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Inhibition of Soybean Urease by Triketone Oximes

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Abstract—Competitive inhibition of soybean urease by 15 triketone oximes has been studied at 36°C in aqueous solution (pH 4.95). The studied oximes are supposed chelators for the nickel atom in the urease metallocenter. The inhibition constants of urea hydrolysis (K_i) varied in the range 2.7-248 μM depending on the oxime structure. Analysis of this dependency demonstrates that the optimal inhibitor is the one containing carbonyl group in position 1 of the cycle, the ethoxyimino group and alkyl residue in the substituent in position 2, as well as the methoxycarbonyl group in position 4 of the cycle.

Key words: soybean urease, inhibition, cyclic β-triketones, triketone oximes, nickel chelators, inhibition mechanism

Soybean urease (EC 3.5.1.5) is a homohexameric enzyme (containing six subunits of 90,770 dalton molecular weight and two nickel atoms in each), which catalyzes urea hydrolysis by water with high activity and specificity, resulting in the formation of ammonium cations and carbonate anions [1]. Ureases from various sources are widely used for determination of urea by enzymatic electrodes [2] and in immunochemical analysis as an enzyme label for many antigens [3, 4]. Our laboratory has carried out a detailed investigation of urease immobilization [5], ureolysis kinetics, and catalytic activity and stability of soybean urease in aqueous solutions, water–organic mixtures, and reversed micelles of surface-active compounds (surfactants) in organic solvents [6-10]. Despite the great importance of ureases in biotechnology and medicine and also continuous interest in many laboratories [11], the detailed mechanism of urea hydrolysis by urease is still not clarified, and the structure of the metallocenter containing two nickel atoms is not precisely known [1, 11-13].

An important tool for the investigation of the catalytic mechanism of urease is inhibition of these enzymes with potential inhibitors such as chemical compounds imitating urease substrate (urea) or compounds which are blocking nickel atoms in the active site of the enzyme [11-13]. In recent decades, urease inhibition has gained great significance in medicine because urease is produced by many microorganisms and is connected to such pathologies as duodenal and gastric ulcer and also with a number of human and animal urinary diseases [12-19]. Most of the proteins of such microorganisms as *Helicobacter pylori*, *Klebsiella aerogenes*, and many others are ureases, which in the acidic media of the stomach hydrolyze food urea and create a "comfortable" medium for the survival and reproduction of pathogenic microorganisms [12-19]. It is generally recognized that *Campylobacter*-like microorganisms are one of the main reasons for gastric ulcer diseases [20], while helicobacteriosis is one of the research directions of gastroenterology [21]. The pathogenesis of *H. pylori* in ulcer disease and efficacy of treatment of this pathology via eradication of *H. pylori* have been clearly demonstrated [20, 21]. Many laboratories are now performing comprehensive studies on inhibition of microbial ureases and their action mechanism [11-19].

It is known that inhibition efficacy by the same compounds can vary for ureases from different sources; however, there are fully possible correlations between inhibiting properties of the same compounds for ureases of microbial and plant origin [12, 13, 22]. Therefore, the initial selection of potential inhibitors for ureases is performed easier and faster using soybean urease [12, 13, 22].

All known inhibitors of ureases can be divided into two large groups: inhibitors blocking the active site of the enzyme and inhibitors affecting the mechanism of urea hydrolysis by ureases [11]. By chemical structure, urease inhibitors can be divided into four groups [23].

The first group is formed by thiolic compounds, because thiolate anions react directly with the metallocenters of ureases [12, 13]. Maximal inhibiting activity is

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detected with cystamine (K_i 5.0 μM [22]). The disadvantage of thiols as inhibitors is the non-specificity of their action.

Another large group of urease inhibitors is hydroxamic acid and its derivatives [12-15, 17-19, 22, 24-27]. Inhibitors of this group compete with urea for binding with the urease active site, and their efficacy is comparable to thiols.

The third group of the most effective inhibitors includes substituted phosphorodiamidates [12, 13, 16, 22]:

$$RO-\underset{\underset{O}{\|}}{P}(NH_2)_2 .$$

The highest inhibiting activity was achieved for phenyl phosphorodiamidate (K_i $9.4 \cdot 10^{-11}$ M [22]).

