[Chem. Pharm. Bull. 36(2) 670—675 (1988)]

Monoamine Oxidase Inhibitors from a Fungus, *Emericella navahoensis*

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(Received July 1, 1987)

Norsolorinic acid, averufin and 6,7,8-trihydroxy-3-methylisocoumarin were isolated from *Emericella navahoensis* as metabolites having monoamine oxidase (MAO)-inhibitory action. Norsolorinic acid inhibited MAO in mouse liver non-competitively when kynuramine was used as a substrate, and its IC_{50} was 0.3 μM. The inhibition constants (K_i) for MAO-A and -B in mouse liver were 0.24 and 0.68 μM, respectively. On the other hand, K_i values for MAO-A and -B in mouse brain were 2.9 and 0.32 μM, respectively. Inhibitory potencies towards MAO of some anthraquinone derivatives were also examined.

Keywords——monoamine oxidase; inhibitor; *Emericella navahoensis*; fungal metabolites; norsolorinic acid; averufin; 6,7,8-trihydroxy-3-methylisocoumarin; kinetic study; anthraquinone

Introduction

During a survey of neurotropic metabolites of fungi, 207 strains of 122 species of Ascomycetes[2)] were assayed for inhibitory potency towards monoamine oxidase (MAO; EC 1.4.3.4). Among these species, the ethyl acetate extracts of *E. navahoensis* CHRISTENSEN & STATES IFM 42019 exhibited the most potent effect. In this paper, we describe the isolation of metabolites having an MAO-inhibitory effect from this fungus and also report some results of kinetic studies on the effect.

MAO is known to play an important physiological role by regulating the levels of neurotransmitter monoamines in various organs and a protective role by inactivating potentially toxic exogenous monoamines.[3)] Some MAO inhibitors were the first successful drugs in the treatment of depression, but it soon became apparent that they showed side effects such as hepatotoxicity, hypertensive crises and hypotension.[4)] MAO inhibitors as antidepressant drugs are considered to act by increasing the levels of monoamines, especially norepinephrine and serotonin in the brain. Moreover, it was reported recently that l-deprenyl, which is one of the most potent MAO-B inhibitors, can be effectively used in combination with levodopa for the treatment of Parkinson's disease.[5)] It is therefore expected that MAO inhibitors may be useful in the therapy of psychosis, depression, schizophrenia, *etc.*

Results and Discussion

Crude MAO solution was prepared from the homogenate of mouse liver or brain. The activity of MAO was assayed fluorometrically at 380 nm (4-hydroxyquinoline) according to the method of Kraml[6)] by using kynuramine as a substrate, with a slight modification. Three crystalline compounds, tentatively named ENA-1, ENA-2 and ENA-3, were isolated from the ethyl acetate extracts of moldy rice infected with *E. navahoensis* IFM 42019 by repeated silica gel column chromatography, and the inhibitory effects of these compounds towards MAO were examined. The IC_{50} values of these compounds obtained from the inhibition curves are shown in Table I, and indicated that ENA-1 had the strongest inhibitory potency in mouse

TABLE I. Inhibition of MAO by Compounds from *E. navahoensis*

Compound	Mol. wt.	IC_{50} (μM)
ENA-1	370	0.3
ENA-2	368	54.4
ENA-3	208	817.3

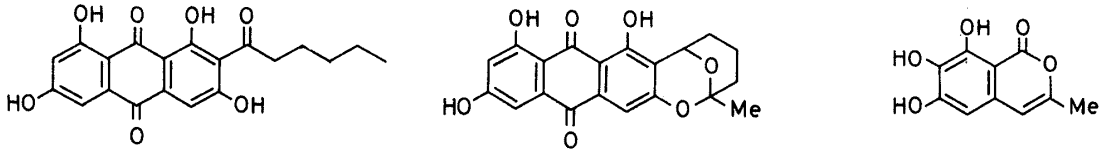

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Fig. 1. Structures of Norsolorinic Acid (**1**), Averufin (**2**) and 6,7,8-Trihydroxy-3-methylisocoumarin (**3**) Isolated from *Emericella navahoensis*

TABLE II. Inhibition of MAO by Anthraquinone Derivatives

Compound	IC_{50} (μM)
Averufin	92.4
Averufin dimethylether	303.0
Averythrin	3.7
Emodin	50.0
Erythroglaucin	—
(+)Flavoskyrin[a)]	—
Iridoskyrin[a)]	—
Islandicin	148.2
(−)Luteoskyrin[a)]	—
Nidurufin	—
Physcion	—
Skyrin[a)]	—
Versicolorin A	—
Versiconol	52.8
Norsolorinic acid	0.3

a) Dimer of anthraquinone. —, IC_{50} not determined.

liver MAO. The inhibitory potency ($IC_{50}=0.3\ \mu$M) of ENA-1 was similar to those of pargyline ($IC_{50}=0.17\ \mu$M) and nialamide ($IC_{50}=0.37\ \mu$M) (unpublished) when kynuramine was used as a substrate of mouse liver MAO.

