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Anti-ureolytic activity of substituted 2,5-diaminobenzoquinones

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A series of 2,5-*bis*(alkyl/arylamino)-1,4-benzoquinones (**1**-**12**) were investigated *in vitro* for their potential to inhibit the activity of jack bean urease. Compounds **1**–**6**, **8**, **9**, **11** and **12** effectively inhibited the jack bean urease activity by 90.8% when tested at 5 μM, whereas **7** and **10** had relatively little effect. The IC_{50} for most compounds was in the nanomolar range (31.4 nM and 36.0 nM for **2** and **8**, respectively). The mechanism of enzyme inhibition shown by **2** and **8** is typical of mixed-type inhibitors, whose affinity for the active site is over 6- and 2-fold higher (*Ki* = 30.0 and 22.8 nM, for **2** and **8**, respectively) than that of an allosteric site. Molecular docking studies revealed that both **2** and **8** establish hydrogen bonds with the amino acids residues Asp494, Met588, His593 and Ala636 in the active site of jack bean urease. These results indicate that such aminoquinones are useful leads for the development of more efficient urease inhibitors of wider utility.

Keywords: Urease inhibitors; Jack bean urease; 2,5-diamino-1,4-benzoquinones; Molecular docking.

Introduction

Urease (E.C.:3.5.1.5) is a well-known nickel metalloenzyme that catalyzes the hydrolysis of urea to ammonia and carbon dioxide.[1] The primary environmental role of ureases is to allow organisms to use urea as nitrogen source. Moreover, urease participates in systemic nitrogen transport pathways in plants, fungi, algae, bacteria and microorganisms.[2] However, the activity of soil ureases leads to nitrogen losses when urea is used as fertilizer for crop production.[3-4] Additionally, urease is a known virulence factor for the bacteria *Helicobacter pylori* and *Staphylococcus saprophyticus*, involved in gastrointestinal diseases and urinary stone formation.[5-6] It is estimated that each human being produces 10 kg of urea per year. Spontaneous degradation of urea in cells is believed to occur with a half-life of *c.a.* 3.6 years,[7] whilst this process becomes 100 trillion-fold faster in the presence of ureases.[8] Since urea hydrolysis yields ammonia,[9] the ammonia-dependent increase of cell pH in the media has medicinal implications, while excessive ammonia emission to atmosphere causes environmental

issues and economic problems due to lower availability of nitrogen to crops.[2, 10] For this reason, the control of urease activity is of considerable clinical and agricultural importance.

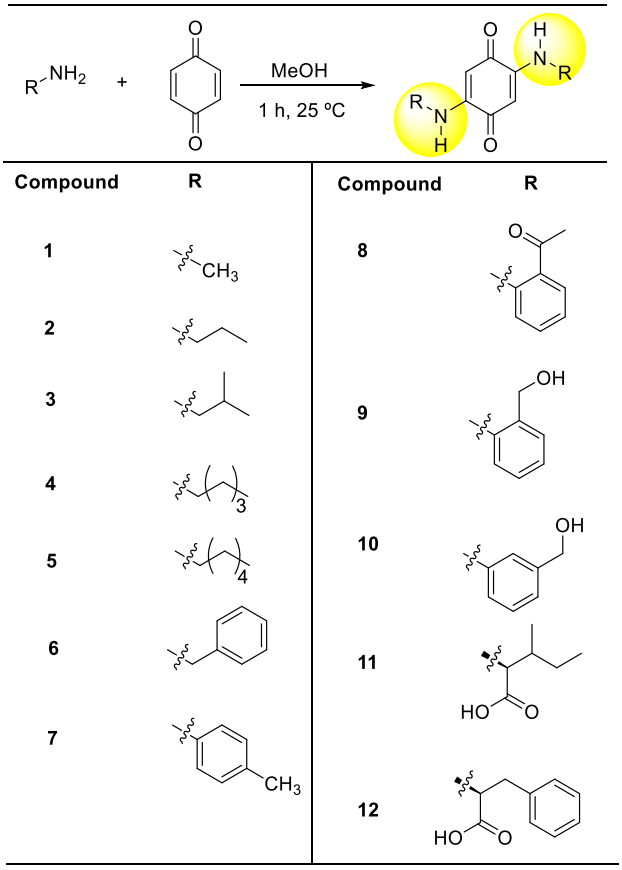
Among all the nutrients used in agriculture, the nitrogen fertilizers are the highest with an annual consumption in 2018/2019 over 107 Mt, of which 55% is represented by urea. It is estimated that 14 – 40% of nitrogen is lost as ammonia depending on both climate and the soil type.[11] The greater part of such losses is due to the activity of urease found in soils. Consequently, finding urease inhibitors that can be so applied with urea is a research field of great economical interest. Among the few urease inhibitors in commercial use is the *N*-butyl thiophosphoric triamide (NBPT), whose agronomic efficiency has been recently reviewed.[11]

