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BINDING AFFINITIES OF 3-(3-PHENYLISOXAZOL-5-YL)METHYLIDENE-1-AZABICYCLES TO ACETYLCHOLINE RECEPTORS

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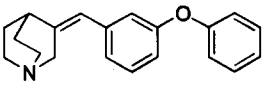
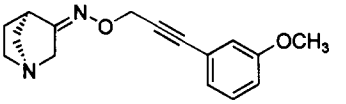
Abstract: A series of 3-(3-phenylisoxazol-5-yl)methylidene-1-azabicycles synthesized showed different binding characteristics to acetylcholine receptors depending on the substituents on the phenyl ring. Small polar substituents gave preferential binding affinity to nicotinic receptors, and large hydrophobic substituents to muscarinic receptors.

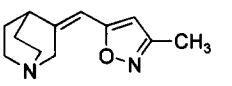
Acetylcholine receptors have been attracting the attention of scientists as therapeutic targets in the treatment of neural disorders such as Alzheimer's disease, Parkinson's disease and Tourette's syndrome. As ligands acting on the acetylcholine receptors, various 1-azabicyclic compounds were prepared and evaluated. KST-5452 (**1**)[1] and CI-1017 (**2**),[2] muscarinic M_1 agonists, and the compound of Novo Nordisk **3**,[3] a nicotinic agonist, are such examples.



KST-5452 (**1**)
(Kanegafuchi)



CI-1017 (**2**)
(Parke-Davis)



3
(Novo Nordisk)

From the structures of the above compounds, we found that muscarinic agonists have rather longer linear structures as compared to the compound of Novo Nordisk **3**, a nicotinic agonist. Separately we were working on the 1-azabicyclic compounds bearing isoxazolyl substituents, and became curious about what the biological outcome of the compound of Novo Nordisk **3** would be when its structure was lengthened. The 3-methyl group of the isoxazole was substituted by a phenyl group, and the effect of substituents on the phenyl ring given to the binding affinity to acetylcholine receptors was investigated. In the present report the synthesis and *in vitro*

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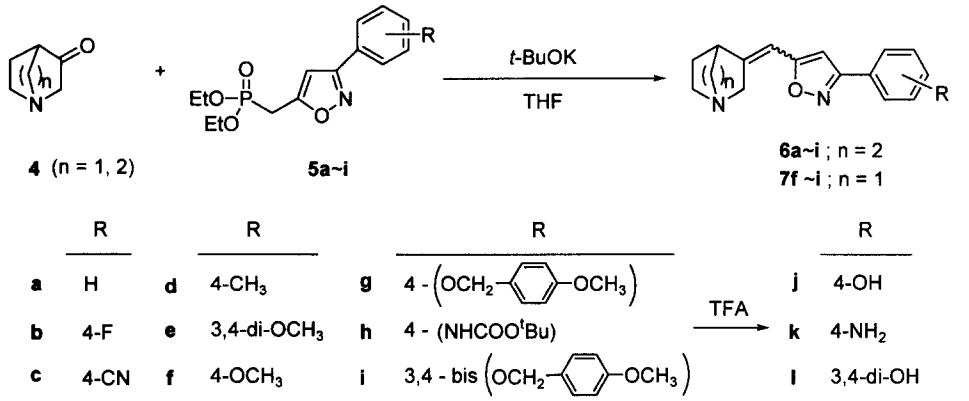
binding affinities of 3-(3-phenylisoxazol-5-yl)methylidene-1-azabicycles to muscarinic and nicotinic receptors are described.

Chemistry: Preparation of 3-(3-phenylisoxazol-5-yl)methylidene-1-azabicycles, the compounds **6a~i** (n=2) and **7f~i** (n=1), was made *via* Horner-Emmons reaction of 1-azabicyclic ketone **4** with various 3-phenylisoxazol-5-ylmethylphosphonates **5a~i** in the presence of potassium *tert*-butoxide (Scheme 1). The resulting regioisomeric mixtures (*Z*:*E* = 6:4 ~ 7:3) were separated by flash column chromatography to give respective isomers. The relative configuration of each isomer was assigned by the NOE ^{1}H NMR experiment. The ketone **4**, which was synthesized according to a known method,[4] was a racemate when n = 1 and thus each of the products (*Z*)-**7** and (*E*)-**7** was a racemate. Further reaction with trifluoroacetic acid added to the compounds **6g~i** and **7g~i** yielded **6j~l** and **7j~l**, respectively (**g** corresponds to **j**, **h** to **k**, and **i** to **l**).

