Synthesis and Structure–Activity Studies of a Series of Spirooxazolidine-2,4-diones: 4-Oxa Analogues of the Muscarinic Agonist 2-Ethyl-8-methyl-2,8-diazaspiro[4.5]decane-1,3-dione

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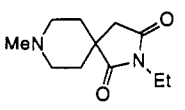
A series of spirooxazolidine-2,4-dione derivatives related to the putative M_1 agonist 2-ethyl-8-methyl-2,8-diazaspiro[4.5]decane-1,3-dione (RS86; 1) were synthesized. The compounds were evaluated as cholinergic agents in in vitro binding assays and in in vivo pharmacological tests including antiamnesic effects using scopolamine-treated mice, hypothermia, and salivation in mice. Four compounds (**5a**,**c**,**f** and **17a**) exhibited affinity for cortical M_1 receptors and reversed scopolamine-induced impairment of mouse passive avoidance tasks, as did 1. Among these compounds, only **5a** exhibited M_1-receptor stimulating activity in pithed rats. Structural requirements for muscarinic activity in this series of spirooxazolidine-2,4-dione derivatives were as strict as those reported for spirosuccinimide derivatives including 1. The antiamnesic dose of 3-ethyl-8-methyl-1-oxa-3,8-diazaspiro[4.5]decane-2,4-dione (**5a**) was 2 orders of magnitude lower than the doses inducing hypothermia and salivation, in contrast to 1 for which the former dose was only 5–10-fold lower than the latter. These results suggest that the 8-azaspiro[4.5]decane skeleton represents a useful template for designing new muscarinic agonists as antidementia drugs.

Muscarinic agonists have attracted much interest as candidate therapeutic agents for cognitive disorders in senile dementia of Alzheimer's type (SDAT).[1,2] One of the characteristic changes in the brains of patients with SDAT is the reduced activity of choline acetyltransferase (ChAT),[3-5] a marker of presynaptic cholinergic function. The degree of this decrease in ChAT activity is closely correlated with the severity of the dementia.[6,7] In contrast, post synaptic muscarinic receptors, particularly the M_1 subtype (high affinity for pirenzepine), are reported to be well preserved.[8] M_1 muscarinic receptors are highly distributed in the hippocampus and cerebral cortex, both of which are associated with memory and learning.[9] Thus, among muscarinic agonists, selective M_1 agonists may have potential as highly safe and beneficial antidementia drugs.

In clinical studies in SDAT, the putative M_1 agonist 2-ethyl-8-methyl-2,8-diazaspiro[4.5]decane-1,3-dione (RS86; 1) has been reported to show significant, albeit weak, improvements in cognitive deficits, while inducing cardiovascular and abdominal side effects[10] which are considered to be mediated by M_2 receptors (low affinity for pirenzepine). These findings demonstrate the need for M_1 agonists more selective than 1.



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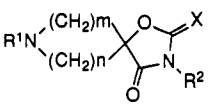

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(m, n) = (2, 2), (2, 1), (3, 1)

X = O, S

Although many reports[11-17] described arecoline- and oxotremorine-based compounds, few studies[18-20] have investigated muscarinic agonists with a spiro-skeleton such as 1 and AF30. Bolliger et al.[20] reported that very strict structural requirements for muscarinic activity exist in a series of analogues of the spirosuccinimide 1. In our synthesis project aimed at producing selective, centrally acting M_1 agonists, we chose 1 as a mother compound because of its selectivity for M_1 receptors over M_2 receptors in in vitro binding studies (Table III)[21] and focused on altering its five-membered imide ring which might be an alternative to the ester group of acetyl choline. In the present paper, we describe the synthesis and biological evaluation of a novel series of spirooxazolidine-2,4-diones and their 2-thioxo analogues (2) including the 4-oxa analogue (**5a**) of 1. Their affinity for M_1 receptors and their antiamnesic effects in scopolamine-treated mice were investigated. Selectivity for M_1 over M_2 receptors, as well as selectivity in in vivo muscarinic effects (antiamnesic effect, hypothermia, and salivation) were also examined for selected compounds. Compound **5a** was found to have more selective antiamnesic effects in three in vivo muscarinic effects than 1 but to be less potent than 1 in affinity for M_1 receptors.

To get information about structure–activity relationships, substituents at 2-, 3-, and 8-positions were altered as shown in Tables I and II according to the following concepts: (1) replacement with a hydrogen atom, lower alkyl groups (R^1 and R^2 in 2) or a sulfur atom (X in 2), and alteration of the spiro-skeleton, the results of which could be compared with those of spirosuccinimide derivatives,[20] (2) substitution with a donor or an acceptor of hydrogen bond such as an ester group, an amide group, or a morpholino group, which were assumed to interact with a hydrogen-bond acceptor or donor which might exist in the vicinity of muscarinic agonist recognition sites, (3) introduction of a substructure of [4-[[*N*-(3-chlorophenyl)-carbamoyl]oxy]-2-butynyl]trimethylammonium chloride (McN-A-343, 3), quaternary M_1 muscarinic agonist,[22] which might improve M_1 selectivity.

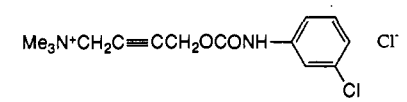

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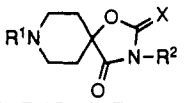
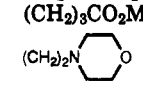
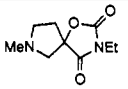
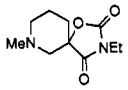
Table I. Structures and Activities for 1-oxa-3,8-diazaspiro[4.5]decan-2,4-diones and Their 2-Thioxo Analogues



compd	R^1	R^2	X	[^{3}H]PZ displacement[a] K_i, μM[c]	antiamnesic effect[b] MED, mg/kg, sc
6a·HCl	Me	H	O	>10 (0%)	>30
5e·HCl	Me	Me	O	16 (16–17)	>30
5a·HCl	Me	Et	O	5.0 (4.5–5.7)	0.3
5b·HCl	Me	t-Bu	O	1.5 (1.4–1.7)	>30
5j	Me	$CH_2CH{=}CH_2$	O	5.1 (4.7–5.6)	>30
5g	Me	CH_2CO_2Me	O	>10 (40%)	>30
5k	Me	CH_2CONH_2	O	>10 (36%)	>30
5h·HCl	Me	$(CH_2)_3CO_2Me$	O	6.4 (5.9–6.9)	>30
5i·2(HCl)	Me		O	>10 (10%)	>30
12	Me	3-Cl-Ph	O	12 (11–13)	0.3
6b·HCl	Me	H	S	>10 (0%)	30
5f	Me	Et	S	0.89 (0.82–0.96)	0.3
5c·HCl	Me	t-Bu	S	0.21 (0.20–0.23)	0.3
5d	Me	Ph	S	4.0 (3.9–4.2)	>30
17a·HCl	H	Et	O	7.8 (6.3–9.6)	0.3
16a·HCl	$PhCH_2$	Et	O	>10 (2%)	30
18·HCl	3-Cl-$PhNHCO_2CH_2C{\equiv}CCH_2$	Et	O	>10 (0%)	>30
19·HCl	$MeOCO(CH_2)_3$	Et	O	>10 (0%)	30
1·HBr				0.46 (0.45–0.46)	0.1

[a] Displacement of [^{3}H]pirenzepine from rat cortex membrane preparations. [b] Improvement of scopolamine-induced impairment of mouse passive avoidance tasks. [c] K_i values were determined by a single experiment performed in triplicate. Values in parentheses are 95% confidence intervals or inhibition rates at 10^{-5} M.

