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A New *Bacillus pasteurii* Urease Inhibitor from *Euphorbia decipiens*

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Abstract

Inhibition of *Bacillus pasteurii* urease enzyme by 3,7,15-tri-*O*-acetyl-5-*O*-nicotinoyl-13,14-dihydroxymyrsinol (**1**), a diterpene ester with a myrsinol-type skeleton, isolated from *Euphorbia decipiens* Boiss. & Buhse, was un-competitive consistent with the molecular docking results. The *Ki* value was 117.40 ± 0.7 μM.

Keywords: *Euphorbia decipiens*, *Bacillus pasteurii* Urease, Inhibition, Kinetics, Molecular Docking

Introduction

Euphorbia plants, ranging from herbs and shrubs to trees, occur in tropical and temperate regions all over the world [1] and have multiple medicinal uses. The paste of rhizomes of *E. acaulis* Roxh. has been used by the Tharu tribes of the Kheri district of central India as a cure for inflammatory disorders [2]. The extracts of *E. esula* L. have been widely used in folk medicine to treat various cancers, swellings and warts [3]. *E. hirta* L. is often employed in traditional medicine in many parts of Africa and Asia for the treatment of gastrointestinal disorders, intestinal parasitosis, bronchial and ocular diseases [4]. The roots of *E. kansui* Liou, known as 'Kan Sui' in Chinese folklore, are used as herbal remedies for oedema, ascites and cancer [5]. *E. nivulia* Buch-Ham is considered as a remedy for enlargement of liver and spleen, syphilis, dropsy, general anasarca, leprosy, whooping cough, dyspepsia, jaundice, rheumatism, colic and bronchitis [6]. We have already reported the inhibitory activity of the myrsinol-type diterpene ester **1** (Figure 1), isolated from *E. decipiens* Boiss. & Buhse, against jack bean urease [7] and in continuation of this work we report here the inhibition kinetics and molecular docking of **1** against *Bacillus pasteurii* urease, which is a new inhibitor of this enzyme.

Urease (urea amidohydrolase, EC: 3.5.1.5) occurs throughout the animal and plant kingdom. Many microorganisms use this enzyme to provide a source of nitrogen for growth, and it plays an important role in plant nitrogen metabolism during the germination process [8,9]. The presence of urease activity in soils is exploited in the widespread agricultural practice of urea-based fertilizer application for enhancing crop yields. Unfortunately, excessive levels of soil urease can degrade the fertilizer urea too rapidly and result in phytopathic effects and loss of volatilized ammonia [10]. Of medical and veterinary interest, urease is a virulence factor in certain human and animal pathogens; it participates in the development of kidney stones, pyelonephritis, peptic ulcers, and other disease states [11]. The obvious remedy for treating bacterial infection with antimicrobials, however, has often proven futile [12] and only a few

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◆Dedicated to the memory of Dr. Mohammad Hussain Panjwani (1940–1992), a renowned philanthropist and scholar.

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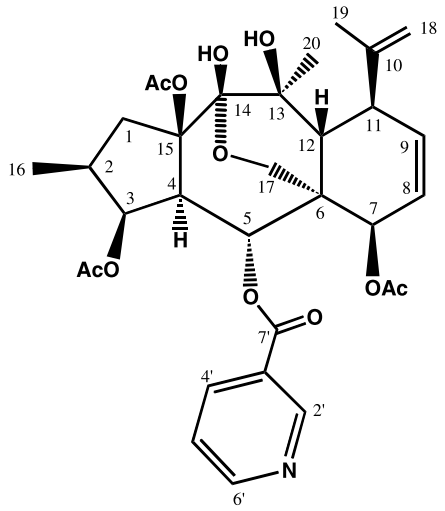


Figure 1. Diterpene 1.

combination regimens have reached clinical practice. Thus the need for alternative or novel treatment is evident. Consequently the discovery of potent and safe urease inhibitors has been a very important area of pharmaceutical research due to the involvement of ureases in different pathological conditions. We previously have reported a number of novel synthetic and natural inhibitors of urease, their inhibition kinetics and structure-activity relationship studies [13,14,7,15]. In continuation of this work to discover new and potent inhibitors of medicinally important enzymes through high-throughput screening assays, we identified the diterpene **1** as having appreciable efficacy against urease. The objectives of the current investigation are to explore the possible binding interactions of **1** in the target protein in the search for an effective inhibitor of urease.

