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Inhibition of Copper-Containing Amine Oxidase by Oximes

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The inhibitory effects of twelve oxime compounds on the activity of bovine serum monoamine oxidase (copper-containing amine oxidase, E.C. 1.4.3.6) were assayed *in vitro*. The most potent inhibitor among the compounds examined was 2-butanone oxime, of which the 50% inhibiting concentration in the reaction mixture was 7×10^{-6} M. Acetone oxime, acetoaldehyde oxime, salicylaldehyde oxime and formamide oxime were less potent inhibitors, while 2,3-butanedione monooxime, 2,3-butanedione dioxime, D-camphor oxime, α-furil dioxime, pyridine-2-aldehyde oxime, pyridine-4-aldehyde oxime, *syn*-2-pyridilaldehyde oxime and di-2-pyridilketone oxime exhibited virtually no inhibitory effect on the enzyme activity. The type of inhibition by 2-butanone oxime was non-competitive, and the inhibitor constant and K_m value with respect to allylamine as the substrate were estimated graphically to be 6.7×10^{-6} and 3.9×10^{-4} M, respectively.

Keywords——serum monoamine oxidase; copper-containing amine oxidase; inhibitor; non-competitive inhibition; oxime; 2-butanone oxime

Copper-containing amine oxidase (E.C. 1.4.3.6) is an enzyme which, like flavin-containing amine oxidase (E.C. 1.4.3.4) in the mitochondrial membrane, catalyzes the oxidative deamination of a variety of amines to yield the corresponding aldehydes. It is contained in the highest concentration, along with lysyl oxidase (E.C. 1.4.3.13), in mammalian (*e.g.* bovine,[1] pig,[2] rabbit,[3] rat and human,[4] *etc.*) connective tissues, although considerable amounts of the enzyme, termed serum monoamine oxidase (serum MAO), are present in the blood plasma.[5-9] In the field of human clinical biochemistry, elevation of the serum MAO activity level has been considered as a marker of hepatic fibrosis,[10] arteriosclerosis,[11] *etc.*, since increased biosynthesis of this enzyme is generally associated with pathological conditions accompanied with acceleration of collagen metabolism within the corresponding organs. The precise physiological roles of the enzyme are not clear at present, but it has been shown that the activity of this enzyme including serum MAO is greatly reduced by various neuropsychiatric agents, such as iproniazid (an anti-tuberculous drug with an anti-depressant activity),[12] phenylhydrazine (an anti-depressant drug),[13] penicillamine (an anti-Wilson disease drug),[14] levodopa (an anti-Parkinson disease drug)[15] and carbidopa (an inhibitor of β-(3,4-dihydroxyphenyl)alanine (DOPA)-carboxylase),[16] as well as by steroids,[17] aminoacetonitrile,[18] semicarbazide,[19] *etc.* In order to obtain new neurologically active substances, therefore the authors have been carrying out screening tests of various substances and crude drug extracts employing inhibitory effects on the activity of this enzyme as a marker. We recently found that some oxime compounds, especially 2-butanone oxime, strongly inactivated bovine serum MAO.[20] This paper deals with the properties of these compounds which are reported here for the first time as inhibitors of this enzyme.

Materials and Methods

Materials——Oximes were purchased from Wako Pure Chemicals (*syn*-2-pyridilaldehyde oxime) or Sigma

Company (the other compounds).

Assays of Inhibitory Effects——The inhibitory effects of the samples on copper-containing amine oxidase activity were estimated by the colorimetric method using a Determiner MAO (Kyowa Medics), a clinical laboratory test kit for the measurement of human serum MAO activity. This kit has five components: (a) the pretreatment reagents: 2.0 U/ml of lipoprotein lipase, 5.0 U/ml of ascorbate oxidase and 3.0 U/ml of peroxidase, (b) the color developing reagent: 52 μM 10-*N*-methylcarbamoyl-3,7-dimethyl-10*H*-phenothiazine, (c) two substrate buffer solutions (75 ml each) for dissolving (a) and (b), respectively: 30 mM allylamine hydrochloride and 0.53 mM phenol dissolved in 25 mM piperazine-*N*,*N*-bis(ethanesulfonate) (PIPES) buffer adjusted to pH 6.75 with NaOH, (d) the reaction stopper: 8.9 mM sodium diethylcarbamate, and (e) the standard enzyme: a vial containing lyophilized bovine serum to be dissolved in 5 ml of deionized water expressing a known activity level of serum MAO (*e.g.* 1.64 U/l) in the assay. According to Kyowa Medics' manual, the MAO activity of clinical serum specimens can be measured as follows. Fifty microliters each of deionized water (as a blank), the above-mentioned aqueous solution of (e) (as the standard enzyme), and clinical sera to be measured for MAO activity levels are placed in test tubes, and 1.5 ml each of the (a) solution dissolved in one of the (c) containers is then added to them. The resultant mixtures are left at 37 °C for 5 min, and 1.5 ml each of the (b) solution dissolved in another (c) container is then added, followed by incubation at 37 °C for 30 min. The reaction is terminated by adding 50 μl each of (d), and the differences in the 660 nm absorbance between the samples (the standard enzyme and clinical specimens) and a blank are measured. The values of the 660 nm absorbance differences correspond linearly to the contents of H_2O_2 produced during the process of oxidative deamination of allylamine by serum MAO, and the MAO activity of the tested sera (U/l) can therefore be finally calculated by comparison of their values with that of the standard enzyme. In the present study, the following slight modifications were introduced in order to examine the inhibitory effects of the oximes on the enzyme activity. (1) All assay mixtures except for the blank contained (e) instead clinical specimens. (2) The compounds examined were dissolved in dimethyl sulfoxide (DMSO) at various final concentrations (1×10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 0 M DMSO alone as a control), and 50 μl of each of them was injected into the reaction mixtures using an Ames Diluter System, at the same time as the addition of (b) dissolved in (c). (3) The inhibition rates of the individual compounds examined were expressed as the % decrease in the enzyme from the control.

