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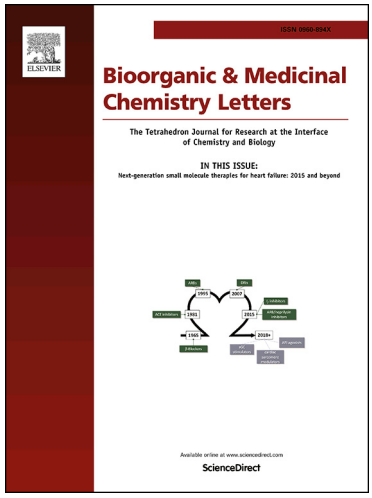

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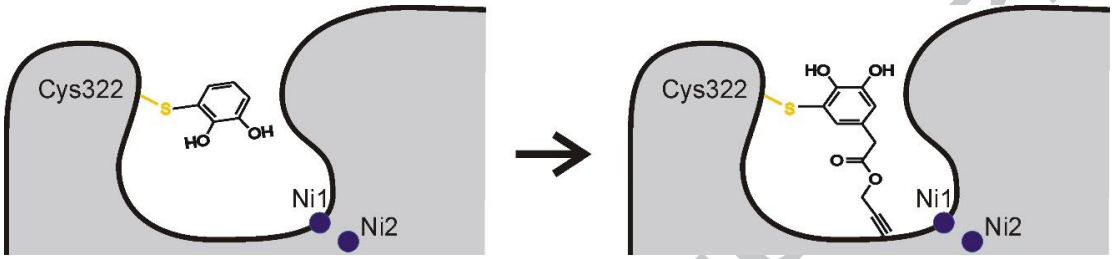

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Catechol-based inhibitors of bacterial urease

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ABSTRACT

Targeted covalent inhibitors of urease were developed on the basis of the catechol structure. Forty amide and ester derivatives of 3,4-dihydroxyphenylacetic acid, caffeic acid, ferulic acid and gallic acid were obtained and screened against *Sporosarcinia pasteurii* urease. The most active compound, namely propargyl ester of 3,4-dihydroxyphenylacetic acid exhibited IC_{50} = 518 nM and k_{inact}/K_i = 1379 $M^{-1}s^{-1}$. Inhibitory activity of this compound was better and toxicity lower than those obtained for the starting compound – catechol. The molecular modelling studies revealed a mode of binding consistent with structure-activity relationships.

Targeted covalent inhibitors of enzymes are an emerging class of biologically active compounds, which offer several key advantages [1, 2]. In particular, higher potency, selectivity and duration of action are expected in comparison to noncovalent binders. Most typically, a cysteine residue is targeted, but other amino acid residues are also explored in this context. Covalent inhibition can lead to complete inactivation of the target, what is particularly valuable in the case of antibacterial compounds. There are numerous known antibiotics exhibiting such a mode of action including beta-lactams and phosphomycin.

One of bacterial enzymes, which could be targeted by covalent inhibitors is urease. Urease is a virulence factor of *Helicobacter pylori* and *Proteus* spp., bacteria that commonly infect gastroduodenal and urinary tracts, respectively [3,4]. This enzyme catalyzes the reaction of urea hydrolysis to carbamate and ammonia [5]. The released ammonia allows *H. pylori* colonization of the stomach by creating a micro-environment with elevated pH values [6] and, by degrading stomach mucosa [7,8]. The enzymatic release of ammonia in the urinary tract during infections involving *Proteus* spp. causes alkalinization of urine and subsequently, rapid formation of urinary stones [9,10].

The active site of urease is built with two nickel ions coordinated by six histidine residues and carbamoylated lysine (a posttranslationally modified residue) [11,12]. During the reaction the active site is closed by a movable loop that contains cysteine, which could be important for inhibitor design. The substrate – urea bound by nickel ions and a net of hydrogen bonds is attacked by hydroxyl coordinated also by the two nickel ions.

There are several classes of urease inhibitors [13-16], which can be roughly divided to: (a) nickel ions coordinating compounds, and (b) cysteine-binding structures. Hydroxamic acids [17], phosphordiamidates [18], phosphonates [19], phosphinates [20] and some heterocycles [21] represent the first mentioned group of inhibitors, while unsaturated compounds [22], ebselen derivatives [23], quinones [24] and heavy metal ions [25] are members of the second set.

