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# Oxidation of a Putrescine–Glyoxylate Adduct Catalyzed by D-Amino Acid Oxidase Gives the Product-Inhibitor 2-Carboxy-4,5,6,7-tetrahydro-1,3-diazepine

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Results are reported which indicate that the D-amino acid oxidase-catalyzed oxidation of a solution containing putrescine (1,4-diaminobutane) and glyoxylate leads to the formation of 2-carboxy-4,5,6,7-tetrahydro-1,3-diazepine (**7**) as the product. This unusual monoamidine derivative of oxalic acid was independently synthesized and shown to be identical to the enzymic product. Compound **7** is not only a product of the enzymic reaction, but is also an effective competitive inhibitor of D-amino acid oxidase, with a $K_i$ of ca. 20 $\mu$M. The possibility that this putative new metabolite of putrescine may be involved in the mechanism controlling cell proliferation is considered. 

## INTRODUCTION

In several recent articles (*1–9*) we have reported that adducts of various nucleophiles and glyoxylate are excellent substrates for the peroxisomal enzymes, D-amino acid oxidase, D-aspartate oxidase, and L-hydroxy acid oxidase. Furthermore, we have summarized a considerable amount of evidence suggesting that such adducts are the normal physiological substrates for these enzymes and that the products of the reactions may play an important role in the control of animal metabolism (*8–19*), including possibly acting as intracellular mediators for hormones such as insulin and insulin-like growth factors. In most cases the specific reactions catalyzed, and the products formed in the oxidase reactions, have been fully characterized. However, in one case, in which D-amino acid oxidase catalyzes the oxidation of an adduct of putrescine (1,4-diaminobutane) and glyoxylate (*1, 8*), the course of the reaction and the product that is formed have remained in doubt. In the present article we report results that clarify the nature of this reaction. In addition, we present evidence that the product is an effective inhibitor of the reaction. Since putrescine is believed to participate in the control of several aspects of animal metabolism, especially the control of cell growth and proliferation (*20, 21*), the current findings could have considerable metabolic significance.

The adducts of putrescine and glyoxylate that have structures suitable for them to be potential substrates for D-amino acid oxidase are illustrated in Scheme 1, along with the enzymic reactions that would result in each case. The initial carbinolamine adduct **1** would be formed in both *R* and *S*-configurations and the *R*-



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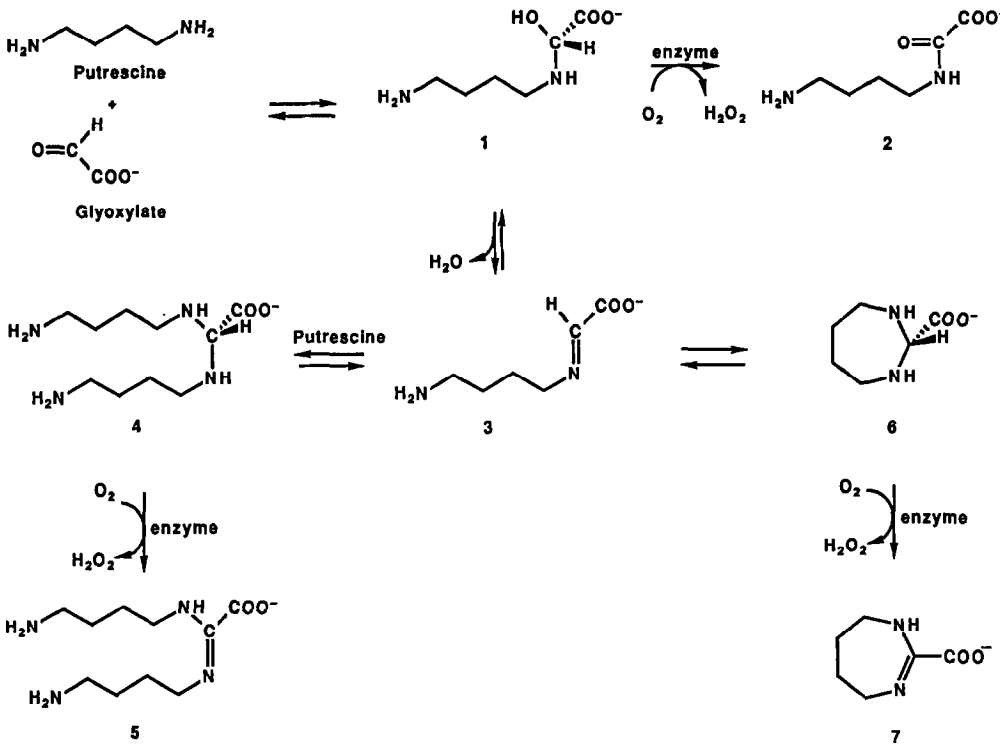



