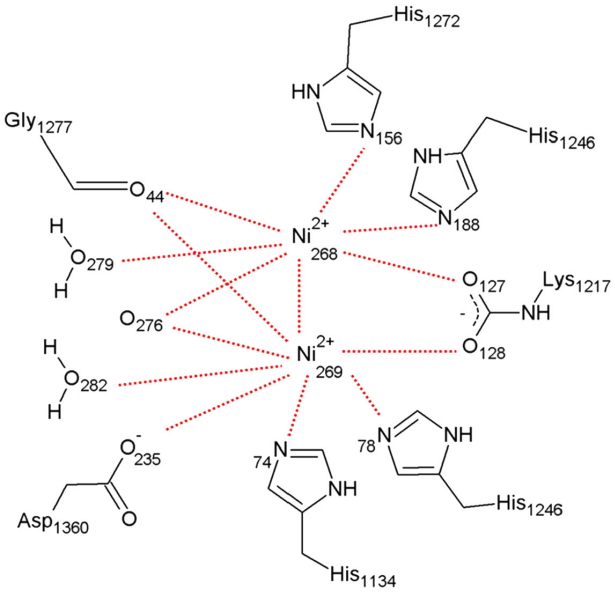

Figure 1. Selected distances between Ni (II) ions and electron donor atoms in 1EJW urease active site

2.2. Molecular docking

The compounds used in this work were divided into two groups: compounds with available urease inhibitory activity data, synthesized by Vassiliou and colleagues in 2008 (Figure 2), which were used to calibrate of free energy models for activity prediction; and compounds proposed by our group, which had their activities predicted by the models developed with the previous series (Figure 3). Compounds of the first group, which contain acidic hydrogen atoms, were considered in the anionic form.

These compounds were proposed by our group because phosphorylhydrazones could possibly act as a structural analogue of the phosphinic acid group, which is involved in interactions with the catalytic nickel (II) ions located in the urease active site, such as the oxygens attached to the phosphorus atom and the nitrogens of the hydrazone group.

All compounds were constructed and optimized with the Spartan'08 program[19] in two steps: first, a conformer distribution was generated with the Monte Carlo (MC)

