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Synthesis and pharmacology of the putative novel muscarinic agonist (*S*)-4-F-MePyMcN [(*S*)-4-(pyrrolidino)-1-methyl-2-butynyl-N-(4-fluorophenyl) carbamate oxalate]

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Abstract – (*S*)-4-F-MePyMcN [(*S*)-4-(pyrrolidino)-1-methyl-2-butynyl-N-(4-fluorophenyl) carbamate oxalate] has been suggested to be a selective agonist at the M_1 subtype of muscarinic receptor [Lambrecht G. et al., Life Sci. 56 (1995) 815–822]. We synthesized the compound and tested its selectivity for different muscarinic receptors with binding experiments using rat cerebral cortex synaptosomal membranes and cloned human m1 to m5 receptors and by functional experiments on rabbit vas deferens preparations. There was little difference in affinity for the compound at the different cloned muscarinic receptors (IC_{50}s for displacement of ^{3}H-*N*-methylscopolamine were 0.7–1.0 μM). On rabbit vas deferens preparations, (*S*)-4-F-MePyMcN did reduce twitch responses to electrical stimulation like the known M_1 agonist McN-A-343, but unlike McN-A-343 the compound reduced postsynaptic sensitivity to noradrenaline, ATP and KCl. Because of these additional actions, (*S*)-4-F-MePyMcN may not be suitable as a tool to study M_1 muscarinic receptors selectively. © Elsevier, Paris

muscarinic receptors / selective agonists / ligand binding / McN-A-343 / N-methylscopolamine

1. Introduction

Muscarinic receptors have been divided into M_1, M_2, M_3 and M_4 subtypes on the basis of pharmacological studies, and there are five genes that direct synthesis of five subtypes of receptor protein, m1 to m5. All of the muscarinic receptor subtypes have been demonstrated to exist in the CNS [1, 2]. The pharmacological classification of muscarinic receptors was developed on the basis of data derived from experiments with the M_1-selective agonist {4-{[N-(3-chlorophenyl)carbamoyl]oxy}-2 butynyl}trimethylammonium chloride (McN-A-343) **1** (see *figure 1*), and the M_1-selective antagonist pirenzepine [3]. Within the brain, M_1 receptors are found especially in the cortex and the hippocampus, where they are thought to play an important role in learning and memory processes. Centrally-acting, selective M_1 receptor agonists may be useful for the treatment of the cholinergic deficit involved in Alzheimer's disease [4].

Lambrecht et al. [4] synthesised and pharmacologically characterised a series of novel McN-A-343 analogues, for example **2** *(figure 1)*. They hoped to enhance the functional selectivity of McN-A-343 in favour of the M_1 subtype and to discover tertiary M_1-selective agonists capable of crossing the blood–brain barrier. These new compounds were assayed for muscarinic activity on the rabbit vas deferens preparation (putative M_1 receptors) as well as on the guinea-pig atria (M_2 receptors) and ileum (M_3 receptors). Two of the McN-A-343 derivatives prepared by Lambrecht et al. [4] were 4-F-PyMcN **3** and 4-F-PyMcN$^+$ **4** *(figure 1)*. Subsequently, (*S*)-4-F-MePyMcN **5** *(figure 1)* was prepared and tested [5]. The details of the synthesis were not published, but the pharmacological results indicated that the compound was a potent and selective partial agonist at M_1 receptors and an antagonist at M_2 and M_3 receptors. Because of the

