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Comparative Studies of the Effects of RS-8359 and Safrazine on Monoamine Oxidase In-vitro and In-vivo in Mouse Brain

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Abstract—The effect of RS-8359, (±)-4-(4-cyanophenyl)amino-6,7-dihydro-7-hydroxy-5H-cyclopenta[d]-pyrimidine on monoamine oxidase (MAO) has been compared with a hydrazinic MAO inhibitor, safrazine (β-piperonylisopropylhydrazine hydrochloride,) which is a MAO inhibitor used clinically. In-vitro radiochemical determination of MAO activity showed that the IC50 of RS-8359 was 0·52 μM for the deamination of 5-hydroxytryptamine (5-HT) in the mouse brain mitochondrial preparation, while β-phenylethylamine (PEA) deamination was inhibited by only 20% at 100 μM of the drug. 5-HT deamination in the brain homogenate prepared from mice killed 60 min after administration of RS-8359 was inhibited significantly by 14 and 48%, at 30 and 100 mg kg^{-1} (p.o.), respectively, while deamination of PEA was little affected at the same doses. On the other hand, safrazine strongly inhibited both 5-HT and PEA deaminations, but showed no selectivity toward the substrate used. The extent of MAO inhibition by RS-8359, measured fluorometrically with kynuramine as a substrate in the brain homogenate, was independent of preincubation up to 80 min. In contrast, the inhibitory potency of safrazine was strengthened by preincubation in a time-dependent manner. Oral administration of RS-8359 (3–30 mg kg^{-1}) caused a dose-dependent increase in endogenous monoamines in mouse brain, which disappeared a few hours after its administration. Increase in monoamine content caused by safrazine lasted for at least 24 h. These results indicate that RS-8359 is a reversible and specific inhibitor of MAO-A, while safrazine is an irreversible and non-specific MAO inhibitor, in-vivo and in-vitro in mouse brain.

Many mental and motor abnormalities that appear in patients with depression, Parkinson's disease, cerebral vascular disorders or Alzheimer's disease are believed to relate to dysfunctions, at least in part, in the transmission of monoaminergic neurons (Schildkraut 1956; Ehringer & Hornykiewicz 1960; Murphy et al 1978; Bowen et al 1983; Agid et al 1984; Arai et al 1984). On the other hand, the endogenous and synthetic monoamines are well known to be oxidatively metabolized in mammalian brain by the two types (type A and B) of monoamine oxidase (MAO, EC 1.4.3.4. monoamine:oxygen oxidoreductase (deaminating, flavin-containing)) with different substrate and inhibitor specificities. 5-Hydroxytryptamine (5-HT) is mainly metabolized by the A-type of MAO (MAO-A) which is selectively inhibited by low concentration of clorgyline, whereas β-phenylethylamine (PEA) and benzylamine are mainly metabolized by the B-type of MAO (MAO-B) which is selectively inhibited by low concentrations of selegiline ((−)-deprenyl), Dopamine (DA) and kynuramine (KYN) are metabolized by both types of enzyme (Johnston 1968; Hall et al 1969; Knoll & Magyar 1972; Neff & Yang 1974). Although the substrate specificities of MAO-A and MAO-B have been found to vary somewhat with the tissues and species used, noradrenaline (NA) appears to be a common substrate for both forms of MAO in the human brain (Fowler & Ross 1984). Thus, the inhibitors of MAO are expected to have various therapeutic effects for the treatments of the disorders described above by accumulation of endogenous monoamines in the synaptic cleft and by increase in their utility at the receptor sites. In fact, MAO-A and MAO-B inhibitors are effective for depression and Parkinson's disease, respectively (Lipper et al 1979; Mendis et al 1981).

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Recently, we have reported that RS-2232, 4-(4-cyanophenyl)amino-6,7-dihydro-5H-cyclopenta[d]pyrimidine, caused the dose-dependent accumulation of NA, DA and 5-HT in the mouse brain following its acute administration (Yokoyama et al 1987a). This effect of RS-2232 was proved to be produced by the reversible and specific inhibition of MAO-A in mouse brain (Yokoyama et al 1987b). Since RS-8359 (I) is a 7-hydroxylated metabolite of RS-2232 and has potent behavioural effects such as antagonism against reserpine-induced hypothermy and ptosis, as well as potentiation of hyperactivity elicited by 1-3,4-dihydroxyphenyl-alanine (L-dopa) or 1-5-hydroxytryptophan (1-5-HTP) in mice (Iwata & Yokoyama 1986), the effects of RS-8359 (I) on mouse brain MAO were investigated and compared with a hydrazinic MAO inhibitor, safrazine (II), which is an MAO inhibitor used for treatment of the depression (Nishimura 1963).

