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Structure–Activity Relationships in 2,2-Diphenyl-2-ethylthioacetic Acid Esters: Unexpected Agonistic Activity in a Series of Muscarinic Antagonists

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Abstract—As a continuation of previous research on anticholinergic drugs derived from 2,2-diphenyl-2-ethylthioacetic acid, several 5,5-diphenyl-5-ethylthio-2-pentynamines (**2**–**11**) were synthetised and their antimuscarinic activity on M_{1-4} receptor subtypes was evaluated by functional tests and binding experiments. One of the compounds obtained showed unexpected agonistic activity in functional experiments on M_2 receptors. Since the compound carried a phenylpiperazine moiety, other similar compounds (**12**–**17**) were prepared and found to be endowed with similar behaviour. These ligands, although possessing the bulky structure characterising muscarinic antagonists, display agonistic activity at M_2 subtypes while, as expected, behaving as antagonists on M_3 and M_4 subtypes. On M_1 subtypes, they show agonistic activity which, however, is not blocked by atropine. The peculiar pharmacological profile of these compounds is of interest for studying muscarinic receptor subtypes.

Introduction

For some time, we have been engaged in a research on muscarinic antagonists derived from 2,2-diphenyl-2-ethylthioacetic acid exemplified by compound **1**, one of the most potent and selective among those studied.[1–3] In an effort to obtain more potent and selective compounds and to improve SARs in this class of muscarinic antagonists, we designed and synthetised the alkyne analogues (**2**–**11**) shown in Figure 1. Obtained compounds were tested with binding experiments and functional assays. Much to our surprise, one of the prepared compounds (**11**), a methyl iodide, behaved as an agonist in functional tests on guinea pig atria, while being an antagonist on the other muscarinic receptor models studied. Unlike the other members of the family, **11** carries a phenylpiperazine ring. We therefore designed the analogues **12**–**17** to verify whether this feature was responsible for this unexpected behaviour and found that, indeed, these compounds show agonistic activity on the same M_2 receptor model. In the present paper, we report the synthesis, binding and functional activity of the compounds shown in Figure 1 as well as a preliminary characterisation of the in vitro pharmacological profile of the members with agonistic activity.

Chemistry

The compounds of the alkyne series (**2**–**11**) were obtained as shown in Scheme 1. Diphenylmethane was submitted to a double metallation with butyllithium to introduce in the benzylic position a thioethyl (**18**) and then a propargyl group (**19**). The acetylenic compound obtained was transformed into the desired amines (**2**, **4**, **6**, **8**, **10**) by Mannich reaction. Compounds **3**, **5**, **7**, **9** and **11** were obtained from the corresponding amines with methyl iodide. Their chemical and physical characteristics are reported in Table 1.

The esters of 2,2-diphenyl-2-ethylthioacetic acid (**12**–**17**) were obtained by standard esterification of compounds **20**–**22**[4,5] with 2,2-diphenyl-2-ethylthioacetic acid chloride,[2] according to Scheme 2. Their chemical and physical characteristics are reported in Table 2. The chemical

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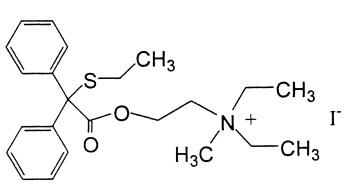

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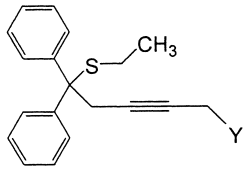
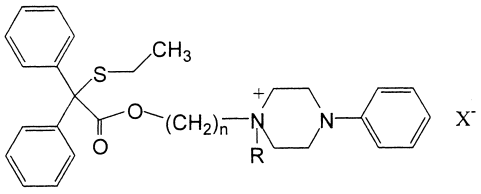


2-11 (For the meaning of Y see Table 1)



12-17 n = 2-4
R = H, CH_3
X = Cl, I

Figure 1.

stability of compounds **11** and **12–17** under experimental conditions was evaluated on samples kept at 30 °C in DMSO, or in a DMSO/H_2O solution adjusted to pH = 7.5, running ^{1}H NMR spectra from time to time (up to 24 h). Under these conditions, no changes in the spectra were observed.

Pharmacology

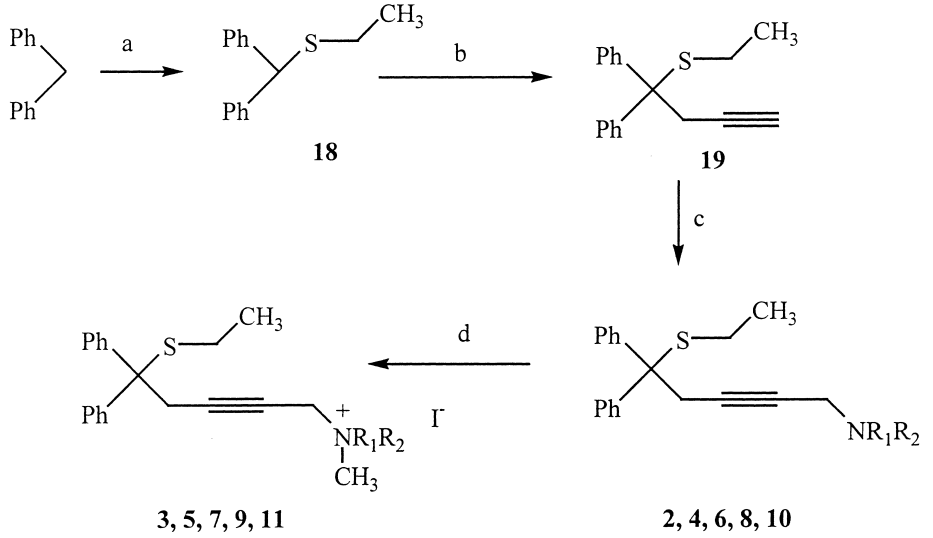
All the compounds were tested on M_1 (rabbit vas deferens), M_2 (guinea-pig atria), M_3 (guinea-pig ileum) and M_4 (guinea-pig lung) muscarinic receptor subtypes for their muscarinic and antimuscarinic activity, using p-Cl-McN-A-343 and arecaidine propargyl ester (APE) as agonists at M_1 and M_{2-4} subtypes, respectively. Agonist and antagonist potencies were expressed as $-\log ED_{50}$ and as $-\log$ of dissociation constant (pK_b), respectively. The muscarinic binding properties of the compounds were evaluated on rat cerebral cortex and domestic pig ventricle homogenates using ^{3}[H]-NMS and ^{3}[H]-OXO as labelled ligand and expressed as K_i.

