MONOAMINE OXIDASE INHIBITORS ISOLATED FROM FERMENTED BROTHS

TOMIO TAKEUCHI, KEIJI OGAWA, HIRONOBU IINUMA, HIROYUKI SUDA, KATSUKO UKITA, TOSHIHARU NAGATSU*, MASAAKI KATO and HAMAO UMEZAWA

Institute of Microbial Chemistry, Shinagawa-ku, Tokyo, Japan

OSAMU TANABE

Central Institute of Takara Shuzo Co., Ltd., Kyoto, Japan

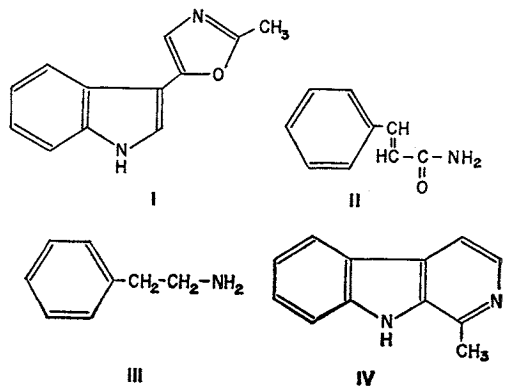
(Received for publication December 26, 1972)

Cultured broths were screened by measuring the inhibition of monoamine oxidase. Using serotonin as the substrate, two active agents were isolated from *Actinomycetes* and shown to be pimprinine and *trans*-cinnamic acid amide, and a compound isolated from a mushroom was identified to be harman. Using benzylamine as the substrate, an inhibitor was isolated from a *Streptomyces* and shown to be phenethylamine.

As reported by UMEZAWA[1] antibiotic research has been extended to the study of enzyme inhibitors produced by microorganisms. In this paper, the results obtained by screening for monoamine-oxidase inhibitors are reported.

A crude monoamine oxidase (E.C. 1.4.3.4) was prepared from rat liver and its inhibition by culture filtrates was tested. In part of the study, 2-^{14}C-serotonin was used as the substrate and the radioactivity of the reaction product which was extracted with butyl acetate at acid pH was determined. In another part, benzylamine was used as the substrate and the reaction product was extracted with cyclohexane and measured by its optical density at 242 nm.

Five hundred strains of Actinomycetes were shake-cultured, and two of them showed inhibition of monoamine oxidase action on serotonin. An active compound from the strain MD 211-C 4 (the strain number in the Institute of Microbial Chemistry) was extracted with butyl acetate and purified by silica gel chromatography and its identity with pimprinine (5,3′-indolyl-2-methyloxazole, I) was confirmed by elemental analysis, molecular peak of mass spectroscopy, ultraviolet spectrum, infrared spectrum and nmr analysis. Pimprinine was first isolated by BHATE *et al.*[2] and chemically synthesized by JOSHI *et al.*[3]



* Department of Biochemistry, and Department of Pharmacology, School of Dentistry, Aichi-Gakuin University, Nagoya, Japan.

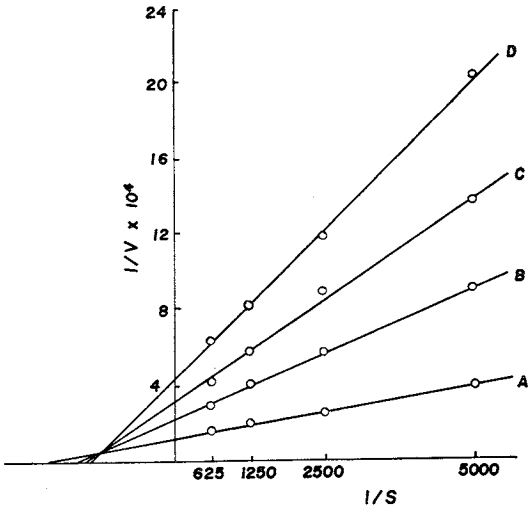
NARASIMHAN *et al.*[4] reported the protection of mice by pimprinine from convulsions caused by electric shock. Compounds structurally relating to pimprinine, 3-substituted indoles, have been studied for monoamine-oxidase inhibition[5]. LINEWEAVER-BURK plots of the results indicate a noncompetitive relation (mixed type) with serotonin as shown in Fig. 1.