During the last two decades a large array of metals and organic substances have been found to inhibit urease. Amongst synthetic organic compounds, the most actives include phosphoramidates, thioureas, dimeric vanadium complexes, benzothiazoles, coumarinyl pyrazolinyl thiomides and dihydropyrimidin(thi)one.[11–15] Several classes of plant-derived compounds, including terpenoids, phenolic acids, flavonoids, stilbenes, xantones, coumarins alkaloids and quinones, among others, have preceding last years, special attention has focused on quinones due to their wide array of biological properties, specially anti-ureolytic activity. Quinones also play important roles in the electron transfer chain of photosynthesis[18–20] and cell respiration[19-20] . For the latter, coenzymes Q is involved in centers of reduction and in the translocation of protons across the inner mitochondrial membrane via quinone/quinol (Q/H_2Q) redox couple turnover.[19] The ability of 1,4-benzoquinone related compounds to inhibit *H. pylori* or *Canavalia ensiformis* (jack bean) ureases is also important.[21–25] The inhibition of urease by naphthoquinones and their mechanism of action have been elucidated.[23, 26]

Although the anti-ureolytic potency of 1,4-benzoquinones has been attributed to the presence of electron-withdrawing chlorine groups,[21] quinones bearing electron-donating amino groups (2,5-bis(alkylamino)-1,4-benzoquinones derivatives) were also shown to inhibit urease with IC_{50} values as low as 27.3 μM.[27] Furthermore, our group has previously shown that substituted 1,4-benzoquinones are promising phytotoxic and algicidal agents.[28–32] Therefore, considering the limited information on the anti-ureolytic activity of 2,5-diamino-1,4-benzoquinones, this work reports the effect of a series of such compounds[29] on the activity of jack bean type III urease and the interaction between these compounds and urease (PDB ID: 4H9M) by molecular docking studies.

Results and Discussion

A series of twelve 2,5-*bis*(alkyl/arylamino)-1,4-benzoquinones (**1**-**12**) were prepared employing a protocol developed and reported by our group.[29] In short, the compounds were obtained from the aza-Michael addition of primary amines to 1,4-benzoquinone (1:3 equiv. ratio) in methanolic solution, followed by spontaneous oxidation (**1**-**12**; Scheme 1). The synthesis of compound **12** required the addition of $NaHCO_3$ to promote the deprotonation of the zwitterion, even though the yield was only 46%, much lower in comparison with the yields obtained for the aliphatic amines derivatives 66-93%. All compounds were fully characterized by their physical and spectroscopic data, that were in full agreement with the literature.[29]



Scheme 1. Structure of 2,5-*bis*(alky/arylamino)-1,4-benzoquinones used in this study.

Anti-ureolytic Activity

The benzoquinones **1**-**12** were subjected to an *in vitro* assay using a purified *Canavalia ensiformis* (jack bean) type III urease to assess their anti-ureolytic potential. Hydroxyurea (**HU**), commonly used in *in vitro* screening,[14] was employed as a reference urease inhibitor. The percentages of urease inhibition caused by all compounds at 5 µM are displayed in Table 1.

All 2,5-*bis*(alkyl/arylamino)-1,4-benzoquinones tested, except for **7** and **10**, inhibited the activity of jack bean urease by 90.8 ± 0.8 % (on average) while the inhibitor-reference **HU** barely affected the urease activity under the same experimental conditions (Table 1). Notably, the only 2,5-diamino-1,4-benzoquinones previously reported as urease inhibitors were shown to inhibit *Helicobacter pylori* urease by up to 81.6% when tested at 100 µM – a concentration 20 times higher than the one used in the current work.[27]

Table 1. Inhibition of *Canavalia ensiformis* (jack bean) type III urease by 2,5-*bis*(alkyl/arylamino)-1,4-benzoquinones **1**-**12**.