Results

The results obtained in binding and functional experiments are reported in Tables 3–6.

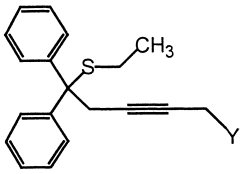
Muscarinic antagonism

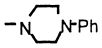
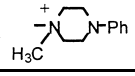
Alkyne derivatives **2–10** possess rather modest antagonistic activity on M_1–M_3 receptors (Table 3) which is even lower as far as M_4 receptors are concerned. The most interesting compounds are **5**, which shows selectivity for M_1 receptors ($M_1/M_2 = 76$; $M_1/M_3 = 20$ and $M_1/M_4 = 95$), and **7** which shows selectivity for M_1 and M_3 receptors ($M_1/M_2 = 16$; $M_1/M_4 = 37$; $M_3/M_2 = 20$ and $M_3/M_4 = 44$). With regard to antagonism, esters **12–14** (Table 4) show the same behaviour on M_2, M_3 and



Scheme 1. (a) BuLi, Et_2S_2; (b) BuLi, propargylbromide; (c) CH_2O, NHR_1R_2; (d) CH_3I.

Table 1. Chemical and physical characteristics of compounds **2–11**

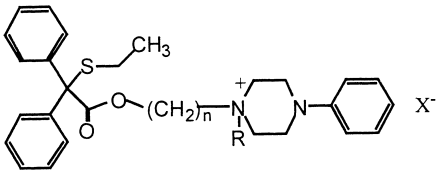


N	Y	Salt	Mp (°C) (recr. solv.)[a]	Analysis[b]
2		Oxalate	90–92 (A)	$C_{25}H_{31}NO_4S$
3		Iodide	80–82 (B)	$C_{24}H_{32}INS$
4		Hydrochloride	134–137 (A)	$C_{23}H_{28}ClNS$
5		Iodide	157–160 (B)	$C_{24}H_{30}INS$
6		Hydrochloride	143–145 (A)	$C_{24}H_{30}ClNS$
7		Iodide	171–173 (B)	$C_{25}H_{32}INS$
8		Hydrochloride	141–142 (A)	$C_{23}H_{28}ClNOS$
9		Iodide	175–178 (B)	$C_{24}H_{30}INOS$
10		Hydrochloride	145–148 (A)	$C_{29}H_{33}ClN_2S$
11		Iodide	171–173 (B)	$C_{30}H_{35}IN_2S$

[a](A) Abs ethanol + anhydrous ether. (B) Abs ethanol.
[b]All compounds were analysed for C, H and N and the results are within 0.4% of the theoretical value. Their IR and ^{1}H NMR spectra are consistent with the proposed structures.

M_4 receptors where they are modest antagonists with poor selectivity. In general, the pharmacological profile of all compounds studied in the present work as antagonists is much less interesting than that of **1** and of the esters reported previously.[1–3] The binding experiments on rat cerebral cortex confirmed the modest affinity of such compounds (Table 5).

Table 2. Physical characteristics of compounds **12–17**



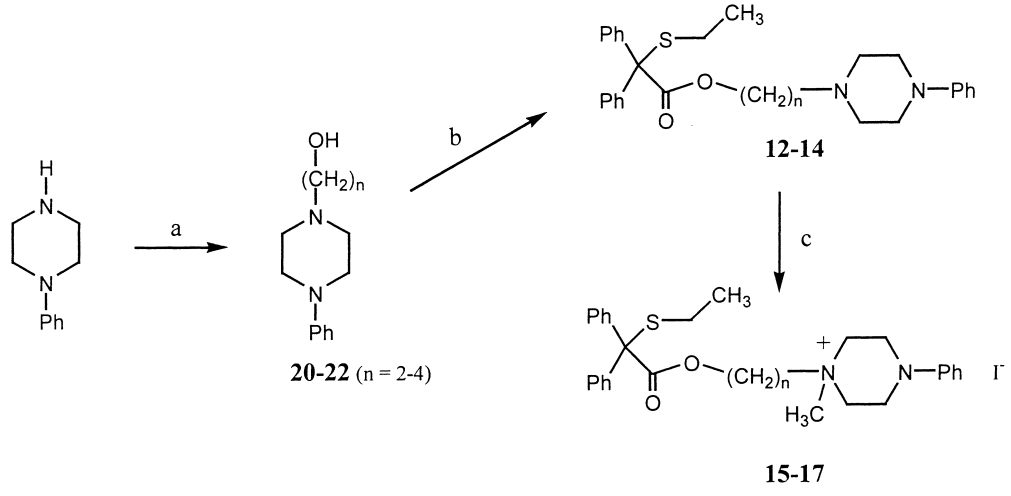
N	*n*	R	X	Mp (°C) (recr. solv.)[a]	Analysis[b]
12	2	H	Cl	149–151 (A)	$C_{28}H_{33}ClN_2O_2S$
13	3	H	Cl	178 dec. (A)	$C_{29}H_{35}ClN_2O_2S$
14	4	H	Cl	169–172 (A)	$C_{30}H_{37}ClN_2O_2S$
15	2	CH_3	I	191–193 (B)	$C_{29}H_{35}IN_2O_2S$
16	3	CH_3	I	99–102 (B)	$C_{30}H_{37}IN_2O_2S$
17	4	CH_3	I	78–80 (B)	$C_{31}H_{39}IN_2O_2S$

[a](A) Abs ethanol + anhydrous ether. (B) Abs ethanol.
[b]All compounds were analysed for C, H and N and the results are within 0.4% of the theoretical value. Their IR and ^{1}H NMR spectra are consistent with the proposed structures.