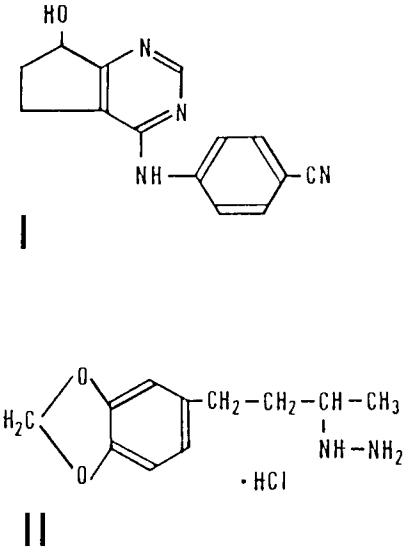
Methods

Animals

Male ddY mice ca 30 g, were used in the experiments. Animals were housed with free access to food and water and kept for at least one week before use.

Radiochemical assay of MAO activity

Mice were decapitated and the whole brain, except for the olfactory bulbs, quickly removed and homogenized in cold 10 mM sodium phosphate buffer (pH 7·4) containing 0·32 M sucrose. The mitochondrial fraction was obtained with a differential centrifugation technique. MAO activity was



I, RS-8359 and II, safrazine.

determined according to the radiochemical method essentially as described by Wurtmann & Axelrod (1963). In brief, the mitochondrial fraction was incubated with [^{14}C]5-HT (final concn 100 μM) or [^{14}C]PEA (final concn 20 μM) as substrates for MAO-A and MAO-B, respectively, in 10 mM sodium phosphate buffer (pH 7·4) at 38°C. Then their ^{14}C-metabolites were extracted into an organic solvent and counted for radioactivity by liquid scintillation spectrometry.

In the other experiment, mice were given orally RS-8359 or safrazine suspended in saline containing 0·3% sodium carboxymethylcellulose (CMC), and 1 h later, they were decapitated. The whole brain, except for the olfactory bulbs, was homogenized (1:10 w/v) in the same buffer and the homogenate was used as an enzyme preparation. The MAO activity in the homogenate was measured as described above, except for the reaction times, 5 and 3 min, for 5-HT and PEA, respectively.

Fluorometric assay of MAO activity

Mice were decapitated and the whole brain, except for the olfractory bulbs, homogenized (1:10 w/v) in 10 nM sodium phosphate buffer (pH 7·4). This homogenate was used as an enzyme preparation and MAO activity was determined fluorometrically according to Morian & Garratt (1985), using kynuramine dihydrobromide as a substrate. RS-8359, or safrazine, was preincubated at 37°C for various periods with the homogenate, then kynuramine dihydrobromide (final concn 100 μM) was added to the mixture and the incubation was continued for another 15 min. After the reaction was stopped by acidifying the mixture, the fluorescence of 4-hydroxyquinoline formed by MAO was measured at 380 nm with an excitation wavelength of 315 nm.

MAO activity was measured under atmospheric conditions. The activity determined was linear for both the preincubation time and tissue concentration used in the present experiments.

Measurement of the contents of endogenous monoamines and their related metabolites in mouse brain

RS-8359 (3, 10, 30 mg kg^{-1}) or safrazine (3, 10, 30 mg kg^{-1}) suspended in saline containing 0·3% (w/v) CMC was administered orally to mice, and 1 h later the mice were decapitated. In the time-course experiment, mice were killed (between 12 00 am and 2 00 pm) at various times after oral administration of RS-8359 (10 mg kg^{-1}) or safrazine (10 mg kg^{-1}). After decapitation, the whole brain, except for the olfactory bulbs, was quickly removed, frozen on dry-ice and stored at −80°C until used. Endogenous monoamines such as NA, DA and 5-HT, and their metabolites such as 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) were separated by the use of Amberlite CG-50 according to Oka et al (1984). They were measured by the use of high-performance liquid chromatography with electrochemical detection.

