Original 和漢医薬学会誌 5, 116－122, 1988

Identification of the inhibitor for monoamine oxidase B in the extract from deer antler (Rokujo)

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(Received March 8, 1988. Accepted May 9, 1988.)

Abstract

Two fractions with high potency for inhibiting the activity of monoamine oxidase B (MAO-B) *in vitro* have been fractionated from an ethanol extract of the pilose antler of *Cervus nippon* TEMMINCK var. *mantchuricus Swinhoe* (Rokujo). The inhibitory effect of one fraction extracted with diethylether was increased with the incubation period, but it was easily reversed by washing the membrane preparation after an incubation with the fraction. On the other hand, the action of butanol-extracted fraction was persistent after the removal of the extract, and selective to MAO-B in comparison with MAO-A. However, further purification revealed that the ether fraction merely contained cholesterol, several known fatty acids and lipids, and that the active substance included in the butanol fraction was, unexpectedly, hypoxanthine. Therefore these Rokujo ingredients may exert the inhibitory action for MAO-B in a non-competitive manner against the biological amines *in vitro*, and also *in vivo* as reported previously.

Key words Rokujo, *Cervus nippon* TEMMINCK var. *mantchuricus Swinhoe*, monoamine oxidase B, MAO inhibitor, purification, aging.

Abbreviations MAO, monoamine oxidase ; MAO-A and MAO-B, types of A and B of MAO ; 5-HT, serotonin ; TA, tyramine ; PEA, phenylethylamine ; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine ; Rokujo, 鹿茸.

Introduction

The oxidative deamination of neurotransmitter amines and other monoamines is now considered to be accomplished by two or more functionally different forms of monoamine oxidase (MAO). The membrane-bound MAO is generally divided into two subtypes, type A and type B, based on their distinct selectivity to the substrates with the aid of specific inhibitors.[1] Namely, type A enzyme (MAO-A) is responsible for the metabolism of serotonin (5-HT) and sensitive to clorgyline ; on the other hand, phenylethylamine (PEA) is a good substrate of type B enzyme (MAO-B) which is inhibited by deprenyl.[2] The activity of MAO in various mammalian organs has been known to increase with aging.[3] Especially, it was demonstrated that the proportion of MAO-B activity in the liver and brain increases during

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Journal of Medical and Pharmaceutical Society for WAKAN-YAKU **5**, 116－122, 1988

post-natal development of rats throughout the life span.[4] Therefore measuring the activity of MAO-B may be one of the markers for evaluating the degree of senescence.

We previously reported[5] that a water extract of pilose antler of Chinese deer (Rokujo) showed significant effects on several biochemical degenerations related to the aging process of the senescence-accelerated mouse when orally administered for 8 successive days. Among these effects, reduction of MAO-B activity was apparent both in senescent and control groups, accordingly, we speculated that the Rokujo extract included some MAO-B-inhibiting substances whith would exert their action *in vitro* as well as *in vivo*.[5] In the present study, purification and identification of such MAO-B inhibitors from Rokujo extract were carried out by evaluating the effects of fractionated materials *in vitro* on the degradation of isotope-labeled subtrates which was added to the mitochondrial fraction from adult mouse liver used as the MAO-B preparation.

Materials and Methods

Extraction and purification from Rokujo : Unossified pilose antler of Manshu-shika (*Cervus nippon* TEMMINCK var. *mantchuricus Swinhoe*)

