17:3] to afford **3** (6.2 g, 88%): mp 190–192 °C; $[\alpha]^{21}_D$ 0°; UV λ_{max} (MeOH) 229 (log ϵ 4.42), 289 (4.35), 329 (sh) nm (3.85); IR ν_{max} (KBr) 3412, 1637, 1590, 1519, 1474, 1270, 1155, 1131, 1083 cm^{-1}; ^1H NMR (DMSO-d_6, 360 MHz) δ 3.79 (s, OMe), 4.83 (1 H, d, J = 11 Hz, H-3), 5.04 (1 H, d, J = 11 Hz, H-2), 5.89, 5.95 (1 H each, d, J = 2 Hz, H-6, H-8), 6.91 (1 H, d, J = 8 Hz, H-6′), 6.94 (1 H, br s, H-2), 6.96 (1 H, d, J = 8 Hz, H-5′); ^{13}C NMR (DMSO-d_6, 90.8 MHz) δ 55.7 (OCH_3), 71.6 (C-3), 82.9 (C-2), 95.1 (C-6), 96.1 (C-8), 100.5 (C-10), 111.7 (C-5′), 115.1 (C-2′), 119.3 (C-6′), 129.7 (C-1′), 146.2 (C-3′), 148.0 (C-4′), 162.5 (C-9), 163.5 (C-5), 166.8 (C-7), 197.7 (C-4); measured mass 318.0739 ($C_{16}H_{14}O_7$ requires 318.0733).

Sensory Evaluation of Compounds 1–3. Prior to being evaluated by a human taste panel consisting of three persons, compounds **1–3** were shown to be pure in several TLC systems. Also, these substances were found to be not acutely toxic for mice, when administered by oral intubation at a dose of 1 g/kg body weight, according to a previously described protocol.[14] Compounds **1–3** were not mutagenic for *Salmonella typhimurium* strain TM677, both in the presence and absence of a metabolic activating system, at various concentrations ranging up to 1.38 μg/mL, when tested as described previously.[15]

Compounds **1–3** were dissolved in 3% EtOH–H_2O and were found to match the sweetness intensity of 20 000 ppm sucrose (in 3% EtOH–H_2O) at 250, 50, and 500 ppm, respectively. No bitterness was ascribed to the taste sensation exhibited by the most intensely sweet of these derivatives, compound **2**.

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2,4-Dihydro-3*H*-1,2,4-triazole-3-thiones as Potential Antidepressant Agents

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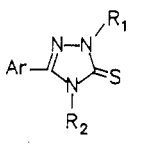
A series of 5-aryl-2,4-dihydro-3*H*-1,2,4-triazole-3-thiones was prepared and evaluated for potential antidepressant activity. Members of this series were generally prepared by the alkaline ring closures of the corresponding 1-aroylthiosemicarbazides. Several members of this series were potent antagonists of both RO 4-1284-induced hypothermia and reserpine-induced ptosis in mice. In general the more active members of this series were substituted by haloaryl groups at the 5-position of the triazole nucleus and by methyl groups at the 2- and 4-positions. Exchange of the thiocarbonyl group at the 3-position for a carbonyl group resulted in the complete loss of activity. Biochemical evaluation of the more active members of this series indicated that the aforementioned activities were not a consequence of either norepinephrine (NE) uptake or monoamine oxidase inhibition. In an attempt to determine a mechanism of action, one member of this series, compound **22**, was selected for further evaluation in an electrophysiological model where it was found to reduce norepinephrine function in the cerebellum as measured by the NE augmentation of GABA inhibition of Purkinje neurons.

Drug intervention in the treatment of depression has traditionally been accomplished by the use of either monoamine oxidase inhibitors or biogenic amine reuptake inhibitors.[1] Unfortunately both therapies are associated with a number of undesirable side effects[2–6] and neither has shown a rapid onset of action.[7,8] More recently, a structurally diverse array of compounds has been described as potential second-generation antidepressants.[9–11] The mechanisms of action of these compounds appear to differ from those of their predecessors, which has spawned the hope that more efficacious therapies might be close at hand. We have recently described an unusual cleavage reaction of a diaryl ketone which afforded 2,4-dihydro-2,4-dimethyl-5-phenyl-3*H*-1,2,4-triazole-3-thione (**1**).[12] Biological evaluation of **1** demonstrated potent effects in



Ar=C_6H_5, R_1=R_2=CH_3

1

several test systems designed to detect potential antidepressant activity. In order to more systematically inves-

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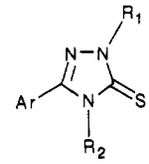
Table I. 2,4-Dihydro-3*H*-1,2,4-triazole-3-thiones