Compounds

[^{14}C]5-HT (5-hydroxy [side chain 2-^{14}C]trypatamine creatinine sulphate) and [^{14}C]PEA (2-phenyl [1-^{14}C]ethylamine hydrochloride) were obtained from Radiochemical Centre (Amersham, UK). Kynuramine dihydrobromide was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals used were of analytical grade.

Data analysis

Results were expressed as mean ± s.e.m. unless specifically stated. All group differences were evaluated by means of the Student's *t*-test.

Results

Concentration-dependent inhibition of MAO activity by RS-8359 in-vitro in mouse brain mitochondrial preparation

As shown in Fig. 1, RS-8359 inhibited 5-HT deamination, in a concentration-dependent manner, in the mouse brain mitochondrial preparation in-vitro between 10^{-7} and 10^{-4}M,

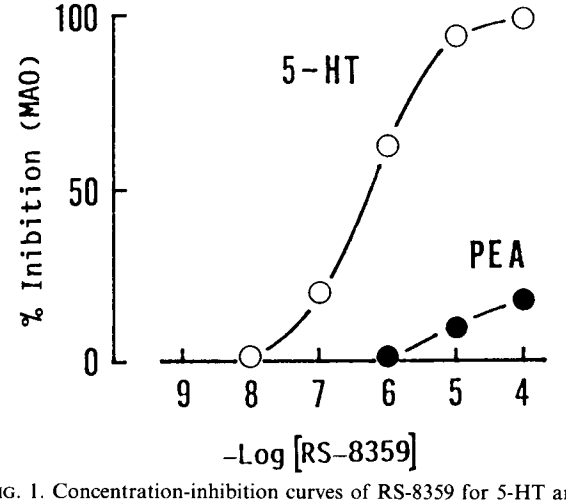


FIG. 1. Concentration-inhibition curves of RS-8359 for 5-HT and PEA deamination in mouse brain mitochondrial preparation in-vitro. Various amounts of RS-8359 were preincubated with mouse brain mitochondrial preparation at 38°C for 20 min at pH 7·4. Then [^{14}C]5-HT (final concn 100 μM) or [^{14}C]PEA (final concn 20 μM) was added to the mixture and incubation was continued for another 20 min. After termination of the reaction by acidifying the mixture, the ^{14}C-metabolites were extracted into an organic solvent and were counted for radioactivity. Each symbol represents the mean of the triplicate determination.

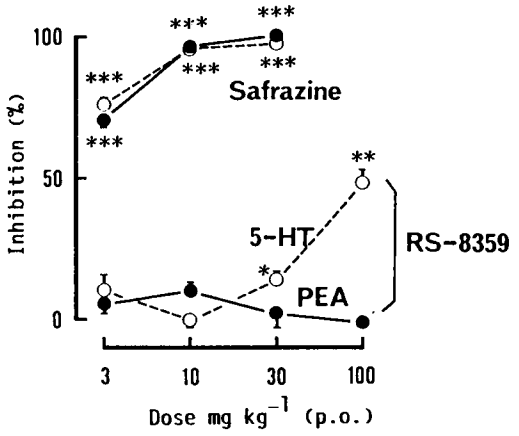


FIG. 2. Effects of systemically administered RS-8359 and safrazine on MAO activity in mouse brain. One hour after administration of RS-8359 or safrazine, mice were decapitated. Their whole brains were removed and homogenized. The MAO activity in the homogenate was measured with [^{14}C]5-HT and [^{14}C]PEA as substrates. The assay condition was the same as shown in Fig. 1, except for the reaction times of 5 and 3 min, for 5-HT and PEA, respectively. Each symbol represents the mean ± s.e. of four determinations. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, compared with the control.

while PEA deamination was little affected. The IC50 of RS-8359 was 0·52 μM for 5-HT deamination, while that for PEA was not determined because only 20% of the reduction of PEA deamination was observed even at the highest concentration (10^{-4}M) of the compound.

