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INHIBITION OF COPPER/QUINOPROTEIN AMINE OXIDASES FROM *ASPERGILLUS NIGER* BY BENZOPHENANTHRIDINE ALKALOIDS

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Inhibition of copper/quinoprotein amine oxidases (EC 1.4.3.6), AO-I (dimer 2×75 kDa) and AO-II (monomer 80 kDa), from the fungus *Aspergillus niger* by benzophenanthridine alkaloids sanguinarine, chelerythrine, and fagaronine were studied. For both amine oxidases the alkaloids showed reversible noncompetitive inhibition of *n*-hexylamine oxidation with K_i 0.6, 0.9 and 2.8 mM for sanguinarine, chelerythrine, and fagaronine, respectively. The values of the inhibition constants corresponded to pK_{R+} values for the iminium ion/pseudobase equilibrium of the alkaloids. Since thio-compounds protected the enzymes against this inhibition, the inhibition effect was ascribed to the interaction with a sulfhydryl group essential for the enzymatic activity.

KEY WORDS: Amine oxidase, *Aspergillus niger*, quinoprotein, benzophenanthridine alkaloids, non-competitive inhibition

INTRODUCTION

The amine oxidase of *Aspergillus niger* was discovered in the middle 1960's, when it was first purified and crystallized.[1,2] Copper and an undetermined carbonyl cofactor were found to function in the catalytic cycle.[2–4] Recently, the importance of the fungal amine oxidase has arisen, since the enzyme can be used to monitor histamine contents in fish products,[5] and a massive production of the enzyme on an industrial scale is the focus of current interest.

We have found two distinct quinoprotein amine oxidases (EC 1.4.3.6), AO-I and AO-II, in *Aspergillus niger* mycelia grown on *n*-butylamine medium.[6,7] AO-I is the

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enzyme previously reported, consisting of two 75 kDa subunits, while AO-II is a novel one of 80 kDa. The enzymes oxidize amines to aldehydes, hydrogen peroxide and ammonia with a stichiometry.

$$CH_3\text{–}(CH_2)_4\text{–}CH_2\text{–}NH_2 + O_2 + H_2O \xrightarrow{AO} CH_3\text{–}(CH_2)_4\text{–}CHO + H_2O_2 + NH_3$$

n-hexylamine

Topaquinone has been confirmed as the cofactor for both amine oxidases.[8] AO-I and AO-II show no significant differences in substrate specificity, but the specific activity of the latter is on average four times lower. Benzylamine, histamine, phenylethylamine and aliphatic *n*-monoamines (*n*-butylamine, *n*-amylamine and *n*-hexylamine)[3,6] are the best substrates for both enzymes.

AO-I has been found to be inhibited by copper chelating agents diethyldithiocarbamate, cuprizone, *o*-phenanthroline, 2,2′-bipyridyle, 8-hydroxyquinoline,[3,9] by carbonyl reagents semicarbazide, guanidine, hydroxylamine, phenylhydrazine, izoniazide and iproniazide,[4] by ethylamine[4] and ethylenediamine,[10,11] heavy metal ions and sulfhydryl-group-seeking reagents[12] and by a mechanism-based inhibitor β-bromoethylamine.[13] The interaction of AO-I and AO-II with active site covalent modifiers has been also studied. Inhibition by 1,4-diamino-2-butyne has been determined as mechanism-based due to the formation of pyrrole covalently bound to the lysyl residue in the active site of AO-I.[14]

Benzophenanthridine alkaloids possess a wide variety of pharmacological and biological activities[15,16] including analgesic, antiinflammatory, antimicrobial and antifungal effects. They are known as potent inhibitors of enzymes containing an essential active site sulfhydryl group, e.g. alanine aminotransferase (EC 2.6.1.2) and aspartate aminotransferase[17] (EC 2.6.1.1), Na^+/K^+ ATPase[18] (EC 3.6.1.37), Ca^{2+} ATPase[19] (EC 3.6.1.38) and pyruvate dehydrogenase complex[20] (EC 1.2.4.1, 2.3.1.12 and 3.1.3.43). Recently, fagaronine has been shown to inhibit topoisomerase I and II[21] and HIV-1 and -2 reverse transcriptase.[22]