An active agent in the culture filtrate of strain MD 235-C 2 was extracted with ethyl acetate and purified by silica gel chromatography, and the identity with *trans*-cinnamic acid amide (II) was confirmed by elemental analysis, molecular peak in mass spectrum, ultraviolet and infrared spectra and nmr analysis. Isolation of cinnamic acid amide from *Streptomyces* was reported by SEKIZAWA[6]. The tranquilizer effect of derivatives of cinnamic acid has been reported[7]. However, this is the first report that *trans*-cinnamic acid amide inhibits monoamine oxidase. LINEWEAVER-BURK plots of the results, indicating the noncompetitive relation (mixed type) with serotonin, are shown in Fig. 2.

Testing the activity of culture filtrates of 500 strains of *Actinomyces* in inhibiting oxidation of benzylamine by monoamine oxidase, a culture filtrate of strain MB 699-A 3 was active. The active agent was obtained by carbon adsorption and by carbon chromatography followed by Amberlite CG-50 chromatography. The identity with phenethylamine (III) was confirmed by nmr and by comparison of its infrared spectum with that of an authentic sample. It is the first isolation of phenethylamine from culture filtrates of *Actinomyces*. However, the activity of phenethylamine in inhibiting monoamine oxidase has been reported by GOMI *et al.*[8]

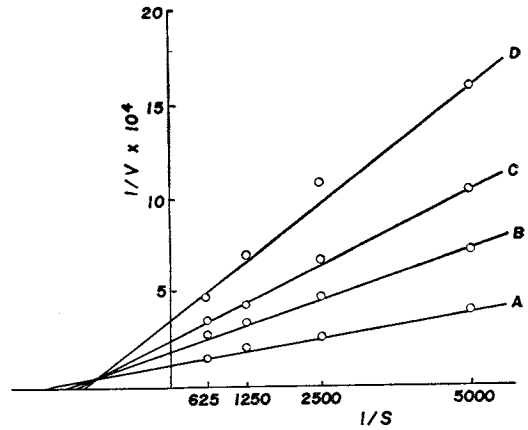
Mushrooms were shake-cultured in a medium (pH 5.8) consisting of glucose 2.0 %,

Fig. 1. LINEWEAVER-BURK plots of serotonin concentration against rate of oxidative deamination with and without pimprinine.



The velocities are expressed as the counts in the product formed.
A : Enzyme alone
B : Enzyme with 2.5×10^{-5} M pimprinine
C : Enzyme with 5.0×10^{-5} M pimprinine
D : Enzyme with 1.0×10^{-4} M pimprinine
Incubation : 60 minutes.

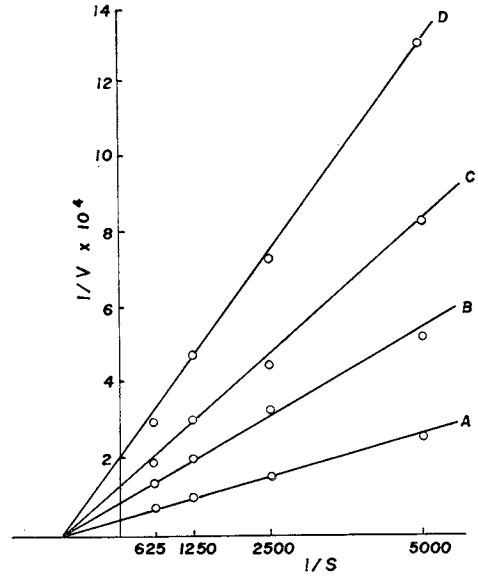
Fig. 2. LINEWEAVER-BURK plots of serotonin concentration against rate of oxidative deamination with and without *trans*-cinnamic acid amide.



The velocities are expressed as the counts in the product formed.
A : Enzyme alone
B : Enzyme with 3.40×10^{-4} M *t*-cinnamic acid amide
C : Enzyme with 6.80×10^{-4} M *t*-cinnamic acid amide
D : Enzyme with 1.36×10^{-3} M *t*-cinnamic acid amide
Incubation : 60 minutes.