Compound	Urease inhibition (%)[a]	Compound	Urease inhibition (%)
1	89.5 ± 0.8	**8**	91.6 ± 0.4
2	89.7 ± 1.0	**9**	97.7 ± 0.1
3	88.4 ± 0.6	**10**	18.9 ± 6.6
4	89.1 ± 1.0	**11**	89.1 ± 0.8
5	90.1 ± 0.7	**12**	91.6 ± 1.3
6	91.4 ± 0.8	**HU**	5.5 ± 0.8
7	13.7 ± 6.7		

[a]Compounds were tested at 5 μM in the presence of 10 mM urea. HU, hydroxyurea (reference of urease inhibitor). Data are the means (n = 4) ± SD.

Although the number of compounds and structural variations are somehow limited, the data clearly show that quinones derived from aliphatic amines (**1**-**6**, **11**, **12**) are very active. In general, quinones **1**–**6**, **8**, **9**, **11** and **12** were approximately 16.5-fold more potent than the inhibitor-reference **HU**. Indeed, the aminobenzoquinones tested were very active, regardless of the nature of the *N*-alkyl or *N*-benzyl groups. Amongst the products derived from substituted anilines (**7**-**10**), those bearing a substituent at an *ortho* position are very potent (>90% inhibition) whilst those with substituents either at the *para* (**7**) or *meta* (**10**) positions were ineffective at 5 μM (<20% inhibition). Although a detailed computational calculation has not been carried out, the presence of a group at *ortho* position on the phenyl ring clearly interferes with the relative conformation of the rings and with the conjugation degree of the nitrogen lone electron pair with the quinone core, probably impacting the biological potency. Further investigation is required to fully understand the effect of *ortho* substituents on the measured bioactivity.

Based on the screening results (Table 1), compounds **2** and **8**, typical examples of *N*-alkyl and *N*-arylamino derivatives, respectively, were randomly selected out of the 10 equally highly active aminobenzoquinones to further explore the mechanism of urease inhibition. Various concentration of each compound (up to 100 nM) were used in *in vitro* experiments carried out with 10 mM urea and 12.5 mU urease to determine the corresponding concentrations necessary to inhibit the enzyme by 50% (IC_{50}). Such data served as a guide for choosing appropriate concentrations (< IC_{50}) for the kinetics studies. The IC_{50} values for compounds **2** and **8** were found to be 31.4 nM and 36.0 nM, respectively. These IC_{50} values are approximately eighteen times lower than those found for the most active 2,3,5,6-tetrachloro-1,4-benzoquinone (IC_{50} = 600 nM) evaluated against the same urease.[21]

Based on the IC_{50} values found for **2** and **8**, concentrations of 10, 20 and 30 nM were chosen for the kinetics assay. As observed on Figures A and C, both aminobenzoquinones caused increments in the urea K_M and

decreases in the urease V_{max}. The analyses of the Lineweaver-Burk plots for **2** and **8** (Figures 1B and 1D) show that both compounds function as mixed-inhibitors as the lines intersect one with another in the second quadrant. These results indicate that the aminobenzoquinones tested are eligible to bind to either free urease to yield an EI complex or urease-urea complex to furnish an ESI complex. The equilibrium dissociation constants for urease-compound **2** and urease-compound **8** complexes (K_i) and for urease-urea-compound **2** and urease-urea-compound **8** complexes (K_i') were 30.0 ± 0.9 nM, 22.8 ± 3.7 nM, 191.4 ± 96.1 nM and 61.6 ± 13.7, respectively. This clearly demonstrates the greater affinity of **2** and **8** to the active site of jack bean urease than to an allosteric site. Indeed, the affinity of **2** to the urease active site is 6.4-fold higher than that of other sites while the affinity of **8** to an allosteric site is only 2.7-fold lower compared to that of the active site. Other quinones such as 1,4-benzoquinone, 2,5-dimethyl-1,4-benzoquinone, tetrachloro-1,2-benzoquinone and tetrachloro-1,4-benzoquinone act as competitive slow-binding inhibitors of jack bean urease, yielding K_i values of 45 nM, 1,200 nM, 2.4 × 10^{-6} nM and 0,45 nM, respectively, in reactions (pH 7.0) containing 15 μg mL^{-1} urease.[1, 33]

