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Potent covalent inhibitors of bacterial urease identified by activity-reactivity profiling

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ABSTRACT

Covalent enzyme inhibitors constitute a highly important group of biologically active compounds, with numerous drugs available on the market. Although the discovery of inhibitors of urease, a urea hydrolyzing enzyme crucial for the survival of some human pathogens, is a field of medicinal chemistry that has grown in recent years, covalent urease inhibitors have been rarely investigated until now. Forty Michael acceptor-type compounds were screened for their inhibitory activities against bacterial urease, and several structures exhibited high potency in the nanomolar range. The correlation between chemical reactivity towards thiols and inhibitory potency indicated the most valuable compound — acetylenedicarboxylic acid, with $K_i^* = 42.5$ nM and $\log k_{GSH} = -2.14$. Molecular modelling studies revealed that acetylenedicarboxylic acid is the first example of highly effective mode of binding based on simultaneous bonding to a cysteine residue and interaction with nickel ions present in the active site. Activity-reactivity profiling of reversible covalent enzyme inhibitors is a general method for the identification of valuable drug candidates.

Inhibitors binding covalently to the residues present in the enzyme active site constitute a large group of biologically active compounds of significant value.[1,2] Numerous covalent drugs, ranging from aspirin (discovered at the end of nineteenth century) to ibrutinib (approved by the FDA in 2013 for Mantle Cell Lymphoma treatment and in 2014–2016 for various leukemias), are available on the market.[3] Although covalent enzyme inhibitors exhibit numerous crucial advantages resulting from their strong binding to the target, they are often considered less attractive as drug candidates in comparison to non-covalent binding compounds because of drawbacks in the later stages of drug studies; in particular, specificity and toxicity issues are of great concern. These problems are often not predictable and are observed only for some patients.[4]

Michael acceptor-based enzyme inhibitors reacting with a cysteine residue in the active sites of proteins constitute one of the most widely studied groups of covalent drug candidates. Although cysteine proteases can be considered the primary target for this type of molecules,[5] inactivation of non-catalytic cysteine residues is also of interest.[6] Recently, reversibly binding covalent inhibitors have received attention because they are considered to be less prone to off-target modifications.[7–9] However, the main factor governing the nonspecific binding and related toxicity and/or side effects is the chemical reactivity of Michael acceptors towards the –SH group.[10–12]

Urease, an enzyme catalyzing the hydrolysis of urea,[13] is an interesting target for cysteine-binding compounds, although this type of urease inhibitor has been rarely explored. Urease is related to the development of infections caused by pathogenic bacteria, such as *Helicobacter pylori* (gastroduodenal tract) and *Proteus mirabilis* (urinary tract).[14,15] *H. pylori* produces large quantities of both intra- and extracellular urease to increase the pH of its microenvironment by ammonia released during enzymatic urease hydrolysis.[16,17] Some urinary tract colonizing bacteria (e.g., *Proteus mirabilis*) also produce urease, which causes rapid alkalinization of urine and precipitation of inorganic salts, forming stones (struvites and/or apatites).[18–20] The goal of combatting the above-men-

Abbreviations: AHA, acetohydroxamic acid; GHS, glutathione; PBMC, peripheral blood mononuclear cells; PI, propidium iodide; SRB, sulforhodamine B; Tol, tollyl.

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tioned pathogens stimulated research on urease inhibitors[21–23] and led to the discovery compounds such as phosphoramidates,[24–28] hydroxamates,[29–32] phosphinates,[33–36] and heterocyclic compounds.[37,38] Currently, only one urease inhibitor available on the market, namely, acetohydroxamic acid,[39] shows significant side effects, including teratogenicity.[40]

The active site of urease contains two nickel ions coordinated by four histidine residues (His137, His139, His249, His275), carbamoylated lysine (Kcx220) and aspartate (Asp363).[41–43] The active site is of relatively small volume (related to the size of urea) and is covered by a movable flap (Fig. S8). This flap contains a cysteine residue that could be targeted by inhibitors.[44] Currently, only a limited number of urease inhibitors possess this mode of action, including benzoquinone, ebselen derivatives, cyclohexanone and cyclopentenone.[45–50] The product of reaction of urease and benzoquinone has been recently characterized structurally.[51]

In this paper, we aimed to establish a general methodology to evaluate the activity-reactivity profile of reversible Michael acceptor-type inhibitors based on studies of urease inhibitors. The various types of Michael acceptors were extensively screened for their inhibitory properties against bacterial urease.