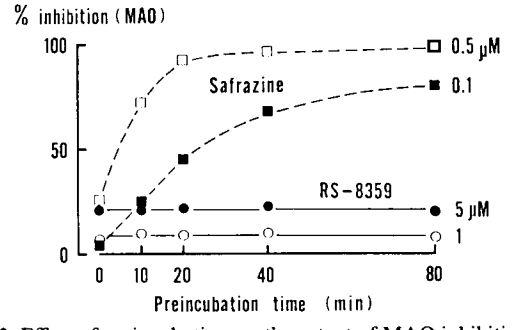


FIG. 3. Effect of preincubation on the extent of MAO inhibition by RS-8359 and safrazine. Either drug was preincubated with mouse brain homogenate at 37°C for various times at pH 7·4. Reaction was started by adding kynuramine dihydrobromide (final concn 100 μM) and incubation was continued for another 15 min. The amount of 4-hydroxyquinoline formed was measured fluorometrically. Each point is the mean value for triplicate determination. The values of s.e.m. of the data were within the range of the symbol's radius.

Effects of systematically administered RS-8359 and safrazine on MAO activity in mouse brain

Since RS-8359 was found to be a specific MAO-A inhibitor in-vitro, it was further investigated whether RS-8359 showed specificity toward MAO-A in-vivo in the mouse brain. As shown in Fig. 2, 5-HT deamination was decreased significantly by 14 and 42% in the brain homogenates prepared from the mice treated with 30 and 100 mg kg^{-1} (p.o.) of RS-8359, respectively, at 60 min after admininistration. In contrast, PEA deamination was not changed even at 100 mg kg^{-1} of the compound. As for safrazine, both 5-HT and PEA deaminations were inhibited significantly at only 3 mg kg^{-1} (p.o.) by 77 and 71%, respectively. Almost complete reduction in both deaminations was marked at 10 and 30 mg kg^{-1} of the drug, although specificity toward substrates was not observed.

Effect of preincubation time on the extent of MAO inhibition by RS-8359 or safrazine in brain homogenate in-vitro

As shown in Fig. 3, 1 and 5 μM of RS-8359 inhibited MAO activity by 7 and 23%, respectively in-vitro in brain homogenate without preincubation. In a preliminary experiment, MAO activity in the homogenate measured with KYN as a substrate was composed of 25% of clorgyline sensitive and 75% of deprenyl sensitive part. This suggests that almost complete inhibition of MAO-A was caused by 5 μM of RS-8359. This inhibitory effect of RS-8359 was not influenced at all by the preceding preincubation up to 80 min. In contrast, the inhibition by safrazine was augmented clearly by the prolonged preincubation period and it attained the maximum when the preincubation was for 20 min at a concn of 0·5 μM.

Changes in the levels of endogenous monoamines and their related metabolites in mouse brain

As summarized in Table 1, endogenous brain monoamines increased, in a dose dependent manner, 60 min after the administration of RS-8359 (3, 10 and 30 mg kg^{-1} p.o.). Both NA and DA increased significantly at 10 mg kg^{-1} or more, while increase in 5-HT content reached a statistically significant level at only 3 mg kg^{-1} of the compound. As for monoamine metabolites, DOPAC content was reduced significantly at 3 mg kg^{-1} or more. HVA and 5-HIAA tended to be reduced in their content at 30 mg kg^{-1} of the compound. With safrazine (Table 2), NA and 5-HT

Table 1. Effects of RS-8359 on the levels of endogenous monoamines and their related metabolites in the mouse brain.

		RS-8359 (mg kg^{-1} p.o.)		
	Control	3	10	30
NA	2·85 ± 0·12 (100)	3·12 ± 0·16 (109)	3·22 ± 0·09* (113)	3·45 ± 0·13*** (121)
DA	6·51 ± 0·11 (100)	7·23 ± 0·45 (111)	7·50 ± 0·22*** (115)	7·38 ± 0·07**** (113)
5-HT	3·23 ± 0·44 (100)	4·35 ± 0·19* (135)	4·77 ± 0·37* (148)	4·89 ± 0·36** (151)
DOPAC	0·49 ± 0·03 (100)	0·35 ± 0·04* (71)	0·27 ± 0·05*** (55)	0·17 ± 0·03**** (35)
HVA	0·87 ± 0·09 (100)	0·77 ± 0·08 (89)	0·71 ± 0·11 (82)	0·67 ± 0·08 (77)
5-HIAA	0·73 ± 0·16 (100)	0·56 ± 0·14 (77)	0·82 ± 0·15 (112)	0·43 ± 0·05 (59)

Mice were decapitated 60 min after oral administration of the drug. All the values expressed as nmol g^{-1} wet tissue are mean ± s.e. of five to six determinations. The number in parentheses represent percent of control. * $P<0.05$, ** $P<0.02$, *** $P<0.01$, **** $P<0.001$, compared with control.