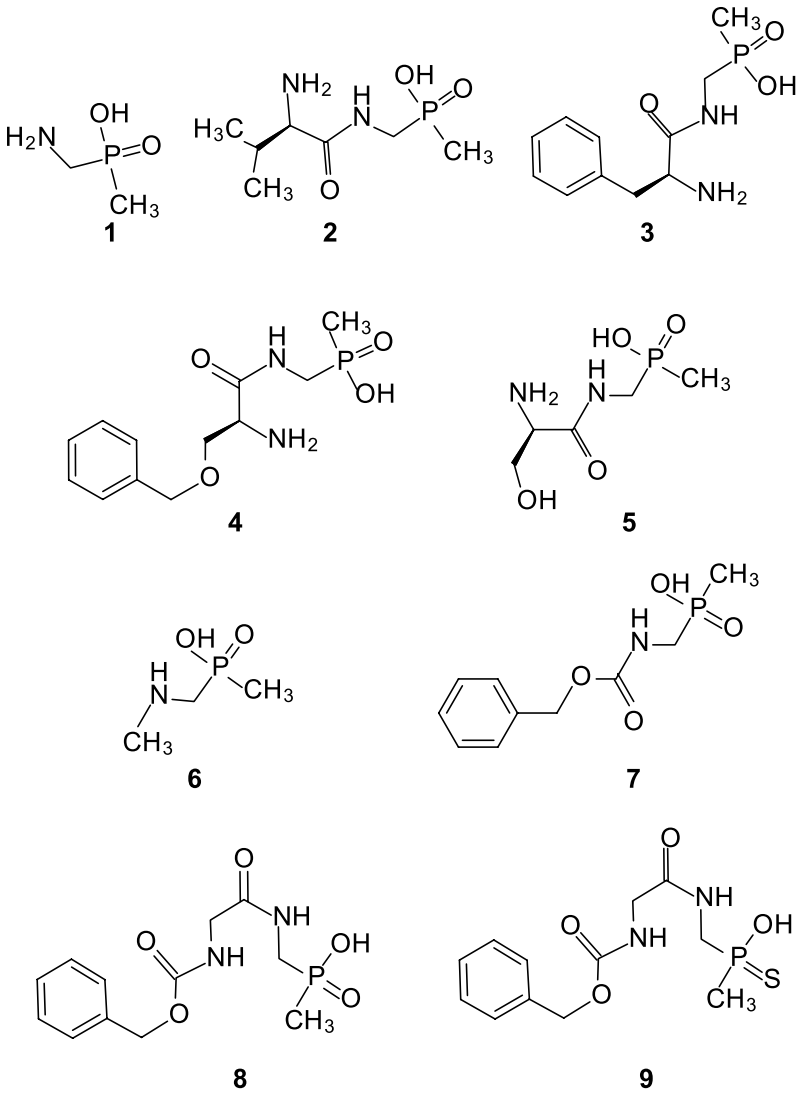

Figure 2. P-methyl phosphinic acids synthesized by Vassiliou *et al* (2008)

method with *MMFF* (*Merck Molecular Force Field*) with standard parameters and convergence criteria[20]. In Spartan, MC calculations use a simulated annealing method to generate conformations of a molecule. This procedure randomly rotates bonds and bends rings until a preferential (minimum energy) geometry is attained. Each rotatable bond has a fold number in Spartan. For example, sp^3-sp^3 bonds have a default fold number of 3 because these bonds usually have 3 minima 120 degrees apart. For the MC method the default number of cycles in Spartan is the square of the sum of all folds.

Next, the most stable conformer identified by this process was reoptimized with the semiempirical molecular orbital approach. We observed differences in the quality of the optimized structures associated to the different charge states of the compounds, so different Hamiltonians had to be applied to each series to get better results: the PM3 method[21] for the phosphoramidate neutral compounds (second group) and the AM1 method[22] for the ionized *P*-methyl phosphinic acids (first group). In the case of molecule **9**, which contain a chiral P atom, both enantiomers were considered during docking.

The *Bacilus pasteurii* urease structure 4UBP deposited in the PDB was selected for the docking procedure. The structure, which contains an acetohydroxamic acid molecule co-crystallized in the enzyme's active site, has a resolution of 1.55 Å [23]. The program used for the docking of the ligands was GOLD 5.1 (CCDC)[24]. Hydrogen atoms were added to the protein according to ionization and tautomeric states defined by the program. In the course of the searching procedure, 100,000 genetic operations (crossover, migration, mutation) were used for each docking run. To evaluate the pose prediction efficiency of each scoring function available in GOLD 5.1, a redocking study was carried out with the 4UBP structure. The redocking experiments were implemented with the four scoring functions available in GOLD: ChemPLP, Goldscore, Chemscore, and ASP.[25–28] The scoring functions were then evaluated according to the RMSD value between the docked and experimental structures.