The measured inhibitory activities were correlated with the chemical reactivity (towards a model compound, glutathione) of the studied compounds to find molecules that exhibit favorable profiles related to specific interactions with the enzyme. Moreover, the cytotoxicity (towards mouse fibroblasts BALB/3T3) of the chosen compounds was analyzed. The most important findings were also rationalized by modelling the structures of the inhibitor-enzyme complexes.

A group of forty compounds of non-extended, Michael acceptor-type compounds was selected for screening. These compounds include molecules containing unsaturated functional groups of various geometries: E and Z isomers of substituted double bonds and linear triple bonds or allenes. All groups controlling the chemical reactivity of double/triple bonds contained carbonyl groups but showed significant differences in activating potency. Ketone and ester functional groups are considered to activate strongly, whereas carboxylates are much less reactive. Moreover, analogues of known inhibitors, cyclopentenone (**10a**) and cyclohexanone (**9a**), were also included in the study for comparison.

All compounds were tested against model bacterial urease purified from *Sporosarcina pasteurii* CCM 2056^{TM} with specific activity of 2451 U/mg. The values of the kinetic parameters ($K_M = 4.92 \pm 0.31$ mM and $v_{max} = 4.224 \pm 0.060$ μM s^{-1}) of highly purified urease in an enzymatic reaction were determined by fitting the initial reaction velocities measured over a range of urea concentrations to the Michaelis-Menten equation by nonlinear regression. The majority of the assayed compounds exhibited inhibitory activity in the micromolar or nanomolar range (Table 1). The nonlinear progress curves indicated that Michael acceptors produced time-dependent inhibition of urease activity, in which steady-state velocity (v_s) was attained slowly with both the initial (v_i) and steady-state velocity (v_s) decreasing with an increase in inhibitor concentration over the examined ranges. The linear replots of $1/v_i$ and $1/v_s$ showed a slow binding mode of action according to mechanism B, in which the initial *EI* complex undergoes conformational change into the final EI^* complex. The steady state inhibition constants (K^*_{iLB}) ranged from 0.00977 to 64.2 μM for compounds **8a** and **3e**, respectively. Importantly, the potency of some of the assayed compounds (**5**, **6a**, **8a**, **8b**, **8c**, **9a**, **9b**, **9c**, **9f**, **10a** and **10b**) exhibited higher activities than the reference inhibitor, acetohydroxamic acid (AHA). All inhibitors demonstrated competitive and reversible type inhibition, as confirmed by Lineweaver-Burk plots and fast dilution assay (data not shown). This mode of action of the studied inhibitors is consistent with the design assumption concerning the reaction with the cysteine residue present at the entrance to the active site. Most probably, the presence of neighboring histidine residue (His323), which could act as a base, makes the covalent modifications of cysteine residue reversible.

There are several interesting structure-activity relationships visible in the study. First, the geometry of the central unsaturated bond has a significant influence on the activity. E isomers of substituted double bond- and triple bond-containing compounds are capable of urease inhibition, whereas Z isomers are completely inactive against the target enzyme (e.g., **3a** and **8a** *versus* **4b** or **3h** and **8c** *versus* **4a**).

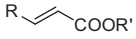
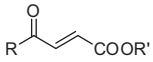
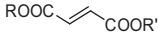
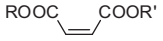
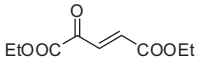
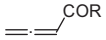
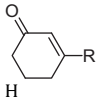
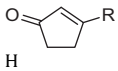
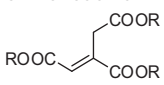
Second, carboxylic acids showed similar, or in some cases even higher, activity than analogous alkyl esters (e.g., **1a** *versus* **1b**, **3a** *versus* **3h**, **8a** *versus* **8c**). This finding is of primary importance because carboxylic acid-containing Michael acceptors are generally much less chemically reactive towards –SH groups than analogous esters. Therefore, the high activity of acids is related to their specific interactions with the enzyme (see the following section).

Third, in most cases, the substituents of phenyl groups or changes in ester functional groups have minor influence on the activity (e.g., **1b** *versus* **1d** *versus* **1e** or **3a-3f**). This observation suggests that these groups do not influence inhibitor binding.