Table 2. Effects of safrazine on the levels of endogenous monoamines and their related metabolites in the mouse brain.

		Safrazine (mg kg^{-1} p.o.)		
	Control	3	10	30
NA	2·56±0·14 (100)	2·65±0·11 (104)	2·88±0·04 (113)	3·06±0·08** (120)
DA	6·17±0·23 (100)	6·32±0·42 (102)	6·32±0·29 (102)	6·93±0·27 (112)
5-HT	4·30±0·53 (100)	4·89±0·17 (114)	5·94±0·20* (138)	5·90±0·36* (137)
DOPAC	0·89±0·09 (100)	0·59±0·09* (66)	0·45±0·03*** (51)	0·54±0·08** (61)
HVA	1·39±0·15 (100)	0·95±0·09* (68)	0·58±0·07**** (42)	0·58±0·07*** (42)
5-HIAA	0·81±0·12 (100)	0·69±0·08 (85)	0·54±0·14 (67)	0·35±0·06*** (42)

Mice were decapitated 60 min after oral administration of the drug. All the values expressed as nmol g^{-1} wet tissue are mean±s.e. of five to six determinations. The number in parentheses represent percent of control. * $P<0·05$, ** $P<0·02$, *** $P<0·01$, **** $P<0·001$, compared with control.

increased significantly at 30 and 10 mg kg^{-1} or more, respectively, while DA was not changed significantly even at 30 mg kg^{-1} of the drug. Both DOPAC and HVA were decreased significantly at 3 mg kg^{-1} or more while 5-HIAA content was decreased significantly at 30 mg kg^{-1} of safrazine.

Time-course experiment of RS-8359 and safrazine on monoamine metabolism

As shown in Fig. 4, the levels of NA, DA and 5-HT in mouse brain were the maximum (113, 115 and 148% of the control, respectively) at 60 min after administration of RS-8359 (10 mg kg^{-1} p.o.), followed by a gradual decline, and reached their control values at 2, 4 and 8 h, respectively. On the contrary, safrazine (10 mg kg^{-1} p.o.) maintained its effects on monoamine metabolism for at least 24 h after administration.

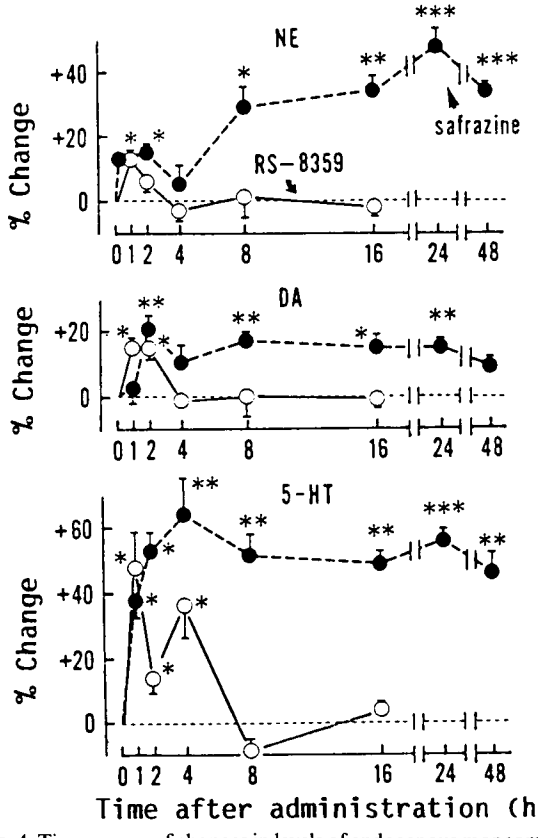


FIG. 4. Time-course of changes in levels of endogenous monoamines in mouse brain after administration of RS-8359 and safrazine. Mice were decapitated at various times after the administration of RS-8359 or safrazine (10 mg kg^{-1}). Each symbol represents the mean±s.e. of five to six determinations.
* $P<0·05$, ** $P<0·01$, *** $P<0·001$ compared with the control rats (CMC alone treated).