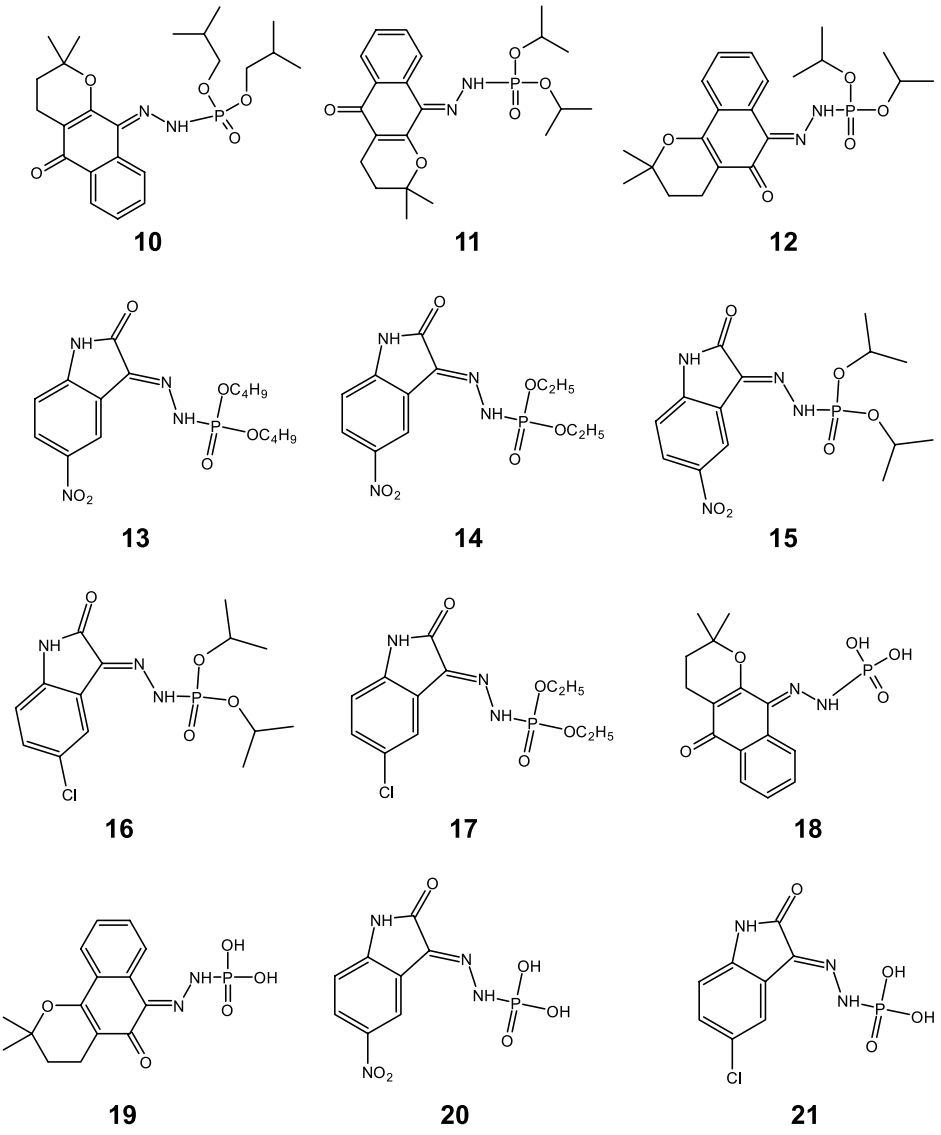

Figure 3. phosphorylhydrazone proposed by our group

The optimized ligands (Figure 2 and 3) were then docked into the 4UBP structure binding site using the scoring function selected based on the redocking study. The complexes containing the highest score poses for each compound were exported and used to prepare the input files for semiempirical calculations. All amino acid residues with at least one atom located within a radius of 6.0 Å sphere around the nickel atoms were selected with RasMol[9] as input data for the semiempirical calculations. Prior to these calculations, the necessary hydrogen atoms were added with Babel.[14]

2.3. Construction of free energy model for prediction of urease inhibitory activities

Considering that the ligands are initially in the aqueous phase, ΔG_{bind} can be conceptually divided into two terms: one term associated with the transport of the inhibitor from the aqueous phase to the protein's interior, ΔG_{trans}, and the other associated with the interaction between the inhibitor and the active site inside the enzyme, ΔG_{int}, so an equation relating the experimental K_i values with ΔG_{bind} can be written as