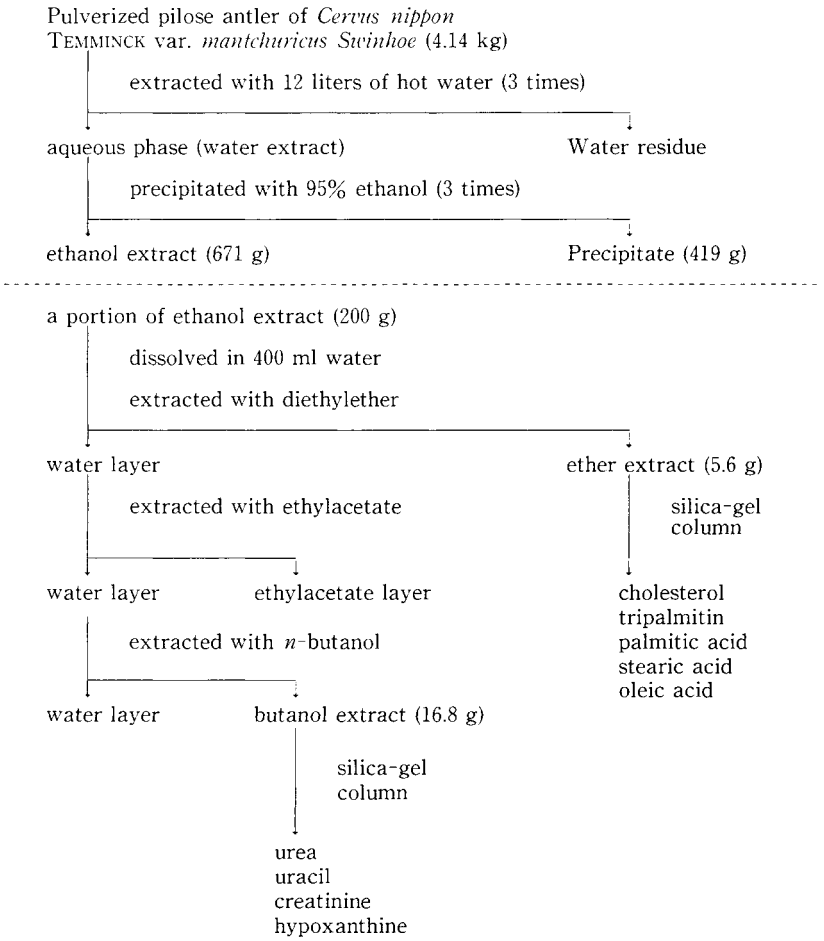

Fig. 1 Extraction and purification procedures from Rokujo.

was purchased from Jilin (吉林) province of China. General procedures for the extraction and purification from the sliced deer antler (Rokujo) are shown in Fig. 1. The upper part of Fig. 1 indicates the first extraction with hot water followed by the second extraction with ethanol. These extracts were almost identical with the water and ethanol extracts we had used for the subchronic administration to senile mice in our previous reports.[5,6] As shown in the lower part of Fig. 1, a portion of the ethanol extract was subjected to a series of extraction with diethylether, ethylacetate, and *n*-butanol, respectively. Final separation of the ether and butanol extracts were done through a silica-gel column, and the identification was carried out using thin-layer chromatography. A single batch of these fractions were used in common in the present study.

Enzyme preparation : Immediately after the decapitation of male ddY mice (30 g), the liver was rapidly removed and homogenized with 10 volume of ice-cold 0.32 M sucrose buffered with 10 mM sodium phosphate (pH 7.4). The following procedures were carried out at 4℃. The homogenate was centrifuged at 600 *g* for 10 min and the supernatant was further centrifuged at 15,000 *g* for 10 min. Obtained pellet was washed once, resuspended with the buffer to yield a protein concentration about 1 mg/ml, and stored at −20℃ until use.

Chemicals : 2-Phenyl [1-^{14}C]ethylamine hydrochloride ([^{14}C]PEA, 60 mCi/mmol), [7-^{14}C]tyramine hydrochloride ([^{14}C]TA, 56 mCi/mmol), and 5-hydroxy [side-chain-2-^{14}C]tryptamine creatinesulfate ([^{14}C]5-HT, 57 mCi/mmol) were from Amersham.

Measurement of MAO-activity : Enzyme stock solution was diluted with 8 volume of 50 mM sodium phosphate (pH 7.4) and preincubated at 37℃ for 10 min in the presence or adsence of the Rokujo extracts, as indicated in each experiment. An aliquot (270 μl) of the membrane preparation was incubated at 37℃ for 20 min with 30 μl of a diluted radiolabeled substrate ([^{14}C]PEA, [^{14}C]-TA or [^{14}C]5-HT) solution. The substrate concentration in the reaction mixture was 3.3 μM for PEA, and 125 μM for TA and 5-HT, respectively. The reaction was terminated by an addition of 1 N HCl (200 μl). The metabolites were extracted with 2 ml of toluene (for PEA and TA), or diethylether (for 5-HT) by a vigorous shaking for 1 min, and the mixture was left at 4℃ overnight. A 1 ml portion of the upper organic phase was added to 10 ml of scintillation cocktail and the radioactivity was measured by a Beckmann LS-250 liquid scintillation spectrometer. All experiments were carried out with duplicate incubations. Every value is indicated as the relative percentage of MAO-B activity. Each point in the figures is the average from two tubes.