The fourth group consists of ligands and chelators of nickel, the most notable of which are F^- anion [28, 29] and certain peptides exhibiting a moderate inhibiting activity ($K_i = (3.0\text{-}4.7) \cdot 10^{-5}$ M [30]).

Earlier we investigated the following urease inhibitors: urea and thiourea polydisulfides competing with substrate for binding with urease active sites; amides of thiophosphoric acid, which are analogs of the substituted phosphorodiamidates [23]; cyclic β-triketones (CTK) and fluoride anion, which are ligands and chelators of organic and inorganic nickel [31]. We investigated the inhibiting action of polycarbonyl compounds with different number of carbonyl groups on urease: oxalyldihydrazide with two carbonyl groups, its polydisulfide with 28 carbonyl groups, and 10 substituted CTK with three carbonyl groups [32]. The efficacy of the best of the investigated inhibitors is on the acceptable level; however, not all of them are nontoxic, which can be an obstacle for their application as medical drugs. Therefore, the search for potential urease inhibitors continues.

The goal of this work was a comparative kinetic study of soybean urease inhibition by organic chelators of nickel, 15 triketone oximes whose chemical structures are presented below, and also the comparison of the obtained inhibition constants (K_i) with the structure of the compounds used in this and previous investigations [23, 31, 32].

MATERIALS AND METHODS

Reagents. The following reagents were used in this work: soybean urease from Biolar (Olaine, Latvia) with initial activity of 1032 Sumner units per g, chemically pure urea as a substrate, EDTA, and dimethylformamide (DMF) from Reakhim (Russia). Urease concentration was calculated assuming the enzyme absorption coefficient $A^{1\%}_{1\ cm}$ at 280 nm is equal to 6.2 [33]. As a pH indicator, we used bromocresol purple from Reakhim, the solutions being prepared as described in [34]. DMF was purified by distillation before use.

Urease inhibitors. Triketone oximes I-III, V, VI, IX, X, XIII were synthesized according to the established techniques described in [35]; IV, XI according to [36]; VII according to [37], VIII, XIV according to [38], XII according to [39], and XV according to [40]. Compounds I-XV (see below) were characterized by conventional techniques, their characteristics being presented in [35-40].

Determination of urease catalytic activity in the presence and absence of inhibitors. To fulfill the defined goals, it was necessary to measure the rate of urea hydrolysis with high precision at low urease concentrations under conditions when its activity is suppressed by inhibitors. This could only be done by the spectrophotometric monitoring of urease activity using pH indicators [4, 8-10].

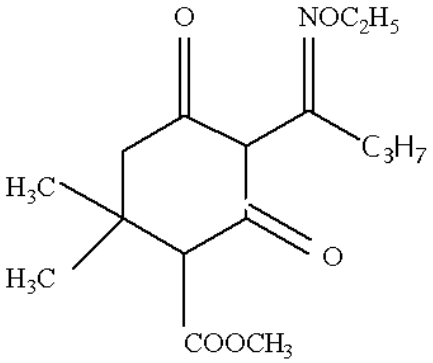
For the preparation of substrate mixtures, EDTA was added to 100 ml of urea aqueous solution (0.03 M) to final concentration of 0.05 mM to bind ions of heavy metals. According to [34], bromocresol purple was dissolved in the mixture of 0.2 ml of 0.05 M NaOH and 0.3 ml of H_2O. The dye solution was added to the urea solution and pH of the mixture was adjusted to the required initial value (pH 4.95) using HCl and 0.05 M NaOH.

Urea hydrolysis in the presence and absence of the inhibitors (In) was performed at 36°C. The total volume of the mixture was 0.8 ml. To 0.69 ml of the original substrate mixture solution, 0.1 ml of urease aqueous solution was added (its pH was adjusted to the required value) and 0.01 ml of inhibitor solution in DMF. The final concentrations of reagents in the reaction mixture were: 5.5 nM urease, 38.2 μM bromocresol purple, and 0.026 M urea. Final concentrations of triketone oxime (TKO) inhibitors I-XIV ranged within 10-100 μM, and TKO XV within 10-400 μM.

During the hydrolysis of urea in the presence and absence of the inhibitors, changes in absorbance of the pH indicator were monitored at 591 nm; kinetic curves were plotted in "absorbance–time" coordinates. All measurements were performed on a Specol-221 spectrophotometer (Carl Zeiss, Germany). Zero absorbance was adjusted by monitoring light absorption by the substrate mixture without inhibitors.

