EFFECT OF 5-HYDROXY- AND 5,8-DIHYDROXY-1,4-NAPHTHOQUINONES ON THE HYDROLYTIC ACTIVITY OF α-GALACTOSIDASE

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The effect of natural and synthetic polyhydroxy-1,4-naphthoquinones on the hydrolytic activity of α-galactosidase from marine bacteria was studied. It was shown that the inhibiting properties relative to the enzyme depended on the nature of the substituents, their number, and their position in the structure of these compounds.

Key words: 5-hydroxy-1,4-naphthoquinones, 5,8-dihydroxy-1,4-naphthoquinones, echinochrome, inhibitors, α-galactosidase.

α-Galactosidases (α-D-galactoside hydrolases [EC 3.2.1.22]) play an important role in the vital activity of many micro- and macroorganisms. They catalyze the cleavage of α-bonded galactose units from the unreducible terminus of oligo- and polysaccharides and participate in degradation of various plant saccharides in bacteria, yeast, and fungi that act as carbon and energy sources for growth and vital activity of the organism [1]. Modification or blocking of these processes by powerful selective inhibitors provides a bssis for treating several infectious diseases, cancerous tumors, and genetic mutations [2].

α-Galactosidases are rather widely distributed in marine bacteria that are living independently in water and soil and are associated with animals and algae [3]. Intracellular α-galactosidase from the marine bacterium *Pseudoalteromonas* sp. KMM 701 is interesting for practical application in medicine and the food industry. The enzyme exhibits maximum activity at pH 7.0-7.5, does not lose activity at 20°C over one day, is not metal-dependent, and has a free SH group that is important for the catalytic activity [4].

The goal of our work was to study the effect of 5-hydroxy- and 5,8-dihydroxy-1,4-naphthoquinones on the hydrolytic activity of α-galactosidase using it as a very simple model for elucidating the structure—function properties of these compounds.

Table 1 presents the results from the study of the effect of natural and synthetic functionally substituted 5-hydroxy- and 5,8-dihydroxy-1,4-naphthoquinones on the activity of α-galactosidase from the marine γ-proteobacterium *P.* sp. KMM 701. The structures of the naphthazarine derivatives (5,8-dihydroxy-1,4-naphthoquinones) are given for only one of all possible tautomers if not otherwise specified.

Table 1 lists the studied compounds in the order of increasing inhibiting ability. The results enabled the tested compounds to be divided into three groups: **I**, β-methoxy derivatives of 5,8-dihydroxy-1,4-naphthoquinone (**1**-**6**) that do not affect the activity of α-galactosidase under the studied conditions at concentrations of $3.2\text{-}4.6\cdot10^{-4}$ M; **II**, compounds **7**-**16** that suppress the activity of the enzyme by 50% at concentrations (C_{50}) of $10^{-4}\text{-}10^{-5}$ M; and **III**, polyhalogenated naphthazarines **17**-**21** that are most effective at inhibiting the enzyme ($C_{50} \sim 10^{-6}$ M).

Table 1 shows that all compounds with free β-OH groups either did not inhibit the enzyme, like echinochrome A (**2**), or became inhibiting at rather high concentrations, like **16** ($C_{50} = 2.3\cdot10^{-5}$ M). The hydroxyls of these compounds, which were located in the β-positions of the quinoid ring of the bicyclic structure, were fragments of vinylogous carboxylic acids, owing to which they exhibited all properties typical of ordinary aliphatic carboxylic acids. The O–H bond of these groups in aqueous solutions at physiological pH values undergoes readily heterolytic dissociation to form mono- or dianions [5, 6].

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TABLE 1. Effect of Natural and Synthetic 5-Hydroxy- and 5,8-Dihydroxy-1,4-naphthoquinones on the Activity of α-Galactosidase from the Marine Bacterium *Pseudoalteromonas* sp. KMM 701

Compound	Origin	MW	$C \times 10^4$, mol/L	Compound	Origin	MW	$C \times 10^4$, mol/L
Lack of inhibition				**10**	Natural	278.3	3.5
1	Synthetic	218.2	4.6	**11**	Natural	278.3	2.0
2	Natural	266.2	3.8	**12**	Synthetic	287.1	2.0±1.0
3	Synthetic	306.3	3.3	**13**	Synthetic	303.1	1.6
4	Synthetic	312.7	3.2	**14**	Natural	250.2	1.3
5	Synthetic	312.7	3.2	**15**	Commercial	190.2	0.41
6	Synthetic	308.3	3.2	**16**	Natural	250.2	0.23
Reduction of enzyme activity				**17**	Synthetic	317.1	0.053
by 50% of initial				**18**	Synthetic	259.1	0.039±0.005
7	Natural	222.2	4.5*	**19**	Synthetic	321.5	0.029±0.005
8	Synthetic	275.0	4.3±1.2**	**20**	Synthetic	337.9	0.015
9	Natural	250.2	4.0	**21**	Synthetic	293.6	0.016±0.005