$$2.303RT \log K_i = \Delta G_{trans} + \Delta G_{int} \quad (1)$$

The evaluation of ΔG_{trans} is not an easy task because the environment changes continually from the border of the protein to the active site. When assuming a continuum model, the most widely used value for the dielectric constant in the interior of proteins is 4.0.[29] As a simple solution, we considered ΔG_{trans} as a polynomial function of $\Delta G_{A\text{-}E}$, the Gibbs free energy required to transport the inhibitor from the aqueous phase (ε = 78.4) into the enzyme (ε = 4.0):

$$\Delta G_{A-E} = (H_{4.0} - TS_{4.0}) - (H_{78.4} - TS_{78.4}) \quad (2)$$

where the entropy (S) and enthalpy (H) values were obtained

using the semiempirical PM6 method after optimization of inhibitors with two dielectric constants, 78.4 and 4.0. Linear and quadratic forms were evaluated for the polynomial function in the search of the best correlation with the activity data, and best results were obtained with a quadratic function.

The term ΔG_{int} consists of an enthalpic term and an entropic term, the latter associated with variations in translational, rotational and conformational entropies of the ligands after interaction with the cavity. The entropic term was considered to be proportional to the number of rotatable bonds N_{RB} that became "frozen" as a result of the interaction of the inhibitors with the active site, as proposed by others.[12] The enthalpic term was calculated with the Mopac 2009 program[15] with the semiempirical PM6 method[16]. The following equilibrium occurring inside the active site was considered for the calculations:

Inhibitor + [Urease-HO$^-$] dupla [Urease-Inhibitor] + HO$^-$

The presence of the hydroxide ion was used to keep the number and type of electron pairs on both sides of the equilibrium, minimizing the errors in the estimation of the interaction enthalpy with the semiempirical method. The interaction enthalpy (ΔH_{int}) was calculated by the following equation:

$$\Delta H_{int} = \Delta H_{EI} + \Delta H_{OH} - (\Delta H_{EOH} + \Delta H_I) \quad (3)$$

where ΔH_{EI} is the enthalpy of the enzyme-inhibitor complex; ΔH_{OH} is the enthalpy of a hydroxide ion; ΔH_{EOH} is the enthalpy of the enzyme/hydroxide complex; and ΔH_I is the enthalpy of the inhibitor. Thus, one can rewrite (1) as follows:

$$\log K_i = c_1(\Delta G_{A-E} + c_2)^2 + c_3\Delta H_{int} + c_4 N_{RB} + c_5 \quad (4)$$

The coefficients c_1-c_5 can be determined by fitting the equation by a multiple linear regression procedure to the experimental values of logK_i for the series of compounds from the literature. The coefficient c_5 is the value where the line intersects the axis of the dependent variable.

3. Results and Discussion

3.1. Study of the spin multiplicity of the nickel (II) ions and the protonation state of the oxygen atom between the nickel (II) ions

After optimization, active sites of urease structures 1FWJ and 1EJW with the different of spin multiplicity and protonation states were analyzed to determine which one had an optimized geometry more similar to the geometry of the original crystal structure. To evaluate the results, bond distances of some atoms able to coordinate to Ni ion (II) atoms were chosen (Figure 2). The values of root mean square deviation (RMSD) are shown in Table 1.

Table 1. Values of the root mean square deviation (RMSD) for distances between selected atoms and Ni (II) ions

Spin state	singlet		quintet	
Urease	RHF/HO$^-$	RHF/H_2O	UHF/HO$^-$	UHF/H_2O
1EJW	0.34	0.56	0.13	0.14
1FWJ	0.55	0.72	0.39	0.39

In all cases the use of the quintet state resulted in structures that match better the experimental results than the singlet state ones. Accordingly to our semiempirical results, a quintet state was previously obtained by Suarez and colleagues in a B3LYP density functional study of dinickel complexes relevant to the catalytic hydrolysis of urea exerted by urease.[17] The effect of the protonation of the oxygen atom located between the Ni(II) ions was significant in the singlet state and better results were obtained with a hydroxide ion; there was also some structural improvement with the quintet state when the hydroxide ion was present, but the effect was almost negligible. Results obtained by others indicated that a hydroxide ion is compatible with the observed weak antiferromagnetic coupling in urease.[17] Based on these results, we choose the hydroxide/quintet combination for the remaining urease complexes modeled in this work.