Table II. Structures and Activities for Spiro-3-ethyloxazolidine-2,4-diones

compd	structure	[^{3}H]PZ displacement[a] K_i, μM[c]	antiamnesic effect[b] MED, mg/kg, sc
20·HCl		>10 (1%)	>30
21·HCl		>10 (4%)	>30
5a·HCl		5.0 (4.5–5.7)	0.3

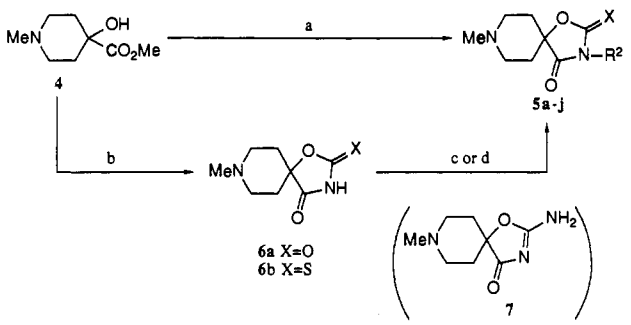
[a–c] See footnotes for Table I.

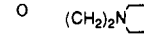
Chemistry

Synthesis of oxazolidine-2,4-dione derivatives has been well studied.[23] Concerning the spirooxazolidine-2,4-diones, Nagai et al.[24] have reported the preparation of 8-benzyl-1-oxa-3,8-diazaspiro[4.5]decane-2,4-dione derivatives. Their synthetic methods were applied to the synthesis of our target compounds.

8-Methyl-1-oxa-3,8-diazaspiro[4.5]decane-2,4-dione derivatives and their 2-thioxo analogues were prepared by the following methods (Scheme I). The known hydroxy ester **4**[25] was treated with ethyl isocyanate in the presence of sodium hydride in tetrahydrofuran at 60 °C to give **5a**, the 4-oxa analogue of RS86, in 90% yield. The same treatment of hydroxy ester **4** with *tert*-butyl isocyanate, *tert*-butyl isothiocyanate, and phenyl isothiocyanate gave the corresponding spiro compounds **5b–d** in good yields. Condensation reaction of hydroxy ester **4** with urea in the presence of sodium ethoxide in ethanol gave 8-methyl-1-oxa-3,8-diazaspiro[4.5]decane-2,4-dione (**6a**) quantitatively. In contrast, the same treatment of **4** with thiourea gave 8-methyl-2-thioxo-1-oxa-3,8-diazaspiro[4.5]decane-4-one (**6b**) in only 12% yield. In this case, 2-amino-8-methyl-1-oxa-3,8-diazaspiro[4.5]decan-2-ene-4-one (**7**) was obtained as a byproduct in 18% yield. 3-Substituted 8-methyl-1-oxa-3,8-diazaspiro[4.5]decane-2,4-diones (**5e,g–j**) were prepared by alkylation of **6a** with appropriate alkyl halides or dialkyl sulfates. 2-Thioxo-4-oxazolidinone **6b** also yielded the desired *N*-alkylated product **5f** in 32% yield by alkylation with diethyl sulfate. Methyl ester **5g** was treated with aqueous ammonia to afford amide **5k** (Table I).

Scheme I[a]

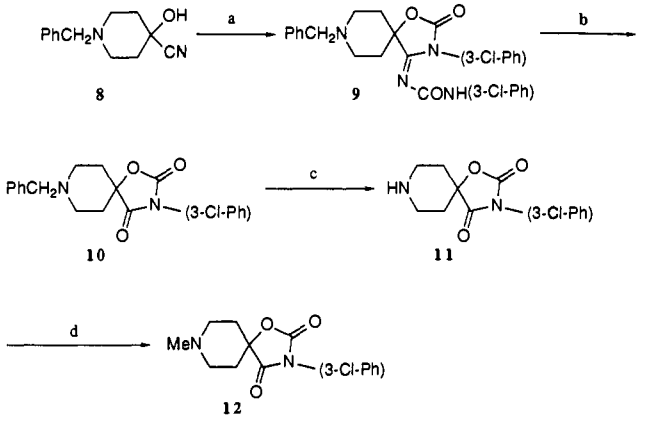


	X	R^2	Method		X	R^2	Method
5a	O	Et	a	**5g**	O	CH_2CO_2Me	d
5b	O	t-Bu	a	**5h**	O	$(CH_2)_3CO_2Me$	d
5c	S	t-Bu	a	**5i**	O		d
5d	S	Ph	a				
5e	O	Me	c	**5j**	O	$CH_2CH{=}CH_2$	d
5f	S	Et	c				

[a] Reagents: (a) NaH, R^2NCO, or R^2NCS; (b) NaOEt, EtOH, NH_2CONH_2, or NH_2CSNH_2; (c) K_2CO_3, $(R^2O)_2SO_2$; (d) NaH, R^2X (X = Cl or Br).

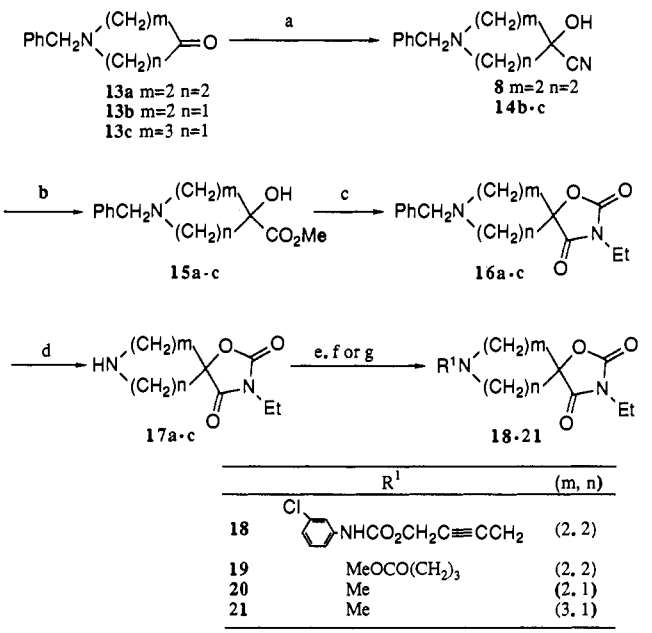
Compound **12** with a 3-chlorophenyl group, which is a substructure of **3**, at the 3-position was prepared by the method[24] reported for 8-benzyl-3-(4-chlorophenyl)-1-oxa-3,8-diazaspiro[4.5]decane-2,4-dione (Scheme II). Treatment of cyanohydrin **8** with 2 equiv of 3-chlorophenyl isocyanate according to Patton's procedure[26] gave 8-benzyl-3-(3-chlorophenyl)-4-[[(3-chlorophenyl)carbamoyl]imino]-1-oxa-3,8-diazaspiro[4.5]decan-2-one (**9**), which was con-

Scheme II[a]



[a] Reagents: (a) 3-ClPhNCO, TEA; (b) concd HCl; (c) H_2, Pd/C; (d) NaH, MeI.