peptone 0.5%, yeast extract 0.3%, KH_2PO_4 0.3%, $MgSO_4 \cdot 7H_2O$ 0.1%, and the activity of the culture filtrates in inhibiting monoamine oxidase action on serotonin was tested. Filtrate from 4 cultures were active, and the inhibitors were non-dialyzable. However, when 200 cultures were grown in a medium (pH 6.0) consisting of glucose 2.0%, lactose 1.0%, corn steep liquor 6.0%, ammonium nitrate 0.2% and $CaCO_3$ 0.4%, 23 culture filtrates contained inhibitors. The active agent of *Coriolus maximus* was extracted with butyl acetate and purified by silica gel chromatography, and the identity with harman (IV) was confirmed by elemental analysis, and infrared and ultraviolet spectra, compared to literature data. The active compounds produced by the other 22 strains were extracted and identity with harman was suggested by thin-layer chromatography using $CHCl_3$-MeOH (10:1) (Rf 0.27~0.28) and the ultraviolet spectrum. Thus, production of harman by various mushrooms, *Coriolus*, *Pogonomyces*, *Lentinus* was suggested. Harman was isolated first by PERKIN and ROBINOSON[9] from plants and later by TAKASE *et al.*[10] from Saké. The monoamine oxidase-inhibiting activity of β-carbolines structurally related to harman has been studied in detail.[5,11] LINEWEAVER-BURK plots of the results indicating the noncompetitive relation are indicated in Fig. 3.

Fig. 3. LINEWEAVER-BURK plots of serotonin concentration against rate of oxidative deamination with and without harman.



The velocities are expressed as the counts in the product formed.
A : Enzyme alone
B : Enzyme with 1.1×10^{-6} M harman
C : Enzyme with 2.2×10^{-6} M harman
D : Enzyme with 4.4×10^{-6} M harman
Incubation : 60 minutes.

The concentrations of pimprinine, phenethylamine, *trans*-cinnamic acid amide and harman in the system described in this paper exhibiting 50% inhibition are shown in Table 1. Pimprinine and harman showed stronger inhibition of serotonin deamination than of benzylamine, and phenethylamine showed stronger effect on benzylamine deamination. Phenethylamine inhibition was noncompetitive (mixed type) with serotonin and competitive with benzylamine.

The results described above indicate that the screening method described is useful in finding and isolating monoamine oxidase inhibitors and depending on different substrates used different active compunds are obtained.

Table 1. IC_{50} values of inhibitors

Substrate	Inhibitor			
	Pimprinine	Phenethylamine	*trans*-Cinnamic acid amide	Harman
Benzylamine	7.6×10^{-4} M	3.8×10^{-5} M	2.3×10^{-4} M	4.18×10^{-4} M
Serotonin	4.8×10^{-5} M	6.6×10^{-4} M	6.4×10^{-4} M	1.59×10^{-6} M

IC_{50} : Concentration for 50% inhibition.

Experimental

Preparation of crude monoamine oxidase:

Rat liver was homogenized with 6 times volume of 0.25 M sucrose by Teflon homogenizer. The homogenate was centrifuged at $700\times g$ for 10 minutes and the supernatant at $15{,}000\times g$ for 10 minutes. The precipitate was suspended in 0.25 M sucrose and again centrifuged at $15{,}000\times g$ for 10 minutes. The precipitate was suspended in 0.25 M sucrose (4.0 ml/g of liver) and stored in the frozen state. It was used as the enzyme preparation.

Method of testing the inhibition against monoamine oxidase using serotonin as the substrate:

A reaction mixture consisted of 0.2 ml of the enzyme preparation, 0.14 ml of 1 M phosphate buffer, pH 7.4, 0.1 ml of 1.6×10^{-2} M serotonin sulfate containing 0.5 μCi 2-^{14}C-serotonin oxalate, 0.52 ml of water and 0.04 ml of a test material. The substrate was added to start the reaction. After 90 minutes at 37°C it was heated at 90°C for 3 minutes to cease the reaction. To the reaction mixture 0.15 ml of 1 N HCl was added and the mixture was shaken with 3.0 ml of butyl acetate. The butyl acetate was separated by centrifugation and the radioactivity of 1.5 ml was determined by liquid scintilation counter. Percent inhibition was calculated as follows:

$$\%=[(c-s)/(c-b)]\times100$$

where c is the count without test material; s with the test material; b without the test material and the enzyme, but with the heat-denatured (3 minutes at 90°C) enzyme.