*Compound concentration at which enzyme activity was reduced by 50% in the presence of the compound for 30 min; **averages are given of 3-5 independent experiments.

Because echinochrome A (**2**) existed under the studied conditions as a dianion, it carried a large negative charge. It was seen from our results that **2** did not affect the enzyme activity. Thus, it was hypothesized that the active center of α-galactosidase at pH 7.3 is electrostatically negative and its charge can completely prevent binding of **2**.

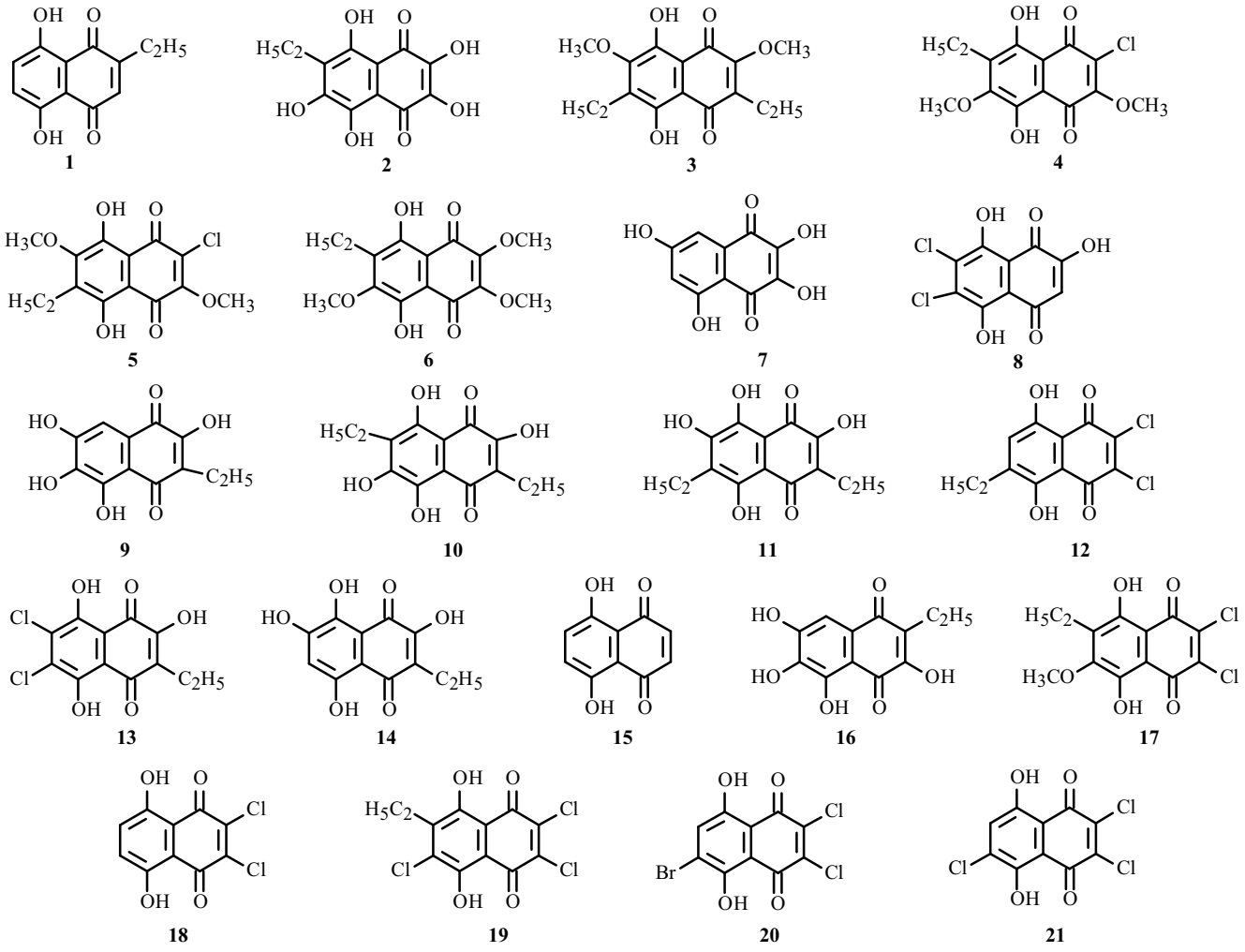
The significant differences among negatively charged compounds on the enzyme activity may be due to the different activity of the β-OH in the benzene rings of the bicyclic structure of these compounds. The studied compounds are strongly bound systems. Therefore, subtle electronic effects of the substituents are distributed over all atoms of the bicyclic system. One ring or another of the compound can have a different effect on the enzyme depending on the nature of the substituent and its location.

