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Cysteine based novel noncompetitive inhibitors of urease(s)—Distinctive inhibition susceptibility of microbial and plant ureases

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Abstract—Based on the catalysis mechanism of urease, a homologous series of 10 cysteine derivatives (CysDs) was designed and synthesized, and their inhibitory activities were evaluated for microbial ureases (*Bacillus pasteurii,* BPU, and *Proteus mirabilis,* PMU) and for a plant urease [jack bean (*Cavavalia ensiformis*), JBU]. As already described, thiol-compounds might inhibit urease activity by chelating the nickel atoms involved in the catalysis process. In contrast to cysteine, which has been reported to be a very weak urease inhibitor, we verified a potential inhibitory activity of these CysDs. The kinetic data demonstrate that thiol derivatives are more effective than the respective thioether derivatives. Besides, thiol-CysDs had a reduced activity in acidic pH (5.0). Lineweaver–Burk plots indicated that the nature of inhibition was of noncompetitive type for all 10 compounds, with the minimum K_i value of 2 μM for *N*,*N*-dimethyl L-cysteine. It is proposed that these classes of compounds are more potent inhibitors of the bacterial ureases, compared with the plant-originated urease. Since microbial urease is directly involved in the infection process of many pathological organisms, this work demonstrates that thiol-CysDs represent a class of new potential urease inhibitors.

1. Introduction

Ureases (urea amidohydrolase; EC 3.5.1.5) are nickel-dependent enzymes that catalyze the hydrolysis of urea to form ammonia and carbon dioxide.[1,2] Ureases have been isolated from a wide variety of organisms, including plants, fungi, and bacteria.[3,4] Structural studies of the enzymes from *Klebsiella aerogenes*, *Bacillus pasteurii*, and *Helicobacter pylori* have revealed a dinuclear Ni active site with a modified amino acid side chain-containing a carbamylated lysine residue that bridges the deeply buried metal atoms.[5–7] Although only bacterial ureases have had their 3D crystallographic structure successfully resolved, the high sequence similarity of all ureases indicates they are variants of the same ancestral protein, and are consequently likely to possess similar tertiary structures and catalytic mechanisms.[8]

The primary physiological role of urease is to allow the organism to use externally and internally generated urea as a nitrogen source (for reviews, see 4 and 9). Urease has also been described as defense protein against predators and phytopathogenic organisms.[3,10,11] Urease is known to be a major cause of pathologies induced by *H. pylori*, which allows the bacteria to survive at the low pH of the stomach during colonization and, there-

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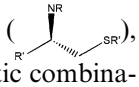
fore, plays an important role in the pathogenesis of gastric and peptic ulcers (including cancer).[8] In infections with *Proteus mirabilis* and *Yersinia enterocolitica*, urease has been implicated in urolithiasis (stone formation) and contributes to the development of acute pyelonephritis and infection-induced reactive arthritis, respectively.[8,12,13] In agriculture, high urease activity causes significant environmental and economic problems by releasing abnormally large amounts of ammonia into the atmosphere during urea fertilization. Moreover, it induces plant damage primarily by depriving plants of their essential nutrients and secondarily by ammonia toxicity, increasing the pH of the soil.[8,14]

Given the diverse functions of this enzyme, its inhibition by potent and specific compounds could provide an invaluable addition for the treatment of infections caused by urease-producing bacteria. Although several potent inhibitors of this enzyme have been reported. [15–17] as the first line of treatment for infections caused by urease-producing bacteria, more effective inhibitors with safe and more potent profile are considered necessary for the control of urease-related ailments and to explore any novel aspects of mechanism of action of ureases isolated from different sources.

Based on the previous data of urease inhibition properties of thiol-compounds,[18] 10 different cysteine derivatives (CysDs) with cysteine-like scaffold (), were designed and synthesized from synthetic combinatorial library. CysDs were then tested for urease-inhibitory activity against two bacterial and one plant-derived enzyme, as well as for the inhibition of growth of bacteria and yeast. Structure–activity relationship (SAR) of the active compounds identified a novel pharmacophore model for urease inhibitors.