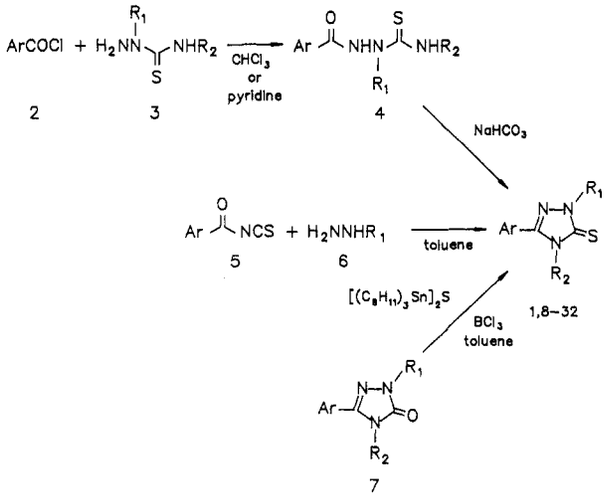
compd	Ar	R_1	R_2	mp, °C	method	crystallization solvent[a]	% yield	formula[b]
1	C_6H_5	CH_3	CH_3	134–135[c]	1a	A	50	$C_{10}H_{11}N_3S$
8	C_6H_5	H	CH_3	164–166[d]	2c	A	60	$C_9H_9N_3S$
9	C_6H_5	CH_3	C_2H_5	358/746 mm[e]	2b		71	$C_{11}H_{13}N_3S$
10	$C_{10}H_7$	CH_3	CH_3	173–175	2d	B	76	$C_{14}H_{13}N_3S$
11	2-FC_6H_4	H	CH_3	137–139	1c	C	40	$C_9H_8FN_3S$
12	2-FC_6H_4	CH_3	CH_3	106–108	1a	B	50	$C_{10}H_{10}FN_3S$
13	3-FC_6H_4	CH_3	CH_3	126–128	1a	B	47	$C_{10}H_{10}FN_3S$
14	4-FC_6H_4	H	H	269–272 dec	2c	D	54	$C_8H_6FN_3S$
15	4-FC_6H_4	CH_3	CH_3	130–132[f]	1a	B	50	$C_{10}H_{10}FN_3S$
16	2,4-$F_2C_6H_3$	CH_3	CH_3	102–104	2b	E	55	$C_{10}H_9F_2N_3S$
17	2,6-$F_2C_6H_3$	CH_3	CH_3	158–160	1a	B	43	$C_{10}H_9F_2N_3S$
18	2-ClC_6H_4	CH_3	CH_3	138–140	1a	B	38	$C_{10}H_{10}ClN_3S$
19	4-ClC_6H_4	H	H	295–297 dec[g]	2e	F	48	$C_8H_6ClN_3S$
20	4-ClC_6H_4	H	CH_3	210–212[h]	1c	B	31	$C_9H_8ClN_3S$
21	4-ClC_6H_4	CH_3	H	269–271[i]		A	55	$C_9H_8ClN_3S$
22	4-ClC_6H_4	CH_3	CH_3	113–115[j]	2a	B	73	$C_{10}H_{10}ClN_3S$
23	4-ClC_6H_4	C_2H_5	CH_3	118–120[k]		B	65	$C_{11}H_{12}ClN_3S$
24	4-ClC_6H_4	CH_3	C_2H_5	113–115	1a	B	45	$C_{11}H_{12}ClN_3S$
25	4-ClC_6H_4	CH_3	n-C_3H_7	240–250/0.55 mm	2b		58	$C_{12}H_{14}ClN_3S$
26	2,4-$Cl_2C_6H_3$	CH_3	CH_3	135–137	1a	B	54	$C_{10}H_9Cl_2N_3S$
27	2,6-$Cl_2C_6H_3$	CH_3	CH_3	115–116	1a[l]	B	29	$C_{10}H_9Cl_2N_3S$
28	3,4-$Cl_2C_6H_3$	CH_3	CH_3	161–163	1a	B	66	$C_{10}H_9Cl_2N_3S$
29	4-$CH_3C_6H_4$	CH_3	CH_3	94–96	1a	B	38	$C_{11}H_{13}N_3S$
30	3-$CF_3C_6H_4$	CH_3	CH_3	73–75	1b	E	50	$C_{11}H_{10}F_3N_3S$
31	4-$CH_3OC_6H_4$	CH_3	CH_3	96–98	1a	B	49	$C_{11}H_{13}N_3OS$
32	3,4-$OCH_2OC_6H_3$	CH_3	CH_3	142–144	1a	B	30	$C_{11}H_{11}N_3O_2S$