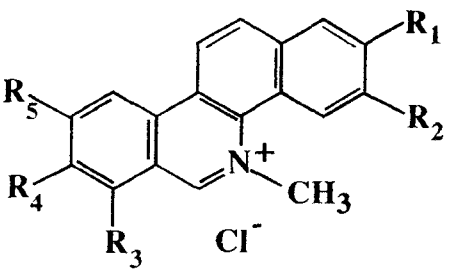
This paper describes our investigation on the inhibition of *Aspergillus* amine oxidases by sanguinarine, chelerythrine and fagaronine. Structures of these alkaloids are shown in Figure 1.

MATERIALS AND METHODS

Enzyme, Alkaloids and Chemicals

Cultivation of *Aspergillus niger* AKU 3302 mycelia in nitrate medium and induction of amine oxidase by *n*-butylamine were achieved using routine methods.[1,2] Amine oxidases were isolated as described previously[6] and the protein content was determined by Bradford's method.[23] Specific activities, measured as an oxygen consumption,[24] were 46 nkat.mg^{-1} for AO-I and 12 nkat.mg^{-1} for AO-II, with *n*-hexylamine as the substrate in 0.1 M phosphate buffer, pH 6.8, at 30°C.

Sanguinarine chloride (M_r 332.3, m.p. 279–281°C) and chelerythrine chloride (M_r 383.5, m.p. 200–204°C) were isolated from *Macleaya cordata* (Papaveraceae),[25] fagaronine chloride (M_r 385.84, m.p. 203–206°C) was synthesized[26] in house.



$R_1 + R_2 = R_3 + R_4 = OCH_2O, R_5 = H$ **Sanguinarine**

$R_1 + R_2 = OCH_2O, R_3 = R_4 = OCH_3, R_5 = H$ **Chelerythrine**

$R_1 = OH, R_2 = R_4 = R_5 = OCH_3, R_3 = H$ **Fagaronine**

FIGURE 1 Structures of the benzophenanthridine alkaloids studied.

Catalase from bovine liver was obtained as a crystalline suspension from Reanal (Budapest, Hungary) with a specific activity of 2,000 Bergmeyer's units.mg^{-1} of protein.

Hexylamine hydrochloride was obtained from Fluka (Basel).

Amine Oxidase Activity Assay

The assay was carried out using a modification of a method already described[24] measuring the oxygen consumption with a Clark oxygen electrode E 5047 (Radiometer, Copenhagen). The reaction mixture of the total volume of 3.5 ml contained 0.1 M phosphate buffer, pH 6.8, 25 μg of catalase (50 Bergmeyer's units) 3% ethanol and appropriate concentrations of amine oxidase and the inhibitor. The mixture was placed in a cell thermostated at 30°C equipped with magnetic stirrer and Clark oxygen electrode, saturated with oxygen by aeration (0.772 mM O_2 at 30°C and 760 Torr) and incubated for 10 min. Then the reaction was initiated by the addition of an appropriate concentration of *n*-hexylamine and the initial rate of the oxygen consumption was measured.

Reversibility of Inhibition

The amine oxidase (25 nkat) was dissolved in 5 ml of 0.1 M phosphate buffer. pH 6.8, containing the inhibitor in a final concentration of 1.0–1.5 mM for sanguinarine and chelerythine and 5 mM for fagaronine. After standing for 30 min at room temperature, the activity was reduced to 20–30%. The solution was then dialyzed against the same buffer at 4°C for 24 h. The activity was assayed as described above and a blank sample without the inhibitor was processed in the same way.

Protective Effect of Thio-compounds

For measuring the protective effect of thio-compounds, cysteine, mercaptoethanol and glutathione were added to the assay mixture at final concentrations of 0.2 M together with 0.8–1.0 M inhibitor. After incubation at 30°C for 10 min, the activity was assayed.