Scheme III[a]



	R[1]	(m, n)
18		(2, 2)
19	$MeOCO(CH_2)_3$	(2, 2)
20	Me	(2, 1)
21	Me	(3, 1)

[a] Reagents: (a) KCN; (b) HCl, MeOH; (c) NaH, EtNCO; (d) H_2, Pd/C; (e) TEA, R^1Cl; (f) HCHO, $NaBH_3CN$; (g) NaH, MeI.

verted to the 4-oxo compound **10** by acidic hydrolysis. Catalytic hydrogenation of **10** over palladium on carbon followed by usual methylation gave compound **12**.

Compound **18** with a 4-[[*N*-(3-chlorophenyl)carbamoyl]-oxy]-2-butynyl group, which is a substructure of **3**, and **19** with a 3-(methoxycarbonyl)propyl group as a hydrogen-bond acceptor at the 8-position were synthesized by alkylation of **17a**[24] with the corresponding alkyl chlorides (Scheme III).

3-Ethyl-7-methyl-1-oxa-3,7-diazaspiro[4.4]nonane-2,4-dione (**20**) and 3-ethyl-7-methyl-1-oxa-3,7-diazaspiro[4.5]-decane-2,4-dione (**21**) were synthesized according to the method for 1-oxa-3,8-diazaspiro[4.5]decane-2,4-dione derivatives (Scheme III). 3-Pyrrolidinone (**13b**) and 3-piperidinone (**13c**) were converted to their respective cyanohydrins under aqueous conditions,[19] and these cyanohydrins were used immediately after extraction because of their instability. The crude cyanohydrins were treated with hydrogen chloride in methanol–ether followed by hydrolysis to afford hydroxy esters **15b** and **15c**. Treatment of **15b** and **15c** with ethyl isocyanate in the presence of sodium hydride gave oxazolidine-2,4-diones **16b** and **16c**, respectively, debenzylation of which was achieved by hydrogenolysis over palladium on carbon to give **17b** and **17c**. Usual methylation of **17b** and **17c** yielded the target compounds **20** and **21**.

Pharmacological Method

Each compound was assessed in in vitro binding assays and observations of in vivo muscarinic effects including hypothermia, salivation, and passive avoidance tasks. The affinities of compounds to cortical M_1 receptors were evaluated in receptor binding studies using [^{3}H]pirenzepine (PZ) as an M_1 selective ligand and rat cerebral cortex as receptor preparations[27] and are shown in Tables I and II as K_i values. As muscarinic agonists are known to induce hypothermia, suggested to be associated with central M_2 receptors,[28] and to increase salivary secretion via peripheral muscarinic receptors, the effects of test compounds on rectal temperature and salivation were observed using mice as described in the Experimental Section. Salivation- and hypothermia-inducing effects were used as indices of cholinergic side effects in anticipation of future clinical use. Antiamnesic effects were evaluated as the ability to ameliorate scopolamine-induced impairments in one-trial passive avoidance tasks using mice. The procedure is described in the Experimental Section.

Compounds exhibiting both high affinity for M_1 receptors and antiamnesic effects were assessed by their affinity for M_2 receptors. The ability of the compounds to displace [^{3}H]quinuclidinyl benzilate (QNB) from rat cerebellum membrane was measured, and the results are presented in Table III. Muscarinic receptors on cerebellum membrane are reported to be mostly of the M_2 subtype.[29] Some compounds were tested for M_1-receptor stimulating activity by recording blood pressure responses in pithed rats as described in the Experimental Section.[22]

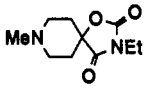
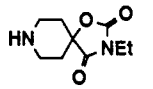
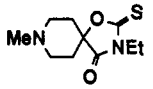
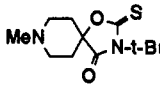
Results and Discussion

Biological results are given in Tables I–III. As none of the compounds exhibiting K_i values of more than 10 μM in [^{3}H]PZ binding experiments induced salivation and hypothermia at doses under 30 mg/kg sc, the results of both tests are shown for selected compounds only in Table III.

The 4-oxa analogue (**5a**) of **1** exhibited both affinity for M_1 receptors in the micromolar range and antiamnesic effects; this compound was, however, 11 times less potent in M_1 receptor binding affinity and three times less potent in antiamnesic effects than **1**. Replacement of the ethyl group at the 3-position with smaller groups (methyl, **5e**; hydrogen, **6a**) decreased affinity for M_1 receptors. Though compounds with bulkier groups (*tert*-butyl, **5b**; allyl, **5j**) at the 3-position seemed to retain M_1 receptor binding affinity, both compounds did not improve scopolamine-induced amnesia. Compound **17a** with a hydrogen atom instead of the methyl group at the 8-position was as potent as **5a** in both tests. Substitution with a benzyl group (**16a**) decreased affinity for M_1 receptors. These strict structural requirements for muscarinic activity in substituents at the 3- and 8-positions of spirooxazolidine-2,4-diones are similar to those for spirosuccinimides as reported by Bolliger et al.[20]

2-Thioxo analogues **5f** and **5c** exhibited higher affinities for M_1 receptors than the corresponding 2-oxo analogues **5a** and **5b**. These findings are consistent with similar observations in spirosuccinimide derivatives where the

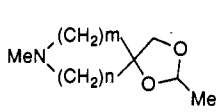
Table III. Pharmacological Profile of Spirooxazolidine-2,4-diones and Spiro-2-thioxooxazolidin-4-ones

compd	structure	binding data: K_i, μM [^{3}H]PZ[b]	binding data: K_i, μM [^{3}H]QNB[c]	M_2/M_1 index[d]	antiamnesic effect[a] MED, mg/kg sc	hypothermia $ED_{\Delta 2°C}$, mg/kg sc	salivation ED_{30mg}, mg/kg sc
5a·HCl		5.0	7.8 (7.0–8.7)	1.5	0.3	>30	30
17a·HCl		7.8	NT[e]		0.3	>30	>30
5f		0.89	4.3 (4.1–4.6)	4.8	0.3	30	30
5c·HCl		0.21	2.1 (1.5–2.9)	10.0	0.3	30	>30
1·HBr		0.46	0.97 (0.95–1.00)	2.1	0.1	1.0	0.5

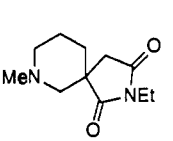
[a,b] See footnotes for Table I. [c] Displacement of [^{3}H]quinuclidinyl benzilate from rat cerebellum membrane. K_i values were determined by a single experiment performed in triplicate. Values in parentheses are 95% confidence intervals. [d] The ratio of QNB/PZ, K_i's. [e] Not tested.

