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Inhibition of pig kidney diamine oxidase by nazlinin and nazlinin derivatives

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Abstract

Nazlinin (1-(4-butylamino)-1,2,3,4-tetrahydro- β -carboline) (**1**), an alkaloid recently isolated from *Nitraria schoberi*, and its two derivatives, 1-(4-butylamino)-3,4-dihydro- β -carboline (**2**) and 1-(4-butylamino)- β -carboline (**3**), were synthesized and their interaction with pig kidney diamine oxidase (PKDO) was studied. Nazlinin appeared to be a very poor substrate while **3** was a good substrate with an apparent K_m of $9.3 \cdot 10^{-5}$ M. The enzyme was inhibited by **1** and **2**. With both compounds the mode of inhibition found was non-competitive and inhibition constants calculated from the slopes and intercepts of double-reciprocal plots show that **2** is a much more potent inhibitor than the natural product. The relationship between the structure of these compounds and the results found is discussed.

Keywords: Diamine oxidase; Inhibition; Alkaloid; (Porcine); (Kidney)

1. Introduction

Pig kidney diamine oxidase (PKDO) [EC 1.4.3.6] is a member of the family of copper-containing diamine oxidases that contains 6-hydroxydopa quinone (topaquinone) as prosthetic group essential for activity [1]. The enzyme catalyses readily the oxidation of diamines like cadaverine (1,4-diaminobutane), putrescine (1,3-diaminopropane) and histamine using molecular oxygen as oxidant according to the following equation:

Nazlinin is an alkaloid recently isolated by Üstünes and Özer [2] from *Nitraria schoberi*, that possesses serotonergic activity. Although structure **4** suggested by these workers agrees with most of their characterization data, based on biosynthetic principles we suggested that structure **1** is a more likely structure for nazlinin [3], because it possesses more chemical stability and also agrees with the characterization data reported by those workers. This assumption was verified via synthesis of **1** [3].

Our interest in the synthesis of lupine and *Nitraria* alkaloids based on biosynthetic pathways (Wanner and Koomen [4] and [5]) and the fact that diamine oxidases play an important role in these biosynthetic pathways ([6] and [7]) led us to examine the interaction of diamine oxidase with the recently isolated alkaloid nazlinin (1-(4-butylamino)-1,2,3,4-tetrahydro- β -carboline) (**1**) and its derivatives 1-(4-butylamino)-3,4-dihydro- β -carboline (**2**) and 1-(4-butylamino)- β -carboline (**3**). (see Fig. 1.)

2. Materials and methods

Nazlinin and derivatives were prepared according to references [3] and [8], pig kidney diamine oxidase [EC 1.4.3.6.] and cadaverine (1,5-diaminopentane) dihydrochloride were purchased from Sigma, horseradish peroxidase [EC 1.11.1.7., grade II (lyophilized)] was from Boehringer Mannheim, 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate and 3-(dimethylamino)benzoic acid were purchased from Aldrich.

2.1. Purification of the enzyme

The enzyme was purified in a Neobar AQ4 column using a gradient of 0.05 M Tris-HCl buffer (pH 8.0)

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¹ Deceased.

Fig. 1. Structures of nazlinin and its analogues.

(solution A) and 0.05 M Tris-HCl buffer (pH 8.0) containing 1 M NaCl (solution B). The fractions containing the higher enzyme activity (eluted with 15% solution B) were collected, concentrated by ultrafiltration, passed over a Sephadex G-25 column using 0.05 M potassium phosphate buffer (pH 7.0) as eluent and concentrated again by ultrafiltration. The specific activity of the final solution was 6.4 U/mg of protein (1 international unit of enzyme is defined as the amount of enzyme that catalyses the oxidation of 1 μmol of cadaverine min⁻¹ at room temperature). Before the purification the specific activity was 0.46 U/mg protein. The protein content of the enzyme preparation was measured by the Bradford method using bovine serum albumin as standard [9].

2.2. Assay conditions

The activity of the enzyme was determined spectrophotometrically using the peroxidase-coupled assay [10]. The method is based on the oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 3-(dimethylamino)benzoic acid (DMAB) in the presence of peroxidase and the hydrogen peroxide released during the reaction with PKDO. The formation of a compound with deep-blue colour is monitored at 595 nm. All experiments were conducted in 0.05 M phosphate buffer (pH 7.0) at room temperature in a final volume of 1 ml and using cadaverine as substrate. Each assay contained 0.67 U of the enzyme. The inhibition was measured by preincubating the enzyme for 20 min with the appropriate alkaloid of a suitable concentration and the reactions were started with the addition of the substrate.

2.3. Kinetics of inhibition of PKDO

According to Cleland [11] the double reciprocal plots of a two-substrate enzymatic reaction is given by the reaction:

$$1/v = K_a/V(1/A) + 1/V(1 + K_b/B)$$

where A is the variable substrate (diamine), B the fixed

substrate (oxygen), K_a and K_b are the Michaelis constants and V is the velocity when A is infinite. In the presence of an inhibitor, when both slope and intercept effects are present, we have a non-competitive inhibition. The replot of the slope or intercept versus concentration of inhibitor as well as Dixon plots can give parabolas, indicating that two molecules of the inhibitors can combine with the enzyme. In the present study, where inhibition was observed, parabolic non-competitive inhibition was seen. This type of inhibition can be represented by:

$$1/v = K_a/V(1/A)(1 + aI + bI^2) + 1/V(1 + K_b/B)(1 + \alpha I + \beta I^2)$$

where

$$a = (K_{i \text{ slope } 1})^{-1}, \quad b = (K_{i \text{ slope } 1} K_{i \text{ slope } 2})^{-1}$$

and

$$\alpha = (K_{i \text{ intercept } 1})^{-1}, \quad \beta = (K_{i \text{ intercept } 1} K_{i \text{ intercept } 2})^{-1}.$$