RESULTS AND DISCUSSION

The interactions of both amine oxidases with alkaloids were investigated by kinetic methods. It was found necessary to incubate the alkaloids with the enzymes for at least 10 min before the addition of the substrate in order to achieve the maximum inhibition. Kinetic data obtained under these conditions in the air-saturated reaction mixture were analyzed graphically according to Lineweaver and Burk (1/v versus 1/[S]), Dixon (1/v versus [I]) and Hunter and Downs ($[I].v_i/(v-v_i)$ versus [S]). All three methods gave comparable results.

It was found that the alkaloids studied inhibited the oxidation of *n*-hexylamine by either AO-I or AO-II noncompetitively, but these effects were fully reversible. After removing the inhibitor from the reaction mixture by dialysis, virtually 100% of the activity was restored when compared to the blank sample without inhibitor.

The double reciprocal plots of the initial rate of enzymatic reaction versus substrate concentration for the inhibitions of AO-I by fagaronine and chelerythrine are shown in Figure 2. The same plots for the inhibitions of AO-I and AO-II by sanguinarine are shown in Figure 3. In all cases, the straightlines intersected on the abscisa indicating noncompetitive inhibition. All replots of slopes and intercepts of the straightlines versus inhibitor concentration were linear.

The values of the inhibition constants K_i and the change of the standard Gibbs energy ΔG° for the interaction $E+I \rightleftharpoons EI$ calculated from the equation: $\Delta G^\circ = -2.303.R.T.\log K_i$ are given in Table 1. It is evident from the values measured that both amine oxidases are most sensitive to chelerythrine and sanguinarine, but only weakly to fagaronine.

It was found that pK_{R+} values of iminium ion/pseudobase equilibrium of the alkaloids increase in the same order as their inhibition constants. The pK_{R+} values represent the ability of the carbon neighboring the quaternary nitrogen in the structure of the alkaloids to react with a nucleophile and the stability of the heteroaromatic system. Fagaronine with the highest K_i and highest pK_{R+} shows the weakest interaction with such a nucleophile. The weak interaction of fagaronine is caused by a more stable character of its molecule. Similar interaction of these alkaloids with sulfhydryl groups as such nucleophiles were observed for several enzymes.[17–20] To confirm whether these alkaloids interacted with -SH groups also contained in AO-I and AO-II, the protective effects of thio-compounds were examined. As shown in Table 2, mercaptoethanol and glutathione significantly protected against the inhibition, while cysteine was almost without effect.

From the results obtained, the inhibition of *Aspergillus niger* amine oxidase by benzophenanthridine alkaloids can be ascribed to the interaction with an -SH group