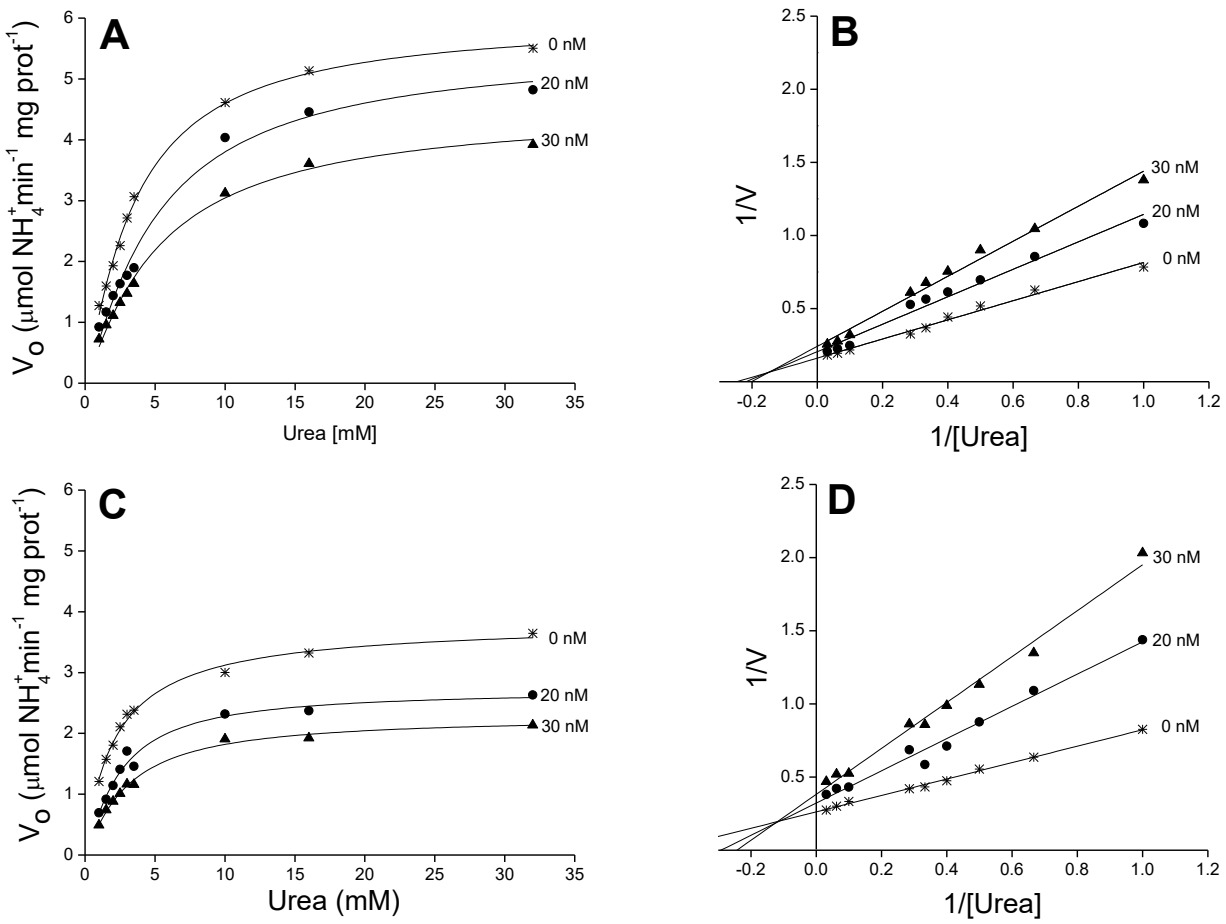


Figure 1. Effects of aminobenzoquinones **2** and **8** on the kinetics of jack bean urease (type III). Increasing amounts of urea (0 – 32 mM) were incubated for 5 min with jack bean urease in the presence of the indicated concentrations of inhibitors. Plots are representative of independent experiments done in triplicates. Michaelis-Menten hyperbolas (**A** and **C**) and Lineweaver-Burk plots (**B** and **D**) for the results originated from reactions performed with **2** and **8**, respectively.