3-thioxo analogue of 1 has higher affinity for M_1 receptors than 1.[20] Compound **5f** with an ethyl group at the 3-position was six times more potent in binding assay than compound **5a** and equipotent in antiamnesic effect to this compound. Compound **5c** with a *tert*-butyl group had the highest affinity for M_1 receptors in this series and was equipotent in antiamnesic effect to compound **5a**. This is in contrast to compound **5b**, the 2-oxo analogue with a *tert*-butyl group described above. Since these in vivo effects would be affected not only by affinity for M_1 receptors but also by efficacy as an M_1 agonist, distribution, and metabolism, etc., further experiments will be necessary for assessment of these factors.

To alter the relative orientation and distance of the nitrogen atom and oxazolidine ring, 1-oxa-3,7-diazaspiro-[4.4]nonane-2,4-dione **20** and 1-oxa-3,7-diazaspiro[4.5]-decane-2,4-dione **21** were synthesized. As shown in Table II, however, both compounds exhibited marked reductions in the affinities and antiamnesic activities. Similar observations to those for compound **21** have been reported for 2,8-dimethyl-1,3-dioxa-8-azaspiro[4.5]decane (**22a**) and



22a m=2 n=2
22b m=3 n=1



23

2,7-dimethyl-1,3-dioxa-7-azaspiro[4.5]decane (**22b**),[19] as well as for 1 and its 2,7-diazaspiro[4.5]decane analogue (**23**).[20] In these cases, 8-azaspiro[4.5]decane analogues (**1**, **5a**, and **22a**) were more potent than the corresponding 7-azaspiro[4.5]decane analogues (**23**, **21**, and **22b**) in binding affinity for muscarinic receptors, respectively. The spiro[4.4]nonane and spiro[4.5]decane structures have not been compared in the literature. Although it is not clear how muscarinic receptors differentiate these close structures, the 8-azaspiro[4.5]decane skeleton seems to fit the fine features of the recognition sites of muscarinic receptors in comparison with the 7-azaspiro[4.5]decane and 7-azaspiro[4.4]nonane skeletons.

Both substitution with a hydrogen-bond donor or acceptor at the 3- or 8-position (**5g–i,k** and **19**) and introduction of substructures of **3** (**5d**, **12**, and **18**) seemed not to increase affinity for M_1 receptors (Table I). The derivatives substituted at the 3-position (**5g,h,k** and **12**) retained some affinity, whereas the 8-substituted derivatives (**18** and **19**) exhibited remarkably decreased affinity. Thus, there seems to be some tolerance for various substituents at the 3-position, while these results do not support the existence of a hydrogen-bond donor or acceptor, which could strengthen the interaction of the ligands and the receptors, in the vicinity of the recognition sites.

Affinities for M_2 receptors and in vivo muscarinic effects of hypothermia and salivation of the four compounds (**5a**, **17a**, **5f,c**) exhibiting both affinity for M_1 receptors and antiamnesic effects are presented in Table III. In receptor binding data, **5f** and **5c** exhibited some selectivity for M_1 receptors (QNB/PZ, K_i's). The selectivity of **5a** was low in comparison with **1**. Compounds **5a,f,c** induced hypothermia, increased salivary secretion, or both at 30 mg/kg sc, with 30-fold less potency than **1**. Dose ratios, $ED_{\Delta 2°C}$ (hypothermia)/MED(antiamnesic effect) and ED_{30mg} (salivation)/MED(antiamnesic effect), indicate therapeutic indices. The ratios of **5a,f,c** were about 100, while those for **1** were 5–10. Thus, **5a,f,c** were more selective for antiamnesic effects than **1**. Compound **17a** was not active in either hypothermia or salivation tests at up to 30 mg/kg sc, suggesting low intrinsic activity of **17a** as a muscarinic agonist. The antiamnesic effects of the four compounds did not parallel their affinities for M_1 receptors. This result may be explained by differences in efficacy as M_1 agonists, distribution, or metabolism. To evaluate the efficacy as M_1 agonists, compounds **5a,f,c** were tested for their effects on blood pressure of pithed rats.[22] Intravenous administration of **5a** and **1** to pithed rats increased blood pressure (**5a**: 24 mmHg increase at 3 mg/kg iv; **1**: 12 mmHg increase at 0.1 mg/kg iv), and these pressor responses were antagonized by PZ (35 μg/kg iv). But the same treatment with **5f** and **5c** had no effect on blood pressure. These results suggest that **5a** and **1** but not **5f** and **5c** have M_1-receptor stimulating activity. The relatively potent antiamnesic activity of **5a** than **5f** and **5c** in view of their M_1-receptor binding affinities may reflect the differences in efficacy. Thus, replacement of the ethyl group at the 3-position with a *tert*-butyl group and that of the oxygen atom at the 2-position with a sulfur atom seem to decrease

efficacy, though these substitutions increased the M_1 selectivity in affinity.

The aim of our research is to identify M_1-selective muscarinic agonists useful in the treatment of SDAT. Compound **5a** seems to fulfill some criteria, though not enough to be sufficient. Compound **5a** has affinity for M_1 receptors, M_1-receptor stimulating activity, and antiamnesic activity separated from hypothermia- and salivation-inducing activity. Though the M_1 selectivity of **5a** based on binding experiments is low, further studies will be necessary to assess the selectivity in M_1- and M_2-receptor stimulating activity. In particular, assays measuring the stimulation of phosphoinositide turnover for M_1 and the inhibition of adenylate cyclase activity for M_2 will help this evaluation.

In summary, we synthesized a series of spirooxazolidine-2,4-diones and found that compound **5a** had muscarinic effects similar to 1 in in vitro and in vivo tests. Structural requirements for M_1 receptor binding in substituents at the 2-, 3-, and 8-positions and in the position of a basic nitrogen atom of the spirooxazolidine-2,4-diones were strict, a finding also obtained for spirosuccinimides.[20] These results suggest that an oxazolidine-2,4-dione represents a bioisostere for the succinimide of 1 in eliciting M_1 agonistic effects and that the 8-azaspiro[4.5]decane skeleton represents a useful template for designing new muscarinic agonists. The finding that compound **5a** and related analogues were more selective for antiamnesic effects in three in vivo cholinergic effects than 1 is interesting from a clinical point of view. Further investigations focused on alteration of the five-membered ring of **5a** to obtain more selective and potent M_1 agonists are currently in progress.