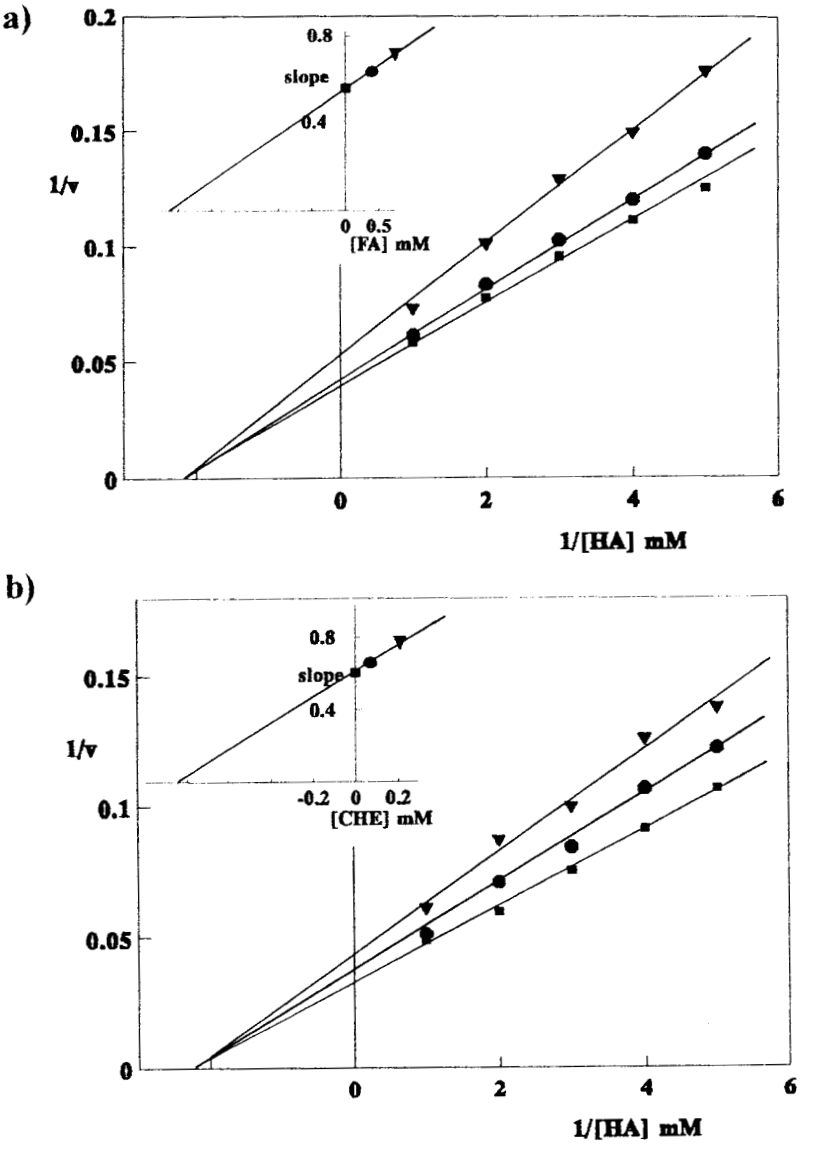


FIGURE 2a Noncompetitive inhibition of AO-I by fagaronine (FA). Initial rate is given in nmol of O_2 consumed per second with *n*-hexylamine (HA) as the substrate. The Lieweaver-Burk plot is shown in the main graph; the insert shows the replot of slopes as a function of the inhibitor concentration. The assays were carried out in 0.1 mM potassium phosphate buffer, pH 6.8, with 25.6 nkat of the enzyme, at 30°C, in the absence (■) and with 0.37 (●) and 0.74 mM (▼) of the inhibitor. The inhibition constant was 2.8 mM. **2b** Noncompetitive inhibition of AO-I by chelerythrine (CHE). The assays were carried out with 31.0 nkat of the enzyme as described above, in the absence (■) and with 0.177 (●) and 0.530 mM (▼) of the inhibitor. The inhibition constant was 0.9 $mmol.l^{-1}$.