ENA-1 was obtained as red needles, mp 256—257 °C, $C_{20}H_{18}O_7$. The physicochemical properties of ENA-1 indicated the identity of this compound with norsolorinic acid (**1**) which was previously isolated from *Solorina crocea* (L.) ACH.[7)] (lichen) and *Aspergillus versicolor* (VUILL.) TIRABOSCHI.[8)] ENA-2 and ENA-3 were also identified as averufin[9)] (**2**) and 6,7,8-trihydroxy-3-methylisocoumarin[10)] (**3**) respectively, by comparison of the physical and spectral data with those reported (Fig. 1). The isolation of these three compounds from *E. navahoensis* has not so far been reported, to our knowledge.

As norsolorinic acid and averufin contain an anthraquinone skeleton, the inhibitory potencies towards MAO of some other fungal anthraquinone derivatives were examined. The most active compound found in this experiment was averythrin[11)] (Table II). Some anthraquinones also exhibited activity, but much weaker.

These results suggested that the presence of an anthraquinone skeleton and a side chain of about six carbons with a conjugated π electron system at the α-position would be required

for inhibitory potency, although there are insufficient data to discuss the role of functional groups in the structure in relation to MAO-inhibitory potency.

Kinetics of Inhibition by Norsolorinic Acid

Kinetic studies were carried out in order to determine the type of inhibition and the inhibition constant (K_i) of norsolorinic acid towards MAO.

It is known that MAO is localized in the outer mitochondrial membrane and exists in two forms, types A (MAO-A) and B (MAO-B). MAO-A has been shown to deaminate preferentially substrates such as serotonin and norepinephrine. On the other hand MAO-B oxidizes β-phenylethylamine and benzylamine. MAO-A is usually distinguished by being inhibited preferentially by a low concentration of clorgyline,[12] whereas MAO-B is inhibited by a low concentration of l-deprenyl.[13]

IC_{50} and K_i values of norsolorinic acid were obtained towards MAO-A and MAO-B in mouse liver and brain homogenate in the presence of l-deprenyl and clorgyline (Table III).

TABLE III. Kinetic Data on the Inhibition of MAO by Norsolorinic Acid

Enzyme source	K_i (μM)	IC_{50} (μM)
Mouse liver homogenate		
Untreated	0.27	0.3
MAO-A (deprenyl-treated)	0.24	0.4
MAO-B (clorgyline-treated)	0.68	0.32
Mouse brain homogenate		
Untreated	0.95	8.1
MAO-A (deprenyl-treated)	2.9	4.0
MAO-B (clorgyline-treated)	0.32	0.59

K_i values were calculated from the results of assays in the absence and presence of norsolorinic acid (0.27, 0.54, 1.08, 1.62 and 2.16 μM). Assays were performed at three concentrations of kynuramine (0.125—0.5 mM) as a substrate.

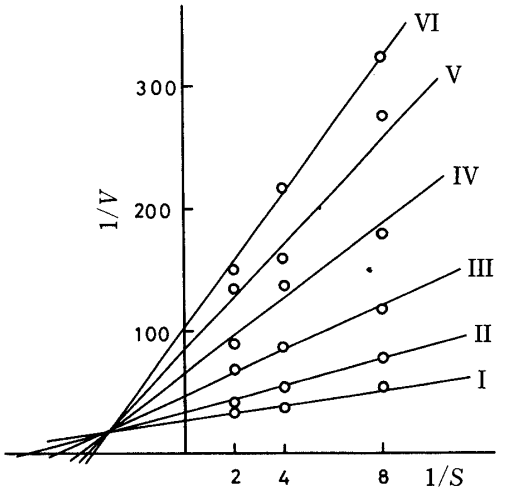

Fig. 2. Lineweaver-Bruk Plots of MAO-A Activity in Mouse Liver Homogenate in the Absence and Presence of Norsolorinic Acid

The norsolorinic acid concentrations were 0 (I), 0.54 (II), 1.08 (III), 1.62 (IV), 2.16 (V) and 2.70 μM (VI). The MAO-A activity in homogenate (10—15 mg protein) was measured in the presence of l-deprenyl (1 μM), using kynuramine as a substrate. Ordinate: 1/{initial velocity (nmol/mg protein/min)}. Abscissa: 1/{kynuramine concentration (mM)}.