3.2. Molecular docking

The redocking study showed that all GOLD 5.1 scoring functions were efficient in predicting the cocrystallized ligand pose, but ChemPLP, which is the default fitness function, presented a slightly better result than the others. The root mean square deviation (RMSD) values were 1.26, 1.44, 1.84, and 1.85 Å for the scoring functions ChemPLP, Goldscore, Chemscore, and ASP, respectively.

The optimized ligands (Figure 2 and 3) were then docked into the urease binding site using the ChemPLP scoring function. The best poses obtained by docking process were selected for the development of the free energy model. This was not the case for compound **9**, for which it was suggested that the good inhibitory activities were related to the presence of the S atom that could establish effective interactions with both Ni(II) ions, similarly to what is observed in the crystal structure of the β-mercaptoethanol/urease complex.[7] None of the docking poses of compound **9** presented the S atom located near the Ni(II) ions. As the absence of the expected structures could be a result of a limitation of the fitness function to predict such interactions, we decided to construct two poses containing the expected interactions by simply editing the poses obtained for ligands **7** and **8**, replacing the O atom located between the Ni(II) ions by a S atom. The geometries of the best docking complexes, including those with the edited poses of ligand **9**,

were subsequently optimized with the semiempirical PM6 method for determination of the interaction enthalpy, as described below.

3.3. Construction of the activity prediction models for urease inhibitors

Molecular docking methods are generally known to be effective in reproducing interaction geometries, but simplifications adopted in their scoring functions may reduce the level of correlation between the scoring data and experimental information related to the ligand-enzyme interaction. A better method for the determination of the ligand-enzyme interaction energetics was then sought. Semiempirical quantum mechanical methods are fast enough to be applied to systems containing hundreds of atoms. PM6 was the first semiempirical molecular orbital method available in the MOPAC program that could be used to treat systems containing transition metals. In this method, the enthalpies of formation are better represented, and the geometries present a significant increase in accuracy, when compared to older parametric methods. PM6 introduces many modifications to the NDDO core-core interaction term and to the parametric optimization method. The average error for the enthalpies of formation calculated by PM6 of 1373 molecules used as reference, containing biologically relevant elements, is only 4.4 kcal.mol^{-1}, surpassing even some higher-level theoretical methods.[16]

In Table 2 are listed the data regarding the *P*-methyl phosphinic acids: the Gibbs free energy required for transport of a molecule from the aqueous medium to the active site of the enzyme ($\Delta G_{A\text{-}E}$), the number of rotatable bonds that are "frozen" (N_{RB}) after fitting of these inhibitors into the enzyme's active site; and the enthalpy of interaction (ΔH_{int}) between the inhibitor and urease, calculated using the semiempirical method PM6, according to (3).

The multiple regression analysis of the calculated terms in Table 2 applied in equation 5 led to a model with good correlation parameters (r^2=0.84; SD=0.55; N=9):

$$\log K_i = -1.62(\Delta G_{A-E} + 0.38)^2 + 0.02\Delta H_{int} + 0.12N_{RB} + 2.18 \quad (5)$$

Table 2. Logarithm of the experimental K_i values and theoretical parameters calculated for the first series of inhibitors

Structure	log(K_i)	$\Delta G_{A\text{-}E}$*	N_{RB}	ΔH_{int}*
1	2.53	-0.11	2	24.50
2	2.08	0.20	5	-16.44
3	2.33	-0.33	6	-57.81
4	1.40	0.24	8	-22.61
5	1.57	-1.58	6	32.41
6	1.26	0.21	2	-19.21
7	1.63	0.96	5	71.00
8	2.13	-0.54	7	-49.44
9	-0.77	-1.90	7	11.02

*$\Delta G_{A\text{-}E}$ e ΔH_{int} are expressed in kcal.mol^{-1}

The model (5) involved the contribution of compounds with a satisfactory range of experimental K_i values (0.17 to 340 μM). An analysis of the contributions of the parameters used in this model demonstrated that the three parameters contribute proportionally (Table 3). Application of (5) to calculate the activities of *P*-methyl phosphinic acids lead to predicted activity data that correlated quite well with the experimental activities (r = 0.92; SD = 0.39).