Experimental Section

Chemical Methods. All melting points were determined with a Yanaco MP-3 melting point apparatus and are uncorrected. ^{1}H NMR spectra were measured with either a JEOL FX90Q or a FX100 spectrometer; chemical shifts are recorded in δ units using tetramethylsilane or 3-(trimethylsilyl)propionic acid sodium salt as the internal standard (in NMR description s = singlet, d = doublet, t = triplet, q = quartet, and m = multiplet). Mass spectra were recorded with a Hitachi M-80 and JEOL JMS-DX300 spectrometer. Infrared spectra were recorded on a Hitachi 270-30 infrared spectrophotometer. Where elemental analyses are indicated only by symbols of the elements, analytical results obtained for these elements were within ±0.4% of theoretical values except where otherwise stated. All solutions were dried over anhydrous magnesium sulfate.

3-Ethyl-8-methyl-1-oxa-3,8-diazaspiro[4.5]decane-2,4-dione Hydrochloride (5a·HCl). NaH (60% mineral oil dispersion, 0.29 g, 7.2 mmol) was added portionwise to a mixture of 4-(methoxycarbonyl)-1-methyl-4-piperidinol[25] (**4**, 2.5 g, 14.4 mmol) and ethyl isocyanate (2.05 g, 29 mmol) in 5 mL of anhydrous tetrahydrofuran. The mixture was stirred at 60 °C for 1 h, cooled to 0 °C, neutralized with acetic acid, and concentrated in vacuo. The residue was purified through a silica gel column ($CHCl_3$/MeOH (97/3)) to give **5a** (2.8 g, 13.2 mmol) in a yield of 92%. **5a·HCl**: mp 290–295 °C (MeOH); IR (KBr) 1816, 1730 cm^{-1}; NMR (D_2O) δ 1.24 (t, 3H), 2.32 (m, 4H), 3.00 (s, 3H), 3.20–3.80 (m, 6H); MS *m/e* 212 (M^+), 197, 183. Anal. ($C_{10}H_{16}N_2O_3$·HCl) C, H, N.

Compound **5b,c,d** were prepared according to the method for **5a**.

3-*tert*-Butyl-8-methyl-1-oxa-3,8-diazaspiro[4.5]decane-2,4-dione hydrochloride (5b·HCl): 80% yield; mp 195–200 °C (dec, EtOH); IR (KBr) 1800, 1710, 1460 cm^{-1}; NMR (D_2O) δ 1.56 (s, 9H), 2.10–2.50 (m, 4H), 2.96 (s, 3H), 3.10–3.80 (m, 4H); MS *m/e* 240 (M^+), 225, 196. Anal. ($C_{12}H_{20}N_2O_3$·HCl) C, H, N, Cl.

3-*tert*-Butyl-8-methyl-2-thioxo-1-oxa-3,8-diazaspiro[4.5]decan-4-one hydrochloride (5c·HCl): 15% yield; mp 225–226 °C (dec, MeOH); IR (KBr) 1750, 1470 cm^{-1}; NMR (D_2O) δ 1.76 (s, 9H), 2.10–2.40 (m, 4H), 2.96 (s, 3H), 3.20–3.80 (m, 4H); MS *m/e* 256 (M^+), 199, 167. Anal. ($C_{12}H_{20}N_2O_2S$·HCl) C, H, N.

8-Methyl-3-phenyl-2-thioxo-1-oxa-3,8-diazaspiro[4.5]decan-4-one (5d): 86% yield; mp 171–173 °C ($CHCl_3$–ether); IR (KBr) 1750, 1590 cm^{-1}; MS *m/e* 276 (M^+), 216, 172. Anal. ($C_{14}H_{16}N_2O_2S$) C, H, N.

8-Methyl-1-oxa-3,8-diazaspiro[4.5]decane-2,4-dione Hydrochloride (6a·HCl). A mixture of compound **4** (17.8 g, 103 mmol) and urea (7.16 g, 119 mmol) in EtOH (110 mL) containing sodium ethoxide (9.2 g, 136 mmol) was heated under reflux for 10 h. The mixture was concentrated, and 50 mL of cold water was added to the residue. After the solution was acidified with 5 N HCl, the resulting precipitate was collected and recrystallized from aqueous EtOH to give **6a**·HCl (22.5 g, 102 mmol) in a yield of 99%. **6a**·HCl: mp >300 °C; IR (KBr) 1805, 1730 cm^{-1}; NMR (D_2O) δ 2.20–2.50 (m, 4H), 2.96 (s, 3H), 3.10–3.80 (m, 4H); MS *m/e* 184 (M^+), 169, 155. Anal. ($C_8H_{12}N_2O_3$·HCl·H_2O) C, H, N, Cl.

8-Methyl-2-thioxo-1-oxa-3,8-diazaspiro[4.5]decan-4-one Hydrochloride (6b·HCl). Similar treatment using thiourea as that for **6a** gave compound **6b** in a yield of 12% and 2-amino-8-methyl-1-oxa-3,8-diazaspiro[4.5]deca-2-ene-4-one (**7**) in a yield of 18%. **6b**·HCl: mp 275–278 °C (MeOH); IR (KBr) 1740, 1440 cm^{-1}; NMR (D_2O) δ 2.20–2.60 (m, 4H), 3.00 (s, 3H), 3.10–3.90 (m, 4H); MS *m/e* 200 (M^+), 139, 96. Anal. ($C_8H_{12}N_2O_2S$·HCl) C, H, N, Cl. The structure of **7** was confirmed by comparison with an authentic sample prepared by the reported method.[30] **7**·HCl: mp >300 °C. Anal. ($C_8H_{14}N_3O_2$·HCl·0.5H_2O) C, H, N, Cl.

3,8-Dimethyl-1-oxa-3,8-diazaspiro[4.5]decane-2,4-dione Hydrochloride (5e·HCl). A mixture of **6a** (1.32 g, 6.0 mmol) and K_2CO_3 (3.31 g, 24 mmol) in acetone (20 mL) was stirred for 2 h at room temperature, and dimethyl sulfate (0.76 g, 6 mmol) was added dropwise. The resulting mixture was heated under reflux for 3.5 h and cooled to room temperature. The precipitate was removed by filtration, and the filtrate was concentrated. The residue was treated with methanolic HCl and recrystallized from MeOH–ether to give **5e**·HCl (0.56 g, 40% yield) as a crystal: mp 295–300 °C dec; IR (KBr) 1805, 1720 cm^{-1}; NMR (D_2O) δ 2.20–2.50 (m, 4H), 2.96 (s, 3H), 3.08 (s, 3H), 3.30–3.80 (m, 4H); MS *m/e* 198 (M^+), 183, 169. Anal. ($C_8H_{14}N_2O_3$·HCl) C, H, N.