2. Results and discussion

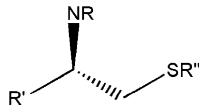
Several thiol compounds have been reported to be competitive inhibitors of ureases. Todd and Hausinger (1989) demonstrated the susceptibility of *K. aerogenes* urease to thiol compounds with K_i values in the range of 0.01 mM (cysteamine) to >100 mM (2-propanethiol and 3-mercaptopropionate).[18] In addition, the absorption spectrums in the presence of such inhibitors are similar to those seen with known thiolate–nickel complexes, suggesting the mechanism of inhibition might involve an interaction with Ni atoms in catalytic site.[18,19] The K_i for cysteamine decreases linearly as pH is increased, with a slope of 0 at pH values above its pK_a (8.1), indicating that the deprotonated thiol is acting as the inhibitory species. Thiol compounds that contain an anionic carboxyl group are typically poor inhibitors at pH 7.75, such as cysteine (K_i ~95 mM) and 3-mercaptopropionate (K_i > 100 mM).

Cysteine derivatives containing a carboxyl group have been previously shown as poor inhibitors of ureases. For instance, cysteamine (K_i = 0.01 mM) with no carboxyl group is more potent urease inhibitor than cysteine methyl ester ($COOCH_3$; K_i = 0.12 mM) which is more potent than cysteine (COOH; K_i = 95 mM). A 12- to 9500-fold increase in urease inhibitory potency of cysteine was observed for cysteine methyl ester and cysteamine, respectively. This observation makes carboxyl group a limiting factor while designing any cysteine based inhibitors. On the other hand, it had also been reported that thiol compounds such as ethanethiol and 2-propanethiol, that do not contain any carboxyl group, display a very weak inhibitory activity.[18]

Taken these findings together and in order to find new potential inhibitors for microbial ureases, we decided to design CysDs, comprised of carboxyl and thiol groups alone as well a chimera of both groups. A series of 10 different L-cysteine (alkylated, acidic, and alcoholic) derivatives was designed (Table 1) synthesized (Fig. 1) and checked for the kinetic parameters of urease inhibition (Table 2). Thiol derivatives were found to be more potent than thioether derivatives in inhibiting the activity of both *P. mirabilis* urease (PMU) and *B. pasteurii* urease (BPU) (Fig. 2A). Compounds **4** and **7** were 2–4-fold potent inhibitors of PMU than BPU, whereas compounds **1**–**3** selectively inhibited BPU over PMU with 1.5–2-fold magnitude. In general, PMU was more susceptible to thioether CysDs than BPU. Neutralization of the terminal SH group in CysDs (by CH_3I, CH_3COOH treatment; Fig. 1) or SH-terminal extension (by C_2H_5, CH_3COOH; Fig. 1) altered the inhibitory effects, suggesting a determinant role of substituted thiol-terminal residues in binding to urease, although not necessarily to the active site. Based on the preliminary structure–activity relationships, we speculate that the electronegative sulfhydryl group of CysDs acts as a ligand chelator (probably to Ni atoms, similar to other thiol compounds) to form subsequent complexes with the enzyme. Formation of the initial E·I complex may involve coordination of the –SH- anion that has pK_a value around 8.7 (as proposed for other anions; e.g., phosphates). This hypothesis agrees with the observation in the present study that K_i is pH-dependent. Sequence similarity between PBU and PMU indicates 60.3% identity of amino acid residues for these microbial ureases. In addition, all residues involved in catalytic site, that is, D_{362}, H_{274}, H_{248}, K_{219}, H_{136} and H_{138} (in BPU), are conserved. The findings with CysDs indicate the other unnoticed important structural differences at or near the active sites of these very conserved bacterial ureases that affect their susceptibility to CysDs implicating that rational development of inhibitors for these enzymes must to consider these aspects.

BPU inhibition by **4**, **7**, **8**, and **10** was dependent on the pH of the pre-incubation mixture (Fig. 2B), with a decrease in K_i values at pH 8.2. In contrast, compound 1 showed an increasing K_i at pH 8.2. Out of these compounds, thiol-CysDs (**7**, **8**, and **10**) are more potent in inhibiting BPU at pH 8.2 than the respective thioether CysDs (**2**, **3**, and **4**). This difference in their inhibition might be explained by the thiolate formation at pH 8.2 as described for *K. aerogenes* urease inhibition by thiol compounds.[18]

Table 1. Design of Cysteine Derivatives (CysDs) (with cysteine-like scaffold) and their observed urease inhibitory effects