Materials and methods

Our research group has already published the isolation and structure elucidation of myrsinol-type diterpene ester **1** from *E. decipiens* [7].

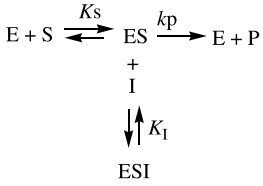
Urease assay and inhibition:

Reaction mixtures comprising 25 μL (40 units per mL) of enzyme (*Bacillus pasteurii* urease) solution and 55 μL of buffers containing urea (2-24 mM for *Bacillus pasteurii* urease) were incubated with 5 μL of **1** at 30 °C for 15 min in 96-well plates. The increased absorbance at 560 nm was measured after 10 min using a microplate reader (Molecular Device, USA). All reactions were performed in triplicate in a final volume of 200 μL. The results (change in absorbance per min) were processed using SoftMax Pro software (Molecular Device, USA). All the assays were performed at pH 6.8 (3 mM sodium phosphate buffer) and 7 μg of phenol red per mL as indicator. Percentage inhibitions were calculated from 100-($OD_{testwell}/OD_{control}$) × 100. Thiourea was used as the standard inhibitor of urease.

Determination of kinetic parameters

The concentration of compound **1** that inhibited the hydrolysis of substrate by 50% (IC_{50}) was determined by monitoring the inhibition effect of various concentrations of the compound **1** in the assay. The IC_{50} values were then calculated using the EZ-Fit Enzyme Kinetics program (*Perrella Scientific Inc., Amherst*, USA).

The interaction of **1** with *Bacillus pasteurii* urease (B.P.) can be described by the following scheme:



Scheme 1. Uncompetitive Inhibition.

where ES is the *B. pasteurii* urease-urea complex and P is the product. K_I is the inhibition constant reflecting the interactions of inhibitor with the free *B. pasteurii* urease and *B. pasteurii* urease-urea complex.

Statistical analysis

Graphs were plotted using the GraFit program [16]. Values of the correlation coefficients, slopes, intercepts and their standard errors were obtained by the linear regression analysis using the same program. The correlation for all the lines of all graphs was found > 0.99. Each point in the constructed graphs represents the mean of three experiments.

Molecular docking simulations

The accurate prediction of protein-ligand interaction geometries is essential for the success of structure-based drug design. It requires docking tools that are able to generate suitable configurations and conformations of a ligand within a protein binding site and scoring functions that appropriately translate interaction geometries into an energetic measure describing the quality of the interaction. In our present study the molecular docking study was conducted using FlexX. The three dimensional structures of **1** were constructed using the SYBYL program [17]. The docking studies were carried out using FlexX [18] docking software. For FlexX energy minimization was performed using the tripos force field with a distance gradient algorithm with convergence

Table I. Kinetic constants for the inhibition of *B. pasteurii* by 1.

Enzyme	Compd.	*K*i (μM) ± SEM	*K*m (mM)	*K*m (mM) *app*	*V*max (μM/min)	*V*maxapp	Type of Inhibition
Urease (BP)	**1**	117.40 ± 0.7	5.1	3.8	160	121	uncompetitive

*K*i (inhibition constant) was determined from nonlinear regression analysis by Dixon and secondary Lineweaver- Burk plots at various concentrations, *K*m (Michaelis-Menten constant) is equal to the reciprocal of the x-axis intersection, *V*max (maximal velocity) is equal to the reciprocal of the y-axis intersection of each line for each concentration in the Lineweaver-Burk plot. The *V*maxapp is equal to the reciprocal of the y-axis intersection of each line for each concentration in the Dixon plot Each point in the Lineweaver-Burk plot represents the mean of three determinations. Urease (BP) = *Bacillus pasteurii*.

criterion of 0.05 kCal/(molÅ) and maximum 1000 interactions, respectively. FlexX software is a fast and flexible algorithm for docking small ligands into binding sites of enzymes, using an incremental construction algorithm that actually builds the ligands in the binding site [17]. The software incorporates protein-ligand interactions, placement of the ligand core and rebuilding the complete ligand. As docking algorithm a Monte Carlo simulated annealing search process was used starting at a temperature corresponding to RT = 1200 cal/mol, which was reduced by a factor of 0.90 after each cycle. A cycle consisted of a maximum of 30,000 accepted or rejected steps, where a step corresponds to the random changes in translational, rotational, and torsional degrees of freedom of the ligand. One hundred cycles were performed per docking experiment, and for each ligand 100 independent dockings were carried out. The charges on the ligand were obtained using the standard RESP procedure [18]. The necessary *ab initio* calculations were performed with GAUSSIAN98 [18]. Docking results were analyzed by VMD [19] and LIGPLOT [20].