Initial reaction rate, v_0, was expressed in arbitrary units of absorbance changes per second and taken as 100% in the absence of inhibitors.

Quantitative characteristics of urease inhibitor efficacy. Inhibition constants K_i were determined according to Dixon [41] from dependences of $1/v_0$ on $[In]_0$, where $[In]_0$ is increasing concentration of the inhibitor at different initial concentrations of urea. The reversible competitive character of urease inhibition in the presence of CTK and TKO was earlier revealed by us [31, 32]. Hence,



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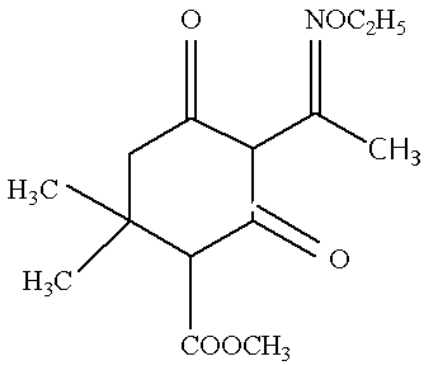

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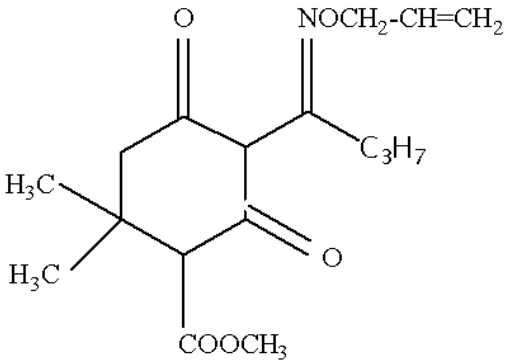

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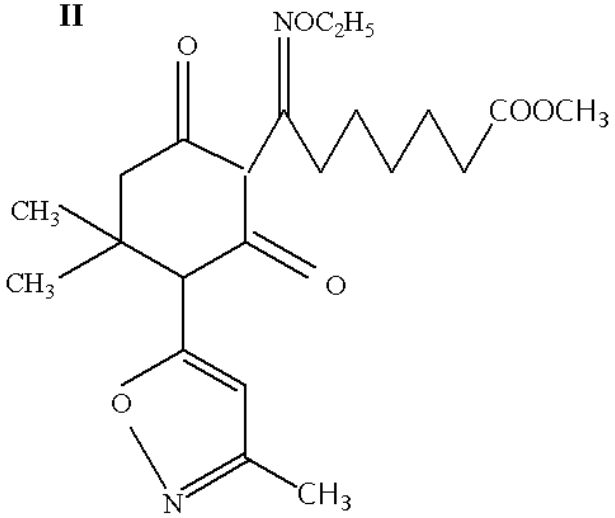

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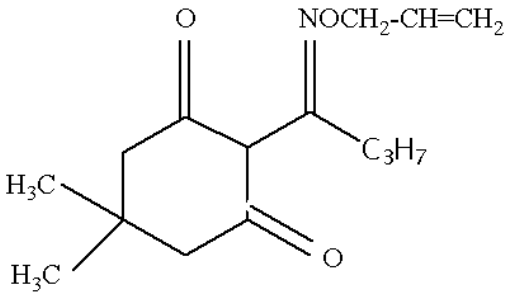

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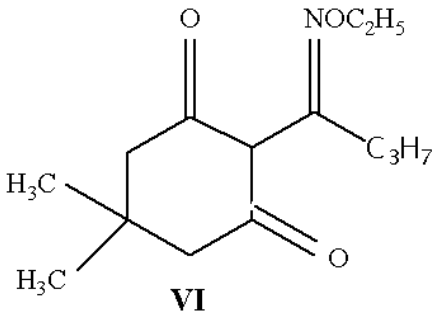

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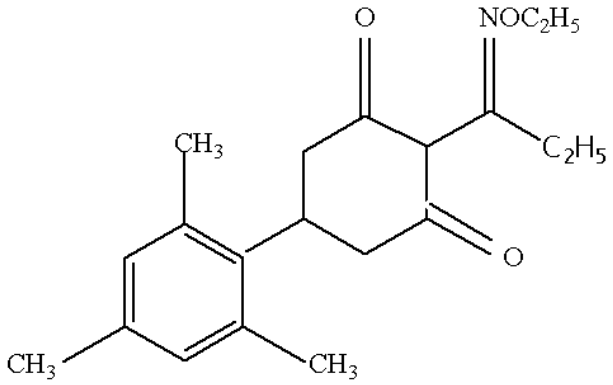