Molecular Docking Analysis

Since kinetics studies have shown preferential interaction with the active urease site, we analyzed compounds **2** and **8** through molecular docking using the jack bean enzyme to shed more light on the mechanism of urease inhibition, revealing biophysical interactions that play a fundamental role in this process.

The jack bean urease PDB ID: 4H9M was chosen since the crystal that originated its X-ray diffraction data was available with resolution of 1.5 Å. Acetohydroxamic acid (**AHA**) was used as a reference ligand, showing a root-mean-square deviation of atomic positions (RMSD) of 0.57 with docking score of -4.1 kcal/mol. The docking scores for **2** and **8** were -5.1 and -8.1 kcal/mol, respectively, lower than that of **AHA**. The lower docking score shows a stronger interaction between the aminoquinones and the enzyme active site, compared with the standard **AHA**, in agreement with the experimental data, in which **2** and **8** are more potent than **HU**. The binding mode of compounds **2** and **8** is shown in Figure 2, serving as a representative template for the binding mode of the series of aminoquinones tested in this study.

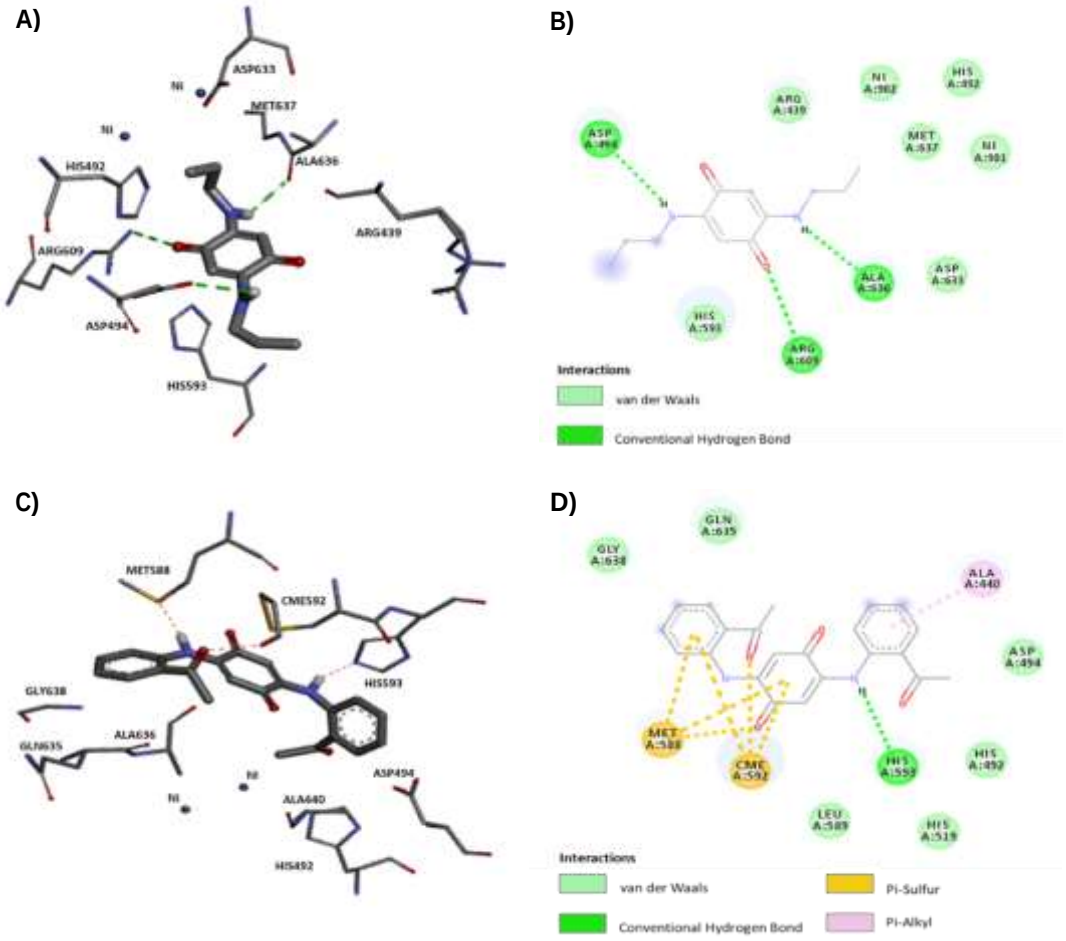


Figure 2. Docking of aminoquinone **2** (green dash) into the active site of *Canavalia ensiformis* urease (PDB 4H9M; **A**) and a 2-D diagram of **2** (**B**). Docking of aminoquinone **8** (red dash) into the active site of *Canavalia ensiformis* urease (PDB 4H9M; **C**) and a 2-D diagram of **8** (**D**).