Our recent studies showed that covalent inhibitors of urease could be highly active, but, surprisingly, most of the studied compounds exhibited reversible mode of action [22,23,19]. Ebselen with K_i = 2.1 nM and acetylenedicarboxylic acid with K_i = 42.5 nM are the most representative examples. Ciurli and co-workers have recently shown that catechol is an irreversible urease inhibitor that binds to the cysteine residue placed on the movable flap [26]. The radical-based autocatalytic reaction mechanism is proposed to explain this mode of action. The studies are illustrated by the crystal structure of catechol-inhibited urease from *Sporosarcinia pasteurii* (Figure 1). Analysis this crystal structure indicates that catechol is a good starting point for development of effective urease inhibitors. Sufficient space around this molecule in the active site of the enzyme gives a chance to introduce fragments that should enhance the specific binding to the protein.

In this article, we describe exploration of numerous derivatives of catechol that contain carboxylic acid or ester/amide functionalities as inhibitors of a bacterial urease. This class of compounds was selected on the basis of previous studies which shown that numerous carboxylic acid and their derivatives were active against urease. Synthesis and evaluation of over forty derivatives allowed to indicate compounds with good inhibitory

profile, that were further characterized by reactivity with glutathione and toxicity evaluation.

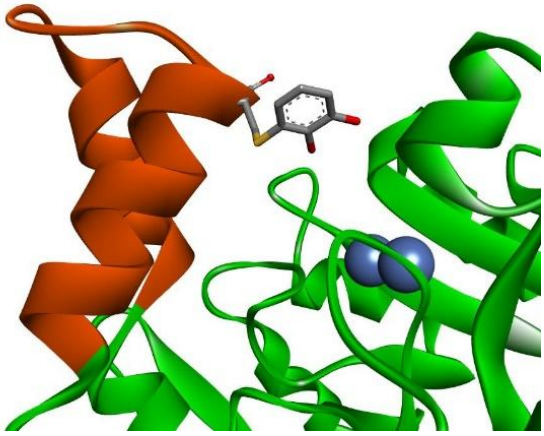
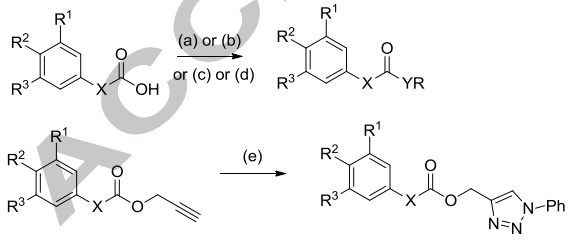


Figure 1. Crystal structure of catechol-inhibited *S. pasteurii* urease (PDB id. 5G4H) [26]. Protein is shown as green ribbon with movable flap marked in orange. Nickel ions are shown as dark blue spheres, and catechol-modified Cys322 residue as sticks.

Forty derivatives of 3,4-dihydroxyphenylacetic acid **2**, caffeic acid **25**, ferulic acid **33** and gallic acid **41** (compounds **3**−**45**) were prepared, among which twenty six were synthesized for the first time (Scheme 1). Diverse methods were used for the synthesis of these derivatives. Esters **3**-**15**, **26**-**28**, **34**-**35** and **42**-**43** were conveniently obtained in very good yields and purities by alkylation (method **A**) using the corresponding halide and sodium carbonate (Na_2CO_3) in hexamethyl phosphoramide (HMPA) in the presence of catalytic amount of potassium iodide (KI). Several esters were obtained using thionyl chloride and the corresponding alcohol (method **B**, compounds **16**−**17**, **29**, **36**-**37**, **44**-**45**) in 1,2-dimethoxyethane (DME) as it results in fewer side products compared to the frequently used 1,4-dioxane. Low to moderate but utilizable yields (10–30%) were obtained. Amide derivatives (compounds **19**-**21**, **31**-**32** and **39**-**40**) were synthesized using a condensing agent such as EDC·HCl (method **C**) in good yields. Some derivatives (compounds **22**-**24**) almost unavailable by this method were prepared by $SOCl_2$ acid activation (method **D**).