Discussion

The present experiments clearly demonstrate that RS-8359 inhibited MAO activity in the mouse brain mitochondrial fraction when 5-HT, but not PEA, was used as a substrate. This indicates that RS-8359 is a specific inhibitor of MAO-A. Further, since the inhibition was not affected by prolonged preincubation up to 80 min, the mode of action was suggested to be reversible. In the experiments with Lineweaver-Burk plot analysis, however, a linear relationship was not obtained between 1/(reaction velocity) and 1/(substrate concentration) when RS-8359 was present in the assay system. This is considered to be because RS-8359 is racemic at an -OH position and one form (85%) of the compound is several times more potent than the other form (90%) compound in inhibiting the MAO activity (unpublished observation). Since systematically administered RS-8359 increased monamine concentration in the mouse brain and decreased the concentration of DOPAC, RS-8359 is strongly suggested to be effective as a MAO inhibitor not only in-vitro but also in-vivo. As RS-8359 showed selective reduction in 5-HT deamination in the homogenate prepared from mice given 30 and 100 mg kg^{-1} of the compound orally, the specificity toward MAO-A is suggested to be maintained in-vivo in mouse brain. Further, the fact that the effects of RS-8359 on the monoamine concentration in the mouse brain lasted for less time than those of SAF supports the idea that RS-8359 is a reversible inhibitor.

It is believed that the extent of the increase in monoamine levels in the brain after treatment with a MAO inhibitor might represent the potency of MAO-A inhibition in-vivo (Green & Grahame-Smith 1978). In our experiment, 3 mg kg^{-1} of RS-8359 increased 5-HT content significantly 1 h after the administration. On the other hand, 30 mg kg^{-1} or more of the compound was needed to produce significant changes in 5-HT deamination in ex-vivo determination. The difference between these two effective doses is considered to be due, at least in part, to the fact that the complex of RS-8359 and MAO-A in the brain is possibly dissociated during

the homogenization because of the drug's reversible property, since according to Tipton & Fowler (1984) inhibition by a reversible inhibitor can be reversed by dialysis, gel filtration or dilution.

The accumulation of 5-HT lasted for longer than that of NA and DA after the systemic administration of RS-8359 (Fig. 4). The same results were also obtained with SAF. These findings indicate that accumulation of 5-HT may be a more sensitive marker reflecting the in-vivo effects of MAO-A inhibitors than that of NA or DA. This could be because firstly, the synthesis and turnover of 5-HT in the mammalian brain are more rapid than those of NA and DA (Cooper et al 1982), and secondly, tyrosine hydroxylase, a rate-limiting enzyme for the biosynthesis of NA and DA, is inhibited by NA and DA themselves (end-product inhibition), whereas tryptophan hydroxylase, a rate-limiting enzyme for the biosynthesis of 5-HT, is not inhibited by 5-HT (Nagatsu et al 1964; Cooper et al 1982; Okuno & Fujisawa 1985). Thus, the accumulation of NA and DA, but not 5-HT, in the nerve cells may prevent their own synthesis in-vivo after the administration of MAO inhibitors.

As shown in Fig. 4, an apparently biphasic response of 5-HT to RS-8359 was observed after its systemic administration. Since no active metabolite of RS-8359 was found in the mouse brain (unpublished observation) and the possibility of a temporary decrease in the synthesis of 5-HT by rapid end-product inhibition is low (as described above), the reason of the phenomenon is not clear.

Although a few reversible and specific MAO-A inhibitors have been reported (Benedetti & Dostert 1987), they are not available. On the other hand, since safrazine is one of MAO inhibitor medically used and the mode of action has not been studied, safrazine was chosen as a reference drug in the present experiment. As has been shown, safrazine is an irreversible and non-specific inhibitor of MAO, as was expected from its hydrazinic structure.

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