3-Ethyl-8-methyl-2-thioxo-1-oxa-3,8-diazaspiro[4.5]decan-4-one (5f) was prepared according to the method for **5e** in 31% yield. **5f**: mp 88–89 °C (ether–*n*-hexane); IR (KBr) 1750 cm^{-1}; NMR ($CDCl_3$) δ 1.28 (t, 3H), 2.36 (s, 3H), 1.60–3.00 (m, 8H except the peak at 2.36), 3.87 (q, 2H); MS *m/e* 228 (M^+), 213, 167. Anal. ($C_{10}H_{16}N_2O_2S$) C, H, N.

8-Methyl-3-(2-morpholinoethyl)-1-oxa-3,8-diazaspiro[4.5]decane-2,4-dione Hydrochloride (5i·HCl). Compound **6a** (1.84 g, 10 mmol) was added to a cooled suspension of NaH (60% mineral oil dispersion, 0.8 g, 20 mmol) in DMF (20 mL). After the mixture was stirred at room temperature for 45 min, 4-(2-chloroethyl)morpholine hydrochloride (1.86 g, 10 mmol) was added portionwise. The mixture was stirred at room temperature overnight and at 60 °C for 2.5 h. After the mixture was cooled, the precipitate was removed by filtration. The filtrate was concentrated and purified through a silica gel column ($CHCl_3$/MeOH/27% aqueous NH_3 (90/10/1)) to give **5i** (2.0 g, 6.7 mmol) in a yield of 67%. **5i**·HCl: mp >290 °C (H_2O–EtOH); IR (KBr) 1820, 1730 cm^{-1}; NMR (D_2O) δ 2.30–2.50 (m, 4H), 2.96 (s, 3H), 3.20–3.80 (m, 10H), 3.90–4.10 (m, 6H); MS *m/e* 297 (M^+), 253, 227. Anal. ($C_{14}H_{23}N_3O_4$·2HCl) C, H, N, Cl.

Compounds **5g,h,j** were prepared according to the method for **5i**.

Methyl 2-[2,4-dioxo-8-methyl-1-oxa-3,8-diazaspiro[4.5]decan-3-yl]acetate (5g): 49% yield; mp 65–66 °C (ether–*n*-hexane); IR (KBr) 1805, 1750, 1730 cm^{-1}; NMR ($CDCl_3$) δ 2.36 (s, 3H), 1.70–3.00 (m, 8H except the peak at 2.36), 3.80 (s, 3H), 4.28 (s, 2H); MS *m/e* 257, 241, 225. Anal. ($C_{11}H_{16}N_2O_5$) C, H, N.

Methyl 2-[2,4-dioxo-8-methyl-1-oxa-3,8-diazaspiro[4.5]decan-3-yl]butyrate hydrochloride (5h·HCl): 22% yield; mp 213–215 °C (EtOH); IR (KBr) 1810, 1795, 1710 cm^{-1}; NMR (D_2O) δ 1.80–2.70 (m, 8H), 3.00 (s, 3H), 3.72 (s, 3H), 3.10–3.90 (m, 6H

except the peak at 3.72); MS *m/e* 284 (M^+), 269, 253. Anal. ($C_{18}H_{20}N_2O_5$·HCl) C, H, N, Cl.

3-Allyl-8-methyl-1-oxa-3,8-diazaspiro[4.5]decane-2,4-dione (5j): 19% yield; mp 64–66 °C (ether–*n*-hexane); IR (Nujol) 1810, 1730 cm^{-1}; NMR ($CDCl_3$) δ 1.60–3.00 (m, 11H), 4.10 (m, 2H), 5.10–5.40 (m, 2H), 5.60–6.00 (m, 1H); MS *m/e* 224, 209, 195. Anal. ($C_{11}H_{16}N_2O_3$) C, H, N (calcd, 12.49%; found, 11.92%).

2,4-Dioxo-8-methyl-1-oxa-3,8-diazaspiro[4.5]decan-3-ylacetamide (5k). 27% aqueous NH_3 (1 mL) was added to a solution of compound **5g** (0.63 g, 2.46 mmol) in MeOH (20 mL) cooled in a cold water bath. After being stirred for 4 days at room temperature, the mixture was concentrated and purified by a silica gel column ($CHCl_3$/MeOH (4/1)) to give **5k** (0.36 g, 61% yield) as a crystal: mp 191–192 °C; IR (KBr) 1810, 1740, 1690 cm^{-1}; NMR (CD_3OD) δ 2.32 (s, 3H), 1.80–3.10 (m, 8H except the peak at 2.32), 4.16 (s, 2H); MS *m/e* 241 (M^+), 212, 196. Anal. ($C_{10}H_{15}N_3O_4$) C, H, N.

3-(3-Chlorophenyl)-1-oxa-3,8-diazaspiro[4.5]decane-2,4-dione Hydrochloride (11·HCl) was prepared according to the method reported for 3-(4-chlorophenyl)-1-oxa-3,8-diazaspiro[4.5]-decane-2,4-dione via three intermediates from 1-benzyl-4-cyano-4-piperidinol.[24]

(i) 8-Benzyl-3-(3-chlorophenyl)-4-[[(3-chlorophenyl)carbamoyl]imino]-1-oxa-3,8-diazaspiro[4.5]decan-2-one (9): 59% yield; mp 206–207 °C; IR (KBr) 1780, 1700, 1670, 1590 cm^{-1}. Anal. ($C_{27}H_{24}N_4O_3Cl_2$) C, H, N.

(ii) 8-Benzyl-3-(3-chlorophenyl)-1-oxa-3,8-diazaspiro[4.5]-decane-2,4-dione hydrochloride (10·HCl): 99% yield; mp 269–273 °C (MeOH); IR (KBr) 1810, 1730, 1590, 1580 cm^{-1}. Anal. ($C_{20}H_{19}N_2O_3Cl$·HCl) C, H, N, Cl.

(iii) 3-(3-Chlorophenyl)-1-oxa-3,8-diazaspiro[4.5]decane-2,4-dione hydrochloride (11·HCl): 92% yield; mp 296–299 °C (MeOH); IR (KBr) 1810, 1740, 1590 cm^{-1}. Anal. ($C_{18}H_{13}N_2O_3$-Cl·HCl) C, H, N, Cl.