R = CH_3, CH_2CH_3, $(CH_2)_2CH_3$, $(CH_2)_3CH_3$, $(CH_2)_4CH_3$, $(CH_2)_5CH_3$, $(CH_2)_7CH_3$,$(CH_2)_{15}CH_3$, $CH_2CH{=}CH_2$, C_6H_{11}, CH_2Ph, $(CH_2)_2Ph$, $(CH_2)_2$(4-F-Ph), $(CH_2)_2$(4-NO_2-Ph), $(CH_2)_2$(3-F-Ph), $CH(CH_3)CH_2CH_3$, $CH_2C{\equiv}CH$
R^1, R^2, R^2 = H, OH, OCH_3
X = CH_2, CH=CH; Y = O, NH

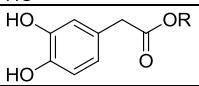
Scheme 1. (a) i) Na_2CO_3, RX, cat. KI, HMPA, rt, 48h, ii) HCl; (b) i) $SOCl_2$, DME, 70°C 4h, ii) ROH, DME, 70°C, 16h; (c) RNH_2, HOBt·H_2O, EDC·HCl, CH_2Cl_2, rt, 24h; (d) $SOCl_2$, 50°C, 3h, ii) RNH_2, Et_3N, CH_2Cl_2, rt, 16h; (e) i) aniline, con. HCl, $NaNO_2$, NaN_3, H_2O 0°C 10 min, rt, 2h, ii) sodium ascorbate, $CuSO_4{\cdot}5H_2O$, *t*-BuOH: H_2O, rt, 24 h.

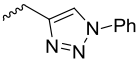
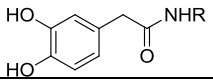
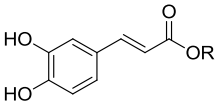
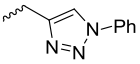
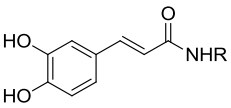
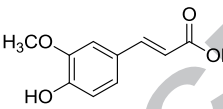
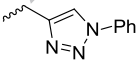
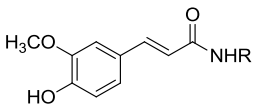
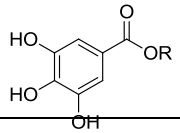
Propargyl esters **12**, **26** and **34** and phenyl azide were used in a click chemistry approach to obtain triazole derivatives (method **E**). Phenyl azide was prepared from aniline by diazotization with sodium nitrite $NaNO_2$ in acidic conditions followed by displacement with sodium azide NaN_3 in quantitative yield. 1,3-Dipolar cycloaddition reaction of propargyl esters with phenylazide in the presence of $CuSO_4{\cdot}5H_2O$ and sodium ascorbate in *t*-BuOH:H_2O (2:1) resulted in formation of triazolyl derivatives (**18**, **30**, **38**) in very good yields (Scheme 1).

All obtained compounds were assayed against urease purified from *Sporosarcinia pasteurii* using the phenol red method. Only the derivatives of 3,4-dihydroxyphenylacetic acid exhibited inhibitory properties similar to catechol. The starting acid **2**, its esters **3**-**18** and amides **19**-**24** showed irreversible inhibition with IC_{50} in micromolar range. The series of aliphatic esters with increasing number of carbon atoms (compounds **2**-**10**) was prepared and clear pattern of the structure-activity relationship was observed. The activity increased from the acid **2** to the butyl ester **6** achieving level substantially higher than catechol (IC_{50} = 0.667 μM for inhibitor **6**) and then, further increase of chain length caused slow decrease of activity up to inactive hexadecyl ester **10**. Highly active derivative was also found among unsaturated esters — propargyl ester **12** exhibited IC_{50} = 0.518 μM and k_{inact}/K_i = 1379 $M^{-1}s^{-1}$ and was the most active in the whole tested set. Cyclohexyl and phenyl-containing esters **13**-**18** showed moderate activity at the level similar to catechol. Amides of 3,4-dihydroxyphenylacetic acid showed moderate activity, in most cases lower than the corresponding esters (e.g. **11** vs **22**, **15** vs **19**, **17** vs **21**).

Surprisingly, although caffeic acid **25** incorporates structural fragment of catechol, the compound itself, as well as its various derivatives (esters **26**-**30** and amides **31**-**32**) did not exhibit any inhibitory activity against urease. Similarly, O-methylated analogues of caffeic acid – compounds **33**-**40** also did not show any potency. Gallic acid **41** was inactive, but its phenyl-containing esters gave moderate inhibition with IC_{50} equal to 972 and 206 μM for compounds **43** and **44**, respectively. However, the inhibition by gallic acid esters was reversible, thus of a different mode in comparison to catechol.