[a] A = ethanol, B = 2-propanol, C = ethyl acetate/hexane, D = water, E cyclohexane, F = ethanol/water. [b] Satisfactory analyses (C, H, and N; ±0.4% of theoretical values) were obtained for all compounds. [c] Literature mp 135.5–136 °C (ref 13). [d] Literature mp 163–164 °C (ref 16). [e] Boiling point determined by differential scanning calorimetry. [f] Literature mp 130–132 °C (ref 15). [g] Literature mp 296–297 °C (ref 17). [h] Literature mp 212–213 °C (ref 18). [i] Literature mp 263–265 °C (ref 19). [j] Literature mp 113–115 °C (ref 15). [k] Literature mp 118–120 °C (ref 15). [l] The procedure was as described in 1a with the exception that 1 M aqueous NaOH was used for the cyclization.

tigate this observation, we have prepared a number of additional examples of this ring system and now report the novel antidepressant activity associated with members of this series.

Chemistry

With only two exceptions, the triazoles described in this study were prepared by modifications of the method of Sandström and Wennerbeck.[13] Thus, reaction of aroyl chlorides **2** and thiosemicarbazides **3** in either chloroform or pyridine gave 1-aroylthiosemicarbazides **4**, which, without purification, were cyclized in refluxing aqueous sodium bicarbonate to yield the desired 2,4-dihydro-3*H*-1,2,4-triazole-3-thiones (Table I). The syntheses of 5-(4-chlorophenyl)-2,4-dihydro-2-methyl-3*H*-1,2,4-triazole-3-thione (**21**) and 5-(4-chlorophenyl)-2,4-dihydro-2-ethyl-4-methyl-3*H*-1,2,4-triazole-3-thione (**23**) by the above method necessitated the preparations of 2-methylthiosemicarbazide (**3**, R_1 = CH_3, R_2 = H) and 2-ethyl-4-methylthiosemicarbazide (**3**, R_1 = C_2H_5, R_2 = CH_3). In practice it was much simpler to prepare **21** by the method of Durant[14] with 4-chlorobenzoyl isothiocyanate (**5**, Ar = 4-ClC_6H_4) and methylhydrazine (**6**, R_1 = CH_3) while **23** was easily prepared by the bis(tricyclohexyltin) sulfide thionation of the corresponding 2,4-dihydro-3*H*-1,2,4-triazol-3-one **7** (Ar = 4-ClC_6H_4, R_1 = C_2H_5, R_2 = CH_3).[15]



Results and Discussion

The potential antidepressant activities of the triazoles listed in Table I were assessed by each compound's ability to antagonize, in mice, both RO 4-1284-induced hypothermia[20] and reserpine-induced ptosis.[21,22] The results

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Table II. Biological Activities for 2,4-Dihydro-3*H*-1,2,4-triazole-3-thiones

compound	estimated LD_{50}, mg/kg ip	ED_{50}, mg/kg ip		% control[c]		
		RO[a]	RSP[b]	NE uptake[d]	MAO[e]	MAO B[e]
1	100–200	0.15	0.13	92 ± 2	104 ± 5	110 ± 6
7[f]	400–800	>50	>200			
8	>800	>50	>200			
9	400–800	0.58	1.20			
10	>800	>50	>200			
11	>800	>25	>200			
12	400–800	0.74	0.23	106 ± 6	72 ± 12[g]	96 ± 3
13	200–400	0.37	0.29	110 ± 8	105 ± 5	96 ± 8
14	400–800	>50	>200			
15	200–400	0.61	1.00	104 ± 5	70 ± 8[g]	90 ± 18
16	400–800	1.00	2.37			
17	400–800	0.44	0.36	105 ± 7	104 ± 3	97 ± 18
18	200–400	0.37	0.58			
19	>800	>50	>200			
20	>800	>50	>200			
21	>800	>50	>200			
22	400–800	1.34	0.27	90 ± 5	109 ± 3	75 ± 10[g]
23	400–800	>50	100–200			
24	>800	>50	21.2			
25	200–400	>12.5	12.5–25			
26	>800	>25				
27	400–800	>50	>100			
28	>800	29.0	4.40			
29	400–800	1.74	4.20			
30	400–800	>50	>200			
31	>800	>25	3.30			
32	>800	>50	>200			
desipramine	75	0.94	1.86	37 ± 5[g]		
imipramine	100–200	0.50	4.89			
nortriptyline	50–100	0.71	4.85			
amitriptyline	100–200	0.36	8.73			
protriptyline	50–100	0.14	0.43			
clorgyline	400–800	2.00	2.04		23 ± 9[g]	
pheniprazine	100–200	1.10	1.61			
pargyline	400–800	13.6	37.1			
deprenyl	200–400	1.8	4.9			13 ± 5[g]