Any compound showing high chemical reactivity with functional groups available in biological systems, in particular –SH groups, has to be avoided in medicinal chemistry due to unpredictable influence on other biomolecules. Unspecific toxicity is correlated with high reactivity of Michael acceptors.[10] Therefore, the preferred profile of the Michael acceptor-based active compound is based on a combination of high inhibitory activity and low reactivity towards thiols. The reactivity of representative compounds was assayed using glutathione (GSH) as a model compound with reactive cysteine residues. The modified Ellman method, where the quantification of unreacted GSH was performed using a spectrophotometric-based concentration-response assay, was applied.[52] The reactions of the extremely reactive Michael acceptor compounds (**3a**, **8a**) with GSH are completed within less than 15 min, whereas compound **1b** demonstrates no reactivity, even after 36 h of reaction (examples of progress curves and ^{1}H NMR spectra of reaction mixtures are shown in Figs. S3–S5, respectively). The tested compounds spanned nearly six orders of magnitude of reactivity, with $\log k_{GSH}$ values between -3.138 ± 0.077 and 2.588 ± 0.088 for ethyl cinnamonate (**1a**) and dimethyl acetylenedicarboxylate (**8a**), respectively (Table S4). The dependence between pK_i and $\log k_{GSH}$, shown graphically in Fig. 1, allows for classification of the studied compounds into three groups. Compounds from the first group show roughly linear dependence between activity and reactivity (**1a**, **8a**, **9a**, **9c**, **10a**, **10b**), which suggests their nonspecific interaction with the enzyme. The second group contains structures with high chemical reactivity and moderate inhibitory activity (**2a**, **2b**, **3a**). In this case, the interactions of the compound with the enzyme are specific but unfavorable due to steric impairment. The third group is of the highest importance because it combines compounds with a preferable profile: high inhibitory potential and low –SH reactivity (**1b**, **3h**, **8c**). In these cases, inhibition of the enzyme is driven not only by –SH reactivity but also specific interactions between the ligand and protein. The specificity of compounds **1b**, **3h**, **8c** was additionally confirmed by complete lack of inhibition of model cysteine-dependent enzyme – papain. On the basis of the discussed graph, compounds of interest are easily indicated; in this particular case, acetylenedicarboxylic acid (**8c**) with $K^*_{iLB} = 42.5$ nM and $\log k_{GSH} = -2.14$ has the most beneficial activity-reactivity profile.

The cytotoxicity towards normal mouse fibroblasts (BALB/3T3) for a group of selected Michael acceptor compounds was

Table 1
Inhibitory activity of Michael acceptor-based compounds against *S. pasteurii* urease (detailed inhibitory characteristics are given in Table S3) and cytotoxicity (LC_{50}) towards mouse fibroblasts (BALB/3T3) of the selected compounds.

no	Compound		K^*_{iLB} [μM]	LC_{50} [μM]
	R	R′		BALB/3T3 SRB assay
				
1a	Ph	Et	NI	100 μM >; 29.09%[a]
1b	Ph	H	21.5 ± 2.1	NT
1c	*p*-Tol	Et	NI	
1d	*p*-Tol	H	34.2 ± 2.8	
1e	*p*-NO_2-C_6H_4-	Et	NI	
1f	*p*-NO_2-C_6H_4-	H	11.9 ± 1.1	
				
2a	Ph	Et	3.956 ± 0.026	24.5 ± 5.4
2b	*p*-Tol	H	6.36 ± 0.45	100 μM >; 31.28%[a]
				
3a	Me	Me	21.3 ± 1.7	39.7 ± 7.0
3b	Et	Et	11.1 ± 1.0	
3c	*i*Pr	*i*Pr	42.4 ± 2.7	
3d	Bu	Bu	53.7 ± 3.7	
3e	*s*Bu	*s*Bu	64.2 ± 3.5	
3f	Et	*t*Bu	22.1 ± 1.8	
3g	Et	H	52.4 ± 5.0	
3h	H	H	36.5 ± 3.2	NT
				
4a	H	H	NI	
4b	Me	Me	NI	
5			0.0928 ± 0.0076	
				
6a	OEt		2.52 ± 0.20	
6b	NHBn		NI	
7a	H	Me	46.0 ± 4.1	
7b	H	H	44.4 ± 3.5	
7c	Me	Et	126 ± 12	
7d	Me	H	109.3 ± 8.7	
7e	Ph	Et	52.3 ± 4.5	
7f	Ph	H	37.1 ± 3.4	
8a	Me	Me	0.00977 ± 0.00084	29.8 ± 1.7
8b	Et	Et	0.01020 ± 0.00095	
8c	H	H	0.0425 ± 0.0036	NT
				
9a	H		0.766 ± 0.063	100 μM >; 29.76%[a]
9b	CH = CHCN		0.784 ± 0.071	
9c	CH = CHCOOMe		0.694 ± 0.064	
9d	CH = CHCOOH		17.8 ± 1.6	
9e	CH = CHC(O)Me		21.2 ± 2.0	
9f	CH = CHC(O)Ph		0.592 ± 0.045	
				