Using structural isomers **9** and **16** as examples, it can be seen that changing the sites of the hydroxyl and ethyl groups in the 2- and 3-positions of the quinoid ring changes significantly the inhibiting activity ($C_{50} = 4.0 \cdot 10^{-4}$ and $2.3 \cdot 10^{-5}$ M, respectively). One of the OH groups in the 6- and 7-positions of **16** is probably more acidic. Therefore, it forms intermolecular H-bonds with proton-acceptor (nucleophilic) groups of the enzyme active center more readily than **9**. The reason for this difference can also be considered a steric factor and/or the formation of an additional H-bond through the OH in the 5-position of the benzene ring. Thus, this OH for **9** and **16** should approach different parts of the active center for the same type of bonding of the whole inhibitor molecule.

The enzyme activity, which was completely suppressed by **16**, was restored to 45% of the initial value during gel filtration, i.e., the inhibition effects by compounds due to the benzene OH groups involved in forming intermolecular associates is reversible. In contrast with **16**, halo-substituted naphthazarine (**19**) inhibits irreversibly, i.e., inactivates the enzyme. Enzyme inactivated by **19** does not regain activity during gel filtration.

Table 1 shows that all compounds with two or more Cl atoms (**17**-**21**) were effective irreversible inhibitors. The possible mechanism of enzyme inactivation by naphthazarine (**15**), which has no β-substituent ($C_{50} = 4.1 \cdot 10^{-5}$ M), should be examined in order to understand these facts.

It was shown previously [4] that sodium *p*-chloromercuribenzoate at a final concentration of 10^{-3} M inactivated completely the studied enzyme; *N*-ethylmaleimide at the same concentration, by 95% of the initial. This confirmed that SH groups were important for the activity of α-galactosidase from *P.* sp. KMM 701. However, arsenate at a concentration of 10^{-2} M did not affect the enzyme activity. This fact suggested that there are no neighboring sulfhydryl groups near the SH group that is important for the activity of α-galactosidase [4]. The mechanism of action of naphthoquinones on the activity of several enzymes involving cysteine units has been studied [7, 8]. A covalently bonded conjugate of naphthazarine and the enzyme is formed by addition (Michael reaction) of the S-nucleophile. We demonstrated previously the ability to carry out reactions of this type under mild conditions using the reaction of 2,3-dichloro-substituted naphthazarine (**17**) and the reduced form of the tripeptide glutathione [9].



Electron-donating substitutes in the quinoid ring of naphthazarine (**15**) should reduce the electrophilicity of C-2 and C-3 and, therefore, its ability to add nucleophiles, which can be seen by comparing the effects of **15** and **1** in addition to **18** and **12**. Electron-accepting substituents in the quinoid ring should have the opposite effect on the reactivity of naphthazarine derivatives, especiallly in those instances where there are two of them. In fact, all compounds of this type **17**-**21** were very effective irreversible enzyme inhibitors due to activation of the 2- and 3-position of the quinoid ring to nucleophilic attack. The presence of electron-donating substituents Et or Me in the benzene ring of these compounds also reduced whereas the presence of electron-accepting substituents Br or Cl increased their inactivating ability. Compounds **19**, **20**, and **21** had the highest cytotoxic activity for developing sea urchin embryos [10] and were the most effective irreversible enzyme inhibitors.