Muscarinic agonism

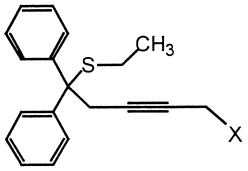
Table 3 shows that compound **11** is a weak antagonist on M_1, M_3, and M_4 muscarinic receptor models and behaves like an agonist on the M_2 receptor model. Much like compound **11**, compounds **15–17** (Table 4) behave as muscarinic agonists on M_2 and as antagonists on M_3 and M_4 receptor subtypes. Agonism on M_2 subtypes is antagonised by atropine. On stimulated rabbit vas deferens (M_1), while compounds **12–14** behave as weak antagonists, compounds **15–17** behave as agonists, but their action is not antagonised by atropine or pirenzepine. The potency of all compounds examined on M_2 (force) is definitely higher when an incubation time of 3 h is adopted (Table 6). In contrast, on M_2 (rate) the same compounds are inactive up to the dose of 10^{-7}–10^{-6} M.

Binding experiments on rat cerebral cortex against [^{3}H]NMS, show that compounds **11** and **15–17** possess affinity for muscarinic receptors comparable with that



Scheme 2. (a) Br–$(CH_2)_n$–OH; (b) 2,2′-diphenyl-2-ethylthioacetic acid chloride; (c) CH_3I.

Table 3. Functional and binding activity of compounds **1** and **2**–**11**



N	Binding[a] K_i±SEM (μM)	Functional activity pK_b±SEM			
		M_1[b]	M_2[c]	M_3[d]	M_4[e]
1[f]		9.97±0.06	9.12±0.03	6.50±0.07	—
2	2.70±0.35	6.35±0.06	6.01±0.06	< 5	< 5
3	0.78±0.13	6.63±0.05	6.96±0.13	6.30±0.25	5.66±0.03
4	0.40±0.038	7.10±0.17	6.79±0.10[g]	6.11±0.11[h]	5.27±0.20[i]
5	0.36±0.039	7.54±0.09[j]	5.71±0.09	6.31±0.19	5.56±0.11
6	4.67±0.89	5.87±0.2	5.30±0.14	6.62±0.20	< 5
7	0.85±0.17	7.01±0.16	5.82±0.20	7.08±0.19[g]	5.44±0.11
8	> 5	< 5	< 5	5.63±0.13[g]	5.11±0.15
9	2.41±0.29	6.43±0.07	5.84±0.15	5.93±0.14[g]	5.58±0.11
10	> 5	< 5	< 5	< 5[k]	5.48±0.20
11	2.38±0.31	< 5	α = 0.92 4.80±0.15 (−log ED_{50})	< 5[l]	5.06±0.08[m]

[a]Rat cerebral cortex homogenate, [^{3}H]NMS. Each experimental value is calculated from at least three determinations performed in duplicate.
[b]Rabbit stimulated vas deferens.
[c]Guinea-pig atria.
[d]Guinea-pig ileum.
[e]Guinea-pig lung strips.
[f]See ref 2.
[g]10% reduction of maximal effect.
[h]25% reduction of maximal effect.
[i]19% reduction of maximal effect.
[j]Non competitive (*n* significantly different from 1).
[k]69% reduction of maximal effect.
[l]At 10^{-5} there is an 85% reduction of maximal effect.
[m]57% reduction of maximal effect.

of carbachol, and pilocarpine,[6] **16** and **17** being the most affinitive. The NMS/OXO ratio, which according to Freedman[7] is an evaluation of efficacy, predicts that compounds **15**–**17**, on rat cerebral cortex as well as on domestic pig ventricle receptors, would behave as antagonists. However the agonistic activity shown by these compounds on M_2 tissue preparations seems to indicate that the method does not have general validity or does not apply to these compounds.

Discussion

Muscarinic antagonism

With regard to antagonism, all the compounds studied show modest potency and selectivity when compared with the compounds reported previously. Therefore, the molecular modifications introduced, in the present work, into the parent compounds[2] are detrimental for their pharmacological profile.

Muscarinic agonism

The muscarinic agonism of **11** and subsequently of **12**–**17** is quite unexpected. As a matter of fact, muscarinic agonists are a fairly well known class of ligands whose molecular characteristics have been studied extensively,[8,9] and it is well known that increasing their size usually results in the appearance of muscarinic antagonism.[10]

However, in the last few years, some potent agonists with somewhat larger size have appeared, and this has required a redefinition of the binding site of muscarinic ligands.[11] Xanomeline,[12] PD151832[13] and SK-946[14] are representative of such compounds (Chart 1). Moreover, a few molecules showing strong muscarinic agonism and characterised by an even larger size have been reported in the literature. Their structures challenge the currently accepted picture of the muscarinic receptor active site and are therefore quite interesting for muscarinic receptor studies. This group of compounds is exemplified by SR-95639A and SR 96095A, which are representative of a series of M_1 selective muscarinic agonists developed by the group of Wermuth[15–17] by the acylhydrazone **23**, claimed to be a M_1/M_3 selective muscarinic agonist,[18] and by clozapine which has been reported to be a potent and selective M_4 receptor agonist[19–21] (Chart 1).