3.4 Prediction of the relative activity from proposed compounds

After developing a model with enough quality, the equation was applied for prediction of urease inhibition by the compounds of series 2, as described in Table 4.

We can see from Table 4 that the interaction enthalpies are predicted as unfavorable for compounds **10** to **17**. In the interaction between these compounds with urease amino acid residues, some hydrophobic interactions could be observed, but they were generally not extensive; there was also the presence of an average of two hydrogen bonds per ligand/urease complex. Probably by steric reasons, in most cases the interaction of any O atom bound to the P atom with Ni (II) ions did not occur. Thus, we can assume that the lack of this type of interaction is the main factor

Table 3. Contribution of each variable term in (5), experimentally measured log*Ki*, log*Ki* calculated by (5), and the difference between the experimental and calculated values of log*Ki*

Structure	$[-1.62(\Delta G_{A\text{-}E} + 0.38)^2]$	$0.02\Delta H_{int}$	$0.12N_{RB}$	$\log K_{i\ exp}$	$\log K_{i\ calc}$	$\Delta \log K_i$
1	-0.12	0.49	0.24	2.53	2.79	-0.26
2	-0.54	-0.33	0.60	2.08	1.91	0.17
3	-0.00	-1.16	0.72	2.33	1.74	0.59
4	-0.62	-0.45	0.96	1.40	2.06	-0.66
5	-2.33	0.65	0.72	1.57	1.21	0.35
6	-0.56	-0.38	0.24	1.26	1.47	-0.21
7	-2.91	1.42	0.60	1.63	1.29	0.34
8	-0.04	-0.99	0.84	2.13	1.99	0.14
9	-3.74	0.22	0.84	-0.77	-0.50	-0.27

Table 4. Theoretical parameters calculated for the second series of compounds

Structure	$\Delta G_{A\text{-}E}$*	N_{LR}	ΔH_{int}*	log$K_{i\ calc}$
10	-1.54	8	66.30	2.29
11	-2.15	6	176.37	1.35
12	-1.59	6	168.12	3.89
13	0.26	10	90.91	4.53
14	-1.27	6	34.90	2.31
15	-0.96	6	62.15	3.60
16	-1.40	6	56.88	2.35
17	-0.68	6	93.68	4.63
18	-1.11	2	7.13	1.70
19	0.22	2	-30.75	1.22
20	-0.83	2	-87.21	0.35
21	0.48	2	-89.16	-0.56

*$\Delta G_{A\text{-}E}$ and ΔH_{int} in kcal mol^{-1}

leading to the unfavorable ΔH_{int} values for the proposed compounds **10** to **17**.

It was also observed that, among the nitroisatin-derived phosphorylhydrazones containing aliphatic chains (**10** to **17**), structure **14** showed the less unfavorable ΔH_{int}, and this can be related to the fact that this structure has small aliphatic chains, causing a better accommodation of the molecule into the urease active site, which allowed the nitro group to stay closer to the Ni (II) ions (Figure 4). The distances between each O atom of the nitro group of the structure and Ni (II) ions were 4.21 and 2.27Å.