[a] Antagonism of RO 4-1284-induced hypothermia in mice. [b] Antagonism of reserpine-induced ptosis in mice. [c] Values shown are means ± SD, $n = 4$. [d] Compounds tested at a final concentration of 1 μM. [e] Compounds tested at a final concentration of 100 μM except clorgyline and deprenyl, which were tested at 1 μM. [f] Ar = C_6H_5, $R_1 = R_2 = CH_3$ (ref 23). [g] $p < 0.05$.

are presented in Table II, where it can be seen that marked activity relative to known antidepressants was found with approximately one-third of the compounds examined. In general, the acute toxicity associated with this series was reasonably low as gauged by LD_{50} estimates indicating very favorable activity/toxicity ratios for the more active members of the series.

Although the correlation between structure and activity in this series was not straightforward, some trends could be observed. The most obvious structural features necessary for antidepressant-like activity were the alkyl groups at the 2- and 4-positions of the triazole nucleus and the thiocarbonyl moiety. Removal of either one or both of the alkyl groups (compounds **8**, **11**, **14**, and **19–21**) caused a pronounced loss of activity. Almost exclusively, the most active members in this series were 2,4-dimethylated (compounds **1**, **13**, **17**, and **18**). Extension of the lengths of the alkyl chains at either position (compounds **9** and **23–25**) caused a decrease in activity, this effect being more pronounced at the 2-position (compound **23**) than at the 4-position (compounds **9**, **24**, and **25**). Replacement of the thiocarbonyl group by a carbonyl group (compound **7**; Ar = C_6H_5, $R_1 = R_2 = CH_3$)[23] resulted in the complete loss of antidepressant-like activity. Less obvious was the relationship between the substitution patterns of the various aryl groups and activity. In general the aryl groups of the more active compounds were either unsubstituted (compounds **1** and **9**) or halogenated (compounds **12**, **13**, **15**, **17**, and **18**). Substitution by one or more fluorine atoms (compounds **12**, **13**, and **15–17**) afforded analogues of good activity seemingly irrespective of either the number or position of these atoms. Substitution by chlorine, on the other hand, was not as universally tolerated and only monosubstitution (compounds **18** and **22**) afforded triazoles of significant activity.

Since most of the known antidepressants which exhibit activity in the aforementioned pharmacologic tests are inhibitors of either norepinephrine (NE) uptake or monoamine oxidase (MAO), several members of this series (compounds **1**, **12**, **13**, **15**, **17**, and **22**) were assessed for these properties (Table II). In this regard it was found that none of the selected compounds, at concentrations up to 1 μM, significantly inhibited [^{3}H]NE uptake into rat cortical synaptosomes. Desipramine (DMI), a potent NE uptake inhibitor,[24] reduced uptake to 37 ± 5% of control at a final concentration of 1 μM. Some of the compounds, however, were weak, selective inhibitors of either MAO A or MAO B at concentrations of 100 μM. Thus, compounds **12** and **15** reduced MAO A activity by 30% while **22** reduced MAO B activity by 25%. None of these compounds, however, were as potent as the specific inhibitors clorgyline or deprenyl, which reduced enzyme activity by 77% and

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87%, respectively, at concentrations of 1 μM.

Since the antidepressant-like effects observed for some of these triazoles were not adequately explained by the inhibition of either NE uptake or MAO and the standard whole animal tests also involved interaction with norepinephrine, their effect in another in vivo test of antidepressant-induced modification of norepinephrine activity was used to help identify a potential mechanism of action. In this regard, it has been shown that the activity of NE on rat cerebellar Purkinje neurons, which receive both GABAergic and noradrenergic inputs, is susceptible to modulation by antidepressants administered chronically but is not altered by acute administration of currently available antidepressants.[25,26] The ability to modulate the effect of NE on Purkinje neuron activity may, therefore, be useful in predicting the antidepressant potential of a particular compound. With this in mind, triazole **22** was compared with DMI in electrophysiological studies to assess NE function on cerebellar Purkinje neurons. Following acute administration, neither **22** nor DMI altered the spontaneous activity of these neurons or the slowing of these neurons produced by iontophoresed NE (Table III). Compound **22**, however, significantly reduced the ability of iontophoresed NE to enhance the inhibition produced by iontophoresed γ-aminobutyric acid (GABA), a robust measure of NE function in these neurons. DMI had no significant effect on this NE response. Following chronic administration (10 mg/kg per day × 21 days), both compounds again failed to alter the NE-evoked slowing of Purkinje neurons. Now, however, both DMI and **22** significantly reduced the NE enhancement of GABA inhibition in these neurons, this blockade being stronger for **22** than for DMI. The fact that **22** acutely blocked the NE enhancement of GABA inhibition in these neurons whereas DMI was not active in this measure until it had been administered chronically suggests that **22** may have an onset of antidepressant activity which is shorter than that of DMI.