SCHEME 1

isomer (illustrated in Scheme 1) is a potential substrate. If it were acted upon by the enzyme, then the amide **2** would be the product. It is known that carbinolamines lose water easily to give imines; if that occurred with **1**, then **3** would be formed. Since imines readily react with nucleophiles, **3** could presumably react with excess putrescine to give the prochiral compound **4**, which again has the correct configuration to be a potential substrate for D-amino acid oxidase. If it is a substrate, then the acyclic amidine **5** would be the expected product. Another possibility is that the imine group of **3** reacts not with excess putrescine, but with its own amino group to give the cyclic compound **6**, and it too is a potential substrate for D-amino acid oxidase. If **6** were a substrate, then the cyclic amidine **7** would result. The present research was mainly concerned with determining which of **2**, **5**, or **7** is the actual product of the enzymic reaction.

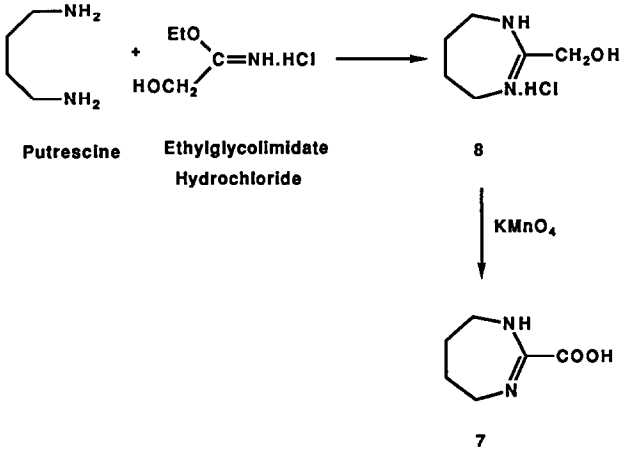
## MATERIALS AND METHODS

### *Materials*

Unless otherwise noted, commercial materials, reagent grade or better, were used as received. Putrescine (1,4-diaminobutane) free base and glyoxylic acid

monohydrate were obtained from Aldrich Chemical Co., glycolic acid nitrile (70% in water) was from Fluka, potassium permanganate was from Baker and Adamson, [1-$^{14}$C]glyoxylic acid was from New England Nuclear, and catalase and porcine kidney D-amino acid oxidase (crystalline suspensions, 5 mg/ml, in 3.2 M $(NH_4)_2SO_4$, pH 6.5) were from Sigma Chemical Co.