Scheme 1. The synthesis of 3-(3-phenylisoxazol-5-yl)methylidene-1-azabicycles[5]



Receptor Binding Study: The abilities of the compounds to displace [^{3}H]*N*-methylscopolamine, a muscarinic receptor antagonist, and [^{3}H]cytisine, a nicotinic receptor agonist, from each receptor were determined[6] by methods described previously with some modification.[7-10]

The inhibition percentages of the compounds **6** were first measured. The results are summarized in Table 1. The electronic effect of the substituent R did not seem to affect the binding affinity significantly to muscarinic receptors. Instead, the compounds **6g** and **6i** bearing a large substituent R showed considerable binding affinities to the M_1 receptor, indicating a certain role of the *p*-methoxybenzyl group. They did not show prominent selectivity for the subtypes of muscarinic receptors.

For nicotinic receptors the tendency was reversed. The compounds having a small polar substituent except hydrogen and fluoro, *e.g.* cyano, methoxy, hydroxy or amino group, exhibited far better binding affinities than the compounds **6g** and **6i**, which did not bind at all. Apparently the size of the substituent R, *tert*-

Table 1. Displacement of radiolabeled compounds by **6** (n=2) from acetylcholine receptors (%)

Compound	R	Muscarinic receptor[a] [^{3}H]*N*-methylscopolamine						Nicotinic receptor[b] [^{3}H]cytisine
		M_1 site		M_2 site		M_3 site		
		1 μM	10 μM	1 μM	10 μM	1 μM	10 μM	10 μM
(*Z*)-**6a**	H	0	13	3	26	5	17	-
(*E*)-**6a**		7	51	0	38	12	48	48
(*Z*)-**6b**	4-F	0	0	13	22	0	10	19
(*E*)-**6b**		0	6	-	-	-	-	0
(*Z*)-**6c**	4-CN	2	22	6	32	9	31	83
(*E*)-**6c**		11	61	0	23	1	26	92
(*Z*)-**6d**	4-CH_3	0	0	0	13	0	0	26
(*E*)-**6d**		7	7	2	14	3	15	70
(*Z*)-**6e**	3,4-di-OMe	3	22	14	22	0	7	58
(*E*)-**6e**		28	81	30	71	6	46	90
(*Z*)-**6f**	4-OMe	0	21	10	19	3	18	25
(*E*)-**6f**		23	79	16	63	14	63	68
(*Z*)-**6g**	4-(OCH_2-Ph-OCH_3)	77	64	55	46	31	37	0
(*E*)-**6g**		89	93	26	83	22	52	0
(*Z*)-**6h**	4- (NHCOO^{t}Bu)	12	59	14	36	5	41	64
(*E*)-**6h**		57	95	16	67	31	87	43
(*Z*)-**6i**	3,4-(OCH_2-Ph-OCH_3)$_2$	85	100	49	92	76	98	0
(*E*)-**6i**		85	100	27	89	44	94	0
(*Z*)-**6j**	4-OH	0	2	0	7	0	0	72
(*E*)-**6j**		6	65	3	17	3	14	93
(*Z*)-**6k**	4-NH_2	0	4	0	5	0	0	77
(*E*)-**6k**		33	86	5	30	1	19	94
(*Z*)-**6l**	3,4-di-OH	0	8	0	7	0	0	88
(*E*)-**6l**		13	59	12	43	5	39	95

a: Human recombinant muscarinic M_1, M_2 and M_3 receptors expressed in CHO cell were used and 1 μM atropine was used for non-specific binding with 1 nM [^{3}H] *N*-methylscopolamine as a radioligand. The extent of displacement by each compound was measured at 1 μM and 10 μM concentrations. b: Synaptic membrane fractions prepared from rat cerebral cortices were used and 10 μM (-)-nicotine was used for non-specific binding with 1.25 nM [^{3}H]cytisine as a radioligand. The extent of displacement by each compound was measured at 10 μM concentration.

butoxycarbonylamino group, of the compound **6h** must be in between the sizes suitable for the two kinds of receptors, large and small. It bound to both muscarinic and nicotinic receptors though not on high levels. Concerning the stereochemistry of the compounds, the (*E*)-isomer was favored over the (*Z*)-isomer for both classes of acetylcholine receptors except for a few cases.