10a	H		0.291 ± 0.016	86.5 ± 5.4
10b	CH = CHCOOMe		0.285 ± 0.026	100 μM >; 35.23%[a]
				
11a	Me		5.21 ± 0.37	
11b	H		6.13 ± 0.48	
AHA			3.30 ± 0.40	
cis-platin				1.92 ± 0.63

NI – no inhibitory activity, NT – not toxic.
[a] Percent of proliferation inhibition at 100 μM.

determined as the concentration required to inhibit 50% proliferation of the cell population (LC_{50}) and is reported in Table 1. As expected, compounds with low reactivity towards glutathione displayed little (**1a**, **9c**, **10b**) to no or almost no (**1b**, **3h**, **8c**) cytotoxic

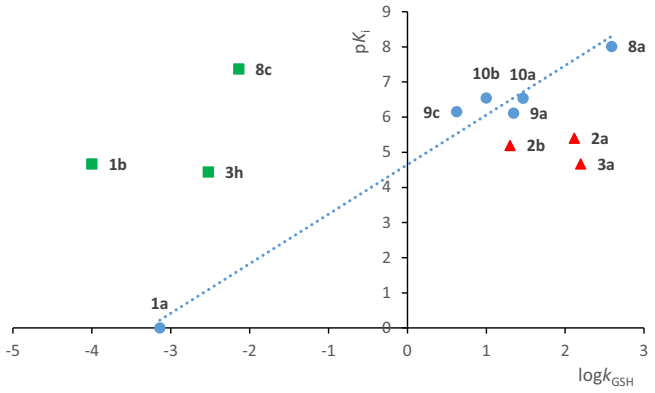


Fig. 1. The inhibitory activity – chemical reactivity profile of selected Michael acceptors (for detailed data, see Table S4).

effects (against BALB/3T3) compared to *cis*-platin as the positive control. However, compounds with the highest chemical reactivity (**2a**, **8a**) demonstrate significant toxicity both in the antiproliferative studies against BALB/3T3. These results are in agreement with previous results on the relationships between various types of toxicity and –SH reactivity.[10,53,54] and confirm that the applied methodology of the selection of compounds of interest by the analysis of their activity-reactivity profile is appropriate.

The biological relevance of the inhibitors was studied *in vitro* against *Proteus mirabilis* strain, which is a urease-positive pathogen of the human urinary tract.[55] *P. mirabils* urease is shows high sequence similarity to *S. pasteurii* enzyme (Fig. S6). The whole-cell urease inhibition studies were performed under conditions corresponding to the acidity of physiological urine (pH 5.5). The effectiveness of urease inhibition against intact cells was evaluated for compounds **1b**, **3h**, **8c** and **1a** (negative control), and compared with well-established acetohydroxamic acid — AHA (Table 2). In the presence of 5 mM urea using 1×10^8 cfu/mL, the IC_{50}^{0} values in non-preincubated assays were in range from 169 to 492 μM (for AHA and compound **1b**, respectively). The IC_{50} parameter after 2 h preincubation of cells with tested inhibitors was lower for all compounds, what means that the degree of inhibition increased with time. The highest activity enhancement was observed for **3h** ($IC_{50}^{2h} = 65.5 \pm 7.2$ μM) and **8c** ($IC_{50}^{2h} = 88.6 \pm 9.3$ μM), this classified them as more potent urease inhibitors than AHA ($IC_{50}^{2h} = 91.4 \pm 8.8$ μM). The non-preincubated system likely requires time to allow the interaction between the enzyme and the inhibitor. This is related to the fact that the studied compounds constitute a time-dependent class of urease inhibitors. Secondly, the *P. mirabilis* urease is located only in the cell cytoplasm, therefore the inhibitory efficiency is strongly dependent on the compound diffusivity. The obtained result show that the newly developed small structures are able to cross bacterial cell membranes and inhibit urease in intact bacterial cells.

Table 2
Whole-cell inhibition (IC_{50}) of native *P. mirabilis* urease.

Compound	IC_{50}^{0} [μM]	IC_{50}^{2h} [μM]
1a	NI	NI
1b	492 ± 54	125 ± 14
3h	182 ± 17	65.5 ± 7.2
8c	326 ± 38	88.6 ± 9.3
AHA	169 ± 15	91.4 ± 8.8

IC_{50}^{0} – parameter assayed without preincubation of cells with inhibitory compounds; IC_{50}^{2h} – parameter assayed using cells subjected to 2 h of preincubation with inhibitors in PBS; NI – no inhibitory activity.