The –NH group and oxygen from quinone moiety may interact with different amino acid residues in the urease active site, probably through hydrogen bonding (Figure 2). For instance, **2** interacts with Asp494 and Ala636 *via* hydrogen bonding through its amino group, while the carbonyl of quinone moiety makes a hydrogen bond with Arg609 residue (Figure 2A). The van de Waals interactions also are observed in the representative Figure 2B, mainly by the amino acids residues Arg439, His492, His593, Asp633, Met637 and the nickel ions Ni901 and Ni902. The amino groups of **8** makes hydrogen bonds with Met588 and His593 and the carbonyl of the acetyl group makes a hydrogen bound with the modified cysteine residue Cme592 (Figure 2C). The aromatic moiety and the benzoquinone core of **8** are engaged in π-sulfur and π-alkyl interactions with Met588, Cme592 and Ala440, respectively (Figure 2D). Overall, the binding mode of the aminoquinones to the active site of jack bean urease resulted in docking scores similar to those obtained for other aminoquinones and the active site of urease from *Helicobacter pylori*.[27] Also, in contrast to the study of urease from *H. pylori*[27], in the current study the aminoquinones revealed no direct interaction with the nickel atoms. Although the docking study has shed some light on the type of interaction that such compounds can have with the amino acid residues at the enzyme active site, a full understanding of their mode of action requires further investigation. Also, compounds **2** and **8** can also bind to allosteric sites of jack bean urease. In the case of a simple 1,4-benquinone (**1,4-BQ**), it has been demonstrated that the inhibition of urease from *Sporosarcina pasteurii* involves a covalent bond formation between the thiol group of Cys322 residue, a key residue found on the mobile flap regulating the substrate access to the active site.[34] The covalent bond formation between **1,4-BQ** and 2,3,5,6-tetrachloro-1,4-benzoquinone (**TC-1,4-BQ**) and thiol groups of cysteine residues from the active site of jack bean urease has also been proposed as a key interaction for their mode of action.[21] Although the aminoquinones herein investigated herein are less electrophilic than a simple **1,4-BQ**, the formation of covalent bonds with thiol groups is not excluded and further investigation is necessary to refute or corroborate this hypothesis.

Conclusions

The synthetic aminoquinones **1**-**6**, **8**, **9**, **11** and **12** are excelent urease inhibitors, showing great performance at nanomolar concentrations. Compounds **2** and **8** exhibit a mixed-type mode of urease inhibition with much more affinity to the enzyme active site. Molecular docking reveals that the oxygen atoms at quinone core and amino groups in compounds **2** and **8** are likely to establish hydrogen bonds with amino acid residues located at the active site of jack bean urease. These results suggest that compounds with an aminoquinone core are excellent candidates for the development of novel urease inhibitors of several interests.

Experimental Section

General Experimental Procedures

Reagents were procured from Sigma-Aldrich (Milwaukee, Wisconsin, USA) and solvents were purchased from Fluka (Rio de Janeiro-Brazil). They were used without further purification. Analytical thin layer chromatography (TLC) was performed on silica gel 60 F_{254} 0.2 mm thick plates (Merck, Rio de Janeiro-Brazil). The plates were visualised under an UV-lamp at a wavelength of 254 nm or by staining with phosphomolibdic acid solution. All compounds were fully characterized by IR, EI-MS, ^{1}H NMR and ^{13}C NMR spectroscopy. Infrared spectra were recorded on a Perkin-Elmer Paragon 1000 FTIR spectrophotometer, preparing the samples as potassium bromide disks (1% w/w). The ^{1}H and ^{13}C-NMR spectra were recorded on a Varian Mercury 300 spectrometer at 300 and 75 MHz, respectively, using $CDCl_3$ as solvent and TMS as internal reference, unless otherwise stated. The melting points were measured with on a MQAPF-301 apparatus and are given uncorrected.

Procedure for the preparation of 2,5-amino-1,4-benzoquinone derivatives (1-12)

Compounds **1-12** were synthesized using methods previously published.[29] The physical and spectroscopic data of all compounds were in agreement with the previously reported[22] and are not repeated here.