The pharmacological profile of compounds **11**–**17** appears fairly complex. So far, our work has been aimed at a better definition of their properties, leaving interpretation of their complex behaviour to further studies.

Since the agonistic action of compounds **11**–**17** is hardly explainable, given their molecular structure, we wanted to be sure that activity was not due to molecular fragmentation as a consequence of poor chemical or metabolic stability. Therefore, the stability of **11**–**17**, under pharmacological experiment conditions, was checked:

Table 4. Functional muscarinic activity (1 h incubation time) of compounds **12**–**17**

N	M_1[a]		M_2[b]				M_3[c]	M_4[d]
			Force[e]		Rate[e]			
	α[f]	$-\log ED_{50}$[g]±SEM	α[f]	$-\log ED_{50}$[g]±SEM	α[f]	$-\log ED_{50}$[g]±SEM	pK_b[h]±SEM	pK_b[h]±SEM
12	0	[i]	0.9	5.54±0.24	1	5.74±0.21	[j]	5.37±0.14
13	0	[i]	1	6.17±0.23	1	6.24±0.20	[k]	5.15±0.01
14	0	[l]	0.7	5.59±0.15	1	5.51±0.11	[m]	5.36±0.02
15	1	5.67±0.19[n]	1	6.07±0.14	1	6.46±0.25	6.78±0.14	5.51±0.06
16	1	6.00±0.20[n]	1	6.54±0.06	1	6.64±0.20	6.66±0.08	5.32±0.05
17	1	5.69±0.23[n]	0.7	4.08±0.24	1	4.51±0.13	5.53±0.12	< 5

[a]Rabbit stimulated vas deferens.
[b]Guinea-pig atria.
[c]Guinea-pig ileum.
[d]Guinea-pig lung strips.
[e]Antagonized by atropine ($pA_2 \sim 9.0$).
[f]α = intrinsic activity, measured by the ratio between the maximum response of the compound and the maximum response of the reference agonist.
[g]ED_{50} = dose producing 50% of maximal effect.
[h]Calculated from the equation $pK_b = \log(DR-1) - \log[B]$.
[i]On this tissue the compound is actually an antagonist with $pK_b < 5$.
[j]On this tissue, at 10^{-5} M, there is a 75% reduction of the maximum effect of APE.
[k]On this tissue, at 10^{-5} M, there is a 53% reduction of the maximum effect of APE.
[l]On this tissue the compound is actually an antagonist with $pK_b = 5.59 \pm 0.02$.
[m]On this tissue, at 10^{-5} M, there is a 76% reduction of the maximum effect of APE.
[n]Not antagonized by atropine nor pirenzepine.

Table 5. Muscarinic binding of compounds **11**–**17**

N	Rat cerebral cortex[a]			Domestic pig ventricle[a]		
	^{3}[H]-NMS K_i±SEM (μM)	^{3}[H]-OXO K_i±SEM (μM)	NMS/OXO	^{3}[H]-NMS K_i±SEM (μM)	^{3}[H]-OXO K_i±SEM (μM)	NMS/OXO
11	2.38±0.31	nd[b]	—	nd	nd	—
12	> 100	—	—	—	—	—
13	> 100	—	—	—	—	—
14	> 10	—	—	—	—	—
15	6.16±0.91	8.47±1.45	0.7	8.78±1.34	19.0±4.6	0.5
16	1.79±0.27	0.96±0.26	1.9	1.99±0.49	0.78±0.20	2.6
17	2.18±0.33	1.37±0.35	1.6	2.98±0.57	0.88±0.23	3.4
Carbachol	41.32±7.0[c]	0.0045±0.0008	> 4000	4.38±0.83	0.0027±0.0005	1622
NMS	0.00016[c]±0.00002	0.00011±0.00002	1.5	0.0002±0.000044	0.000095±0.000023	2
OXO	8.05±1.29[c]	0.00031±0.00006	> 4000	0.22±0.06	0.00058±0.00013	379

[a]K_i values are reported in μM±SEM ($n \geqslant 3$).
[b]nd = not determined.
[c]See ref 6.

apparently the compounds do not undergo any modification after solubilisation (see Chemistry). To exclude metabolic cleavage, agonism experiments were run in the presence of physostigmine, an acetylcholinesterase inhibitor, and we found no differences with previous experiments.

Binding experiments show that only the ammonium salts **11** and **15**–**17** have affinity for muscarinic brain receptors similar to that of carbachol. This is an initial unexpected result, as compounds **12**–**14** do show agonistic activity on M_2 receptors as reported in Table 4. The NMS/OXO ratio,[7] on the other hand, indicates an antagonistic behaviour on the rat cerebral cortex. This result, however, is of little help as their agonistic activity appears only on M_2 receptors while they have antagonistic action on other muscarinic subtypes. For this reason, we tested compounds **11** and **15**–**17** also on domestic pig ventricles where only M_2 receptors are present. In this preparation, the binding affinity appears

Table 6. M_2 muscarinic activity of compounds **11**–**17** after 3 h incubation time

N	M_2[a]			
	Force[b]		Rate	
	α[c]	$-\log ED_{50}$[d]±SEM	α[c]	$-\log ED_{50}$[d]±SEM
11	0.94	8.10±0.17	—	[e]
12	1	7.65±0.08	—	[e]
13	0.9	7.96±0.17	—	[e]
14	0.9	8.59±0.03	—	[e]
15	1	7.62±0.04	—	[e]
16	0.9	8.07±0.19	—	[e]
17	0.9	7.62±0.15	—	[e]

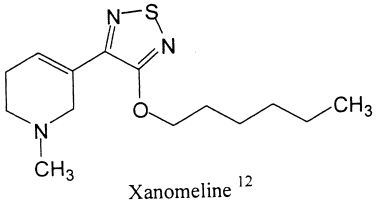
[a]Guinea-pig atria.
[b]Antagonized by atropine ($pA_2 \sim 9.0$).
[c]α = intrinsic activity, measured by the ratio between the maximum response of the compound and the maximum response of APE.
[d]ED_{50} = dose producing 50% of maximal effect.
[e]Inactive up to the dose range of 10^{-7}–10^{-6}M.

to be fairly similar to that on rat brain, but again the NMS/OXO ratio suggests an antagonistic kind of activity. At present, there is not an explanation to account for the peculiar behaviour of this class of drugs.