Taking into account the result of cytotoxicity assay (Table 1) and whole-cell inhibition of native *P. mirabilis* urease for compound **3h** and **8c** (Table 2), it seems that this inhibitors may be clinically useful for the treatment of the human urinary tract infections. Moreover, our results also emphasize the importance of experiments analyzing the influence of the tested compounds on living cells. This type of assay is indispensable step in the screening of chemical compounds enabling to determine the side effects of the tested inhibitors as well as their actual biological activities.

Analysis of the mode of binding for the most active inhibitor of bacterial urease — compound **8c** — was performed using a molecular modelling approach. The detailed analysis of the active site of urease revealed that the cysteine residue (Cys322) located at the movable flap is accompanied by a histidine residue (His323). This residue is expected to catalyze the reaction of the Michael acceptor addition to Cys322. We assume that the mechanism of inhibition is composed of two steps: reaction with Cys322 and rearrangement to an energetically favorable conformation. This mechanism is in agreement with the experimental results indicating slow binding mechanism type B. In the case of compound **8c**, which has a triple bond, the reaction can lead to two potential configurations of the product (E and Z). However, the enzyme is expected to act stereoselectively, and modelling studies indicate the formation of the product with E stereochemistry (both carboxylic acid groups placed at the same side, Fig. 2). This arrangement is supported by two pieces of evidence: (a) His323 (acting as a base) and Cys322 are located at the same side of the inhibitor molecule; therefore, the sulfur and hydrogen atoms should be added to the reacting molecule at the same side (putative reaction mechanism given in Fig. S7); (b) the final conformation of the adduct is thermodynamically more stable in the case of E stereochemistry. Similar mode of binding was observed in crystal structure of citrate-urease complex (PDB id 4AC7).[56] The molecule of citric acid also chelates two nickel ions by one carboxylate and forms hydrogen bonds between the second carboxylate and Arg339 (see Fig. S9 for superimposition of these two structures).

Compound **8c** is the first example of an interesting mode of binding, which combines the formation of a covalent bond with a cysteine residue and interactions with two nickel ions. This interaction pattern is promising because inhibitors acting this way have high activity and selectivity towards bacterial urease.

Covalent inhibitors of urease are a virtually unexplored field, and the presented results considerably increased the number of

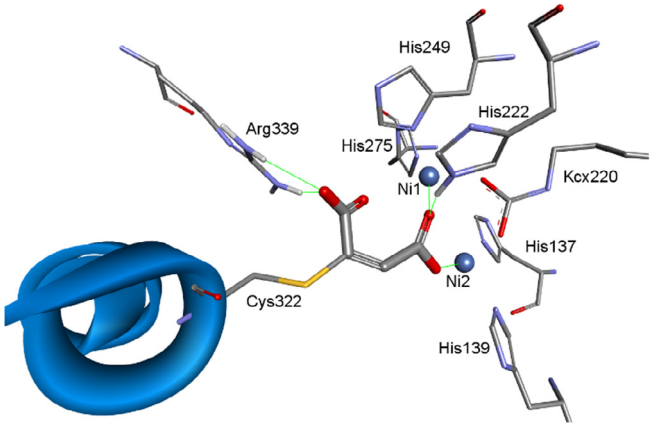


Fig. 2. Modelled structure of the product of addition of compound 8c to *S. pasteurii* urease. The movable flap is shown as a solid ribbon, residues of the active site are presented as sticks, and metal ions are shown as blue spheres. Non-covalent interactions of the inhibitor are shown as solid green lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

known compounds of this type. Although the majority of studied compounds showed significant inhibitory properties, only some of these structures are of interest due to specific reactivity with –SH groups. The analysis of the activity-reactivity profiles of covalent reversible enzyme inhibitors identified the most valuable compounds, which were confirmed by cytotoxicity studies. Compounds with nanomolar inhibitory activity towards highly purified urease and micromolar inhibitory activity against the ureolytically active *P. mirabilis* strain, low reactivity towards glutathione, and no or almost no cytotoxicity were discovered within the studied library of compounds.

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A. Supplementary material

Supplementary data including detailed protocols for the synthesis of compound **9d**, analytical HPLC data for compounds **9b-f** and **10b**, the urease purification, inhibition and reversibility studies, analysis of the mode of inhibition, toxicity studies, inhibitory and reactivity profiles of the studied compounds, an example of a GSH reaction profile, an examples of ^{1}H NMR spectra of Michael acceptor/GSH reaction mixtures, whole-cell urease activity assay and papain activity assay and molecular modelling of inhibitor-urease complex, associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.02.022.

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