Urease inhibition assay

The screening for identifying potential urease inhibitors was done by incubating each 2,5-bis(alkyl/arylamino)-1,4-benzoquinones (**1**-**12**) at 5 µM (5,000 nM) in reactions containing 50 mM phosphate buffer (pH 7.4), urea (10 mM), and 1.25 x 10^{-2} U *Canavalia ensiformis* (jack bean) type III urease (Sigma U-1500-100 kU). Each mixture was incubated for 15 min at 25 °C, and the reactions were interrupted according to Weatherburn,[35] with modifications reported by Brito et al.[36] The ammonium concentration was determined by the phenol hypochloride assay (measured at 636 nm), and the inhibition percentage [INH(%)] was calculated using the equation: INH(%) = 100 – [(AINH/AB) x 100], where AINH and AB are ammonium concentration in the tubes with and without inhibitor, respectively. The inhibitory potential of the compounds was compared with that of the standard-inhibitor hydroxyurea (**HU**; 5 µM). Two compounds (**2** and **8**) were randomly selected among the most potent aminobenzoquinones to perform kinetics assays, in which each inhibitor was added at a fixed concentration (0, 10, 20 or 30 nM) to the reaction media (pH 7.4) containing 12.5 mU jack bean urease and increasing concentrations of urea (1-32 mM). Reactions were stopped after 5 min incubation and analysed for the production of ammonium.

Docking simulations

Molecular docking of 2,5-bis(alkyl/arylamino)-1,4-benzoquinones (**2** and **8**) with the active site of jack bean urease (PDB ID: 4H9M) was performed using the AutoDock Vina program suite.[37-38] Each enzyme's target was prepared for molecular docking simulation by removing water, ligands and cofactors. Hydrogen polar atoms and gasteiger charges were added to each protein atom. The Ni initial parameters were set as r = 1.170 Å, q = +2.0 and van der Waals well depth of 0.100 kcal/mol.[39] Auto-Dock Tools-1.5.6 (ADT) was used to prepare and analyze the docking simulations for the AutoDock 4.2. Coordinates ligands were generated using Chemdraw 14.0 followed by MM2 energy minimization, and non-polar hydrogen atoms were merged using ADT package. The interaction of protein and ligands in binding pocket for AutoDock 4 was defined by a Grid box. The grid box was created using 30 x 35 x 30 points for each direction of x, y, and z, the maps were centered on the Ni atom in the catalytic site of the protein. AutoGrid4 was used to produce grid maps for docking calculations where the search space size utilized grid points of 1.0 Å. Each docking experiment was performed 50 times, yielding 50 docked conformations. The best docking pose was picked on the best binding energy. The docking procedure of aminoquinones **2** and **8**) with the active site of jack bean urease was performed as described.

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Author Contribution Statement

Amalyn Nain-Perez carried out literature reviews, molecular docking, data analyses, interpretation of the results as well as compilation and preparing the manuscript. Diego Rodríguez-Hernández contributed to the manuscript writing, data analysis, and preparation of graphics. Yane C. C. Mota, and Thamara F. S. Ávila carried out the experiments with urease, performed data analyses and discussed the results. Luiz C. A. Barbosa conceived and coordinated the project "aminoquinone as urease inhibitors", analyzed the data and worked on the manuscript preparation. Luzia V. Modolo supervised all biological assays, discussed the results and helped writing the manuscript while Teodorico C. Ramalho contributed with molecular docking.

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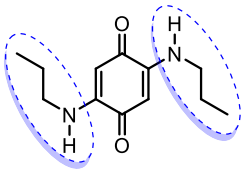
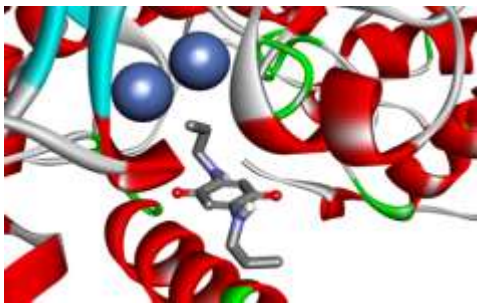


2

anti-ureolytic



Binding site of jack bean urease with compound **2** (IC_{50} = 31.4 nM)