Chemical groups	R, R′ and R″	Urease inhibitory effects
Sulfur derivatives: thioethers versus thiols	R″ = H, CH_3 or CH_2CH_3	Very important [compare **1**–**6** (thioethers) with **7**–**10** (thiols)]
Oxygen derivatives	R′ = COOH, CH_2OH, CH_2OCH_3	Important (compare **2** → **3** → **4** with **7** → **9** and **7** → **10**)
Amine derivatives: primary amines versus tertiary amines	R = H or CH_3	Not so important (compare **1** → **2** with **5**→ **6**)

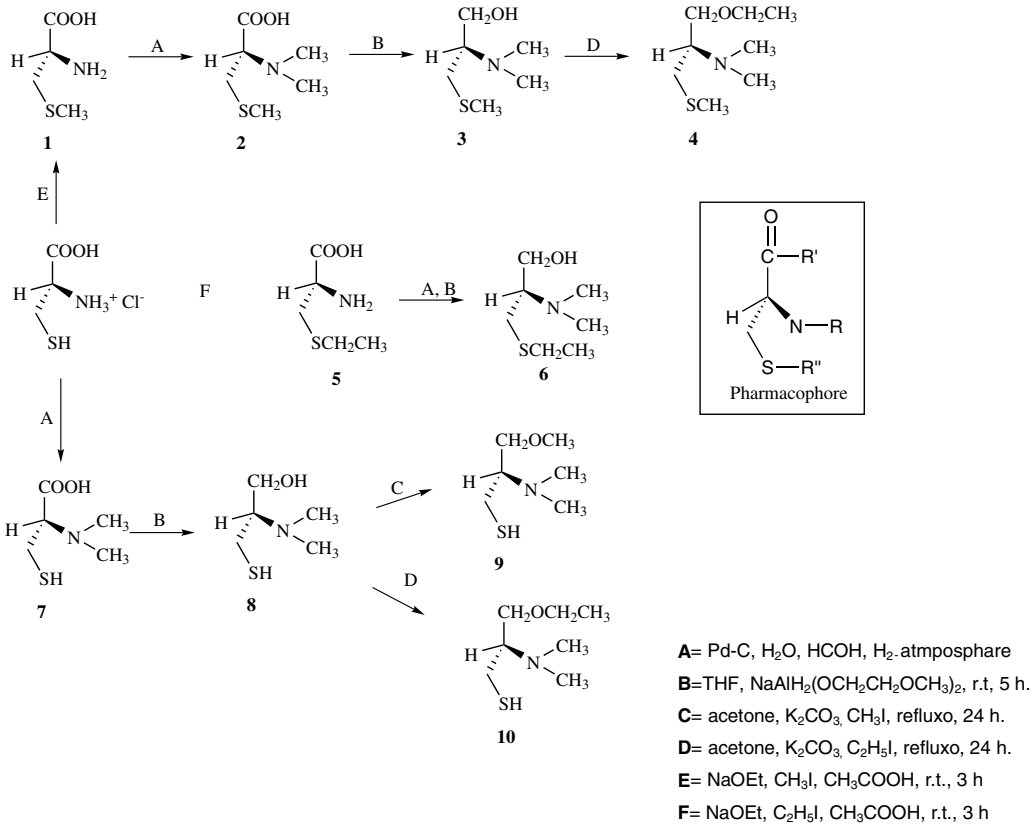
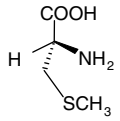
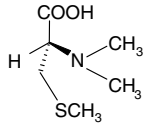
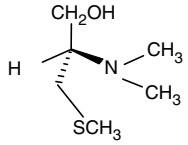
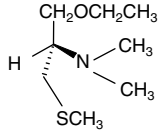
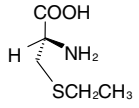
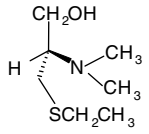
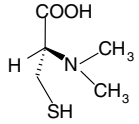
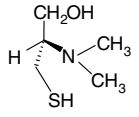
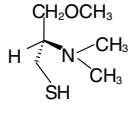
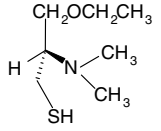


Figure 1. Schematic synthesis of a series of 10 different L-cysteine (alkylated, acidic, and alcoholic) derivatives starting from commercially available amino acid, L-cysteine hydrochloride hydrate.