Changing the 1-azabicycle moiety from 1-azabicyclo[2.2.2]octane to 1-azabicyclo[2.2.1]heptane did not alter the binding tendency of the compounds. Binding affinities of the compounds **7f** ~ **7l** (n=1) to acetylcholine receptors are summarized in Table 2. As was the case with the compound **6**, the compounds **7g** and **7i** containing the *p*-methoxybenzyl substituent showed noticeable binding affinities to muscarinic receptors. For these two compounds, the (*Z*)-isomer was more effective than the (*E*)-isomer in binding to the M_1 receptor.

The IC_{50} values of the selected compounds for the M_1 receptor and nicotinic receptors were measured. The results are summarized in Table 3 and Table 4, respectively. For the muscarinic M_1 receptor, the compound (*Z*)-**7i** showed the highest binding affinity (IC_{50} = 114 nM) of the compounds prepared. It was comparable to pirenzepine (IC_{50} =119 nM), a selective antagonist for the M_1 receptor. Interestingly, the compound (*Z*)-**7i** was

Table 2. Displacement of radiolabeled compounds by **7** (n=1) from acetylcholine receptors (%)

Compound	R	Muscarinic receptor[a] [^{3}H]*N*-methylscopolamine						Nicotinic receptor[b] [^{3}H]cytisine
		M_1 site		M_2 site		M_3 site		
		1 μM	10 μM	1 μM	10 μM	1 μM	10 μM	10 μM
(*Z*)-**7f**	4-OMe	0	34	7	24	0	2	55
(*E*)-**7f**		8	45	15	19	0	0	58
(*Z*)-**7g**	4-(OCH_2-Ph-OCH_3)	69	84	-	-	-	-	0
(*E*)-**7g**		39	66	-	-	-	-	0
(*Z*)-**7h**	4-(NHCOO^{t}Bu)	3	53	10	53	0	53	75
(*E*)-**7h**		0	58	3	23	26	35	74
(*Z*)-**7i**	3,4-(OCH_2-Ph-OCH_3)$_2$	87	100	77	95	88	98	0
(*E*)-**7i**		39	91	24	78	9	82	0
(*Z*)-**7j**	4-OH	0	0	1	10	10	20	36
(*E*)-**7j**		0	26	6	3	0	11	81
(*Z*)-**7k**	4-NH_2	0	2	0	8	18	50	-
(*E*)-**7k**		7	44	1	4	0	10	49
(*Z*)-**7l**	3,4-di-OH	0	0	0	0	0	0	-
(*E*)-**7l**		0	16	6	9	0	0	64

a, b: See the corresponding footnotes in Table 1.

Table 3. IC_{50} of selected compounds for the M_1 receptor[a]

Compound	(*Z*)-**6g**	(*E*)-**6g**	(*Z*)-**6h**	(*E*)-**6h**	(*Z*)-**6i**	(*E*)-**6i**	(*Z*)-**7i**	(*E*)- **7i**	**3**	pirenzepine
IC_{50} (nM)	809 ± 78	250 ± 40	$>10^4$	806 ± 36	260 ± 26	247 ± 11	114 ± 20	1,228 ± 89	$\sim10^4$	119 ± 19

a: Results are expressed as the mean ± SEM (n=4)

Table 4. IC_{50} of selected compounds for nicotinic receptors[a]

Compound	(*Z*)-**6c**	(*E*)-**6c**	(*E*)-**6j**	(*E*)-**6k**	(*E*)- **6l**	**3**	nicotine
IC_{50} (nM)	$>10^3$	803 ± 34	695 ± 23	709 ± 32	486 ± 32	12 ± 2	16 ± 3

a: Results are expressed as the mean ± SEM (n=3)

11 times more effective in binding than (*E*)-**7i**, while binding affinities of the isomers of **6i** were similar to each other.

The compounds prepared in this study were not effective in binding to nicotinic receptors. The compound (*E*)- **6l** showed the highest binding affinity (IC_{50} = 490 nM) to nicotinic receptors, with the value 40 times less effective than the compound of Novo Nordisk **3**, a nicotinic agonist.

Although 3-(3-phenylisoxazol-5-yl)methylidene-1-azabicycles prepared did not show excellent binding affinities to acetylcholine receptors, they exhibited a specific binding tendency according to the substituent R on the phenyl group. Small polar substituents gave preferential binding affinity to nicotinic receptors, while large hydrophobic substituents to muscarinic receptors.