2-Carboxy-4,5,6,7-tetrahydro-1,3-diazepine **(7)** was prepared as outlined in Scheme 2. The reaction of ethyl glycolimidate hydrochloride (*22*) with putrescine by the procedure of Faust *et al.* (*23*) gave a 60% yield of 2-hydroxymethyl-4,5,6,7-tetrahydro-1,3-diazepine hydrochloride **(8)** with a melting point after recrystallization from ethanol–ether of 135–136°C (reported 133–135°C). Compound **8** has the following spectral characteristics: electron impact mass spectrum, *m/e* 128, 99, 71, and 55; $^1$H NMR (in $D_2O$) δ 2.15 (m, 4H), δ 3.64 (m, 4H), and δ 4.4 (s, 2H). After dissolving 3.9 g (0.02 mol) of compound **8** in 30 ml water, adjusting the pH to 11.2 with 6 N KOH, and cooling the solution to 0–5°C, small portions of $KMnO_4$ (total 4.9 g, 0.025 mol) were added with continuous stirring over a 3-h period (during the reaction the pH drops to about 8). The reaction solution was then centrifuged for 15 min at 12,000 rpm, the aqueous solution decanted, and the brown precipitate of manganese dioxide washed twice with 10 ml water. After combining the aqueous solutions, the volume was reduced to 5 ml, 30 ml of ethanol was added, and the white inorganic solids were removed by filtration. Following extraction of the ethanol–water solution several times with chloroform, the chloroform extracts were combined, dried with calcium chloride, and evaporated to dryness to give a white solid, which on recrystallization from 2-propanol gave the neutral form of **7** (mp 196°C dec.) in 50% yield. This compound has the following characteristics: elemental analysis, found (theory in parentheses) C 50.70 (50.85), H 7.04 (7.12), and N 19.72 (19.55); IR (chloroform) 3140 (m), 3100 (m), 3000 (m), 2910 (s), 2825 (m), 1695 (m), 1680 (s), 1580 (m), 1340 (m), and 1322 (s) cm$^{-1}$; $^1$H NMR ($CDCl_3$) δ




SCHEME 2

9.13 (s, 1H), δ 3.5 (m, 4H), and δ 1.94 (m, 4H); electron impact mass spectrum, *m/e* (relative intensity), 142 (3.9), 114 (2.5), 98 (80), 70 (41), and 55 (9).

*Methods*

Many of the methods were similar to those previously described (*1, 5, 10*). Thin-layer chromatography of authentic **7** and the enzymic product was carried out using silica gel 60 $F_{254}$ precoated plates. The compound was visualized using either iodine or potassium permanganate. Authentic **7** has an $R_f$ of 0.50 when the eluting solvent is 1 : 1 chloroform–methanol and an $R_f$ of 0.24 when the solvent is 3 : 1 acetone–methanol.

For the paper electrophoresis experiments, a Savant Model FP22A Flat Plate (water cooled) connected to a Savant Model HV3000 UL-115-3 high voltage power supply was used. The 18 × 18-in. flat plate was first covered with a mylar sheet, followed by a sheet of wetted (with buffer) Whatman No. 3 MM paper streaked with an enzymic reaction mixture containing [1-$^{14}$C]glyoxylate (see below), then another mylar sheet, and finally a ¼-in. glass plate (18 × 18 in.). For the particular experiment described under Results, a 50 mM citrate–phosphate buffer (pH 7.0) was used and the electrophoresis was allowed to proceed for 2.5 h at 1000 V and 30–50 mA. The paper was then dried and sprayed with 0.1% ninhydrin in acetone (to detect free amino groups), and a strip of it cut into 1-cm squares for radioactivity analysis. For counting, each of these was placed in 10 ml of a scintillation cocktail, prepared by dissolving 20 g of 2,5-diphenyloxazole (Fisher) and 0.6 g of 2,2′-*p*-phenylenebis(5-phenyl)oxazole (Mallinkrodt) in 1200 ml 2-methoxyethanol (scintillation grade from Eastman) and diluting with 2000 ml toluene (scintanylized from Fisher).

The enzymic reaction mixture that was analyzed by electrophoresis was prepared as follows. After a 1-ml solution containing 20 mM sodium pyrophosphate buffer (pH 8.3), 10 mM putrescine, 10 mM glyoxylate containing 5 μCi [1-$^{14}$C]glyoxylate, 10 μM FAD, and 0.04 mg/ml catalase had equilibrated at 25°C for 1 h with gentle shaking, 50 μg of D-amino acid oxidase was added and the reaction allowed to proceed for 4 hr. At that time, and again 4 h later, additional 50-μg quantities of D-amino acid oxidase were added to ensure complete reaction over the total 12-h reaction time. This solution was then streaked on the electrophoresis paper for separation and analysis.