Method of testing inhibition against monoamine oxidase using benzylamine as the substrate:

The enzyme preparation described above was diluted 24 times with 0.25 M sucrose solution. The reaction mixture was as follows: 0.7 ml of the enzyme solution (96 ml/g liver), 0.3 ml of 0.3 M phosphate buffer, pH 7.4, 0.4 ml of 8.0×10^{-3} M benzylamine, 0.45 ml of water, 0.15 ml of a test material. The reaction was started by addition of the enzyme and continued for 60 minutes at 37°C. Then 0.15 ml of 60 % perchloric acid was added to stop the reaction. The reaction product was extracted with 2.0 ml of cyclohexane and the optical density of the extract was read at 242 nm. In a test tube, the reaction mixture was incubated without the enzyme and the enzyme was added after the addition of perchloric acid. The value thus obtained was taken as the blank and subtracted from the values obtained from the reaction mixture with the enzyme.

Isolation of pimprinine:

A *Streptomyces*, strain MD 211-C 4, was cultured in a medium (pH 7.4) containing starch 1.0 %, glucose 1.0 %, meat extract 0.75 %, peptone 0.75 %, NaCl 0.3 %, $MgSO_4\cdot7H_2O$ 0.1 %, $CuSO_4\cdot5H_2O$ 7 mg/liter, $FeSO_4\cdot7H_2O$ 1 mg/liter, $MnCl_2\cdot4H_2O$ 8 mg/liter, $ZnSO_4\cdot7H_2O$ 2 mg/liter. After 400 ml of the shake-cultured broth was inoculated, the fermentation in 15-liter medium was carried out in a jar fermenter under aeration of 15 liters/minute and 250 rpm stirring at 28°C for 3 days. The culture filtrate was extracted with butyl acetate (15 liters), and the extract was washed with water and concentrated to a brown syrup. The active agent was purified by silica gel chromatography with benzene - acetone (100 : 5 in volume). The active agent was crystallized by evaporation of the active fraction and recrystallized from benzene - ethyl acetate (10 : 1 in volume). From 28.4 liters of the culture filtrate 58.3 mg of the crystals were obtained. The properties were as follows: m.p. 202°C, $UV_{\lambda max}^{MeOH}$ 224 nm ($\log\varepsilon$ 4.36), 266 nm ($\log\varepsilon$ 4.13), calcd. for $C_{12}H_{10}N_2O$: C 72.71, H 5.09, N 14.13, O 8.07; found: C 72.10, H 5.09, N 13.98, O 8.07; M^+ by mass spectroscopy 198. These data and the infrared spectrum were identical with those of pimprinine.

Isolation of *trans*-cinnamic acid amide:

A *Streptomyces*, strain MD 235-C 2, was cultured in a medium (pH 6.0) consisting of soybean meal 2.5 %, soybean oil 2.0 %, potato starch 0.5 %, glucose 0.5 %, yeast extract

0.2 %. After 400 ml of the shake-cultured broth was inoculated, the fermentation in 15 liters medium was carried out in a jar fermenter at 28°C for 4 days under aeration of 15 liters/minute and stirring at 250 rpm. The active agent showing inhibition in the reaction mixture containing serotonin was extracted with ethyl acetate, the same volume as the filtrate. The ethyl acetate was washed with water and concentrated to a syrup (30 g). The syrup was subjected to silica gel chromatography using chloroform - methanol (100 : 2 in volume). Concentration of the active fraction gave crystals of the active agent. The mother liquor was subjected to silica gel chromatography using benzene - ethyl acetate (1 : 1) and the evaporation of the active fraction gave also crystals of the active agent. The crystals were combined and recrystallized from ethyl acetate. From 13 liters of a culture filtrate 1.79 g crystals was obtained. The properties were as follows: m.p. 145°C, $UV_{\lambda max}^{MeOH}$ 216 nm (log ε 4.32), 273 nm (log ε 4.30); calcd. for C_9H_9NO: C 73.45, H 6.16, N 9.52, O 10.87; found C 71.77, H 5.94, N 9.55, O 10.29; M^+ by mass spectroscopy 147. The identity with *trans*-cinnamic acid amide was confirmed by comparison of the infrared and nmr spectra with those of an authentic sample.

Isolation of phenethylamine:

A *Streptomyces*, strain MB 699-A 3, was cultured in the following medium (pH 7.0): glycerol 3.0 %, NZ-amine 2.0 %, yeast extract 0.2 %, NaCl 0.3 %. After 400 ml of a shake-cultured broth was inoculated, the formentation in 15 liters medium was continued for 3 days at 28°C under aeration of 15 liters/minute, and 250 rpm. The active agent was adsorbed on carbon (2.0 % of the filtrate), and eluted with 50 % acetone (5 liters, 3 times) at pH 2.0. After concentration and drying, a brown powder (51.0 g) was obtained. A 40 g portion was subjected to carbon column chromatography with 80 % methanol followed by Amberlite CG-50 (NH_4^+) chromatography using 0.1 N NH_4OH for elution. The evaporation of the active fraction under reduced pressure gave a crystalline active compound which showed one spot of Rm 1.3 (taking L-alanine as 1.0) in high voltage electrophoresis under 3,000 V. The identity with phenethylamine was suggested by nmr spectrum and finally confirmed by comparion of the infrared spectrum with that of an authentic sample.