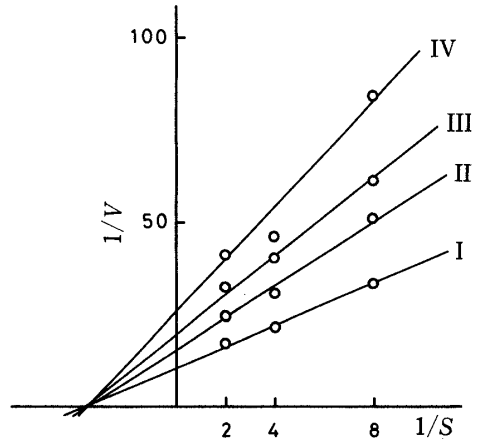

Fig. 3. Lineweaver-Burk Plots of MAO-B Activity in Mouse Liver Homogenate in the Absence and Presence of Norsolorinic Acid

The norsolorinic acid concentrations were 0 (I), 1.08 (II), 1.62 (III) and 2.16 μM (IV). The MAO-B activity in homogenate (10—15 mg protein) was measured in the presence of clorgyline (1 μM), using kynuramine as a substrate. Ordinate: 1/{initial velocity (nmol/mg protein/min)}. Abscissa: 1/{kynuramine concentration (mM)}.

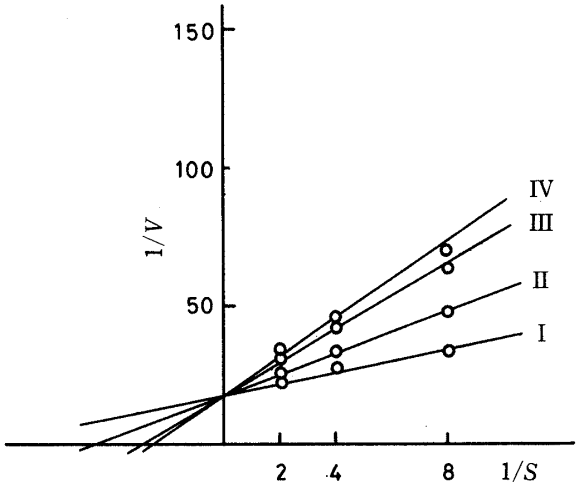

Fig. 4. Lineweaver-Burk Plots of MAO-A Activity in Mouse Brain Homogenate in the Absence and Presence of Norsolorinic Acid

The norsolorinic acid concentrations were 0 (I), 1.08 (II), 2.16 (III) and 2.70 μM (IV). The MAO-A activity in homogenate (3—5 mg protein) was measured in the presence of 1-deprenyl (1 μM), using kynuramine as a substrate. Ordinate: 1/{initial velocity (nmol/mg protein/min)}. Abscissa: 1/{kynuramine concentration (mM)}.

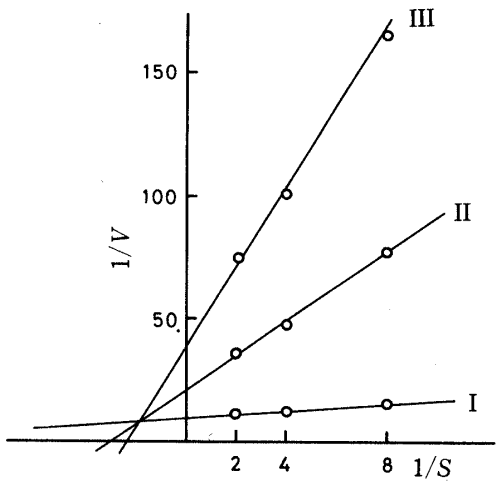

Fig. 5. Lineweaver-Burk Plots of MAO-B Activity in Mouse Brain Homogenate in the Absence and Presence of Norsolorinic Acid

The norsolorinic acid concentrations were 0 (I), 1.08 (II), and 2.16 μM (III). The MAO-B activity in homogenate (3—5 mg protein) was measured in the presence of clorgyline (1 μM), using kynuramine as a substrate. Ordinate: 1/{initial velocity (nmol/mg protein/min)}. Abscissa: 1/{kynuramine concentration (mM)}.