Results and discussion

3,7,15-Tri-*O*-acetyl-5-*O*-nicotinoyl-13,14-dihydroxymyrsinol (**1**), a myrsinol-type diterpene ester, was isolated as a colourless oil from the chloroform soluble fraction of *Euphorbia decipiens* Boiss. & Buhse and its structure was identified through detailed spectral analysis [7].

Urease is an enzyme present in many plants and in soil that catalyzes the hydrolysis of urea to ammonium and carbamate ions, which decompose to carbon dioxide and ammonia. The active site contains two nickel (II) atoms which, as shown by X-ray analysis, are linked by a carbamate bridge; furthermore, two imidazole nitrogen atoms are bound to each nickel atom, and a carboxylate group and a water molecule fill the remaining coordination site of the metal ion [11]. The coordination geometry of the first nickel atom is pseudo tetrahedral, while that of second is roughly trigonal bipyramidal. In order to discriminate among the inhibition capacities of various compounds, it is important to understand the coordination mechanism between the active site of the enzyme and the inhibitor.

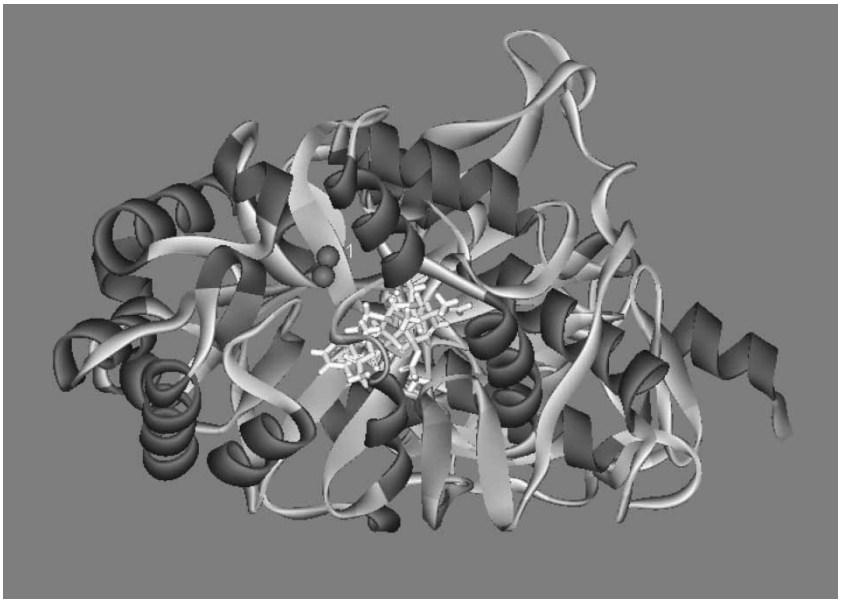


Figure 2. Diterpene **1** (white) in the active site of urease shows that **1** is unable to reach the nickel metal (blue) centre. The enzyme conformation of the (*Bacillus pasteurii*) Urease-Ligand complex (PDB code: 4UBP) was used for docking. Colours: Helix (Red), sheet (blue) and loop (green).