Table 1. Inhibitory activity of catechol derivatives against *S. pasteurii* urease

No	Structure R	Synthetic method[a]	IC_{50} [μM]	k_{inact}/K_i [$M^{-1}s^{-1}$]
1		-	7.40 ± 0.08	346 ± 16
				
2	-H	-	240.4± 2.8	32.0 ± 2.4
3	$-CH_3$	(a)	158.2 ± 2.3	44.0 ± 0.9
4	$-CH_2CH_3$	(a)	106.1 ± 1.3	56.3 ± 3.9
5	$-(CH_2)_2CH_3$	(a)	22.49 ± 0.30	123.5 ± 9.8
6	$-(CH_2)_3CH_3$	(a)	**0.667 ± 0.005**	**571.3 ± 7.1**
7	$-(CH_2)_4CH_3$	(a)	25.64 ± 0.31	101.3 ± 8.4
8	$-(CH_2)_5CH_3$	(a)	62.4 ± 4.8	92.7 ± 6.4
9	$-(CH_2)_7CH_3$	(a)	235.5 ± 3.6	27.56 ± 0.14
10	$-(CH_2)_{15}CH_3$	(a)	NI	
11	$-CH_2CH{=}CH_2$	(a)	47.12 ± 0.32	94.1 ± 35.3
12	$-CH_2C{\equiv}CH$	(a)	**0.518 ± 0.004**	**1379 ± 26**

13	-C_6H_{11}	(a)	50.12 ± 0.40	169.2 ± 7.8
14	-CH_2Ph	(a)	44.09 ± 0.30	99.7 ± 6.9
15	-$(CH_2)_2$Ph	(a)	6.54 ± 0.05	243.3 ± 1.4
16	-$(CH_2)_2$(4-F-Ph)	(b)	106.4 ± 1.1	46.8 ± 2.5
17	-$(CH_2)_2$(4-NO_2-Ph)	(b)	7.85 ± 0.09	379 ± 23
18		(e)	73.1 ± 0.9	113.6 ± 5.1
				
19	-$(CH_2)_2$Ph	(c)	13.69 ± 0.10	247.2 ± 1.7
20	-$(CH_2)_2$(3-F-Ph)	(c)	667.0 ± 7.6	12.12 ± 0.88
21	-$(CH_2)_2$(4-NO_2-Ph)	(c)	296.5 ± 2.2	29.1 ± 1.8
22	-$CH_2CH{=}CH_2$	(d)	89.20 ± 0.94	76.4 ± 4.8
23	-$CH(CH_3)CH_2CH_3$	(d)	33.60 ± 0.25	142 ± 12
24	-C_6H_{11}	(d)	11.33 ± 0.09	221.2 ± 1.2
				
25	-H	-	NI	
26	-$CH_2C{\equiv}CH$	(a)	NI	
27	-$(CH_2)_2$Ph	(a)	NI	
28	-$(CH_2)_2$(4-F-Ph)	(b)	NI	
29	-$(CH_2)_2$(4-NO_2-Ph)	(b)	NI	
30		(e)	NI	
				
31	-$(CH_2)_2$Ph	(c)	NI	
32	-$(CH_2)_2$(3-F-Ph)	(c)	NI	
				
33	-H	-	NI	
34	-$CH_2C{\equiv}CH$	(a)	NI	
35	-$(CH_2)_2$Ph	(a)	NI	
36	-$(CH_2)_2$(4-F-Ph)	(b)	NI	
37	-$(CH_2)_2$(4-NO_2-Ph)	(b)	NI	
38		(e)	NI	
				
39	-$(CH_2)_2$Ph	(c)	NI	
40	-$(CH_2)_2$(3-F-Ph)	(c)	NI	
				
41	-H	-	NI	
42	-$CH_2C{\equiv}CH$	(a)	NI	
43	-$(CH_2)_2$Ph	(a)	972 ± 11	1106 ± 18[b]
44	-$(CH_2)_2$(4-F-Ph)	(b)	205.8 ± 1.7	143.6 ± 2.1[b]
45	-$(CH_2)_2$(4-NO_2-Ph)	(b)	NI	
	AHA	-	14.31 ± 0.85	3.30 ± 0.40[b]

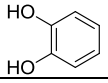
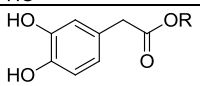
[a] Synthetic methods are defined in Scheme 1.

[b] Reversible inhibitors, K_i [μM] value is given.

The most active identified inhibitors (**1**, **2**, **6**, **12** and **17**) were further evaluated for their chemical reactivity with thiols using a model substrate — glutathione (Table 2). Kinetic parameter describing this reaction i.e. logk_{GSH} was found to be at the similar level of 2.09 – 2.42 for all tested compounds. Thus, differences in observed inhibitory activities were not the result of variable chemical reactivity of studied inhibitors, but are related to specific interactions with the enzyme.