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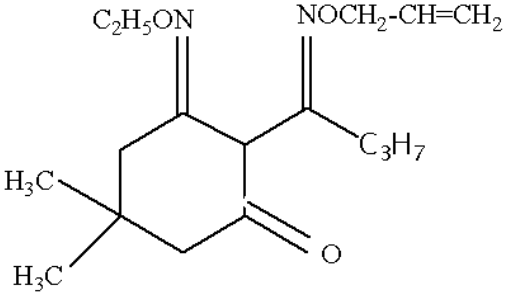

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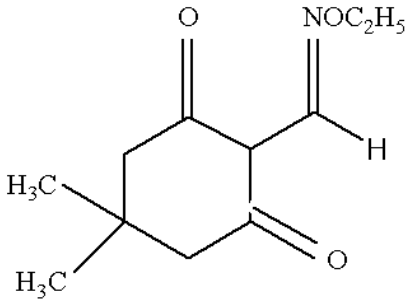

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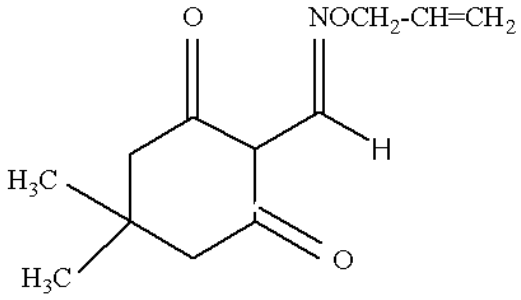

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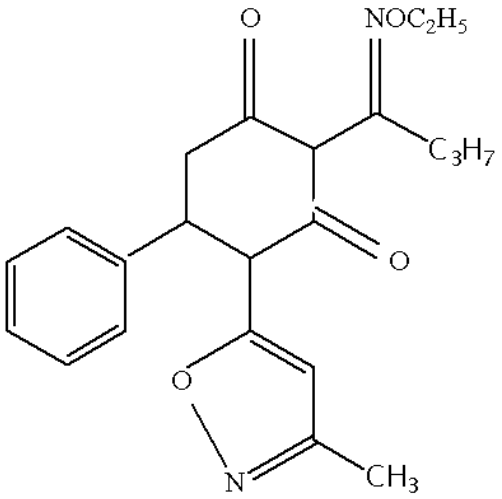

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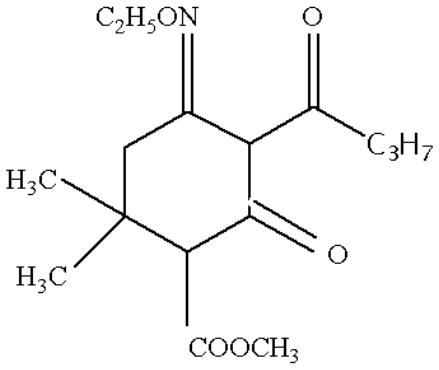

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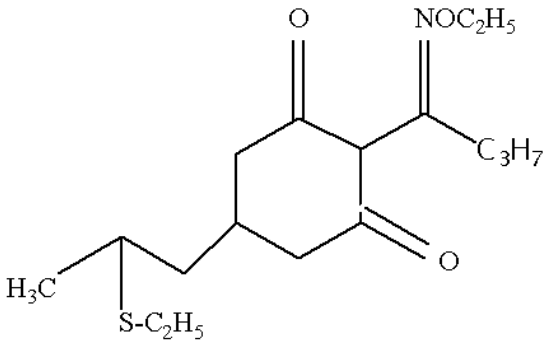

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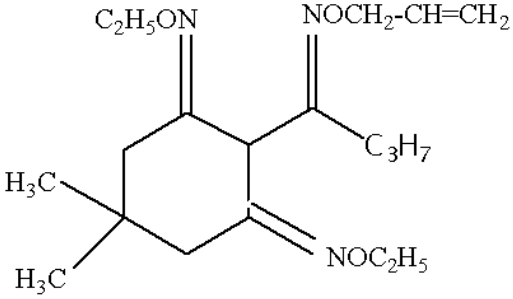