The enzymic product that was analyzed by spectral and TLC methods was prepared and isolated as follows. A 3.3-ml solution containing 18 mM sodium pyrophosphate buffer (pH 7.4), 4.5 mM putrescine, 4.6 mM glyoxylate, 3 μM FAD, and 3 mg D-amino acid oxidase was stirred under $O_2$ for 24 h. After removing the enzyme by ultrafiltration, 15 ml of methanol was added to the aqueous solution and the white precipitate removed by filtration. The product in the methanol solution was purified following concentration by thin-layer chromatography on silica gel 60 $F_{254}$ precoated plates using chloroform and methanol (1 : 1) as the eluant. The silica in the region of $R_f$ 0.50 was scraped off and the enzymic product extracted with 4 : 1 chloroform–methanol. Following evaporation of the solvent the product was subjected to spectral and TLC analysis.

## RESULTS

When an enzymic reaction mixture containing putrescine and [1-$^{14}$C]glyoxylate as reactants was subjected to electrophoresis as outlined under Methods, two radioactive bands were detected, one centered at about +21 cm from the origin and the other at about −3 cm. The band at +21 cm is due to unreacted [1-$^{14}$C] glyoxylate so that the band at −3 cm is presumably due to the enzymic product. Only one ninhydrin-positive band was seen and that was at about −19 cm, in a position identical to that of authentic putrescine; thus, it is presumably due to the unreacted putrescine. These results suggest that the enzymic product is **7.** If it were **2** or **5** it should have been ninhydrin positive. Furthermore, the position of the product band at −3 cm is consistent with it being **7** because one would expect it to be neutral (a zwitterion) under the conditions of the electrophoresis so it should migrate very little. One would expect **2** to be neutral as well so the position of the band alone cannot distinguish **2** from **7.**

In initial efforts to synthesize **7** to confirm that it is the enzymic product, attempts were made to prepare the ethyl ester of **7** by reacting putrescine with ethyl cyanoformate under conditions similar to those used previously to prepare the ethyl ester of thiazoline-2-carboxylate (*19*). However, the only product isolated (*24*) from such a reaction was *N,N'*-dicarboethoxy-1,4-diaminobutane ($EtOOCNH(CH_2)_4NHCOOEt$). Apparently the adducts of putrescine and cyanoformate prefer to lose HCN rather than to cyclize to the seven-membered ring compound.

Compound **7** was successfully synthesized by the method outlined under Materials. This involved reacting putrescine with ethyl glycolimidate hydrochloride to give **8** (*23*), followed by oxidation of **8** with basic potassium permanganate. Both the chemically synthesized and the enzymic products were found to have identical infrared and mass spectra, and they have the same $R_f$ values when subjected to TLC in two different solvent systems. Furthermore, as described below, they are both good inhibitors of D-amino acid oxidase. Thus, there can be no doubt that **7** is the enzymic product.

In early experiments involving the putrescine–glyoxylate adduct as substrate for D-amino acid oxidase, it was noted that the rate of $O_2$ uptake is not linear with time but decreases as the reaction proceeds, even under conditions where the substrate concentrations are not appreciably diminished. Such results suggested that the product is an inhibitor of the reaction. This was qualitatively confirmed when it was found that the rate of the D-amino acid oxidase-catalyzed oxidation of other substrates, for example, of D-alanine, is decreased when an aliquot, taken from an oxidase-catalyzed reaction of the putrescine–glyoxylate adduct that had proceeded for some time, is added. With the synthetic product available, the inhibition of the D-amino acid oxidase reaction was studied in more detail and it was found, using either the D-alanine or the cysteamine–glyoxylate adduct (*1, 10*) as substrate, that **7** is a substrate competitive inhibitor which has an apparent $K_i$ of ca. 20 $\mu$M (at 25°C, pH 7.4 and an air atmosphere).