A comparison of the inhibitory activity of CysDs containing carboxyl group (**2**, **5**, and **7**) with those containing alcohol group (**3**, **6** and **8**, respectively), that is, comparing (**2** and **3**, **5** and **6**, **7** and **8**) did not show any significant difference in their inhibition pattern at either pH values (Fig. 2B). This is in contrast to the reduction in the inhibitory effect of CysDs when its acidic hydrogen (carboxylic or alcoholic hydrogen) is removed by the formation of ether groups (e.g., carboxylic CysDs to ether derivatives, compare: **2** → **4**, **7** → **9**, **7** → **10** and from alcoholic CysDs to ether derivatives, compare: **8** → **9**, **8** → **10**, and **3** → **4**). Since the inhibitory activity of CysDs decreases in the following order **2**–**3** > **4** and **7** > **9** and **7** > **10**. If we assume that carboxyl group is there to impede the urease inhibitory activity of CysDs then there must be some other functional groups which are modulating the inhibitory properties of CysDs. In this scenario sulfhydryl group seems to be very promising not only in overcoming the undesirable effects of carboxyl group but also to enhance the urease inhibitory properties of CysDs. Our data suggest that the presence of thiol group is more critical to urease inhibition than carboxyl group (Table 1).

Although CysDs inhibited the bacterial enzymes potently (BPU IC_{50} = 10–110 μM and PMU IC_{50} = 22–100 μM) than jack bean urease (JBU), of the 10 compounds tested only three were inhibitors of JBU (**2**, **7**, and **10**). For instance compound **2** is more effective against the plant enzyme than BPU (Fig. 3). The present

Table 2. Inhibition effect ($IC_{50} \pm SEM$, μM) of CysDs on microbial ureases (BPU and PMU) and plant urease (JBU) and the influence of two different pH (pH 5.0 and 8.2) on the BPU inhibition by CysDs

Compound	IC_{50} (pH 7.5), μM			K_i (BPU), μM	
	PMU	JBU	BPU	pH 5.0	pH 8.2
Cysteine	—	—	—	$1 \times 10^5 \pm 450$	$1 \times 10^5 \pm 410$
Cysteamine	—	—	—	20 ± 0.9	15 ± 1.6
Hydroxyurea	150 ± 5	100 ± 2.5	140 ± 7	—	—
	50 ± 3.5	N.I.	78 ± 3.4	9 ± 1.4	36 ± 3.5
	50 ± 1.5	78 ± 6.3	110 ± 7.5	6 ± 0.75	8 ± 1.5
	63 ± 4.1	N.I.	95 ± 5.0	5 ± 0.5	15 ± 0.41
	100 ± 4	N.I.	50 ± 6.5	7 ± 0.65	40 ± 4
	35 ± 1.5	N.I.	30 ± 2.5	11 ± 2.5	14 ± 1.5
	40 ± 3	N.I.	29 ± 1.6	18 ± 1.6	17 ± 3
	50 ± 2.5	58 ± 2.5	10 ± 0.02	9 ± 2.0	2.0 ± 0.25
	22 ± 3.1	N.I.	25 ± 1.2	13 ± 1.2	2.0± 0.31
	35 ± 1.5	N.I.	36 ± 4.8	19 ± 4.8	2.0 ± 0.15
	31 ± 2.5	100 ± 5.5	20 ± 1.0	55 ± 1.0	2.0 ± 0.25

Values given are means ± SEM of three independent experiments.
N.I., no inhibitory activity. IC_{50} values are comparable with hydroxyurea (IC_{50} = 100 μM).