The low inhibiting ability of **8**, which also contains two Cl atoms in one ring of the naphthazarine core, can be explained by its existence under the experimental conditions as a monoanion, which is the energentically more favorable tautomer of this molecule. The Cl atoms in the monoanion (**8**) were localized on the benzene ring, where they could not undergo nucleophilic substitution of the RS group through addition of the thiol and subsequent loss of HCl. The same applied to **13**. The lack of inactivating activity for the Cl-substituted naphthazarines **4** and **5** can be explained by the proximity to the Cl atom of a strongly electron-accepting MeO group, which neutralizes the activating effect of the Cl atom for nucleophilic attack in the 2- and 3-positions of the quinoid ring.

Thus, the effect of a wide range of 5-hydroxy- and 5,8-dihydroxy-1,4-naphthoquinones on enzymatic activity of α-galactosidase from the marine γ-proteobacterium *P.* sp. KMM 701 was studied. β-Methoxy and fully hydroxylated derivatives of 5,8-dihydroxy-1,4-naphthoquinones, including echinochrome, did not affect the activity of α-galactosidase. β-Hydroxylated derivatives of juglone exhibited reversible inhibition whereas naphthazarine and its chlorinated derivatives with vinylic halide atoms in the quinoid ring were highly effective irreversible inhibitors of this enzyme. α-Galatosidase was inactivated by naphthazarine and chlorinated derivatives of 5,8-dihydroxy-1,4-naphthoquinones by formation of a covalent bond through

nucleophilic attack of cysteine of the enzyme active center on the activated double bond of the quinoid ring and subsequent addition (Michael reaction) of the S-nucleophile, as a result of which a covalently bonded conjugate of the enzyme and naphazarine or its chloro derivatives was formed. The α-galactosidase used in this work can be considered to be a simple and convenient model for obtaining reliable analytical information for evaluating highly toxic chloro derivatives of naphthazarine, which are micro or even nano impurities in sythetic echinochrome preparations.

EXPERIMENTAL

Preparation and Properties of 1,4-Naphthoquinones. 2,3,5,6,8-Pentahydroxy-7-ethyl-1,4-naphthoquinone (echinochrome A) (**2**), 2,5,6,7-tetrahydroxy-3-ethyl-1,4-naphthoquinone (**9**), 2,5,6,8-tetrahydroxy-3,7-diethyl-1,4-naphthoquinone (**10**), 2,5,7,8-tetrahydroxy-3,6-diethyl-1,4-naphthoquinone (**11**), 2,5,7,8-tetrahydroxy-3-ethyl-1,4-naphthoquinone (**14**), and 3,5,6,7-tetrahydroxy-2-ethyl-1,4-naphthoquinone (**16**) were isolated from sea urchin *Scaphechinus mirabilis* [11]; 2,3,5,7-tetrahydroxy-1,4-naphthoquinone (**7**), from sea urchin *Strongylocentrotus nudus* [12]. Synthetic naphthoquinones were prepared by the previously described methods: 5,8-dihydroxy-2-ethyl-1,4-naphthoquinone (**1**) and 5,8-dihydroxy-2,3-dichloro-6-ethyl-1,4-naphthoquinone (**12**) [13]; 5,8-dihydroxy-3,6-dimethoxy-2-chloro-7-ethyl-1,4-naphthoquinone (**4**), 5,8-dihydroxy-3,7-dimethoxy-2-chloro-6-ethyl-1,4-naphthoquinone (**5**), and 2,5,8-trihydroxy-6,7-dichloro-1,4-naphthoquinone (**8**) [14], 5,8-dihydroxy-2,3,6-trimethoxy-7-ethyl-1,4-naphthoquinone (**6**) and 2,5,8-trihydroxy-6,7-dichloro-3-ethyl-1,4-naphthoquinone (**13**) [15]; 5,8-dihydroxy-2,3-dichloro-1,4-naphthoquinone (**18**) [9]; 5,8-dihydroxy-2,3,6-trichloro-1,4-naphthoquinone (**21**) [16]; 6-bromo-5,8-dihydroxy-2,3-dichloro-1,4-naphthoquinone (**20**) [17]; and 5,8-dihydroxy-2,3,6-trichloro-7-ethyl-1,4-naphthoquinone (**19**) [18]. 5,8-Dihydroxy-2,7-dimethoxy-3,6-diethyl-1,4-naphthoquinone (**3**) and 5,8-dihydroxy-6-methoxy-2,3-dichloro-7-ethyl-1,4-naphthoquinone (**17**) were prepared by treating ether solutions ($0.3 \cdot 10^{-3}$ mol) of the appropriate substrates (**11**) and (**13**) with a solution of diazomethane in ether with subsequent purification of the reaction products on SiO_2 plates.