In conclusion, several 5-aryl-2,4-dialkyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thiones have been shown to be potent antagonists of both RO 4-1284-induced hypothermia and reserpine-induced ptosis, two common test systems used to detect potential antidepressants. Moreover, these effects do not appear to be a consequence of either the inhibition of NE uptake or MAO. While the mechanism of action of these compounds remains unclear from the whole animal tests of antidepressant efficacy, the electrophysiological experiments suggest that these triazoles do interact with the NE neurotransmitter system by reducing the NE enhancement of GABA inhibition of Purkinje neurons.

Experimental Section

Melting points were determined in open capillaries on a Thomas-Hoover apparatus and are uncorrected. Elemental analyses were performed on site and are within ±0.4% of the theoretical values. All compounds were routinely checked by proton NMR (Varian FT80A, EM390, and XL300), IR (Perkin-Elmer 180), and TLC (silica gel).

General Procedures for the Preparation of 2,4-Dihydro-3*H*-1,2,4-triazole-3-thiones. Method 1. To a stirred, room-temperature solution of the thiosemicarbazide (0.050 mol) in $CHCl_3$ (125 mL) was added dropwise the aroyl chloride (0.053 mol). After several hours, the precipitate was collected by filtration, washed with small portions of $CHCl_3$, and dried by suction. The resulting crude aroylthiosemicarbazide was heated to reflux in 1 M aqueous $NaHCO_3$ (500 mL, 0.500 mol) for approximately 17 h.

Choice of Workup. (a) The reaction was cooled in an ice bath and the product was collected by filtration. Purification was then accomplished by a combination of flash chromatography,[27] using mixtures of EtOAc and CH_2Cl_2, and crystallization from the solvents listed in Table I.

(b) The reaction was extracted with EtOAc (three times). The EtOAc extracts were combined, washed with saturated aqueous NaCl, and dried over anhydrous Na_2SO_4. Removal of the drying agent by filtration and evaporation of the filtrate gave the desired product, which was purified as described above.

(c) The reaction mixture was filtered while still hot and the filtrate was cooled in an ice bath. Careful acidification by the dropwise addition of concentrated HCl (46 mL, 0.55 mol) afforded the product, which was collected by filtration. Purification was then accomplished as described above.

Method 2. To a stirred, room-temperature solution of the thiosemicarbazide (0.010 mol) and pyridine (10 mL) was added dropwise the aroyl chloride (0.011 mol). After several hours, the excess pyridine was evaporated at reduced pressure, leaving a mixture of the crude aroylthiosemicarbazide and pyridine hydrochloride.

Choice of Workup. (a) This mixture was heated to reflux in 1 M aqueous $NaHCO_3$ (100 mL, 0.100 mol) for approximately 17 h. The reaction was cooled in an ice bath and the precipitate was collected by filtration. Purification was accomplished by a combination of flash chromatography,[27] using mixtures of EtOAc and CH_2Cl_2, and crystallization from the solvents listed in Table I.

(b) This mixture was heated to reflux in 1 M aqueous $NaHCO_3$ (100 mL, 0.100 mol) for approximately 17 h. The reaction mixture was extracted with EtOAc (three times). The EtOAc extracts were combined, washed with saturated aqueous NaCl, and dried over anhydrous Na_2SO_4. Removal of the drying agent by filtration and evaporation of the filtrate gave the desired product, which was purified as described above.

(c) This mixture was heated to reflux in 1 M aqueous $NaHCO_3$ (100 mL, 0.100 mol) for approximately 17 h. The reaction mixture was filtered while still hot and the filtrate was cooled in an ice bath. Careful acidification by the dropwise addition of concentrated HCl (9.2 mL, 0.11 mol) afforded the product, which was collected by filtration. Purification was then accomplished as described above.

(d) This mixtre was washed with H_2O, removing the pyridine hydrochloride, and the crude aroylthiosemicarbazide was collected by filtration. This was heated to reflux in 1 M aqueous $NaHCO_3$ (100 mL, 0.100 mol) for approximately 17 h. The reaction was cooled in an ice bath and the product was collected by filtration. Purification was then accomplished as described above.