The Lineweaver–Burk plots of inhibition by norsolorinic acid suggested that the type of inhibition of mouse liver MAO by norsolorinic acid was non-competitive. The K_i value was 0.27 μM. In mouse brain, this compound also inhibited MAO non-competitively and the K_i value was 0.95 μM. Norsolorinic acid inhibited MAO-A (1-deprenyl-treated) in a mixed-type mode and MAO-B (clorgyline-treated) non-competitively in mouse liver homogenate (Figs. 2 and 3). The K_i values for types A and B were 0.24 and 0.68 μM, respectively. Thus, norsolorinic acid inhibited MAO-A preferentially in mouse liver. However, it is known that the level of MAO-A is rather low in comparison with that of MAO-B in the mouse liver.[14,15] Moreover the effect in the liver should cause little problem.

In mouse brain homogenate treated with 1-deprenyl, norsolorinic acid inhibited MAO-A competitively (Fig. 4), but in mouse brain treated with clorgyline, this compound inhibited MAO-B in a mixed-type inhibition mode as shown in Fig. 5. The K_i values were 2.9 μM for MAO-A and 0.32 μM for MAO-B. These facts indicated that norsolorinic acid preferentially inhibited MAO-B in brain homogenate MAO (IC_{50} for A type, 4.0 μM; for B type, 0.59 μM).

Conclusion

Norsolorinic acid, which was isolated from *Emericella navahoensis*, exhibited MAO-inhibitory potency. Its inhibition pattern was non-competitive and the inhibitory potency of this compound was found to be similar to those of pargyline ($IC_{50}=0.17$ μM) and nialamide ($IC_{50}=0.37$ μM) in the case of mouse liver MAO. Clorgyline, which is an MAO-A inhibitor, was reported to be an effective antidepressant drug,[16,17] whereas 1-deprenyl, which is an MAO-B inhibitor, was recently re-examined and suggested to be of value in the treatment of Parkinson's disease[18,19] and senile dementia of Alzheimer's type.[20] In our experiment, norsolorinic acid was found to inhibit MAO-B preferentially in mouse brain MAO. The K_i values towards MAO-A and MAO-B are 2.9 and 0.59 μM, respectively. Detailed enzymological and pharmacological studies on norsolorinic acid are still in progress.

Experimental

All melting points were measured on a Yanagimoto micro-melting points apparatus and are uncorrected. Fluorescence was measured with a Hitachi 650—10S fluorophotometer. The ultraviolet (UV) spectra were recorded with a Hitachi 323 recording spectrophotometer, the infrared (IR) spectra with a Hitachi EPI-G3 grating infrared spectrophotometer, the mass spectra (EI-MS) with a Hitachi RMU-7M mass spectrophotometer, the proton nuclear magnetic resonance (^{1}H-NMR) spectra with a JEOL JNM-GX 270 FT-NMR at 270 MHz and the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra at 67.8 MHz.

Chemical shifts are expressed in δ (ppm) value from tetramethylsilane (TMS) as an internal standard. The following abbreviations are used: s, singlet; d, doublet; t, triplet; q, quartet.

Isolation of ENA-1, ENA-2 and ENA-3 from *Emericella navahoensis*——*E. navahoensis* strain IFM 42019 was grown in stationary culture at 25 °C for 21 d on polished rice (100 kg) using Roux flasks containing 200 g of the rice in each flask. After cultivation, the moldy rice was extracted with ethyl acetate. The evaporated residue (607 g) was defatted with hexane and repeatedly chromatographed on silica gel to obtain ENA-1 (25 mg), ENA-2 (6 mg) and ENA-3 (414 mg), successively.

ENA-1 (Norsolorinic Acid)——Red needles from acetone, mp 256—257 °C (dec.). IR (KBr) cm^{-1}: 3390, 1681, 1630, 1623, 1580, 1470, 1405, 1322, 1246. UV_{max}^{EtOH} nm (ε): 235 (16400), 269 (13300), 284 (14600), 297 (16400), 314 (18000), 465 (6400). MS *m/z* (%): 370 (M^+, 31), 352 (14), 341 (2), 327 (62), 314 (35), 299 (100), 272 (23), 215 (23), 187 (13). High-resolution MS: Calcd for $C_{20}H_{18}O_7$, 370.1050. Found: 370.1014. ^{1}H-NMR (DMSO-d_6): 0.87 (3H, t, J=7.3 Hz), 1.32—1.29 (4H), 1.60 (2H, t, J=7.3 Hz), 2.79 (2H, t, J=7.3 Hz), 6.55 (1H, d, J=2.4 Hz), 7.11 (1H, d, J=2.4 Hz), 7.20 (1H, s), 12.04 (1H, s), 12.60 (1H, br s). ^{13}C-NMR (DMSO-d_6): 202.59, 188.55, 180.88, 165.12, 164.19, 161.65, 160.72, 134.77, 134.63, 121.29, 108.80, 108.66, 108.54, 108.09, 43.58, 30.57, 22.50, 21.02, 13.73.