Results

Screening of MAO inhibitors from 6 fractions

Six fractions were obtained during the purification of Rokujo ingredients, and they were screened for the identification of *in vitro* MAO-B inhibitor. As shown in Fig. 2, MAO-B activity in the liver membrane preparation was inhibited dose-dependently in the presence of butanol extract or diethylether extract from Rokujo, how-

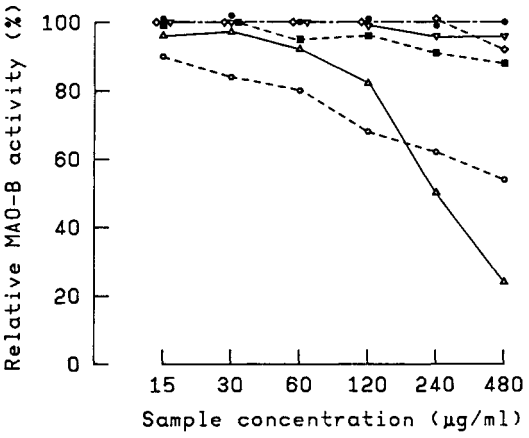

Fig. 2 Effects of six fractions separated from Rokujo on the MAO-B activity in mouse liver mitochondrial membranes *in vitro*.
Radioactivity of formed [^{14}C]PEA metabolites by MAO-B in the liver membranes was determined in the presence of water extract (●), water layer residue (■), ethanol extract (▽), butanol extract (△), diethylether extract (○), or cholesterol (◇). Values are indicated as the relative percentage of MAO-B activity.

ever, their dose-inhibition curves were not parallel each other. Other fractions produced no apparent changes in the MAO-B activity.

Characterization of the MAO-B inhibition by butanol and ether fractions

As shown in Fig. 3 and Fig. 4, the MAO-B inhibiting action of the ether extract was increased with the incubation period, however, it was easily reversed after washing the membrane preparation with the buffer solution. On the other hand, the effect of butanol extract was not so reduced by a removal of the extract from the membranes. However, prolonged incubation of butanol extract with membrane preparation spoiled the suppression of MAO-B activity, suggesting that the active substances included in the butanol extract may inhibit MAO-B enzyme irreversibly, but that it may be inactivated by other membrane enzymes.

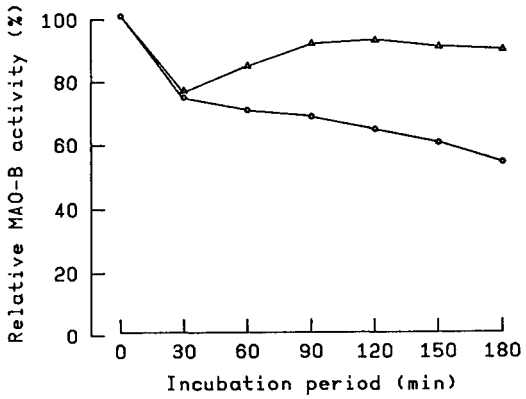

Fig. 3 Time course of the effects of butanol extract (△) and diethylether extract (○) at a concentration of 120 μg/ml on MAO-B activity in mouse liver membranes.

Fig. 5 suggests further difference between the effects of two extracts. When dose-dependence of the effect of butanol extract was examined in the presence of constant concentrations of ether extract, the inhibition of MAO-B activity seemed not additive of the independent effects of these two extracts.

Substrate-specific inhibition by the butanol fraction

Using three substrates for types A and B of MAO, the substrate-selectivity in the action of butanol extract was evaluated (Fig. 6). The butanol extract strongly inhibited the degradation of [^{14}C]PEA and that of [^{14}C]TA in the liver membrane preparation. However, degradation

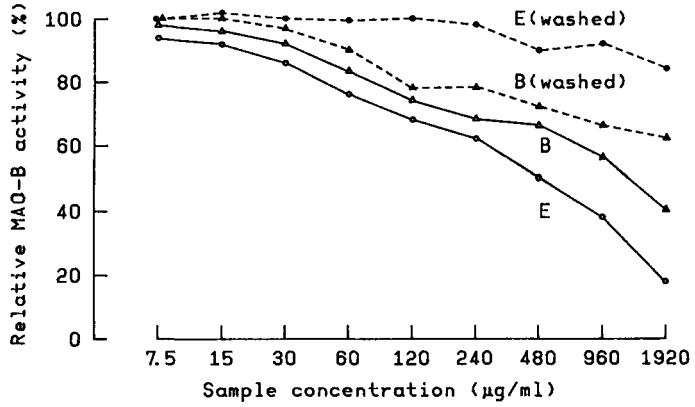

Fig. 4 Effects of the Rokujo extracts before and after the removal of the extracts in mouse liver membranes.