(e) This mixture was washed with H_2O, removing the pyridine hydrochloride, and the crude aroylthiosemicarbazide was collected by filtration. This was heated to reflux in 1 M aqueous $NaHCO_3$ (100 mL, 0.100 mol) for approximately 17 h. The reaction was filtered while still hot and the filtrate was cooled in an ice bath. Careful acidification by the dropwise addition of concentrated HCl (9.2 mL, 0.11 mol) afforded the product, which was collected by filtration. Purification was then accomplished as described above.

The triazole-3-thiones prepared by the above methods were uniformly insoluble in aqueous media and did not form suitable salts.

Antagonism of RO 4-1284-Induced Hypothermia.[20] Groups of five or 10 CD-1 male mice (18–26 g) were housed individually before being administered the test compound ip as a suspension in distilled H_2O/Tween 80. Standard compounds were administered as solutions in distilled H_2O. The Tween 80/H_2O vehicle had no effect in the test systems. Thirty minutes later, RO 4-1284 was administered at a dose of 20 mg/kg ip. The animals were placed in a cold room (ca. 4 °C) for 30 min and then removed to room temperature for an additional 30 min. At this time, the rectal temperatures were recorded with a thermistor probe. The inhibition of hypothermia was considered significant in an individual mouse if the rectal temperature exceeded the mean + 2 standard

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Table III. Effect of **22** and Desipramine (DMI) on Purkinje Neuron Activity

		spontaneous firing rate, Hz	% inhibn caused by NE (20 mA)[a,b]	% of maximal NE-induced augmentation of GABA inhibn[b]
control	acute[c]	41.08 ± 2.75[e] (n = 26)	14.4 ± 7.1[e]	100
	chronic[d]	35.29 ± 3.18 (n = 21)	13.1 ± 3.7	100
DMI	acute[c]	41.28 ± 4.41 (n = 24)	3.5 ± 4.0	68
	chronic[d]	32.18 ± 2.65 (n = 28)	7.8 ± 1.2	49
22	acute[c]	36.49 ± 2.58 (n = 47)	6.3 ± 5.0	33
	chronic[d]	29.35 ± 2.95 (n = 44)	10.9 ± 8.0	0

[a] Norepinephrine and GABA were applied locally onto the neuron by using iontophoresis with the specified ejecting current. [b] Data in these columns represents a range of between seven and 24 neurons. [c] Acute drug treatment consisted of a single ip injection of 10 mg/kg of drug or 1 mL/kg of vehicle for control. [d] Chronic treatment schedule consisted of 10 mg/kg ip of DMI or **22** given once a day for 21 days. Controls received 1 mL/kg per day of H_2O ip for 21 days. [e] Values represent the mean ± SEM.

deviations of 100 control animals treated with RO 4-1284 alone. The ED_{50} was defined as that dose causing a significant inhibition in 50% of the mice and was calculated, where appropriate, with a computer program for analysis of quantal data. For all actual ED_{50} values, 95% confidence limits are within the range 0.5–2 ED_{50}. All ED_{50} values were calculated from the results of at least four doses given to at least four groups of 10 mice.

Antagonism of Reserpine-Induced Ptosis.[21,22] Groups of five or 10 CD-1 male mice (18–26 g) were housed individually before being administered the test compound ip as a suspension in distilled H_2O/Tween 80. Standard compounds were administered as solutions in distilled H_2O. Thirty minutes later, reserpine, prepared as a 0.2 mg/mL solution in dilute HOAc, was administered via the tail vein at a dose of 2.0 mg/kg. Sixty minutes later, the mice were examined individually in a Plexiglas chamber for the presence or absence of ptosis. Ptosis was considered absent if the average closure of both eyes was less than 50% after the mouse was observed for 20 s. the ED_{50} for prevention of ptosis was defined as that dose which significantly prevented ptosis in 50% of the mice and was calculated, where appropriate, with a computer program for the analysis of quantal data. For all actual ED_{50} values, 95% confidence limits are within the range 0.5–2 ED_{50}. All ED_{50} values were calculated from the results of at least four doses given to at least four groups of 10 mice.

Intraperitoneal LD_{50} Estimation. Groups of three CD-1 male mice (18–26 g) were administered graded doses of the test compounds prepared as suspensions in distilled H_2O/Tween 80. Standard compounds were administered as solutions in distilled H_2O. Deaths occurring during the next 5 days were recorded and the dose range encompassing the 50% lethal effect was recorded as the estimated LD_{50}.