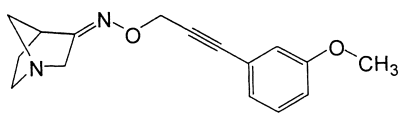
A particular aspect of M_2 muscarinic agonist activity of compounds **11**–**17** is that, when the incubation time is prolonged up to 3 h, the potency of the compounds is definitely higher. In this case, however, the recovery of tissue functionality is reduced to some 40% while recovery ranged around 80% using the more usual 1 h incubation time. The reasons for this behaviour are not understood at present; they could be related to the high lipophilicity of compounds **11**–**17** (which show poor water solubility while being soluble in chloroform).

Despite the complexity of the pharmacological profile of this series of molecules (a fact that obviously requires further studies), there is little doubt that they can mimic agonistic activity on M_2 receptors. Therefore, these compounds should be added to the ligands reported in Chart 1 showing muscarinic agonism, although their molecular structure is odd with respect to the currently-accepted picture of the muscarinic receptor active site.

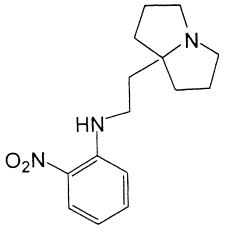
Alternative explanations for the peculiar activity of compounds **11**–**17**, such as an indirect agonistic effect, seem less probable. In fact, since their behaviour is similar on stimulated M_1 and M_2 subtypes and their binding affinity is poor to modest, they could con-



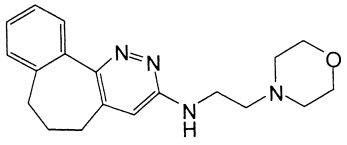
Xanomeline [12]



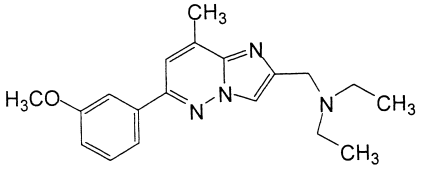
PD 151832 [13]



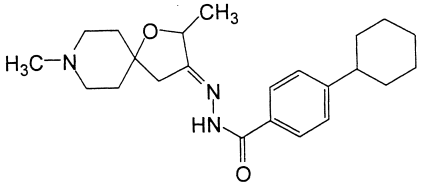
SK-946 [14]



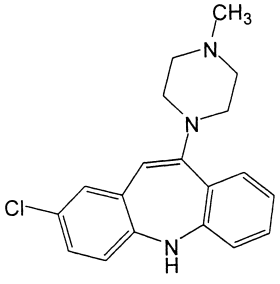
SR-95639A [15-17]



SR 96095A [15-17]



23 [18]



Clozapine [19-21]

Chart 1.

ceivably be involved in the mobilisation of Ca^{++} or ATP, producing a reduction of tissue contraction which can mimic a muscarinic agonistic activity. However, while this explanation fits well for the rabbit vas deferens model, where activity of **15**–**17** is not antagonised by muscarinic antagonists, it is in contrast with the fact that the action of compounds **11**–**17** on atria is blocked by the classical muscarinic antagonist atropine.

From a medicinal chemistry point of view, compounds like **15**–**17** can be useful leads for designing classical and non-classical muscarinic agonists. It is tempting to consider that the phenylpiperazine nucleus, which is present in all active compounds, is responsible for their activity and therefore use it as a pharmacophore for new muscarinic ligands. At present, we are exploring this possibility.

Experimental

Chemistry

All melting points were taken on a Büchi apparatus and are uncorrected. Infrared spectra were recorded with a Perkin-Elmer 681 spectrophotometer in a Nujol mull for solids and neat for liquids. NMR spectra were recorded on a Gemini 200 spectrometer. Chromatographic separations were performed on a silica gel column by gravity chromatography (Kieselgel 40, 0.063–0.200 mm, Merck) or flash chromatography (Kieselgel 40, 0.040–0.063 mm, Merck). Yields are given after purification, unless otherwise stated. Where analyses are indicated by symbols, the analytical results are within ±0.4% of the theoretical values.

Ethylthiodiphenylmethane (18). Diphenylmethane (3 g; 0.018 mol) was dissolved in THF (15 mL) and cooled at 0 °C. Butyllithium in hexane (12.25 mL of a 1.6 M solution) was then added and the mixture stirred for 2 h at 0 °C. Diethyldisulphide (0.02 mol) was then added and the mixture warmed up to room temperature, treated with water, extracted with ether and the organic phase dried. The oily mixture (3.5 g) obtained after evaporation was purified by flash chromatography using cyclohexane/ethyl acetate 98/2 as eluting system. Yield 67% of a thick oil that was used as such in the following reaction. ^{1}H NMR ($CDCl_3$): δ 1.25 (t, 3H, $SCH_2\underline{CH_3}$); 2.42 (q, 2H, $S\underline{CH_2}CH_3$); 5.20 (s, 1H, CH); 7.08–7.32 (m, 6H, aromatics); 7.40–7.51 (m, 4H, aromatics) ppm.