Isolation of harman:

A strain of *Coriolus maximus* was cultured in the following medium (pH 6.0): glucose 2.0 %, lactose 1.0 %, corn steep liquor 6.0 %, NH_4NO_3 0.2 %, $CaCO_3$ 0.4 %. After 4 days shake-cultured broth was inoculated, the fermentation in 15 liters medium was carried out under aeration of 15 liters/minute and stirring at 250 rpm at 28°C for 47 hours. The culture filtrate (12 liters) was extracted with the same volume of butyl acetate at pH 8.0, and after concentration to 4.3 liters under reduced pressure, the active agent inhibiting monoamine oxidase was transferred into 4 liters of water at pH 2.0. The active agent was transferred again into 4.3 liters of butyl acetate at pH 8.0, and then evaporated under reduced pressure to give a white powder (545 mg). The active agent was purified by silica gel column chromatography using chloroform - methanol (100 : 1), and the evaporation of the active fraction gave crystals (86.1 mg) which were recrystallized from chloroform: m.p. 230~232°C, $UV_{\lambda max}^{MeOH}$ 235 nm (log ε 4.74), 241 nm (log ε 4.69), 251 nm (log ε 4.51), 283 nm (shoulder), 289 nm (log ε 4.37), 337 nm (log ε 3.8), 351 nm (log ε 3.8); $UV_{\lambda max}^{MeOH\text{-}HCl}$ 249 nm (log ε 4.62), 302 nm (log ε 4.35). Reviewing monoamine oxidase inhibitors, a close structural relationship with harman, 1-methyl-β-carboline, was suggested. The result of the elemental analysis was as follows: calcd. for $C_{12}H_{10}N_2$: C 79.09, H 5.53, N 15.38; found C 78.85, H 5.46, N 15.40. These data and the infrared spectrum are identical with those described for harman, and identity was also supported by the nmr spectrum.

References

1) Umezawa, H.: Enzyme Inhibitors of Microbial Origin. University of Tokyo Press, 1972
2) Bhate, D. S.; R. K. Hulyalkar & S. K. Menon: Isolation of *iso*-butyropyrrothine along with thiolutin and aureothricin from a *Streptomyces*, sp. Experientia 16 : 504~505, 1960

3) JOSHI, B. S. & W. I. TAYLOR : The structure and synthesis of pimprinine. Tetrahedron 19 : 1437~1439, 1963
4) NARASIMHAN, M. J. & V. G. GANLA : Pimprinine in the treatment of hyperkinetic diseases. Hindustan Antibiot. Bull. 9 : 138~141, 1967
5) HO, B. T.: Monoamine oxidase inhibitors. J. Pharm. Sciences 61 : 821~837, 1972
6) SEKIZAWA, Y.: *trans*-Cinnamic acid amide as a metabolic product of *Streptomyces*. J. Bact. 45 : 9~11, 1958
7) American Cyanamid Company : Preparation of 3, 4, 5-triloweralkoxy-cinnamide. Japanese Patent 37-10781, 1962
8) GOMI, Y.: Studies on monoamine oxidase inhibitors. IV. On the mode of nonspecific inhibitory action. Yakugaku Zasshi 86 : 483~487, 1966
9) PERKIN, W. H. & R. ROBINSON : Harmine and harmalin. J. Chem. Soc. 115 : 967~971, 1919
10) TAKASE, S. & H. MURAKAMI : Studies on the fluorescens of Saké. I. Fluorescens spectrum of saké and identification of harman. Agr. Biol. Chem. 30 : 869~876, 1966
11) HO, B. T.; K. LI, K. E. WALKER, L. WOYNE TANSEY, P. M. KRALIK & W. M. MCISAAC : Inhibitors of monoamine oxidase. VI. Effects of substitution on inhibitory activity of 6 (or 8) substituted β-carbolines. J. Pharm. Sci. 59 : 1445~1448, 1970