As can been seen in Table 4, the removal of the alkyl chains from the phosphorylhydrazone group (compounds **18** to **21**) resulted in a much more favorable ΔH_{int} values with the urease active site; only structure **18**, which contains a α-lapachone-derived group, remains with a positive ΔH_{int}, but the value is less positive than that of **10** and **11**, compounds that also have α-lapachone groups. In the complexes formed between the modified structures and the active site of urease, the phosphorylhydrazone group could now interact with the Ni(II) ions. The distances between the O atom of the phosphorylhydrazone group and the Ni(II) ions in all complexes were around 2.0 Å. A second favorable point is the lower N_{RB} of the proposed structures: they all have only two rotatable bonds, which also contributed to the more favorable values for the predicted activity. As expected, the only unfavorable effect was that the reduced hydrophobicity of the proposed structures resulted in less favorable $\Delta G_{A\text{-}E}$ values. Together, the parameter values of the new structures lead to much more favorable predicted logK_i values, suggesting they as quite promising urease inhibitors, especially structure **21**.

4. Conclusion

Studies with the semiempirical method PM6 indicated that the most probable spin state for urease is the quintet state due to improved reproduction of experimental geometry crystallographic structures of the enzyme. Likewise, studies indicate that the oxygen atom situated between the Ni (II) ions is more likely to be a hydroxide ion.

The model developed in this work, based on enthalpic and entropic factors involved in interactions between the inhibitors and the enzyme, allowed the development

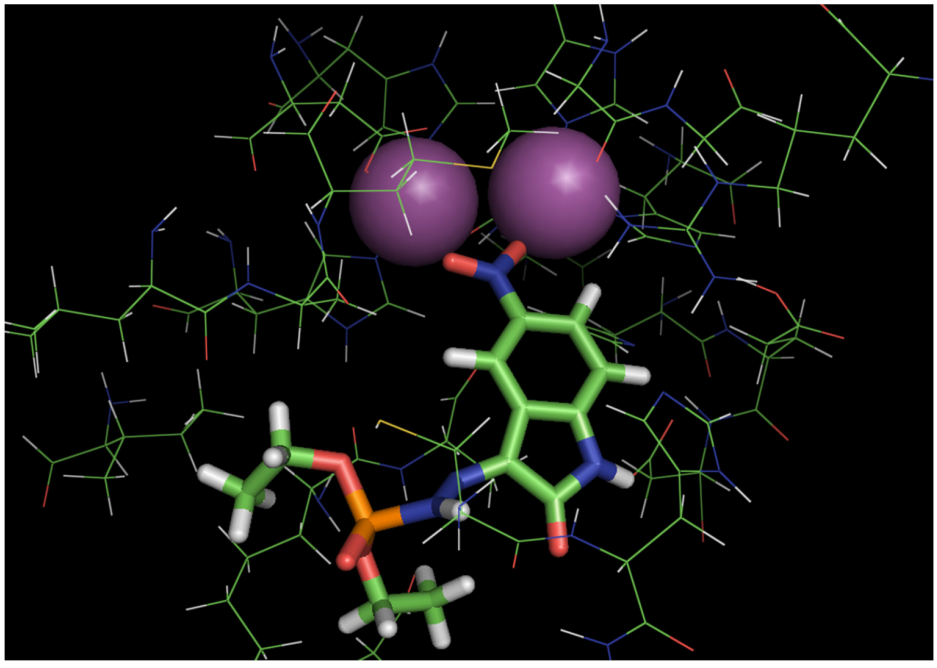

Figure 4. PM6 optimized structure of the complex between **14** (stick mode) and the active site of urease from *B. pasteurii* (4UBP). Nickel ions are presented as spheres. Colour code: carbon, green; hydrogen, white; nitrogen, blue; oxygen, red; phosphorous, orange; nickel, purple

of a thermodynamic equation capable of predicting the corresponding binding free energy. As demonstrated in this study, this method can be used even in systems such as urease, which possesses complicating factors such as the presence of Ni (II) ions in the catalytic site, thanks to the availability of PM6, a semiempirical quantum method with parameterizations for transition metals.

A number of proposed compounds by our group was analyzed according to the model developed and the predicted activities indicated that the absence of the alkyl chains from the phosphorylhydrazone group resulted in a much more favorable ΔH_{int} values with the urease active site.

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