1-Ethylthio-1,1-diphenylbut-3-yne (19). Butyllithium in hexane (3 mL of a 1.6 M solution) was added to 1 g (4.4 mmol) of **18** dissolved in THF (15 mL) and cooled at 0 °C and the mixture stirred for 2 h at 0 °C. Propargylbromide (0.57 g, 4.8 mmol) was then added and the mixture warmed up to room temperature, treated with water, extracted with ether and the organic phase dried. The oily mixture was purified by flash chromatography using CH_2Cl_2/hexane 15/85 as eluting system.

Yield 47% of a thick oil that was used as such in the following reaction. ^{1}H NMR ($CDCl_3$): δ 1.18 (t, 3H, $SCH_2\underline{CH_3}$); 2.07 (t, 1H, CH); 2.35 (q, 2H, $S\underline{CH_2}CH_3$); 3.41 (d, 2H, CH_2); 7.32–7.48 (m, 6H, aromatics); 7.52–7.62 (m, 4H, aromatics) ppm.

***N*,*N*-Diethyl (5-ethylthio-5,5-diphenylpent-2-ynyl)amine (2).** A solution of formaldehyde (0.25 mL of a 40% solution in water), diethylamine (3.0 mmol) and $CuSO_4$ (0.05 g) were added to a solution of **19** (2.3 mmol) in 4 mL of EtOH/H_2O (1/1). The pH of the solution was adjusted to 8 with 50% sulphuric acid. The mixture was heated to reflux for 24 h, then 15 mL of NH_4OH were added and the solution was extracted with Et_2O. The organic phase was dried and evaporated under reduced pressure. The oily mixture was purified by flash chromatography using CH_2Cl_2/MeOH 95/5 as eluting system. Yield 75% of a thick oil. ^{1}H NMR ($CDCl_3$): δ 0.92 (t, 6H, $NCH_2\underline{CH_3}$); 1.18 (t, 3H, ($SCH_2\underline{CH_3}$); 2.35 (q, 2H, $S\underline{CH_2}CH_3$); 2.29 (q, 4H, $N\underline{CH_2}CH_3$); 3.22 (m, 2H, CH_2); 3.31 (m, 2H, CH_2); 7.22–7.33 (m, 6H, aromatics); 7.42–7.50 (m, 4H, aromatics) ppm. The oily amine was transformed into the oxalate and recrystallized from abs. EtOH and dry ether. Mp 90–92 °C. Anal. ($C_{25}H_{31}NO_4S$) C, H, N.

Compounds **4**, **6**, **8**, and **10** were made in the same way, starting from the appropriate amine and transformed into the salt indicated. Their chemical and physical characteristics are reported in Table 1. Their IR and ^{1}H NMR spectra are consistent with the proposed structures.

***N*,*N*-Diethyl-*N*-methyl (5-ethylthio-5,5-diphenylpent-2-ynyl)amonium iodide (3).** An excess of methyl iodide (2 mL) was added to 0.2 g of **2** dissolved in anhydrous ether (10 mL) and the solution left overnight at room temperature in the dark. The pale yellow solid obtained was recrystallised from abs. EtOH; yield 90%. Mp 80–82 °C. ^{1}H NMR($CDCl_3$): δ 1.05 (t, 3H, $SCH_2\underline{CH_3}$); 1.15 (t, 6H, $^+NCH_2\underline{CH_3}$); 2.12 (q, 2H, ($S\underline{CH_2}CH_3$); 3.06 (s, 3H, $^+NCH_3$); 3.18 (q, 4H, $^+N\underline{CH_2}CH_3$); 3.22 (m, 2H, CH_2); 4.12 (m, 2H, CH_2); 7.09–7.22 (m, 10H, aromatics) ppm. Anal. ($C_{24}H_{32}INS$) C, H, N.

Compounds **5**, **7**, **9**, and **11** were made in the same way, starting from the appropriate amine. Their chemical and physical characteristics are reported in Table 1. Their IR and ^{1}H NMR spectra are consistent with the proposed structures.

2-(4-Phenylpyperazinyl)ethyl 2,2-diphenyl-2-ethylthio acetate (12). 2,2-Diphenyl-2-ethylthio acetyl chloride[2] (2 mmol) was refluxed for 8 h with 4 mmol of 2-(4-phenylpyperazinyl)ethanol (**20**)[5] in 20 mL of EtOH-free $CHCl_3$. The solution was washed with 10% Na_2CO_3 solution and the organic layer anhydrified and evaporated to give an oil that was purified by flash chromatography on silica gel (eluent $CHCl_3$/MeOH 99/1). Yield 59%. IR (neat) ν 1740 (CO) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.15 (t, 3H, SCH_2–$\underline{CH_3}$); 2.42 (q, 2H, $S\underline{CH_2}$–CH_3); 2.51–2.58 (m, 4H, piperazine protons); 2.62 (t, 2H, OCH_2–$\underline{CH_2}$–N); 3.09–3.19 (m, 4H, piperazine protons); 4.41 (t, 2H, $O\underline{CH_2}$–CH_2–N); 6.85–6.98 (m, 2H, aromatics); 7.21–7.52 (m, 8H, aromatics) ppm.