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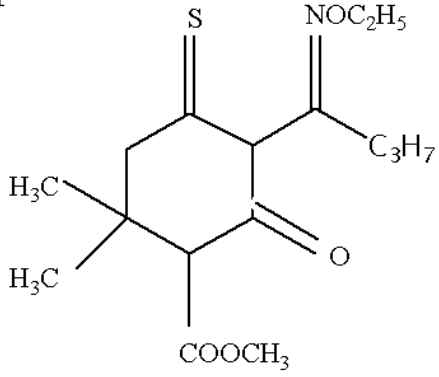


XV

in most of the cases, we plotted the linear dependences in Dixon coordinates, changing In concentration only at one urea concentration and then graphically measuring the length of the line dissected on the X-axis, [In]* (Fig. 1). For reversible competitive inhibition [41]:

$$[In]^* = K_i ([S]_0/K_m + 1). \quad (1)$$

From the Eq. (1), K_i can be calculated via graphically determined [In]* and knowing substrate concentration $[S]_0$ (0.026 M) and Michaelis constant K_m (21.3 mM) in the absence of the inhibitors. Table 1 presents graphically determined [In]* values and calculated K_i values for all investigated TKO.

RESULTS AND DISCUSSION

We have shown many times [5-10, 23, 31, 32] that in the absence of the inhibitors and in the presence of substrate analogs and thiophosphamides [23], as well as cyclic β-triketones [31] and other polycarbonyl compounds [32], the dependences of initial rates of urea hydrolysis by urease on substrate concentration are described by the Michaelis–Menten equation; after its linearization in Lineweaver–Burk coordinates, the maximum hydrolysis rate and Michaelis constants (K_m) were determined.

As an example, Fig. 1 illustrates the dependences of the inverse initial rate of urea hydrolysis on increasing concentration of TKO-I (*1*) and TKO-IV (*2*) inhibitors at 36°C and constant concentrations of urease (5.5 nM) and substrate (0.026 M). Similar dependences were obtained for TKO I-XV, which are presented in the "Materials and Methods" section and in Table 1. Since the inhibition of urea hydrolysis in the presence of CTK and TKO [31, 32] is reversible and competitive, according to Eq. (1) and using graphically determined [In]* values and known urea concentration and K_m in the absence of inhibitors, in all cases the inhibition constants (K_i) were calculated (presented in Table 1). Data in Table 1 show that all studied TKO inhibit urea hydrolysis with different efficiencies, and inhibition constants K_i vary in the 2.7-248 μM range depending on TKO structure. The K_i values vary over two orders of magnitude.

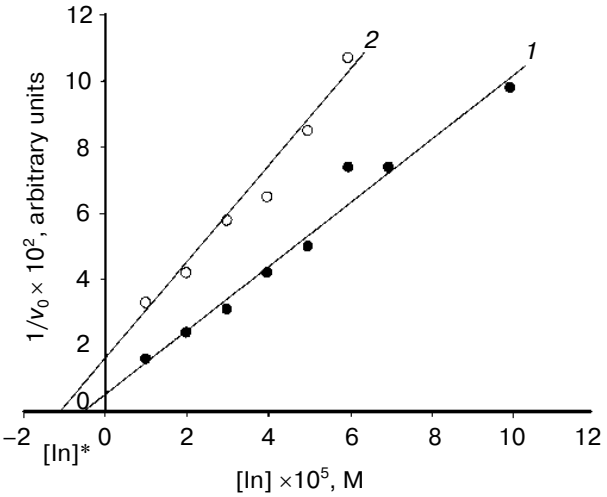


Fig. 1. Dependences of the inverse initial reaction rate of urea (0.026 M) hydrolysis on initial concentration of TKO I (*1*) and TKO IV (*2*) in aqueous solution (pH 4.95, 36°C, urease concentration 5.5 nM).