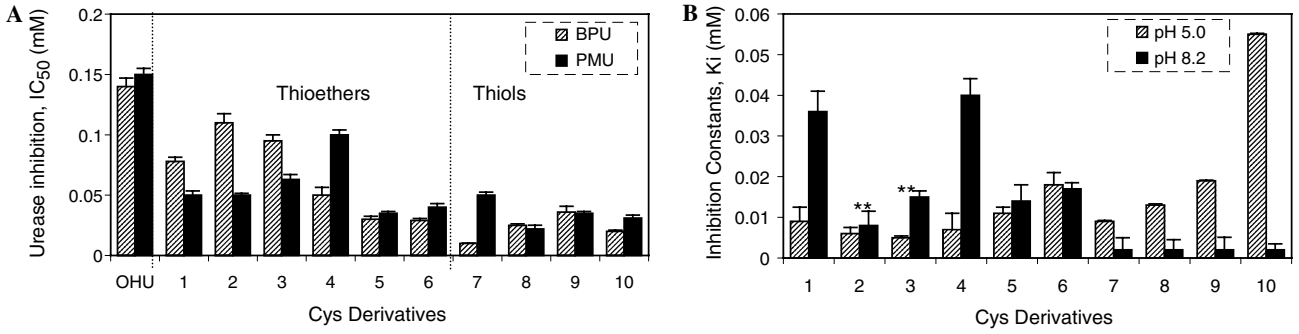


Figure 2. Inhibitory effects of CysDs on microbial ureases. (A) The susceptibility of *Bacillus pasteurii* urease (BPU) and *Proteus mirabilis* urease (PMU) to CysDs was evaluated. Microbial enzymes were incubated in the presence of 5 μl of CysDs (10–100 μM concentration) at 37 °C, pH 7.5, for 30 min and then residual enzyme activity was monitored as described.[31] Values are highly significant, except **2**, **3**, and **10**. (B) Effect of pH (5.0 or 8.2) on the activity of BPU, determined by the inhibitory constant, K_i. Values given are mean ± SEM of three independent experiments. In (A) statistical analyses indicated significant effects except compounds **2**, **3**, and **10**.

observations of different kinetic behavior of bacterial and plant ureases are consistent with the structural differences between the two types of urea-splitting enzymes already reported in the literature.[20,21] The data of kinetic parameters for the CysD inhibitors are summarized in Table 2.

The mode of inhibition of BPU activity by the CysDs was analyzed by Lineweaver–Burk (LWB).[22] Inhibition was found to be noncompetitive (also denoted as mixed inhibition) at both pH values (5.0 or 8.2), Fig. 4; data shown for only compounds **6** and **8**. CysDs were found to decrease V_{max} ranging from 30.5% to 70.4% without producing an appreciable change in the K_m values. The mean K_m values were found to be between 5.04 ± 0.10 and 8.93 ± 0.20 μM. The inhibition pattern observed suggests a modification of any functional group or a conformational change in the environment near the catalytic site rather than in the active site, thus causing a decrease in V_{max} in the presence of inhibitor without any change in K_m values of urease.

N,*N*-Dimethyl L-cysteine (**7**; K_i value 2.0 μM) and *S*,*N*,*N*-trimethyl L-cysteine (**2**; K_i value 6.0 μM) were found to be the most active ones, most probably due to the small R, R′, and R″ groups (Fig. 1). Based on the above data, we propose that the initial binding of the COOH-group core could serve as the driving force for the second complementary interaction, effectively bridging two remote binding domains separated by a spacer of suitable length. The potency of this series was improved through the incorporation of more electronegative and less bulky groups. Moreover, bifunctional inhibitors of this type are expected to exhibit enhanced activity and specificity resulting from a favored intramolecular interaction.

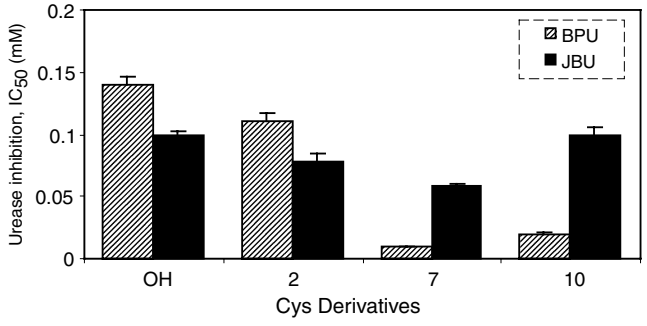


Figure 3. Inhibitory effects of CysDs **2**, **7** and **10** on jack bean urease (JBU) and BPU as described in Fig. 2A. Values given are means ± SEM of three independent experiments.

In addition to establishing the mechanism of urease inhibition at the molecular level, we were also interested in finding the effects of CysDs on the growth of urease-producing bacteria. CysDs inhibited the growth of both BP and PM, however not as strongly as their inhibitory effect seen upon cell-free ureases (Fig. 5). Antibacterial activity of CysDs was assessed in the presence of 0.5 mM NiCl (NiCl is needed to produce activated nickel–urease complex). It is possible that the CysDs bind free metal and the resulting CysDs–Ni complex might be less potent in inhibiting bacterial growth compared with the corresponding metal-free CysDs, because the inhibitors seem to bind to the nickel site of the urease, as has been established above.