Table of elemental analyses of compounds **2**–**17** and **19**

	Calculated			Found		
Comp.	C %	H %	N %	C %	H %	N %
2	68.00	7.08	3.17	67.83	7.31	3.44
3	58.41	6.54	2.84	58.20	6.32	3.07
4	71.57	7.31	3.63	71.81	7.52	3.38
5	58.65	6.15	2.85	58.93	5.90	3.05
6	72.06	7.56	3.50	72.33	7.89	3.30
7	59.40	6.38	2.77	59.71	6.05	2.61
8	68.72	7.02	3.48	68.45	7.38	3.59
9	56.80	5.96	2.76	57.03	5.81	2.97
10	73.01	6.97	5.87	73.31	6.72	5.63
11	61.85	6.06	4.81	62.13	5.84	4.67
12	67.65	6.69	5.64	67.31	6.40	5.31
13	68.15	6.90	5.48	68.41	7.15	5.67
14	68.61	7.10	5.33	68.87	6.85	5.07
15	57.80	5.85	4.65	58.08	5.66	4.37
16	58.44	6.05	4.54	58.71	5.88	4.85
17	59.04	6.23	4.44	58.77	6.51	4.16
19	81.15	6.81		81.40	6.58	

The oily product was transformed into the hydrochloride and crystallised from abs. EtOH and an. ether. Mp 149–151 °C. Anal. ($C_{28}H_{33}ClN_2O_2S$) C, H, N.

3-(4-Phenylpyperazinyl)propyl 2,2-diphenyl-2-ethylthio acetate (13) and 4-(4-phenylpyperazinyl)butyl 2,2-diphenyl-2-Ethylthio acetate (14). These were obtained in the same way from 2,2-diphenyl-2-ethylthio acetyl chloride and the proper alcohols **21** and **22**[4] and transformed into the corresponding hydrochloride. Their chemical and physical characteristics are reported in Table 2. Their IR and ^{1}H NMR spectra are consistent with the proposed structures.

2-(4-Phenylpyperazinyl)ethyl 2,2-diphenyl-2-ethylthio acetate methyliodide (15). An excess (2 mL) of CH_3I was added to 2 mmol of the free base of **12** in 20 mL of anhydrous ether and the solution was left at room temperature overnight in the dark. The white solid was filtered and crystallised from abs. ethanol. Yield 90%. Mp 191–193 °C. IR (nujol) ν 1740 (CO) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.10 (t, 3H, $SCH_2\underline{CH_3}$); 2.32 (q, 2H, $S\underline{CH}CH_3$); 3.15–3.26 (m, 4H, piperazine protons); 3.32 (s, 3H, $^+NCH_3$); 3.42–3.52 (m, 4H, piperazine protons); 4.22–5.00 (m, 2H, $\underline{CH_2}CH_2O$); 4.70–4.78 (m, 2H, $CH_2\underline{CH_2}O$); 6.82 (d, 2H, aromatics); 6.98–7.18 (m, 1H, aromatic); 7.29–7.49 (m, 12H, aromatics) ppm. Anal. ($C_{29}H_{35}IN_2O_2S$) C, H, N.

Compounds **16** and **17** were obtained in the same way starting from **13** and **14**, respectively. Their chemical and physical characteristics are reported in Table 2. Their IR and ^{1}H NMR spectra are consistent with the proposed structures.

Pharmacology

Functional in vitro tests

General considerations. Male guinea pigs (200–300 g) were killed by cervical dislocation. The organs required were set up rapidly under 1 g of tension in 20 mL organ baths containing physiological salt solution (PSS) maintained at an appropriate temperature (see below) and aerated with 5% CO_2–95% O_2. Two dose–response curves were constructed by cumulative addition of the reference agonist. The concentration of agonist in the organ bath was increased approximately 3-fold at each step, with each addition being made only after the response to the previous addition had attained a maximum level and remained steady. When agonism was studied, following 30 min of washing, a new dose–response curve for the agonist under study was obtained. Responses were expressed as a percentage of the maximal response obtained in the control curve. The results are expressed in terms of $-\log ED_{50}$, the concentration of agonist required to produce 50% of the maximum contraction. The dose–response curve for the agonist under study was obtained at two different incubation times, 1 and 3 h, with the cardiac tissue, in order to examine the kinetics of the transduction mechanism which is responsible for the slow tissue response. When a 1 h incubation time with cardiac tissue was used, a 75–80% of functional recovery was observed after 1 h of washing; this percentage drops to 40–45% when a 3 h incubation time and persistent washings were used. When antagonism was studied, following 30 min of washing, tissues were incubated with the antagonist for 1 h, and a new dose–response curve for the agonist was obtained. To quantify antagonist potency, pK_b values were calculated from the equation $pK_b = \log(DR-1) - \log[B]$, where DR is the ratio of ED_{50} values of agonist after and before treatment with one or two antagonist concentrations [B].[22]

Contractions were recorded by means of a force displacement transducer connected to the MacLab system PowerLab/800 and to a two-channel Gemini polygraph (U. Basile).

In all cases, parallel experiments in which tissues received only the reference agonist were run in order to check any variation in sensitivity.

Guinea-pig ileum. Two-cm-long portions of terminal ileum were taken at about 5 cm from the ileum–cecum junction and mounted in PSS, at 37 °C, with the following composition (mM): NaCl 118, $NaHCO_3$ 23.8, KCl 4.7, $MgSO_4.7H_2O$ 1.18, KH_2PO_4 1.18, $CaCl_2$ 2.52, and glucose 11.7. Tension changes were recorded isotonically. Tissues were equilibrated for 30 min and dose–response curves for arecaidine propargyl ester (APE) were obtained at 30 min intervals, the first one being discarded and the second one being taken as the control.

Guinea-pig stimulated left atria. The heart was rapidly removed, and the right and left atria were separately excised. Left atria were mounted in PSS (the same used for ileum) at 30 °C and stimulated through platinum electrodes by square-wave pulses (1 ms, 1 Hz, 5–10 V) (Tetra Stimulus, N. Zagnoni). Inotropic activity was recorded isometrically. Tissues were equilibrated for 2 h and a cumulative dose–response curve using APE was constructed.

Right atria. Right atria were equilibrated for 2 h at the above conditions (see preceding paragraph for PSS and temperature). Contractions were recorded isometrically.