Kinetic Treatment of the Data for 2-Butanone Oxime——The effects of varying the concentration of both the substrate (allylamine) and the inhibitor (2-butanone oxime) were investigated graphically by the use of Lineweaver–Burk and Dixon plots. In these experiments, the contents of (a) and (b) of the Determiner MAO were dissolved respectively in 75 ml each of freshly prepared 25 mM PIPES buffer (adjusted to pH 6.8 with NaOH) containing 0.5, 1, 2, 5, 10 and 30 mM allylamine hydrochloride in addition to 0.53 mM phenol and 0.02% Brij 35, instead of the same volume of (c). On the other hand, 2-butanone oxime was dissolved in DMSO at final concentrations of 1, 2, 5 and 10 μM in the assay mixture. Other procedures were identical to those in the assays described above.

Results and Discussion

The inhibition rates of the examined oximes at various final concentrations in the assay mixtures are listed in Table I. The most potent inhibitor among the examined compounds was 2-butanone oxime which reduced 12.8, 63.4, 78.7, and 86.2% of the enzyme activity in its presence at concentrations of 1×10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M, respectively. As shown in Table I, acetone oxime, acetoaldehyde oxime, salicylaldehyde oxime and formamide oxime acted as inhibitors with less efficiency, while 2,3-butanedione monooxime, 2,3-butanedione dioxime, D-camphor oxime, α-furil dioxime, pyridine-2-aldehyde oxime, pyridine-4-aldehyde oxime, *syn*-2-pyridilaldehyde oxime and di-2-pyridilketone oxime exhibited virtually no inhibitory effect on the enzyme activity. More detailed work is needed to eatablish the precise relations between the inhibition rates and structures of the oximes, although the data in Table I do appear to suggest that the inhibition is not associated with the presence of the NOH group itself and that potent inhibition is exerted by compounds having methyl and/or ethyl group(s) attached to the α-C atom of the oxime.

Kinetic studies were performed to elucidate the characteristics of the inhibition by 2-butanone oxime which was the most potent inhibitor among the examined compounds. The effects of varying the concentrations of both the allylamine (substrate) and 2-butanone oxime were evaluated graphically on the basis of Lineweaver–Burk (Fig. 1) and Dixon (Fig. 2) plots. No change in the K_m value of bovine serum MAO ($-1/-2.55 = 0.39$ mM) was observed on varying the concentration of allylamine (Fig. 1), indicating that the type of inhibition was non-competitive. On the other hand, the inhibitor constant was estimated to be 6.7 μM (Fig.

TABLE I. Values for the Percent Inhibition of the Activity of Serum Monoamine Oxidase by the Examined Oximes at Various Concentrations

Compound	Concentration (1×10^{-x} M)[a]			
	$x=6$	5	4	3
2-Butanone oxime	12.8	63.4	78.7	86.2
Acetone oxime	11.3	48.7	62.0	76.5
Acetoaldehyde oxime	10	23.0	45.6	66.9
Salicylaldehyde oxime	10	12.4	29.8	52.1
Formamide oxime	0	<10	19.4	40.3
2,3-Butanedione monooxime	0	0	0	<10
2,3-Butanedione dioxime	0	0	0	<10
D-Camphor oxime	0	0	0	<10
α-Furil dioxime	0	0	0	<10
Pyridine-2-aldehyde oxime	0	0	0	<10
Pyridine-4-aldehyde oxime	0	0	0	<10
syn-2-Pyridilaldehyde oxime	0	0	0	<10
Di-2-pyridilketone oxime	0	0	0	<10

a) The concentrations of the examined oximes are expressed as the final molar values in the assay mixtures.