Uptake of [^{3}H]Norepinephrine.[28] Male Sprague–Dawley rats (180–200 g) were decapitated and the cortices were removed by dissection. The brain tissues were individually homogenized in 0.32 M isotonic sucrose (2.0 mL) at 0 °C with a Teflon–glass homogenizer. All further procedures were carried out at 0–4 °C. Tissue homogenates were centrifuged at 1000g for 10 min. The supernatant was decanted, diluted with additional sucrose (1.0 mL), and centrifuged at 10000g for 20 min. Partial removal of the microsomal contamination was achieved by resuspending the pellet in sucrose (2.0 mL) and recentrifuging at 10000g for an additional 20 min. This washed homogenate was prepared for incubation by gently resuspending in sucrose (1.5 mL). Final protein concentrations ranged from 1.8 to 2.5 mg/mL. Incubations were performed at 37 °C in Krebs-Ringer phosphate (0.60 mL, pH 7.4) containing 1.0 mM l-ascorbic acid, 8.4 mM d-glucose, and 12 μM nialamide. To this were added the resuspended synaptosomes (100 μL), the test compound (100 μL, concentrations ranging from 0.001 to 1.0 μM) or H_2O (100 μL), and a solution of [^{3}H]NE (50 μL, final concentration 0.2 μM). After a 3-min incubation, uptake was terminated by the addition of cold 0.9% aqueous NaCl (5.0 mL), which contained 100 μM unlabeled NE. Quenching was followed immediately by filtration (Millipore, 0.8 μm). The filters were placed in scintillation vials containing Bray's solution (10.0 mL). Blanks for nonspecific binding were obtained by repeating the uptake procedure, except that quenching with the NaCl solution was performed at zero time.

Monoamine Oxidase Activity. Monoamine oxidase activity was measured in an extract of rat whole brain minus cerebellum. Brains were homogenized in 0.2 M K_2HPO_4 (7.5 mL, pH 7.4) and 0.25 M sucrose. The homogenate was centrifuged at 1000g for 10 min and the pellet discarded. The assay consisted of enzyme (100 μL), H_2O (100 μL) or test compound, 50 μM [^{14}C]serotonin (50 μL) for MAO A or 50 μM [^{14}C]phenylethylamine (50 μL) for MAO B, and 0.143 M K_2HPO_4 (700 μL, pH 7.0). Where needed, the test compounds were solubilized by heating with trace amounts of acetic acid. The reaction was allowed to proceed for 30 min at room temperature before being stopped by the addition of 2 M citric acid (500 μL). Products were extracted into a 50% solution of EtOAc and toluene (5.0 mL), the organic phase was added to Bray's scintillation fluid (5 mL), and the radioactivity was determined.

Electrophysiological Activity. Fifty-three male, Sprague-Dawley rats (180–330 g) were housed in small groups (usually four to a cage) and were allowed access to food and water prior to the experiment. Purkinje neuron activity was recorded extracellularly by using techniques that have been described previously.[29] The action potentials from single Purkinje neurons were recorded with a glass multibarreled micropipet and the firing rates of these neurons were observed during control and drug treatments. Both NE and GABA were administered from adjacent barrels of the micropipet by iontophoresis. Twenty-five rats were administered intravenous doses of either compound **22** as a fine suspension in saline vehicle, DMI, or saline vehicle. The drug was administered via an indwelling catheter placed in the lateral tail vein. In each experiment, the protocol involved first isolating and recording from a single, stable Purkinje neuron for a control period and then administering the drug. Following the injection, the Purkinje neuron was continuously recorded for an additional 30 min during which any changes in the pattern of activity of the cell were noted. Twenty of the rats were chronically treated with the compounds of interest prior to recording. Thus, six animals were given DMI (10 mg/kg per day ip × 21 days), eight were given **22** (10 mg/kg per day ip × 21 days), and six were treated with vehicle (H_2O) for 21 days. No difference in the growth rates for these three groups was observed. The Purkinje neuron activities of all chronically treated rats were recorded on day 21, approximately 2 h after their last injection. Comparisons were subjected to statistical analysis by using the RS1 program (BB&N Software) and were based on the analysis of variance and the Student's t test, with the significance level set at $p < 0.05$.

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6610-29-3; **3** (R^1 = CH_3, R^2 = C_2H_5), 21198-37-8; **3** (R_1 = R_2 = H), 79-19-6; **3** (R^1 = CH_3, R_2 = n-C_3H_7), 21198-40-3; **5** (Ar = p-ClC_6H_4), 16794-67-5; **6** (R^1 = CH_3), 60-34-4; **7** (Ar = p-ClC_6H_4, R^1 = C_2H_5, R_2 = CH_3), 114058-88-7; **8**, 38942-51-7; **9**, 114058-89-8; **10**, 114058-93-4; **11**, 114058-90-1; **12**, 110623-32-0; **13**, 110623-33-1; **14**, 114058-91-2; **15**, 110623-34-2; **16**, 110623-35-3; **17**, 110623-36-4; **18**, 110623-25-1; **19**, 26028-65-9; **20**, 29527-27-3; **21**, 27349-24-2; **22**, 110623-24-0; **23**, 114058-92-3; **24**, 110623-26-2; **25**, 110623-27-3; **26**, 110623-29-5; **27**, 110623-31-9; **28**, 110623-30-8; **29**, 110623-38-6; **30**, 110623-37-5; **31**, 110623-39-7; **32**, 110623-40-0.