Guinea-pig lung strips. The lungs were rapidly removed and strips of peripheral lung tissue were cut either from the body of a lower lobe with the longitudinal axis of the strip parallel to the bronchus or from the peripheral margin of the lobe.[23] The preparations were mounted, with a preload of 0.3 g, in PSS with the following composition (mM): NaCl (118.78), KCl (4.32), $CaCl_2.2H_2O$ (2.52), $MgSO_4.7H_2O$ (1.18), KH_2PO_4 (1.28), $NaHCO_3$ (25), glucose (5.55). Contractions were recorded isotonically at 37 °C after tissues were equilibrated for 1 h, then two cumulative dose–response curves using APE (0.01, 0.1, 1, 10, 100 μM) were obtained at 45 min intervals, the first one being discarded and the second one being taken as the control.

Rabbit stimulated vas deferens. This preparation was set up according to Eltze.[24] Vasa deferentia were carefully dissected free of surrounding tissue and were divided into four segments, two prostatic portions of 1 cm and two epididymal portions approximately 1.5 cm long. The four segments were mounted in PSS with the following composition (mM): NaCl (118.4), KCl (4.7), $CaCl_2$ (2.52), $MgCl_2$ (0.6), KH_2PO_4 (1.18), $NaHCO_3$ (25), glucose (11.1); 10^{-6} M yohimbine and 10^{-8} M tripitramine were included to block alpha$_2$-adrenoceptors and M_2 muscarinic receptors, respectively. The solution was maintained at 30 °C and tissues were stimulated through platinum electrodes by square-wave pulses (0.1 ms, 2 Hz, 10–15 V). Contractions were measured isometrically after tissues were equilibrated for 1 h, then a cumulative dose–response curve using pCl-McN-A-343 was constructed.

Statistical analysis. The results are expressed as the mean + SEM. Student's *t*-test was used to assess the statistical significance of the difference between two means.

Binding tests

Membrane preparation

Rat cerebral cortex. Male rats (Wistar, 200 g) were killed by decapitation and the brains were removed and immediately placed on ice. The brain was dissected, homogenised in 10 vol 0.32 M sucrose with a glass-teflon homogeniser. The homogenate was then centrifuged at 1000*g* for 15 min; the resulting supernatant was further centrifuged at 17,000*g* for 20 min and the pellet was stored at −80 °C until assayed.[7]

Domestic pig ventricles. Preparation of porcine heart homogenates was carried out as described by Burgmer et al.[25] Briefly, porcine hearts were obtained from the local slaughterhouse and stored at −80 °C. Thawed ventricular tissue (40 g) was minced and homogenised in 20 vol of 0.32 M sucrose solution with the aid of Ultra-Turrax T25 IKA and then with a motor driven glass-teflon homogeniser. The homogenate was then centrifuged for 11 min at 300*g* and the resulting supernatant centrifuged for 41 min at 80,000*g*; the pellet was stored at −80 °C.

Binding experiments

Rat cerebral cortex. The muscarinic receptors were labelled with 0.1 nM [^{3}H]N-methyl scopolamine ([^{3}H]NMS, 79.5 Ci/mmol from NEN-Dupont). The frozen pellet was suspended in Krebs-Hepes buffer pH 7.4 (composition, mM: NaCl 118, KCl 4.7, $NaHCO_3$ 5, KH_2PO_4 1.2, glucose 11, $MgSO_4$ 1.2, $CaCl_2$ 2.5 and Hepes 20), at 4 °C, as previously described in Freedman et al.[7] Aliquots of cerebral membranes at a protein concentration of 100–150 μg/mL were incubated at 30 °C for 60 min in a final volume of 1 mL with the marker ligand and with different concentrations of unlabeled ligands (0.1 nM–0.1 mM); the incubation was terminated by filtration through Whatman GF/B glass fiber filters presoaked in 0.1% polyethyleneimine (PEI), using a Brandel M-48R 48 well cell harvester. Filters were washed twice with 10 mL of ice-cold 0.9% saline solution.

For ^{3}[H]oxotremorine (^{3}[H]-OXO, 87.5 Ci/mmol from NEN-DuPont) binding studies, frozen pellet was washed by suspending in Hepes buffer, pH = 7.4, and centrifuged for 15 min at 17,000*g* as described by Freedman et al.[7] Incubations were initiated by adding 100 μL of membrane suspension (1–1.5 mg/mL) with 0.3 nM ^{3}[H]-OXO and different concentrations of cold ligands (0.1 nM–0.1 mM) at 30 °C for 40 min in a final volume of 1 mL. After incubation, membranes were filtered through Whatman GF/C filters strips presoaked in 0.1% PEI solution; the filters were rinsed with 10 mL of ice-cold saline solution. Protein determinations were performed by the method of Bradford[26] using bovine serum albumine as the standard.

Domestic pig ventricles. [^{3}H]NMS (0.2 nM) and ^{3}[H]-OXO (0.3 nM) were carried out using thawed pellet suspended in Krebs–Hepes and in Hepes buffer respectively. Incubation mixtures (1 mL) contained 150–300 μg of membranes in [^{3}H]NMS competition experiments, while with ^{3}[H]-OXO the amount of membranes was 600–800 μg; mixtures were incubated as previously described. Protein concentration was estimated using the Pierce protein assay reagent (Pierce Chemical Co., Rockford, IL) based on the method of Bradford[26] with bovine serum albumine as standard.

In all experiments, the radioactivity retained by filters was measured in a liquid scintillation counter (TRI-CARB 1900TR, Packard) after the addition of 4 mL of scintillation fluid (Filter Count, Packard) and all measurements were obtained in duplicate.

The binding data were evaluated quantitatively with non-linear least-square curve fittings using the computer programs ALLFIT[27] and LIGAND.[28]

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