The liver membranes were preincubated for 10 min with butanol (B) and diethylether (E) extracts. A half of the incubated membranes were once washed with buffer solution and the extracts were removed. MAO-B activities in the washed membranes and other half of the incubated membranes were determined.

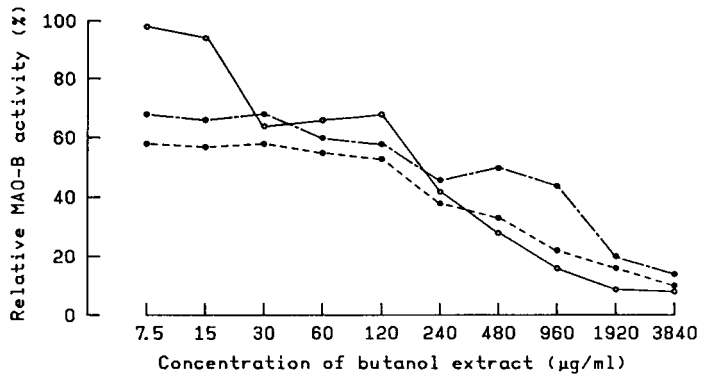

Fig. 5 Interaction between butanol and diethylether extracts on the inhibition of MAO-B activity in mouse liver membranes.
MAO-B activity in the presence of butanol extract alone (○), butanol extract plus 60 μg/ml diethylether extract (○) and butanol extract plus 120 μg/ml diethylether extract (●) are shown.

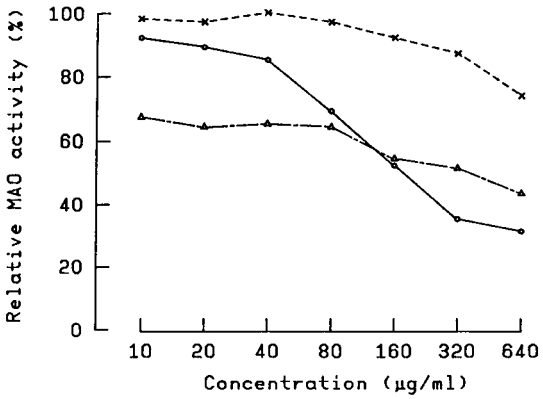

Fig. 6 Substrate-specific inhibition of MAO activity by butanol extract in mouse liver membranes.
[^{14}C]5-HT (×), [^{14}C]TA (△) and [^{14}C]PEA (○) were used for the specific substrates for MAO-A, both MAO-A, B and MAO-B, respectively.

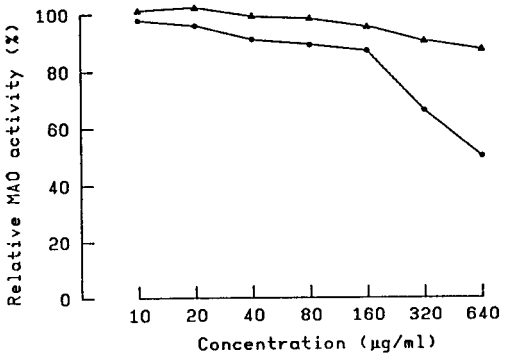

Fig. 7 MAO-A inhibition by hypoxanthine (●) and uracil (△) isolated from the butanol extract of Rokujo.
[^{14}C]5-HT was used for the substrate.

of [^{14}C]5-HT which was considered to be mediated by MAO-A[1,2] was not obviously suppressed in the presence of the butanol extract.