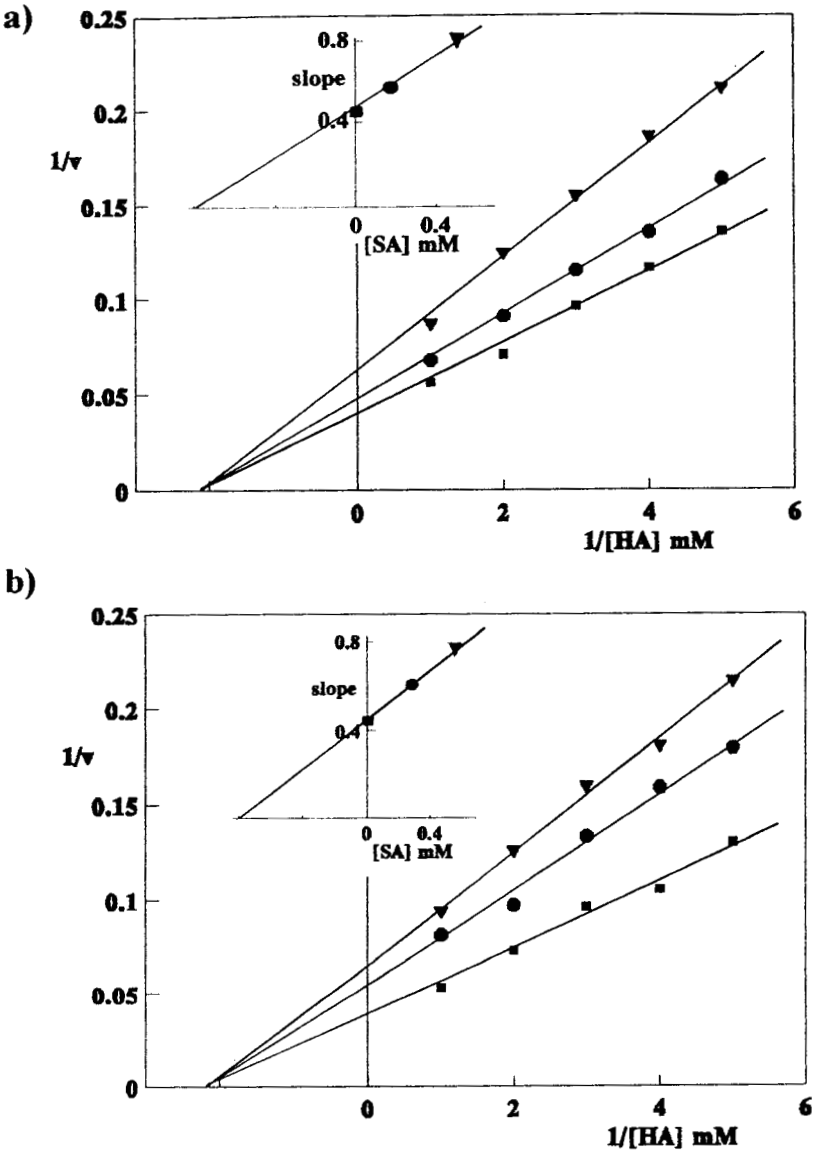


FIGURE 3a Noncompetitive inhibition of AO-I by sanguinarine (SA). Initial rate is given in nmol of O_2 consumed per second with *n*-hexylamine (HA) as the substrate. The Lineweaver-Burk plot is shown in the main graph; the insert shows the replot of slopes as the function of the inhibitor concentration. The assay were carried out in 0.1 mM potassium phosphate buffer, pH 6.8, with 25.0 nkat of the enzyme, at 30°C, in the absence (■) and with 0.064 (●) and 0.193 mM (▼) of the inhibitor. The inhibition constant was 0.6 mM. **3b** Noncompetitive inhibition of AO-II by sanguinarine (SA), under the same conditions, in the absence (■) and with 0.279 (●) and 0.558 mM (▼) of the inhibitor. The inhibition constant was 0.5 $mmol.l^{-1}$.

TABLE 1
Inhibition constants and changes of the standard Gibbs energy ΔG° of interactions of the AO-I and AO-II with benzophenanthridine alkaloids ($-\Delta G^\circ = -2.303.R.T.\log K_i$) related to the pK_{R+} values of the iminium ion/pseudobase equilibrium of the alkaloids.

Alkaloid	AO-I		AO-II		
	K_i(mM)	$-\Delta G^\circ$(kJ.mol^{-1})	K_i(mM)	$-\Delta G^\circ$(kJ.mol^{-1})	$pK_{R+}(H_2O)$
Sanguinarine	0.6	3.5	0.5	3.5	8.2[28]
Chelerythrine	0.9	5.0	0.9	5,0	9.2[28]
Fagaronine	2.8	16.4	2.7	15.8	13.2,[27] 7.57*

*pK_{R+} value for phenolic group at C-2.[27]

TABLE 2
Effect of some thio-compounds on the AO-I inhibition by sanguinarine, chelerythrine and fagaronine.

Compound	Sanguinarine (0.8 mM)		Chelerythrine (0.8 mM)		Fagaronine (1 mM)	
	% inhibition	% protection	% inhibition	% protection	% inhibition	% protection
Cysteine (0.2 M)	73	2–5	68	5	52	2–4
Thioethanol (0.2 M)	20	76	25	68	40	50
Glutathione (0.2 M)	25	70	30	60	38	53

essential for the enzymatic activity. No inhibition was observed with amine oxidases isolated from etiolated pea seedlings and fenugreek isolated as described previously,[29] where the presence of an -SH group was not confirmed.

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