## DISCUSSION

The results reported here show conclusively that **7** is the product formed on oxidation of a putrescine–glyoxylate adduct catalyzed by D-amino acid oxidase. The fact that the synthetic and enzymic products have identical characteristics is the best evidence for this conclusion, but the results from the electrophoresis experiments are consistent with it. Compound **7** has a relatively uncommon structure; it is one of the few known examples (*25–27*) that contain oxalic acid as a monoamidine derivative (the antibiotic kasugamycin (*25*) was the first described example of this class of compounds), and it is the only characterized cyclic monoamidine derivative. Oxalic acid monoamidines exist as zwitterions in neutral aqueous solution (*27*), but the observation that **7** can be extracted with chloroform indicates that the adjacent two oppositely charged groups must effectively cancel each other's charge, possibly with the help of two intramolecular hydrogen bonds.

In many ways it is a surprising result that **7,** which must have arisen from oxidation of **6,** is the enzymic product. One does not expect that the seven-membered ring of **6** would be formed nearly as readily, or in as high concentration, as the six-membered analog that is formed from 1,3-diaminopropane and glyoxylate. Yet the adduct resulting from this latter combination is a very poor substrate for D-amino acid oxidase (*1, 10*). Furthermore, no adduct formed from cadaverine (1,5-diaminopentane) and glyoxylate is a substrate (*1*). These results taken together clearly indicate that the active site of D-amino acid oxidase must be quite specific for the seven-membered ring adduct **6.** Such a conclusion implies that D-amino acid oxidase has evolved a specific binding site for this adduct and thus that **6** may be a normal *in vivo* substrate for the enzyme.

The question remains, however, whether **6** would be formed in large enough amounts *in vivo* to be converted in significant quantities to **7.** The concentration of glyoxylate in cells is believed to range from 0 to 200 $\mu$M (*8*), while that of putrescine is ca. 100 $\mu$M in normal cells and 500 $\mu$M or more in rapidly dividing or transformed cells ((*20, 21*) and references therein). At such concentrations of putrescine and glyoxylate it is not possible to detect significant $O_2$ uptake catalyzed by D-amino acid oxidase (*1, 8*), but presumably there would still be a small amount of **6** present and being converted to **7.** More importantly, whatever small amount of reaction is occurring in normal cells would be increased fivefold or more when the putrescine level rises in transformed or rapidly dividing cells. Given the fact that numerous studies indicate that the putrescine concentration is a marker for rapid cell proliferation (*20, 21*), one must consider the possibility that even small amounts of **7** may be a contributing factor to the proliferation response.

The details of how **7** might participate in cell proliferation remain to be determined, but one possibility that needs to be considered is that it may be related to inhibition of the D-amino acid oxidase reaction itself by **7.** As reported here, **7** is an effective competitive inhibitor of D-amino acid oxidase with a $K_i$ of ca. 20 $\mu$M. This is in the same range as the $K_i$ values of 1 to 100 $\mu$M found for a number of arenecarboxylates that are considered excellent inhibitors of D-amino acid oxidase (*10*). In earlier publications (*1, 5, 8*), evidence has been reported indicating that the main reaction catalyzed by D-amino acid oxidase *in vivo* is probably the

oxidation of the cysteamine–glyoxylate adduct, thiazolidine-2-carboxylate, to thiazoline-2-carboxylate. Furthermore, correlations have been presented (*8–11*) which suggest that this product (or its further metabolites) may somehow be involved in the control of animal metabolism, including possibly acting as an intracellular mediator for some hormones, such as insulin and growth factors. One class of compounds that may be (*19*) subsequent metabolites of thiazoline-2-carboxylate are oxalyl thiolesters ($RSCOCOO^-$), which very recent results (*16, 18*) have indicated are probable cell proliferation inhibitors. Consequently, a possible scenario for the stimulation of cell proliferation by putrescine involving its metabolite **7** is that, by inhibiting D-amino acid oxidase, **7** ultimately leads to a decrease in the cellular concentration of oxalyl thiolesters, the putative cell proliferation inhibitors. Clearly, however, this is only a working hypothesis that needs to be clarified by further investigations.

## ACKNOWLEDGMENTS

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