Table 1. Kinetic parameters for inhibition of urea hydrolysis by triketone oximes (36°C, aqueous solution, pH 4.95, urease concentration 5.5 nM)

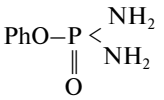
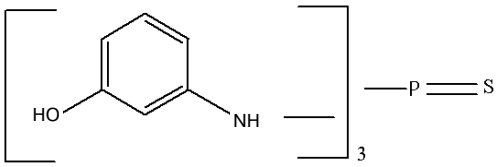
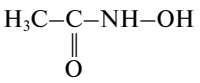
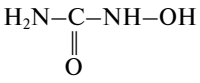
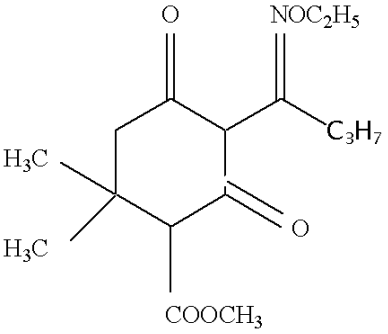
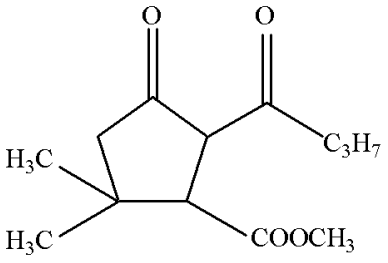
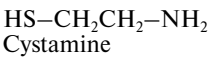
Oximes of triketones	[In]*, μM	K_i, μM
I	6.0	2.7
II	9.0	4.06
III	11.0	4.95
IV	12.0	5.42
V	13.0	5.87
VI	19.0	8.57
VII	35.0	15.80
VIII	36.0	16.20
IX	45.0	20.30
X	45.0	20.30
XI	57.0	25.70
XII	110	49.60
XIII	146	65.90
XIV	217	97.90
XV	550	248.0

From the data presented in Table 1 and the TKO structures as well as based on our earlier results regarding urease inhibition by cyclic β-triketones [31, 32] (see Table 2), it is possible to draw a definite conclusion about the influence of structural changes of the inhibitors on K_i values, since all experiments were performed under strictly identical conditions (36°C, pH 4.95, 5.5 nM urease concentration, bromocresol purple pH indicator).

Oximes of cyclic β-triketones TKO I-VIII with K_i values in the 2.7-16.2 μM range appeared to be more effective inhibitors than cyclic β-triketones themselves, the best of which had K_i in the range of 18.9-29.3 μM [32], i.e., introduction of an ethoxyimino group ($=NOC_2H_5$) in place of one of the carbonyl groups significantly increases the inhibition efficacy, thus resulting in significant improvement in the quality of the inhibitor.

The increase in TKO inhibition efficacy is greatly promoted by the introduction of a methoxycarbonyl group ($-COOCH_3$) either in cycle position 4 (most effec-

Table 2. Structure and K_i values of urease inhibitors

Structure and name of inhibitors	K_i, μM	References
 Phenyl phosphorodiamidate	$9.4 \cdot 10^{-5}$	[22]
 Tri-(N-3-hydroxyphenyl)thiophosphamide	2.8	[23]
 Acetohydroxamic acid	2.6	[13]
 Hydroxyurea	6470	[31]
 Triketone oxime (TKO I)	2.7	this work
 Cyclic β-triketone (CTK V)	56.4	[32]
 Cystamine	5.0	[13]
F- Fluoride anion	36.5	[31]

tive TKO I, II, III) or into the prolonged chain of the substituent in the second position of the cycle (TKO IV). The absence of the methoxycarbonyl group significantly decreases the efficacy of TKO VI compared to TKO I and efficacy of TKO V compared to TKO III.

Significant improvement in inhibiting properties is achieved via increasing alkyl chain length of the substituent in cycle position 2 (from -H to $-C_3H_7$), which is confirmed by comparison of TKO I with TKO II, TKO V with TKO X, and TKO VI with TKO IX.

Furthermore, let us mention structural modifications of TKO leading to the decrease in their inhibition efficacy. An introduction of am aryl substituent in position 5 deteriorates the inhibiting properties of TKO as confirmed by comparison of TKO VI with TKO VII and TKO IV with TKO XI.

Comparison of TKO I with TKO XV shows that replacement of one of carbonyl groups of cyclohexanedione fragment with thiocarbonyl group increases K_i value by nearly two orders of magnitude. Introduction of ethylthiopropyl substituent in TKO XIII greatly decreases inhibition efficacy even comparing with negative effect of aryl substituents in TKO VII and TKO XI.