Additionally, cytotoxicity of selected urease inhibitors towards normal mouse fibroblasts (BALB/3T3) was also measured (Table 2). As expected, catechol showed toxicity at micromolar level very close to its inhibitory activity. The toxicity of 3,4-dihydroxyphenylacetic acid and its derivatives was variable. The acid **2** showed low toxicity (> 100 μM), but it also exhibited poor inhibitory activity against urease. Butyl and 2-(4-nitropehyl)ethyl esters exhibited toxicity similar to catechol, but for propargyl ester **12** this value was one order of magnitude higher (LC_{50} = 53.2 μM). Thus, significant improvement of inhibition/toxicity profile was achieved — in case of compound **12**, two orders of magnitude difference between inhibitory activity and toxicity was observed.

Table 2. Chemical reactivity and cytotoxicity for selected compounds

No	Structure R	logk_{GSH}	LC_{50}^{72H} [μM]
1		2.24 ± 0.17	7.87 ± 2.58
			
2	-H	2.09 ± 0.17	> 100 μM; 22.7 ± 8.0%[a]
6	Bu	2.37 ± 0.19	5.6 ± 1.3
12	-$CH_2C{\equiv}CH$	2.21 ± 0.18	53.2 ± 23.7
17	-$(CH_2)_2$(4-NO_2-Ph)	2.42 ± 0.19	5.4 ± 1.1

[a] Percent of proliferation inhibition at 100 μM.

The mode of binding of the most active compound **12** was modelled using the catechol-inhibited urease crystal structure as the starting point for calculations (Figure 2). There are two possible points of attachment of thiol group on the ring – namely the positions 2 and 5. Unfortunately, on the basis of the radical-type reaction mechanism it is not possible to indicate the preferred reaction point. Therefore, both possibilities were modelled (Figure 2 and S1) and similar mode of binding was found. The space between the movable flap and the rest of the

enzyme is filled with the inhibitor molecule. Ester substituent — propargyl is docked in the shallow pocket formed by Met367, Met318, Pro303 and Arg369. The size of propargyl fragment is well fitting the pocket, which explains the observed structure-activity relationship related to the size of ester substituent.

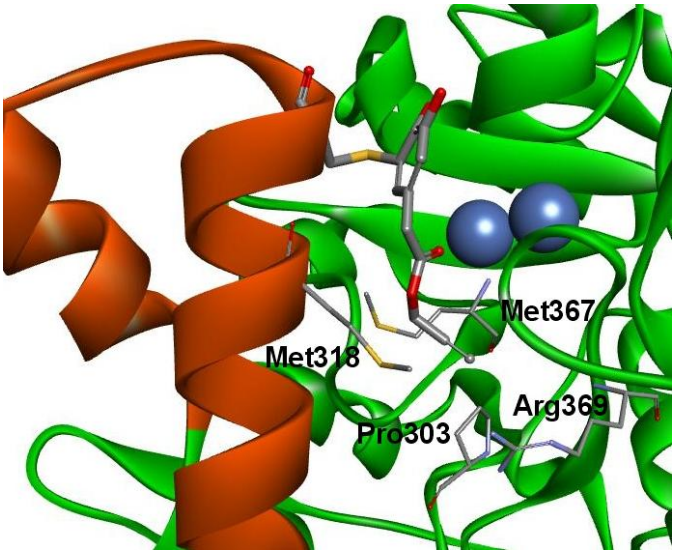


Figure 2. Modelled mode of binding of inhibitor **12** to *S. pasteurii* urease. Protein is shown as green ribbon with movable flap marker in orange. Nickel ions are shown as dark blue spheres, inhibitor **12**-modified Cys322 residue and other residues interacting with inhibitor as sticks.

Structure-activity relationships for phenyl substituted esters **14**-**17** could be also explained by molecular modelling. The longer linker of inhibitor **15** in comparison to compound **14** allows formation of cation-π interaction between Arg369 and phenyl ring (Figure S2), what leads to increased activity. Substitution of phenyl ring with electron-withdrawing fluorine atom decrease cation-π interaction energy, what corresponds to lowered activity of **16** [27]. However, increased activity of nitro-substituted analogue **17** is related to formation of hydrogen bonds between NO_2 group and Arg369 instead of possible weak cation-π interaction (Figure S3).

In summary, a new class of bacterial urease inhibitors based on catechol molecule was discovered. Derivatives of 3,4-dihydroxyphenylacetic acid exhibit irreversible inhibition of the studied enzyme and selected compounds show better inhibitory activity and lower toxicity in comparison to the reference compound. Such mode of inhibition should be particularly effective against microbial pathogens for which urease was identified as virulence factor because it allows complete inactivation of the target protein.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at @.