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5. A typical experimental procedure is as follows: to a stirred solution of potassium *t*-butoxide (215 mg, 1.92 mmol) in dry tetrahydrofuran (3 mL) was added diethyl [3-{4-(4-methoxybenzyloxy)phenyl}isoxazol-5-yl]methylphosphonate (**5g**, 827 mg, 1.92 mmol) in dry tetrahydrofuran (3 mL) for 10 min at 23 °C. After stirring for 30 min, a solution of 3-quinuclidinone (200 mg, 1.60 mmol) in dry tetrahydrofuran (3 mL) was added dropwise for 10 min. The reaction mixture was stirred at 23 °C for 1 h and evaporated under reduced pressure. Then the residue was treated with water (15 mL) and extracted with ethyl acetate (3 x 10 mL), and the combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered and evaporated. The resulting residue was purified by column chromatography on silica (eluent: ethyl acetate/methanol/43% ammonium hydroxide in water: 3/1/2%) to afford (*Z*)-3-{4-(4-methoxybenzyloxy)phenyl}isoxazol-5-yl]methylidene-1-azabicyclo[2.2.2]octane ((*Z*)-**6g**, 250 mg, 39%) and (*E*)-3-{4-(4-methoxybenzyloxy)phenyl}isoxazol-5-yl]methylidene-1-azabicyclo[2.2.2]octane ((*E*)-**6g**, 168 mg, 26%). (*Z*)-**6g**: mp 238-241 °C (oxalate); ^{1}H NMR (300 MHz, $CDCl_3$, δ) 1.80-2.00 (4H, m), 2.60-2.70 (1H, m), 3.00-3.25 (4H, m), 3.84 (3H, s), 3.99 (2H, s), 5.06 (2H, s), 6.29 (1H, s), 6.32 (1H, s), 6.94 (2H, d), 7.05 (2H, d), 7.39 (2H, d), 7.75 (2H, d); ^{13}C NMR (75 MHz, $CDCl_3$, δ) 27.49, 33.69, 47.73, 55.72, 56.10, 70.29, 99.95, 107.56, 114.47, 115.63, 122.19, 128.56, 129.00, 129.66, 153.40, 159.96, 160.60, 162.46, 168.61; HRMS calcd for $C_{26}H_{26}N_2O_3$ (M + H) 403.2021, found 403.2029. (*E*)-**6g**: mp 195-199 °C (oxalate); ^{1}H NMR ($CDCl_3$, δ) 1.85-2.01 (4H, m), 3.00-3.25 (4H, m), 3.69-3.73 (1H, m), 3.76 (2H, s), 3.84 (3H, s), 5.06 (2H, s), 6.19 (1H, s), 6.35 (1H, s), 6.96 (2H, d), 7.06 (2H, d), 7.39 (2H, d), 7.74 (2H, d); ^{13}C NMR ($CDCl_3$, δ) 25.73, 27.31, 47.48, 55.72, 56.02, 70.30, 77.00, 100.93, 107.97, 114.45, 115.64, 121.98, 128.55, 128.94, 129.66, 153.40, 159.98, 160.67, 162.39, 168.10; HRMS calcd for $C_{26}H_{26}N_2O_3$ (M + H) 403.2021, found 403.2015.
6. Competitive muscarinic receptor binding assay was performed by measuring the abilities of compounds to inhibit specific binding of 1 nM [^{3}H]*N*-methylscopolamine, and nonspecific binding was determined in the presence of 1 μM atropine. Receptor source was the human recombinant muscarinic receptor subtypes (M_1, M_2, M_3) expressed in CHO cells (Biosignal). The reaction mixture containing 2 μg of receptor (100 μL suspension) was incubated at 27 °C for 1 h, and analyzed by a conventional filtration assay method. Competitive neuronal nicotinic receptor binding was performed by measuring the abilities of compounds to inhibit specific binding of 1.25 nM [^{3}H]cytisine, and nonspecific binding was determined in the presence of 10 μM (-)-nicotine. Receptor source was a crude synaptic membrane fraction prepared from the rat cerebral cortex according to the method of Zukin et al.[11] with minor modifications. After the addition of the membrane fraction containing approximately 300 μg of protein followed by incubation at 4 °C for 75 min, the reaction mixture was analyzed by a conventional filtration assay method.
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