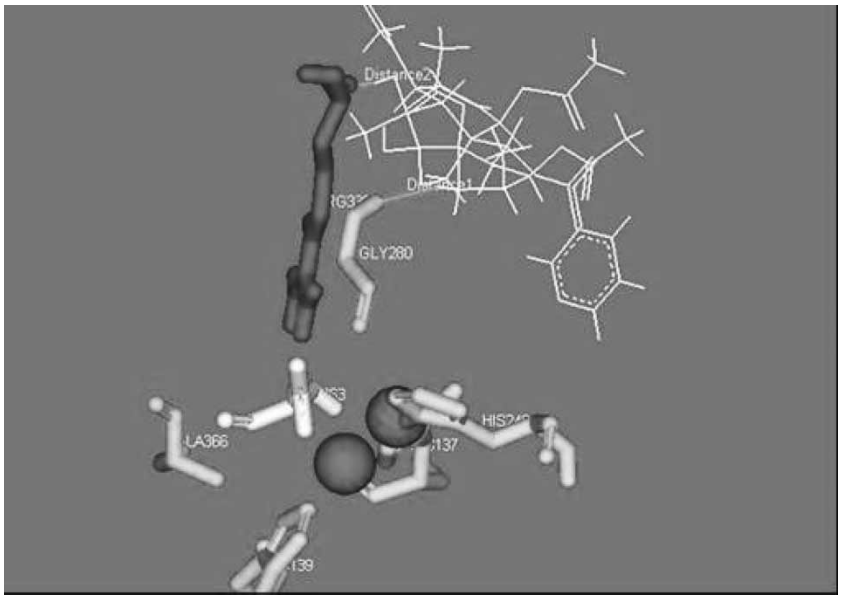


Figure 3. Molecular Docking simulations obtained at lowest energy conformation, highlighting potential hydrogen contacts of the compound, mainly with the binding site residues Gly 280, Arg 338 have been shown. The ligand **1** is shown in white.

The steady-state kinetics analysis of *Bacillus pasteurii* urease inhibition by **1** produces parallel lines in a double reciprocal plot (Figure 4) which suggests either of two interpretations. (1) Classical uncompetitive inhibition which occurs when an inhibitor binds to an enzyme-substrate complex. This type of inhibition is typically observed with multi-substrate enzymes; however, an uncompetitive inhibitor could reasonably interact with hydrolytic sites in the enzyme normally occupied by catalytic water. (2) Parallel double-reciprocal plots may also arise for inhibitors that bind to a form of the enzyme (E*) generated from the active enzyme species (E) during catalysis. Steady-state kinetics data analysis suggested an uncompetitive type of inhibition ($K_i = 117.40 \pm 0.7\,\mu M$, $IC_{50} = 101 \pm 2.07\,\mu M$; thiourea, $K_i = 8.41 \pm 0.66\,\mu M$, $IC_{50} = 13.00 \pm 1.14\,\mu M$) (Table I) and molecular docking also gave consistent results. Principal interactions experienced by the ligand **1** are hydrogen bonding with the Gly 280 (2.51 Å), Arg 338 (1.79 Å) and some hydrophobic interactions with (Glu 166, Lys 169, Ala 170, Met 367, Leu 365 and Ala 366) also stabilized the ligand in the receptor (Figures 2 and 3). These mechanistic studies on inhibitor **1** are expected to provide rational information for the design of a new potential inhibitor of urease.

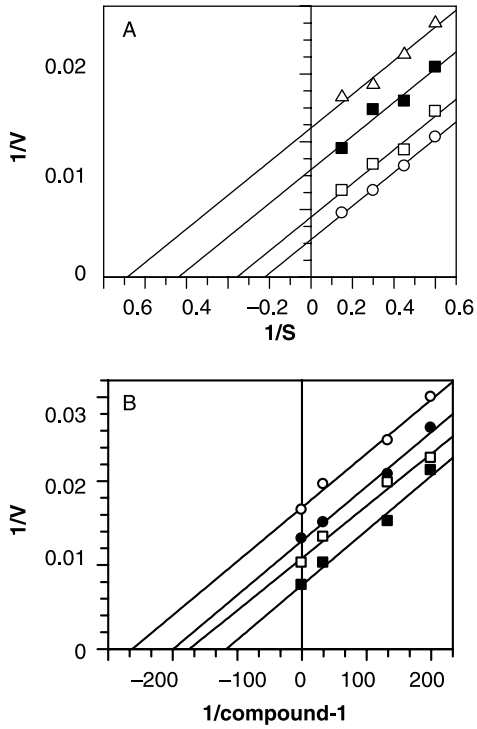


Figure 4. Inhibition of *Bacillus pasteurii* urease by **1**. **A**: Lineweaver– Burk plot of reciprocal of initial velocities versus reciprocal of fixed *Bacillus pasteurii* urease substrate concentration in absence (○) and presence of 75.0 μM (□ or □), 150.0 μM (■), and 225 μM (Δ) of **1**. **B** is the Dixon plot of reciprocal of the initial velocities versus various concentrations of **1** at fixed urea concentrations, (■) 24 μM, (□ or □) 18.0 μM, (●) 6.2 μM and (○) 2.0 μM. The unit is $(\mu M/L/min)^{-1}$ in all cases.

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