Pure compounds were isolated by preparative TLC on plates (20 × 20 cm) with an unfixed layer of activated SiO_2 (5-40 μm) using hexane:acetone (5:1). The purity of the compounds was monitored by TLC on SiO_2 60 F-254 (Merck) plates using benzene:hexane (4:1). PMR spectra in $CDCl_3$ were recorded on an Avance DPX-300 (Bruker) spectrometer with Me_4Si internal standard.

5,8-Dihydroxy-2,7-dimethoxy-3,6-diethyl-1,4-naphthoquinone (3). PMR spectrum (300 MHz, δ, ppm, J/Hz): 1.14 (t, 6H, 2 × CH_3, J = 7.5), 2.69 (q, 4H, 2 ×CH_2, J = 7.5), 4.07 (s, 6H, 2 × OCH_3), 12.91 [s, 1H, C(8)-OH], 13.46 [s, 1H, C(5)-OH].

5,8-Dihydroxy-6-methoxy-2,3-dichloro-7-ethyl-1,4-naphthoquinone (17). PMR spectrum (300 MHz, δ, ppm, J/Hz): 1.14 (t, 3H, CH_3, J = 7.5), 2.66 (q, 2H, CH_2, J = 7.5), 4.17 (s, 3H, OCH_3), 12.90 [s, 1H, C(5)-OH], 13.36 [s, 1H, C(8)-OH].

5,8-Dihydroxy-1,4-naphthoquinone (naphthazarine) (15) (Fluka) was purified by crystallization from acetone and subsequent sublimation at 210-220°C at reduced pressure (5 mm Hg).

Isolation and Purification of Enzyme. α-Galactosidase was isolated from biomass of marine bacterium *P.* sp. KMM 701 cultivated in the Microbiology Laboratory of PIBOC FEB RAS by the literature procedure [4]. The substrate was *p*-nitrophenyl-α-D-galactopyranoside (Sigma).

Standard Method of Inhibition. A solution of enzyme (0.095 mL, 0.70 ± 0.01 unit/mL) in sodium phosphate buffer (0.1 M, pH 7.3) was mixed with a solution of the studied compound (0.005 mL) in ethanol and stored for 30 min. Then, this mixture (0.020 mL) was added to a solution of *p*-nitrophenyl-α-D-galactopyranoside (0.380 mL) at a concentration of $3.3 \cdot 10^{-3}$ M in sodium phosphate buffer (0.1 M, pH 7.3). The reaction was stopped by addition of an aqueous solution of sodium carbonate (1 M, 0.6 mL). The amount of *p*-nitrophenol released during the enzymatic reaction was determined by spectrophotometry at 400 nm (ε = 18,300 mol^{-1} cm^{-1}). The amount of enzyme that catalyzed formation of 10^{-6} mol of *p*-nitrophenol per minute was taken as the activity unit. The initial rate of substrate hydrolysis (ν) was determined from the linear part of D as a function of reaction time subtracting absorption of a control mixture of buffer, compound, and substrate. All inhibition procedures and activity determinations were made at 20°C. The effect of the compounds on enzyme activity was studied in the concentration range 10^{-1} to 10^{-4} g/L. Compounds that did not affect the enzyme at a concentration of 10^{-1} g/L were not further studied. Enzyme in sodium phosphate buffer solution (0.1 M) and enzyme stored in buffer solutions of ethanol (5%) were used in all experiments as controls. The concentration of a compound that reduced enzyme activity by half of the

initial value after 30 min was estimated from a plot of the enzyme activity as a function of concentration (C) of the compound or log C, where C is the concentration expressed in mol/L (M).

Reversibility of inhibition was studied using **16** and **19** as examples and the FPLC (Acta) method on a Superdex 75 HR 10/30 column (Amersham Pharmacia Biotech) equipped with a 0.1-mL loop. Sodium phosphate buffer solution (0.01 M, pH 7.3) containing NaCl (0.1 M) was used for elution at 0.4 mL/min. Enzyme solution (0.18 mL, 1.0 unit/mL) in sodium phosphate buffer solution (0.1 M, pH 7.3) was mixed with a solution of the compound in ethanol (0.02 mL, 1 g/L) and stored for 30 min. An aliquot (0.1 mL) of the mixture was chromatographed. Enzyme activity before and after gel filtration was compared with the activity of enzyme stored in working buffer solution containing ethanol (10%) that was also chromatographed.

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