This observation suggests that the inhibitory effects of CysDs on PMU and BPU activity might be related to their ability to inhibit bacterial growth, consistent with the previous findings that there is a direct relationship between the growth and urease inhibitory actions of such compounds.[21]

In order to evaluate the physiological tolerance of CysDs, various dose levels of the inhibitors were assessed for their mutagenic potential using *Saccharomyces cerevisiae* strains optimized for this purpose. But none of the inhibitors showed any considerable mutagenic effect or did not interfere with the cell-proliferation of the yeasts at nuclear level, except for **1**, **3**, and **4**, which were found to be toxic against both strains in the range of 500–1000 μM. This may be correlated to their cytotoxic effects rather than DNA damage, since according to the principle of the assay, any agent that is cytotoxic (>65% inhibition) to the yeast in the RS322Yplate

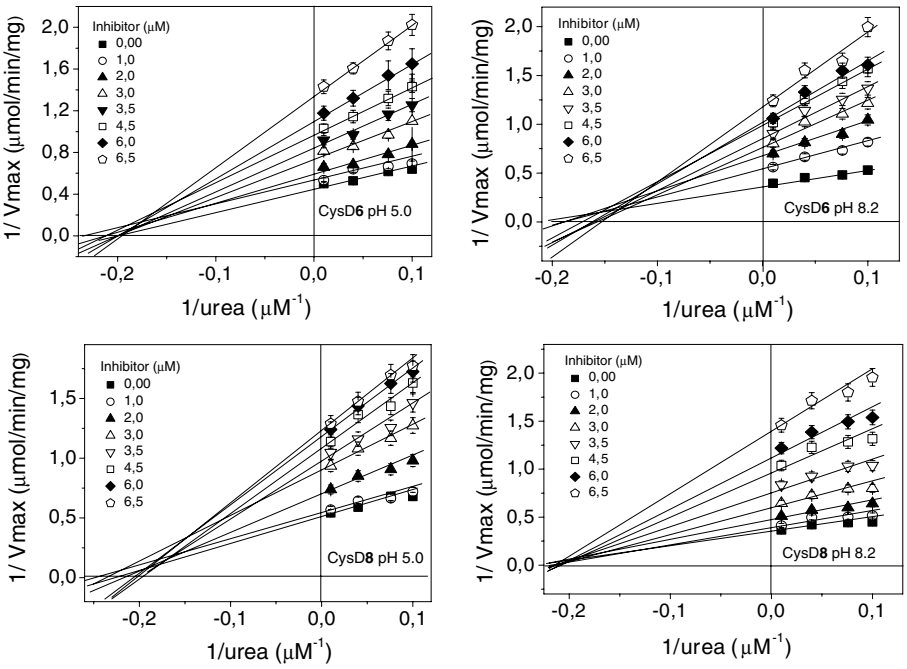


Figure 4. Lineweaver–Burk plots for BPU in the presence of CysDs. Lineweaver–Burk plots representing reciprocal of initial enzyme velocity versus reciprocal of urea concentrations in the absence and presence of inhibitors **6** (above) and **8** (below) (concentrations mentioned in the legend box) at both pH 5.0 (left) and 8.2 (right). Each point represents means ± SEM of three independent observations ($p < 0.05$). K_m of BPU in the absence of inhibitor was found to be 120 μM.

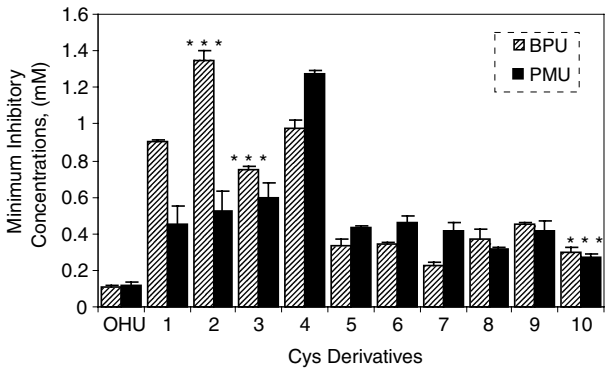


Figure 5. Minimum inhibitory concentrations (MICs) in the presence of 0.1–1.5 mM CysDs after 24 h of incubation. The progression in the turbidity of the broth was used as a measure of bacterial growth at 600 nm in 96-well plates. Statistical analyses indicated significant effects except compounds **2**, **3** and **10**.

(mutated species) and not cytotoxic (<35% inhibition) to the yeast in the LF15 plate (wild type), or whose IC_{50} gives a 3-fold differential in the concentration response assay will be considered genotoxic.