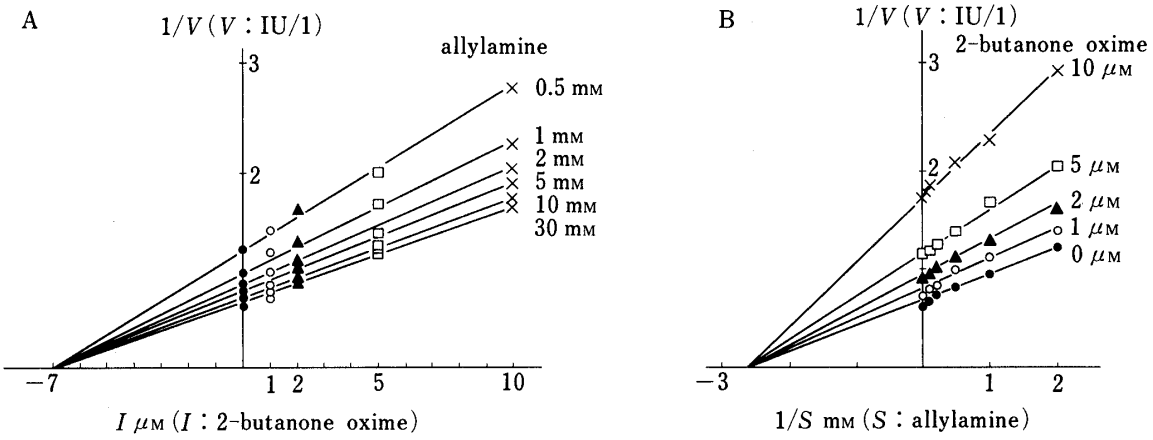

Fig. 1. Lineweaver–Burk Plot (A) and Dixon Plot (B) of the Bovine Serum MAO Activity in the Presence or Absence of 2-Butanone Oxime with Various Concentrations of Both Allylamine (the Substrate) and 2-Butanone Oxime

The assay of bovine serum MAO in the presence or absence of 2-butanone oxime was performed as described under Materials and Methods. The final concentration of 2-butanone oxime in the assay mixtures was as follows: (●) 0 μM as the control, (○) 1 μM, (▲) 2 μM, (□) 5 μM, or (×) 10 μM.

2). The 50% inhibiting concentration (IC_{50}) was *ca.* 7 μM which is similar to those of known potent inhibitors of this enzyme; the IC_{50} values with this assay system for phenylhydrazine, aminoacetonitrile and semicarbazide were *ca.* 2, 5 and 30 μM, respectively. In order to check the enzyme specificity of 2-butanone oxime as an inhibitor, the effects of adding 2-butanone oxime DMSO solutions to various human serum enzymes were investigated using commercial kits for routine laboratory tests to measure their activity levels.

The results showed that the IC_{50} of 2-butanone oxime for the activity of human serum MAO (6.5 μM) was approximately equal to that for the bovine serum MAO employed to

obtain kinetic data in this study. However, 2-butanone oxime exerted little or no inhibitory effect (IC_{50}, more than 100 mM) on glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, γ-glutamyltranspeptidase, lactic dehydrogenase, leucine aminopeptidase, alkaline phosphatase choline esterase, lipase, amylase and creatine kinase. These findings suggested that the inhibition by 2-butanone oxime was highly specific to serum MAO (copper-containing amine oxidase).

As described in the introduction, the present study was undertaken as part of a project to obtain neurologically active substances. Among the neuropsychiatric drugs known to be potent inhibitors of MAO, phenylhydrazine and related compounds with an anti-depressant activity also inhibit flavin-containing amine oxidase,[21] and it is considered that their pharmacological activities are mainly associated with an increase of biogenic amines within the central nervous system caused by inactivation of the flavin-containing amine oxidase in the brain mitochondrial membrane. However, 2-butanone oxime caused virtually no inhibition of the activity of flavin-containing amine oxidase. On the other hand, penicillamine, which has been employed to remove surplus copper from the brain and/or liver of Wilson's disease patients, is thought to express its activity on the basis of the formation of hydrophilic complexes, with heavy metals including copper.[22] Oximes are well known as compounds with a potent affinity for metal ions. Serum MAO contains copper as its prosthetic group.[23] It is assumed therefore that the inhibition of this enzyme by 2-butanone oxime and related compounds could possibly be due to the occurrence of chelate linkages between the oximes and functional copper within the enzyme molecules. More detailed work, mainly based on animal experiments, is needed in order to determine whether the oximes, similar to penicillamine, display neuropharmacological (*e.g.* anti-Wilson's disease) activities. *In vitro* experiments are in progress to asses the changes in inhibition rates on adding various concentrations of copper ion to the assay mixtures containing the inhibitors at various pH values, and to elucidate the effects of these compounds on a variety of copper-containing enzymes including dopamine-β-hydroxylase which is one of the neurologically important enzymes.

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