3-(3-Chlorophenyl)-8-methyl-1-oxa-3,8-diazaspiro[4.5]-decane-2,4-dione (12). Compound 11·HCl (1.27 g, 4.0 mmol) was added portionwise to a suspension of NaH (60% mineral oil dispersion, 320 mg, 8.0 mmol) in DMF (20 mL) at 0 °C. After the mixture was stirred for 20 min at room temperature, a solution of methyl iodide (0.63 g, 4.4 mmol) in DMF (3 mL) was added below 30 °C. The mixture was stirred for 4 h and concentrated. Purification of the residue by a silica gel column ($CHCl_3$/MeOH (97/3)) gave compound **12** (0.56 g, 48% yield) as a crystal: mp 145–146 °C (ether); IR (KBr) 1810, 1740, 1590 cm^{-1}; NMR ($CDCl_3$) δ 2.40 (s, 3H), 1.80–3.00 (m, 8H), 7.36–7.56 (m, 4H); MS *m/e* 294 (M^+), 250, 123. Anal. ($C_{14}H_{15}N_2O_3Cl$) C, H, N.

Compounds **18** and **19** were synthesized by alkylation of 3-ethyl-1-oxa-3,8-diazaspiro[4.5]decane-2,4-dione (**17a**)[24] with yields of 76% and 48%, respectively, using corresponding alkyl chlorides and triethylamine according to the method for compound **12**.

4-(2,4-Dioxo-3-ethyl-1-oxa-3,8-diazaspiro[4.5]decan-8-yl)-2-butynyl *N*-(3-chlorophenyl)carbamate hydrochloride (18·HCl): mp 166–172 °C (MeOH–ether); IR (KBr) 1810, 1720, 1590 cm^{-1}; NMR ($CDCl_3$) δ 1.28 (t, 3H), 3.40 (m, 2H), 3.60 (q, 2H), 4.84 (m, 2H), 6.80–7.60 (m, 5H); MS *m/e* 420 (M^+), 374, 303. Anal. ($C_{20}H_{22}N_3O_5Cl$·HCl) C, H, N.

Methyl 4-(2,4-dioxo-3-ethyl-1-oxa-3,8-diazaspiro[4.5]decan-8-yl)butylate Hydrochloride (19·HCl): mp 233–235 °C (dec, MeOH–ether); IR (KBr) 1800, 1720 cm^{-1}; NMR (D_2O) δ 1.24 (t, 3H), 1.90–2.70 (m, 8H), 3.76 (s, 3H), 3.10–3.90 (m, 8H); MS *m/e* 298 (M^+), 283, 267. Anal. ($C_{14}H_{22}N_2O_5$·HCl) C, H, N.

3-Ethyl-7-methyl-1-oxa-3,7-diazaspiro[4.4]nonane-2,4-dione Hydrochloride (20·HCl). (i) A solution of sodium bisulfite (5.41 g, 52 mmol) in water (15 mL) was added to a solution of 1-benzyl-3-pyrrolidinone (**13b**, 7.0 g, 22 mmol) and potassium cyanide (3.9 g, 60 mmol) in water (14 mL) at 0 °C. The mixture was stirred for 1 h and extracted with $CHCl_3$. The extract was dried and concentrated in vacuo to give the crude 1-benzyl-3-cyano-3-pyrrolidinol (**14b**, 8.23 g). A mixture of MeOH (175 mL) and anhydrous ether (175 mL) was saturated with dry HCl gas at 0 °C and cooled to −70 °C. A solution of compound **14b** in MeOH was added to the mixture at −70 °C, and the mixture was stirred overnight at 4 °C, concentrated, and poured into cold water under stirring. The solution was basified with aqueous NaOH, extracted with $CHCl_3$, dried, and concentrated. The residue was purified through a silica gel column ($CHCl_3$/MeOH (10/1)) to give 1-benzyl-3-(methoxycarbonyl)-3-pyrrolidinol (**15b**, 3.25 g, 14 mmol, 64% from **13b**) as an oil: IR (neat) 1738 cm^{-1}; NMR ($CDCl_3$) δ 1.85–2.11 (m, 2H), 2.26–3.05 (m, 4H), 3.71 (s, 2H), 3.82 (s, 3H), 7.32 (s, 5H); MS *m/e* 235 (M^+), 176.

(ii) Compound **15b** (1.4 g, 6.4 mmol) was treated according to the procedure described for compound **5a** to give 7-benzyl-3-ethyl-1-oxa-3,7-diazaspiro[4.4]nonane-2,4-dione (**16b**, 1.74 g, 99%) as an oil: IR (neat) 1816, 1738 cm^{-1}; NMR ($CDCl_3$) δ 1.24 (t, 3H), 2.23–2.46 (m, 2H), 2.56–3.20 (m, 1H), 3.60 (q, 2H), 3.70 (s, 2H), 7.32 (s, 5H); MS *m/e* 274 (M^+), 230, 183.

(iii) Compound **16b** (2.15 g, 9.1 mmol) in MeOH (50 mL) was catalytically hydrogenated with 10% palladium on carbon (410 mg) under atmospheric pressure. After removal of the catalyst by filtration, the solvent was evaporated away in vacuo. The resulting residue was purified through a silica gel column ($CHCl_3$/MeOH (9/1)) to give 3-ethyl-1-oxa-3,7-diazaspiro[4.4]nonane-2,4-dione (**17b**, 0.92 g, 55%) as a crystal: mp 75–76 °C; MS *m/e* 184 (M^+), 139. Anal. ($C_8H_{12}N_2O_3$) C, H, N.

(iv) Sodium cyanoborohydride (318 mg, 5 mmol) was added portionwise to a solution of compound **17b** (0.62 g, 3.4 mmol) and 37% aqueous formaldehyde (1.26 mL) in acetonitrile (10 mL). After being stirred for 30 min at room temperature, the mixture was neutralized with acetic acid, stirred for 1 h, and evaporated in vacuo. The residue was dissolved in saturated aqueous $NaHCO_3$, extracted with ether, dried, and concentrated in vacuo. The residual oil was purified through a silica gel column ($CHCl_3$/MeOH (95/5)) to give compound **20** (257 mg, 38% yield) as an oil. **20**·HCl: mp 194–196.5 °C; IR (KBr) 1820, 1736 cm^{-1}; NMR ($CDCl_3$) δ 1.30 (t, 3H), 2.58–2.80 (m, 2H), 3.05 (s, 3H), 3.66 (q, 2H), 3.24–4.20 (m, 4H); MS *m/e* 198 (M^+), 154. Anal. ($C_9H_{14}N_2O_3$·HCl) C, H, N, Cl.

3-Ethyl-7-methyl-1-oxa-3,7-diazaspiro[4.5]decane-2,4-dione hydrochloride (21·HCl) was prepared according to the method for compound **20** via the following intermediates. (i) 1-Benzyl-3-(methoxycarbonyl)-3-piperidinol (**15c**): 94% yield; IR (neat) 1738 cm^{-1}; NMR ($CDCl_3$) δ 1.50–1.90 (m, 4H), 1.90–2.30 (m, 2H), 2.70–3.00 (m, 2H), 3.56 (s, 2H), 3.76 (s, 3H), 7.30 (s, 5H); MS *m/e* 249 (M^+), 231, 190.