3. Conclusions

The present study concludes that CysDs inhibit bacterial and plant ureases in a concentration-dependent manner and shows that these compounds are more effective at inhibiting microbial ureases (BPU and PMU) than the plant urease (JBU). The kinetic data suggest that these novel inhibitors act in a noncompetitive (mixed) fashion. CysDs and other similar compounds may provide useful probes for the study of the active site of urease and other hydrolytic enzymes, their catalytic activity, intimate details of the molecular geometry at the active-site, and the kinetics of the binding site of the urease. Additional detailed studies with more potent derivatives of CysDs will further clarify the mechanism of action of this enzyme in urea hydrolysis, and provide new template structures for the design of new inhibitors with an optimized pharmacological profile.

4. Experimental

4.1. Chemical synthesis

The CysDs (**1**–**10**) were synthesized starting from commercially available amino acid, L-cysteine hydrochloride hydrate (Fig. 1).[23–29] Elemental microanalysis (C, H, N percentages), infrared (IR) and mass spectrometry, proton nuclear magnetic resonance (^{1}H NMR), and carbon 13 nuclear magnetic resonance (^{13}C NMR) have been used to determine the structures of compounds.

4.2. Enzymes

Commercially available preparation of *B. pasteurii* urease (100,000 U/g, pH 8.2; Sigma) and Jack bean urease type C-3 (12,000,000 U/g, pH 7.0; Sigma) was used in all experiments without further purification. *P. mirabilis* urease was partially purified as described.[30] The specific activity of the enzyme was 100,000 U/g protein.

4.3. Urease inhibition assay

In order to evaluate the inhibitory properties of CysDs on the urease activities, the purified enzymes were incubated in the presence of 5 μl of CysDs (0.001–0.2 mM concentration) at 37 °C, pH 7.5, for 30 min in 96-well plates. Urease activity was determined by measuring ammonia production using the indophenol method.[31] One unit of urease was defined as the amount of enzyme that releases one μmol ammonia per minute, at 37 °C, pH 7.5. The concentration that inhibited 50% of urease activity (IC_{50}) was determined using the EZ-Fit™ 5.0, software program (Perrella Scientific, Inc. USA). The K_i values (the dissociation/inhibition constant of the urease–CysDs complex into free BPU and CysDs) were also calculated using three different methods. First, the slope of each LWB plot was plotted against concentrations of different CysDs. Second, $1/V_{maxapp}$ was calculated at each concentration of different inhibitors and then plotted against CysD concentration; the K_i was calculated from the abscissa. Third, the K_i were determined directly from the intersections of the line for each substrate concentration on the *x*-axis. Similarly, V_{maxapp} was determined by the intersection of the line for each substrate concentration on the *y*-axis.

4.4. Antibacterial activity

Antibacterial activity was assessed by adding 95 μl of the microbial suspensions of 10^{7-8} colony-forming unit/ml density in 100 μl of broth containing yeast extract (2%), NiCl (1 mM), and urea (2%) in nutrient broth containing 5 μl of test compounds in 96-well plates. The cultures were incubated at their optimum temperatures for 24 h. The progression in the turbidity of the broth was used as a measure of bacterial growth and measured at 600 nm in 96-well Microplate Reader (Molecular Devices, USA). Minimum inhibitory concentrations (MICs): the lowest concentrations of the compound giving >50% inhibition of growth were determined in the presence of 0.1–1.5 mM test compounds after 24 h of incubation.

4.5. DNA damaging microtiter assay

In order to evaluate the potential of the compounds to induce DNA damage, they were incubated with *S. cerevisiae* strains (gifted by Leo F. Faucette of Smith Kline & French Research & Development Labs, PA, USA) in media enriched with YPD that is, yeast extract (1%), peptone (2%), dextrose (2%), and +/− agar (2%) as described.[32]

4.6. Estimation of protein concentration and statistical analysis

The protein content of the enzyme preparation was estimated as described by Lowry et al.[33] using bovine serum albumin (Sigma) as standard. Graphs were plotted by using GraFit program (Version 4.09, Erithacus Software Ltd, Staines, UK). All the values including K_i, K_m, V_{max}, correlation coefficient, slope, intercept, and their standard errors are presented as means ± SEM and obtained by the linear regression analysis using this program. All statistical comparisons were performed by two-tailed *t*-test.

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