Replacement of carbonyl group of the cyclohexanedione moiety with ethoxyimino group ($=NOC_2H_5$) increases K_i in TKO VIII compared with TKO V, while a replacement of both carbonyl groups with ethoxyimino group in TKO XIV results in further decline in inhibition efficacy.

A large influence on inhibition efficacy is provided by mutual disposition of carbonyl and ethoxyimino groups in TKO I and TKO XII: apparently, in the first case $K_i = 2.7$ μM, in the second case only 49.6 μM.

Correlation between the efficacy of inhibitors (K_i values) and their structure reveals that the best among TKO group inhibitors (I) must have a carbonyl group in position 1 of the cycle, ethoxyimino group and alkyl residue in substituent in position 2, and also methoxycarbonyl group in position 4. These structural properties of TKO provide effective inhibition of urea hydrolysis by urease. Any modifications in TKO I structure result in an increase in K_i, i.e., decrease in inhibition activity. Obviously, the presence of three carbonyl groups and ethoxyimino group in TKO I molecule allows its binding to nickel atoms in the urease metallocenter; hydrophobic residue $-C_3H_7$ promotes TKO I binding by the urease active site. Even the change of mutual disposition between carbonyl and ethoxyimino group has a large significance for the favorable binding of inhibitor with urease (compare K_i for TKO I and for TKO XII).

It is a paradox that despite the fact that urease was the first enzyme obtained in crystallized state the mechanism of its action is still not clearly known [11]. Figure 2 presents one of the possible urea hydrolysis schemes; it

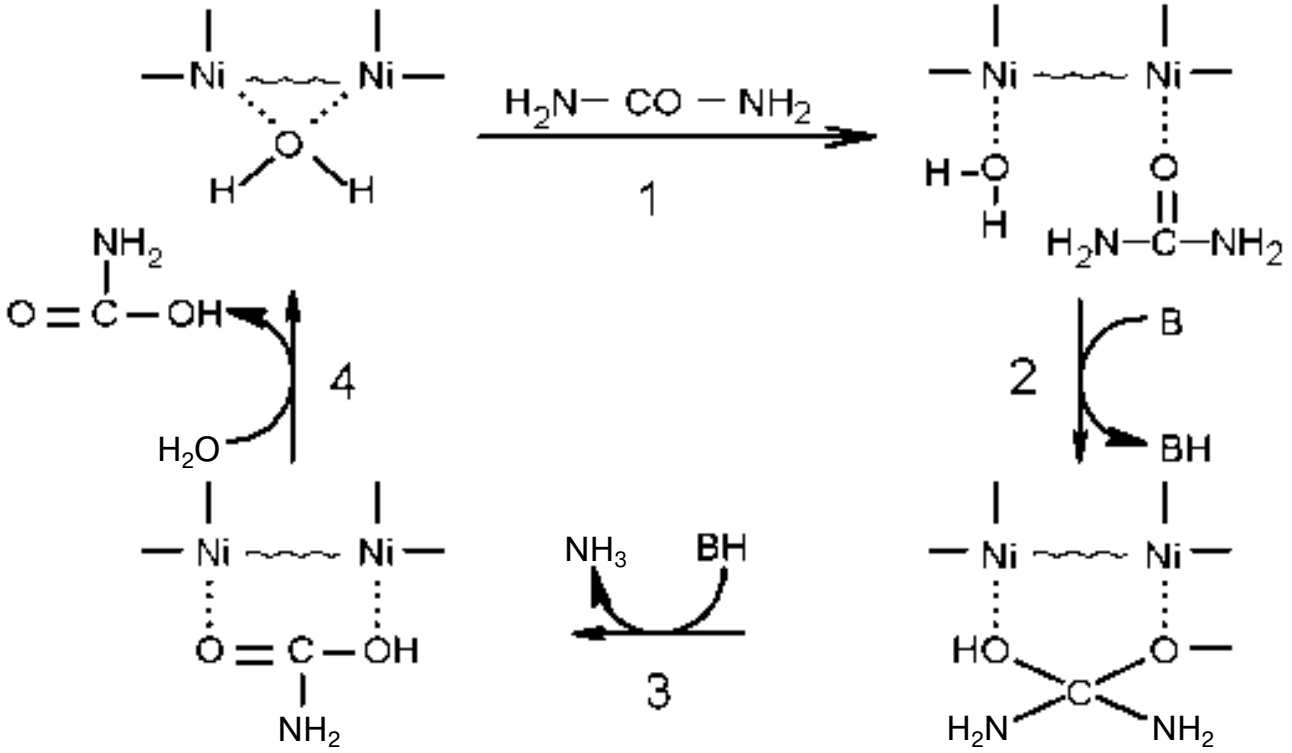


Fig. 2. Putative scheme for the hydrolysis of urea by urease.