3. Results

The double reciprocal plots show that compounds 1 and 2 inhibit PKDO non-competitively (Figs. 2 and 3). With both alkaloids the replots of the slopes and intercepts of

Fig. 2. Double reciprocal plot of inhibition of PKDO by nazlinin (1). The incubations were carried out with 0.67 U of PKDO, 0.05 M phosphate buffer (pH 7.0), at room temperature, in a final volume of 1 ml. (○), no inhibitor, (▲) 0.5 mM, (*) 1.0 mM, (■) 1.5 mM and (×) 2.0 mM inhibitor. The insets show: (a) the replots of slopes (○) and intercepts (■); (b) Dixon plot: (▲) 0.34 mM, (○) 0.68 mM, (■) 0.92 mM and (Δ) 1.1 M cadaverine.

Fig. 3. Double reciprocal plot of inhibition of PKDO by 1-(4-butylamino)-3,4-dihydro- β -carboline (2). The incubations were carried out with 0.67 U of PKDO, 0.05 M phosphate buffer (pH 7.0), at room temperature, in a final volume of 1 ml. (○) No inhibitor, (▲) 20 μ M, (*) 35 μ M, (■) 50 μ M, and (×) 60 μ M inhibitor. The inset shows the replots of slopes (○) and intercepts (■).

the reciprocal velocity versus concentration of inhibitor as well as the Dixon plots ($1/v$ versus $[I]$) are parabolic (Fig. 2a,b and Fig. 3a), suggesting the binding of two alkaloid molecules to the enzyme.

The inhibition constants obtained from the parabolas (Table 1) show that compound 2 is a much more potent inhibitor than compound 1, nazlinin.

Unlike the former two compounds, compound 3 did not inhibit the enzyme, but behaved as a substrate. The appar-

Fig. 4. Double reciprocal plot of 1-(4-butylamino)- β -carboline (3) oxidation by PKDO. The incubations were carried out with 0.67 U of enzyme, 0.05 M phosphate buffer (pH 7.0), at room temperature, in a final volume of 1 ml.

Table 1

Inhibition of PKDO by nazlinin (1-(4-butylamino)-1,2,3,4-tetrahydro- β -carboline) 1 and 1-(4-butylamino)-3,4-dihydro- β -carboline 2

Inhibitor	$K_{i \text{ slope } 1}$ (mM)	$K_{i \text{ intercept } 1}$ (mM)	$K_{i \text{ slope } 2}$ (mM)	$K_{i \text{ intercept } 2}$ (mM)
1	3.42	7.53	1.11	0.58
2	0.27	0.91	0.020	0.0064

ent K_m value calculated from the double reciprocal plot for this compound is $9.3 \cdot 10^{-5}$ M (Fig. 4).

In preliminary studies we observed that nazlinin is also a substrate for PKDO, albeit poor. All our attempts to determine the K_m for this compound — by applying both the peroxidase-coupled method and the glutamate dehydrogenase-coupled method [12] — failed.

It was also observed during inhibition studies that compound 1 is oxidized by exposure to air, probably under formation of compound 2. For this reason, spectroscopic purity control of solutions of compound 1 at 340 nm, where only compound 2 absorbs, was carried out. The nazlinin solutions used contained less than 0.2% of compound 2.

4. Discussion

Our results show that nazlinin (1) as well as its derivatives (2–3) interact with PKDO in different ways: nazlinin is a poor substrate and an inhibitor, 2 is a good inhibitor and 3 is a good substrate.

The only difference in the structures of these three compounds concerning the substrate–enzyme interaction is in the amino group responsible for the interaction with the substrate binding site (notation suggested by Bardsley [14]), that is, the region responsible for the specificity of the enzyme. In nazlinin it is a dialkyl-substituted amino group and in 3 it is an amino group that belongs to an aromatic system. Nazlinin, as expected, appeared to be a poor substrate. *N*-Alkyldiamine derivatives of cadaverine and putrescine are known to be poorer substrates than the non-substituted diamines [13,15,16]. On the other hand

Fig. 5. Molecules, present in the equilibrium mixture of 2 in water.

compound **3**, possibly in analogy with histamine, appeared to be a good substrate. In addition, in compound **3** the β -carboline part of the molecule is flat, which could be facilitating the interaction of the nitrogen with the enzyme. In the presence of **2** no oxidation was observed. It seems reasonable to suppose that in solution this compound is in equilibrium with compound **7** (Fig. 5), having compounds **5** and **6** as possible intermediates. The presence of compound **7** contributes to this effect because tryptamine is not a substrate for PKDO [14]. The piperidine ring of the ring system of compound **2** has an intermediate conformation in relation to the piperidine and pyridine rings of compounds **1** and **3**, respectively.

Very little work has been done on the interaction of alkaloids with PKDO. The enzyme is inhibited non-competitively by some cinchona alkaloids and competitively by lobeline [17]. Some typical monoamine oxidase inhibitors are also non-competitive inhibitors of PKDO [18]. The structure of all these compounds differs considerably. We found that with both compounds **1** and **2** the type of inhibition is parabolic non-competitive. These compounds can form a complex with the enzyme form E (slope effect) and with the enzyme reduced form F (intercept effect) and therefore excess of substrate cannot suppress the inhibition. A parabolic inhibition plot indicates that complexes EI₂ and FI₂ are formed.

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