Facile N-Oxygenation of 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine by the Flavin-Containing Monooxygenase. A Convenient Synthesis of Tritiated [*methyl*-^{3}H]-4-Phenyl-2,3-dihydropyridinium Species

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A rapid, efficient procedure useful for the radiosynthesis of [*Me*-^{3}H]-MPDP$^+$ ([*methyl*-^{3}H]-4-phenyl-2,3-dihydropyridinium species) is described. Hog liver microsomes or the highly purified flavin-containing monooxygenase from hog liver quantitatively biotransforms [*Me*-^{3}H]-MPTP to its corresponding radiolabeled *N*-oxide. For the small-scale synthesis required for radiolabeling procedures, this enzymatic process is superior to H_2O_2-mediated N-oxygenation of MPTP. In the presence of 0.5 mM NADPH, 4.5 mM *n*-octylamine, and 2 μCi [*Me*-^{3}H]-MPTP, the only product detected in extracts from incubations performed with hog liver microsomes or purified hog liver flavin-containing monooxygenase is [*Me*-^{3}H]-MPTP *N*-oxide. [*Me*-^{3}H]-MPTP *N*-oxide is almost completely converted to [*Me*-^{3}H]-MPDP$^+$ by the action of trifluoroacetic anhydride. This procedure has the advantage of using a commercially available tritiated starting material, efficient transformations, and easily accomplished purification to afford a rapid synthesis of [*Me*-^{3}H]-MPDP$^+$.

There is considerable current interest in radiolabeled metabolites of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a thermal decomposition product of a "street narcotic" that is responsible for a parkinsonism-like syndrome in humans.[1-3] A convenient procedure for the synthesis of tritiated 1-methyl-4-phenyl-2,3-dihydropyridinium species (MPDP$^+$) may be useful in delineating the involvement of MPDP$^+$ in the neurotoxicity of MPTP.

The mechanism of neurotoxicity of MPTP is not clear, but it is apparent that any explanation must include the facts that (1) MPTP requires metabolic bioactivation by monoamine oxidase (MAO) to exert its toxic effect,[4-7] (2) the primary initial metabolite of MAO biotransformation of MPTP is MPDP$^+$,[8,9] (3) MPDP$^+$ is reactive to nucleophiles such as cyanide ion and is readily oxidized,[7,10-12] and (4) MPDP$^+$ is further transformed to 1-methyl-4-phenylpyridinium species (MPP$^+$).[7,13]

Processing of MPTP by MAO appears to be critical for the expression of MPTP neurotoxicity. Results from a number of laboratories examining a variety of tissues suggest that high specific binding of MPTP parallels the presence of MAO and especially MAO B in the tissues examined.[4] Specific binding of MPTP to MAO B could result from at least two mechanisms, including (1) covalent binding to MAO B itself, or (2) high affinity and slow enzymatic turnover of MPTP to MPDP$^+$. The evidence is that MAO B is irreversibly inactivated by MPTP[8] and that this process is relatively inefficient: 1 mol of enzyme is inactivated per 17 000 mol of product formed.[9] In contrast, MAO A is only competitively inhibited by MPTP.[8] MAO B catalyzes the oxidation of MPTP 10 times faster than MAO A[8,9] and the rate of MAO B oxidation of MPTP compares favorably with benzylamine, an excellent MAO type B substrate. That the neurotoxicity observed for MPTP requires MAO-catalyzed oxidation is supported by the finding that MAO B inhibitors protect against the neurotoxicity of MPTP.[4,15,16] A number of studies have demonstrated that MPTP or derivatives of MPTP that are neurotoxic are MAO substrates, but, of course, not all MAO substrates are toxic.[5,6] It is clear that MPDP$^+$ is the primary initial metabolite arising from MAO oxidation of MPTP and it is this metabolism that appears to parallel the neurotoxicity of MPTP.[7] MPDP$^+$, the major initial metabolite of MPTP in the brain, was found to serve as an electrophile for cyanide.[11,13] An analogue of MPDP$^+$ stable to oxidation was observed not to react with various

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