(ii) 7-Benzyl-3-ethyl-1-oxa-3,7-diazaspiro[4.5]decane-2,4-dione (**16c**): 60% yield. **16c**·HCl: mp 186–190 °C (CH_2Cl_2–ether); IR (KBr) 1822, 1732 cm^{-1}; NMR ($CDCl_3$) δ 1.24 (t, 3H), 1.80–2.70 (m, 4H), 2.90–4.00 (m, 6H), 4.50 (broad s, 2H), 7.44 (m, 3H), 7.70 (m, 2H); MS *m/e* 288 (M^+), 245, 197. Anal. ($C_{18}H_{20}N_2O_3$·HCl) C, H, N.

(iii) 3-Ethyl-7-methyl-1-oxa-3,7-diazaspiro[4.5]decane-2,4-dione hydrochloride (**21**·HCl). The crude compound **17c** obtained by catalytic hydrogenation of compound **16c** was methylated according to the method for compound **12** to give **21** in 35% yield. **21**·HCl: mp 215–225 °C; IR (KBr) 1826, 1736 cm^{-1}; NMR (D_2O) δ 1.22 (t, 3H), 2.12 (m, 4H), 2.92 (s, 3H), 3.00–3.90 (m, 6H); MS *m/e* 212 (M^+), 168, 124. Anal. ($C_{10}H_{16}N_2O_3$·HCl) C, H, N.

Biological Methods. Doses are expressed in terms of the free base. The following were obtained commercially: scopolamine hydrochloride (Tokyo Kasei Co., Ltd.), [^{3}H]pirenzepine, and [^{3}H]quinuclidinyl benzilate (Du Pont-New England Nuclear).

Receptor Binding Assay. Membrane preparation and tritium-labeled ligand receptor binding assay were described previously.[21] The cerebral cortex and the cerebellum from male Wistar rats were homogenized separately in 0.32 M ice-cold sucrose (1:10 w/v) using a motor-driven Teflon/glass homogenizer. The homogenates were centrifuged at 900*g* for 10 min at 4 °C. The supernatants were then recentrifuged at 11500*g* for 20 min at 4 °C. The pellets thus obtained were washed twice in 5 mM Tris–HCl buffer (pH 7.4) by resuspension and recentrifugation. Membranes were stored at −80 °C until required.

[^{3}H]PZ binding for M_1 receptors was performed according to the method of Watson et al.[27] Frozen rat cerebral cortex membrane was resuspended in an assay buffer (8.3 mM Tris–HCl, 1.25 mM $MnCl_2$, pH 7.4). The membrane suspension, corresponding to 250 μg of protein determined by the method of Lowry with bovine serum albumin as the standard, was incubated with approximately 1.0 nM [^{3}H]PZ at 25 °C for 60 min. Test compounds were added in a volume of 50 μL to give a final assay volume of 1.0 mL. Nonspecific binding was determined using 10 μM atropine. Assays were terminated by rapid filtration under vacuum through a Whatman GF/B filter. The filters were washed

immediately four times with 4 mL each of 50 mM Tris–HCl and 120 mM NaCl buffer (pH 7.4). The filter was placed in a scintillation vial to which 10 mL of NEN Aquasol-2 cocktail was added. Radioactivity retained on the filter was determined by liquid scintillation spectrometry. All receptor binding assays were performed in triplicate. Competition binding data were analyzed by logit-log analysis using an RS1 package (BBN Software Products Corp.) to provide the IC_{50}. The IC_{50} values were corrected for receptor occupancy by [^{3}H]PZ as described by Cheng and Prusoff[31] to give K_i values (concentration of nonlabeled ligand that causes half-maximal receptor occupancy in the absence of [^{3}H]PZ).

[^{3}H]QNB binding for M_2 receptors was carried out according to the method of Yamamura et al.[32] The incubation mixture consisted of 50 mM Na^+/K^+ phosphate buffer (pH 7.4), 0.06 nM [^{3}H]QNB, 150 μg protein of resuspended rat cerebellum membrane, and test drug in a total volume of 4.0 mL. Nonspecific binding was defined with 10 μM atropine. The mixture was incubated for 60 min at 25 °C, and the incubation was terminated by filtration over Whatman GF/B glass filters. The filters were washed three times with 3 mL of 50 mM Na^+/K^+ phosphate buffer (pH 7.4). The estimations of filter-bound radioactivity and the data analyses were similar to those for [^{3}H]PZ binding.

Passive Avoidance Tasks with Scopolamine-Treated Mice. Two groups of 10 male ICR mice (Japan SLC. Inc.) each were used: a scopolamine/vehicle control group and a scopolamine/test compound group. The test compound or vehicle was administered subcutaneously 30 min before the training session simultaneously with scopolamine (1 mg/kg ip). In the training session, each mouse was placed in the light box of a two-compartment passive avoidance apparatus (O'hara Co., Ltd.). When the mouse entered the dark compartment, a foot-shock (60–70 V AC) was applied for 1 s through the metal grid bars of the floor. Retention was assessed 24 h after the training session. Latency to enter the dark compartment was measured up to a maximum cut-off of 300 s. When the latencies of a test compound group were significantly different ($p < 0.05$) from those of a control group by the Mann–Whitney U-test, the compound was evaluated as active.

Each compound was initially tested at 0.3, 3, and 30 mg/kg. When a compound was active at one of these doses, the compound was retested at a 3-fold lower dose and the minimal dose required to improve the impairment of the passive avoidance tasks (MED) thereby determined.

Hypothermia. Male ICR mice (Japan SLC. Inc.) were used. Before and 30 min after the subcutaneous administration of a test compound, rectal temperature was measured with a thermoprobe (MC-111, Omron) and the dose required to reduce the rectal temperature by 2 °C ($ED_{\Delta 2°C}$) was determined. Each test compound was initially tested at 30 mg/kg; the dose was then consecutively decreased to one-third of the preceding dose until hypothermia over 2 °C was not observed. Each dose group contained 3–6 mice.

Salivation. Male ICR mice (Japan SLC. Inc.) were used. Saliva was collected 15 min and 45 min after the subcutaneous administration of a test compound. The dose producing more than 30 mg of saliva as a total amount (ED_{30mg}) was determined in the same manner as in hypothermia. Each dose group contained 3–6 mice.

Blood Pressure Responses in Pithed Rats. Male Wister rats weighing ca. 200–300 g were anesthetized with diethyl ether. The left femoral vein was cannulated for the administration of drugs. Arterial blood pressure was measured from the cannulated left common carotid artery. After catheterization of the trachea, the rats were pithed with a steel rod and artificial respiration with room air was provided (1 mL/100 g of body weight, 60 strokes/min). Body temperature was carefully monitored with a rectal thermometer and was maintained at 37 °C by an overhead heating lamp. All drugs were dissolved in 0.9% saline and given iv.

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