Active substances identified from the butanol fraction

As previously indicated in Fig. 1, the final purification of the butanol extract through a silica-gel column yielded several known compounds which may be concentrated in the deer antler as the metabolites of nucleic acid. These included hypoxanthine, uracil, urea, and creatinine. Therefore these compounds were tested for the MAO-A and MAO-B assays. As shown in Fig. 7 and Fig. 8, it was found that hypoxanthine showed remarkable suppressing effect on the metabolism of [^{14}C]5-HT and [^{14}C]TA in a similar manner to the butanol extract itself. A weaker effect of uracil was also detected, but cre-

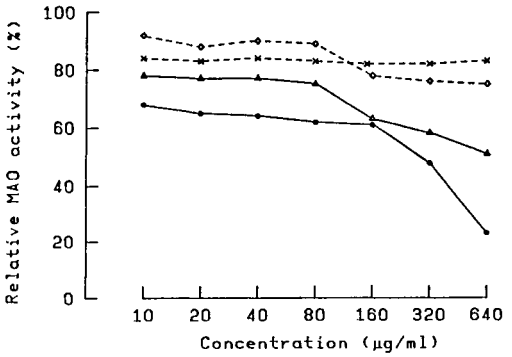

Fig. 8 MAO-B inhibition by hypoxanthine (●), uracil (△), urea (×) and creatinine (◇) isolated from the butanol extract of Rokujo. [^{14}C]PEA was used for the substrate.

atinine and urea showed no obvious effect.

Purification of the ether fraction

From the diethylether extract, cholesterol as a main constituent, together with some fatty acids and lipids, were found. However, we did not further pursue the active substances in the diethylether fraction because of its very small amount and poor recovery.

Discussion

The present results showed that two fractions from Rokujo extract possess high potency for inhibiting the activity of MAO-B in the liver mitochondrial preparation *in vitro*. The potency of butanol extract was detectable under a concentration of 30 μg/ml in the reaction mixture, and a complete suppression was observed at 2 mg/ml or more. The effect was also specific to the B type enzyme of MAO as determined by the substrate-selectivity. The relative low concentration required for the specific inhibition of MAO-B may be of physiological significance in the case of *in vivo* administration, as previously demonstrated in the tissue preparations from age-promoted and the control mice repetitively treated with Rokujo.[5] Long-lasting inhibition of MAO-B activity by a repeated Rokujo treatment may be in part responsible for the anti-aging effect hoped for the chronic pharmacological action of Rokujo.[5,6] In this regard, recent studies have shown that the administration of the inhibitor for MAO-B can prevent the induction of parkinsonism induced by a neurotoxin, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), in rodents[7,8] and primates.[9] The anti-parkinsonism effect of MAO-B inhibitor is now anticipated.

Purification of the active fractions yielded only several known, low-molecular weight compounds. Nevertheless, among these compounds, hypoxanthine exerted a potent inhibition of MAO-B in a similar manner with its source, the butanol extract. Since MAO is a flavin-type enzyme which requires FAD as the coenzyme, hypoxanthine may compete against FAD in the binding site because of their structural similarity. Thus the inhibition manner of hypoxanthine, namely, that of butanol extract from Rokujo, is non-competitive for the substrate, but may be competitive for the coenzyme. We could not identify the active substance in the diethylether fraction. However, the main ingredient of the fraction was cholesterol which in itself showed little MAO-inhibiting activity (see Fig. 2). Some unidentified minor component might be active, but still remained to be clarified.

和文抄録

マンシュウジカ（*Cervus nippon* TEMMINCK var. *mantchuricus Swinhoe*）の幼角（鹿茸）のエタノールエキスから，B型モノアミン酸化酵素（MAO-B）活性を試験管内で強く阻害する二つの画分が得られた。ジエチルエーテルで抽出された一つの画分の阻害活性は，反応時間とともに増大したが，酵素膜標本をこの画分とインキュベートした後に洗浄すると容易に回復した。一方，ブタノールで抽出された画分の作用は，抽出物を洗い流した後でも持続し，またMAO-AよりもMAO-Bに対して選択的であった。しかしながら，さらに精製を進めたところ，エーテル画分はコレステロールや，既知の脂肪酸とか脂質を含んでいるに過ぎないこと，ならびに，ブタノール画分中の活性成分は，予期せぬことにヒポキサンチンであることが判明した。それゆえに，これらの鹿茸含有成分は生体アミンに対して非競合的な様式で，試験管内において，あるいは

また，以前に報告したように生体内において，MAO-B阻害活性を発揮するのであろう。

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