consists of at least four stages [1, 13, 31]. It is believed that in a native enzyme two nickel atoms are positioned in such way that they can coordinate one water molecule. In the first stage of the process the urea coordinates with one of the nickel atoms via its carbonyl group; in the second stage and with the participation of an unidentified organic base B an intermediate cyclic structure is formed, which in the third stage and with the participation of BH loses the first ammonia molecule which in the fourth stage assisted by water molecule turns into an original native state losing carbamine acid, which degrades releasing the second ammonia molecule and CO_2. Apparently, the urea hydrolysis cycle includes acid–base catalysis stages; however, the nature of the carrier of these catalytic properties is unknown.

Urease inhibitors investigated in this work (see "Methods and Materials" and Table 1) and also all compounds mentioned in Table 2 compete in the first stage of the cycle with urea in binding with one or two nickel atoms, partially or completely blocking urea hydrolysis. Interacting with nickel atoms are: oxygen from ≡P=O and >C=O groups, nitrogen atom from $=NOC_2H_5$ group or sulfur atom ≡P=S, and also from sulfhydryl groups of cystamine and other thiols.

The structure of nickel chelators plays an important role in urease inhibition and determines K_i value: according to Table 2 the substitution of a CH_3-group in acetohydroxamic acid with NH_2-group in the hydroxyurea molecule leads to an increase in K_i by more than three orders of magnitude. This fact indicates the immediate participation of the functional groups of inhibitors in their binding with the metallocenter of urease, which is not reflected in the simplest scheme of urease hydrolysis (Fig. 2).

Increasing the number of carbonyl groups in CTK and TKO decreases K_i value of the inhibitor, since it increases the chelating abilities of these compounds toward nickel atoms in urease subunits. We have revealed that poly(disulfidoxalyldihydrazide) containing ~28 carbonyl groups in the polymer molecule is an effective urease inhibitor characterized by K_i = 8.5 μM [32]. However, an important role is also played by the three-dimensional disposition of carbonyl groups: oxalyldihydrazide NH_2-NH-CO-CO-NH-NH_2 having two neighboring carbonyl groups proved to be an unexpectedly weak urease inhibitor with K_i = 3800 μM [32], i.e., the distance between carbonyls which are liganding nickel atoms in urease must be larger than in the oxalyldihydrazide molecule. Indeed, the increase in this distance in cyclic β-triketones (see Table 2) leads to a decrease in K_i to 18.9-56.4 μM depending on CTK structure [31, 32]. Another surprise is associated with the fact that substitution of one carbonyl group with ethoxyimino group ($=NOC_2H_5$) in CTK molecules results in increased inhibiting activity of TKO to K_i = 2.7-25.7 μM (see Table 1).

Hence, the efficacy of inhibiting action of CTK [31, 32] and TKO is determined not only by structural but also by three-dimensional (steric) factors. It could be assumed that hydrophobization of TKO VII and XI molecules by aryl substituents would promote the increased inhibiting activity of these compounds; however, experience has revealed that TKO VII and in particular TKO XI (also containing isoxazole substituent at position 4 of the cycle) appeared to be less effective inhibitors than expected due to the steric factors complicating the chelation of nickel atoms in urease metallocenters.

As follows from Table 2, the most effective urease inhibitors are phosphorodiamidates [22]; however, these compounds as well as thiophosphamides [23] are toxic, while cystamine and other sulfhydryl compounds are nonspecific inhibiting agents since they react with several proteins [22]. Therefore, in our opinion, the most promising from the medical point of view are derivatives of hydroxamic acid and sterically unhampered cyclic β-triketones [31, 32] and their oximes TKO I-VI (Table 1). Obviously, the search for new nontoxic and effective urease inhibitors will continue, as is also confirmed by recent publications [11, 42].

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