ENA-2 (Averufin)——Orange-red laths from acetone, mp 280—283 °C (dec.). ^{1}H-NMR (DMSO-d_6): 1.53 (1H, s), 5.26 (1H, m), 6.57 (1H, d, J=2.2 Hz), 7.01 (1H, s), 7.10 (1H, d, J=2.2 Hz), 12.07 (1H, s), 12.46 (1H, br s). ^{13}C-NMR (DMSO-d_6): 188.8, 180.8, 165.2, 164.2, 159.8, 158.1, 134.8, 133.1, 115.9, 108.8, 108.4, 108.0, 107.0, 101.1, 66.0, 35.1, 27.3, 26.8, 15.3.

ENA-3 (6,7,8-Trihydroxy-3-methylisocoumarin)——Colorless platelets from acetone, mp 213—215 °C. MS *m/z* (%): 208 (M^+, 100), 190 (3), 179 (9), 166 (15), 137 (21), 105 (3), 66 (4), 43 (11). High-resolution MS: Calcd for $C_{10}H_8O_5$, 208.0370. Found: 208.0356. ^{1}H-NMR (DMSO-d_6): 2.19 (3H, s), 6.41 (1H, s), 6.42 (1H, s), 10.86 (1H, br s). ^{13}C-NMR (DMSO-d_6): 165.9 (s), 154.5 (s), 151.4 (s), 148.9 (s), 131.68 (s), 130.0 (s), 104.0 (d), 102.2 (d), 98.4 (s), 18.6 (q).

Preparation of Crude MAO——Mouse liver was homogenized with 4 volumes of 0.15 M KCl and mouse brain was homogenized with 10 volumes of 0.25 M sucrose in a Teflon homogenizer on ice. The homogenates were centrifuged at 1000 × *g* for 10 min to remove cell debris. These supernatants were used for assay.

Assay of MAO Activity——The MAO activity with kynuramine as a substrate was assayed by a modification of the fluorometric method of Kraml.[6] A reaction mixture consisted of 0.5 ml of enzyme preparation, 0.5 ml of 0.3 M phosphate buffer (pH 7.4), 2.3 ml of water and 0.1 ml of test solution. The incubation mixture was preincubated in the water bath at 37 °C for 10 min. The reaction was started by adding 0.1 ml of 5 mM kynuramine as a substrate at 37 °C for 30 min with shaking. The reaction was stopped by adding 0.25 ml of 10% $ZnSO_4$ and 0.05 ml of 1 N NaOH. The reaction mixture was boiled for 5 min and centrifuged at 2500 rpm for 25 min. One milliliter of the supernatant was mixed with 2.0 ml of 1 N NaOH to stabilize the fluorescence of 4-hydroxyquinoline. In the blank, kynuramine was omitted, but was added after the incubation. Fluorescence was measured at 380 nm with excitation at 315 nm. Activity was calculated as follows:

$$\text{inhibitory ratio } (\%) = (A - B)/A \times 100$$

(A = MAO activity in the absence of inhibitor, B = MAO activity in the presence of inhibitor)

MAO-A or -B activity in mouse liver or brain MAO was measured in the presence of 1 μM 1-deprenyl or clorgyline, respectively. Samples were dissolved in dimethylsulfoxide, which was found to have no effect of MAO activity at below 2.8% concentration. The final concentrations were 0.1—1.0 μg/ml (0.27—2.7 μM). Kinetic data were calculated from MAO activity in the absence and the presence of norsolorinic acid, and at three concentrations of kynuramine.

Acknowledgement We are very grateful to Prof. T. Hamasaki, Tottori University, for his kind gift of norsolorinic acid and averufin. We are also grateful to Dr. S. Suzuki of our laboratory, for his advice on the assay of MAO activity. We are also indebted to Dr. O. Suzuki, Hamamatsu University School of Medicine, for the gift of 1-deprenyl and clorgyline.

References and Notes

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