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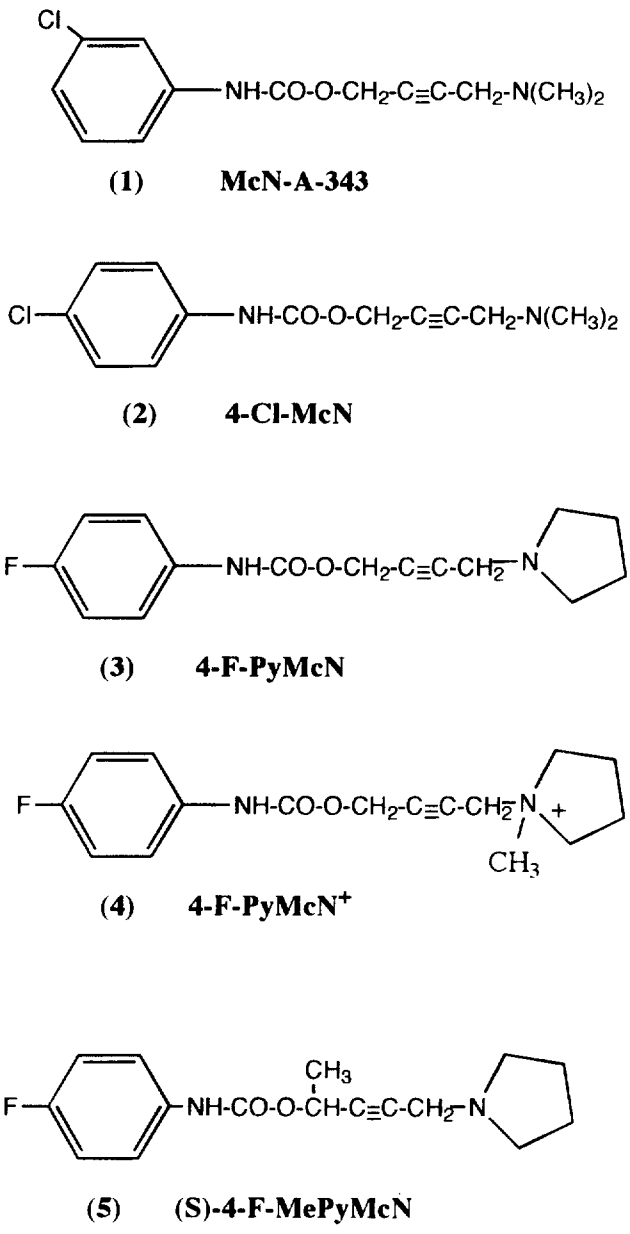

Figure 1. Structure of McN-A-343 and analogues, including (*S*)-4-F-MePyMcN.

potential usefulness of this compound as a selective agonist for the study of M_1 receptors, we undertook its synthesis and its characterisation on human cloned muscarinic receptors and on classical pharmacological preparations.

2. Chemistry

The compound (*S*)-**10**, which served as an intermediate to the enantiomer **11**, was prepared from the resolved 3-butyn-2-ol hydrogen phthalate (*S*)-**9** *(figure 2)*.

The racemic compound **7** consisted of equal parts of (*R*)- and (*S*)-isomers. The resolution of compound **7** was achieved by fractional crystallisation using (*S*)- or (*R*)-α-methylbenzyl amine. Thus, treatment of the racemic product **7** with (*S*)-α-methyl benzyl amine in acetone, after seven recrystallisations, gave the pure isomer (*S*)-3-butyn-2-ol hydrogen phthalate-(*S*)-(α-methylbenzyl) ammonium salt **8**, which upon treatment with 5 molar aqueous hydrogen chloride furnished the desired compound (*S*)-3-butyl-2-ol hydrogen phthalate **9**.

Treatment of the (*S*)-compound **9** with pyrrolidine and paraformaldehyde in the presence of glacial acetic acid and cuprous chloride using dioxane as solvent with heating at 55 °C for 4 h afforded the desired compound (*S*)-4-(pyrrolidino)-1-methyl-2-butyn-1-ol **10** in good yield *(figure 2)*. This compound **10** was treated with 4-fluorophenyl isocyanate in the presence of triethyl amine in tetrahydrofuran with stirring at room temperature for three days to give the target compound **5** which was converted to its oxalate **11** *(figure 3)*.

3. Pharmacology: results and discussion

3.1. Binding experiments

(*S*)-4-F-MePyMcN was tested for its ability to displace the nonselective muscarinic antagonist ^{3}H-*N*-methylscopolamine (NMS) from its binding sites on membranes from rat cerebral cortex. As shown in *figure 4a*, the test compound could displace almost all of the bound ^{3}H-NMS. The IC_{50} was 1.2×10^{-6} M, representing a K_i of 3.1×10^{-7} M. As the muscarinic receptors in cerebral cortex are about 50% M_1 and then mainly a mixture of M_2 and M_4 subtypes [1], the almost complete displacement of ^{3}H-NMS by (*S*)-4-F-MePyMcN suggests that the compound does not clearly discriminate between these receptor subtypes.

To gain further insight into binding properties of (*S*)-4-F-MePyMcN at defined subtypes of muscarinic receptors, competition binding experiments were carried out using ^{3}H-NMS and membranes containing the cloned human m1 to m5 receptors. As shown in *figure 4b*, (*S*)-4-F-MePyMcN caused similar displacements at all five receptor subtypes. The IC_{50}s were not significantly different at any of the subtypes, being between 0.7 and 1.0 μM (K_i between 0.16 and 0.25 μM).

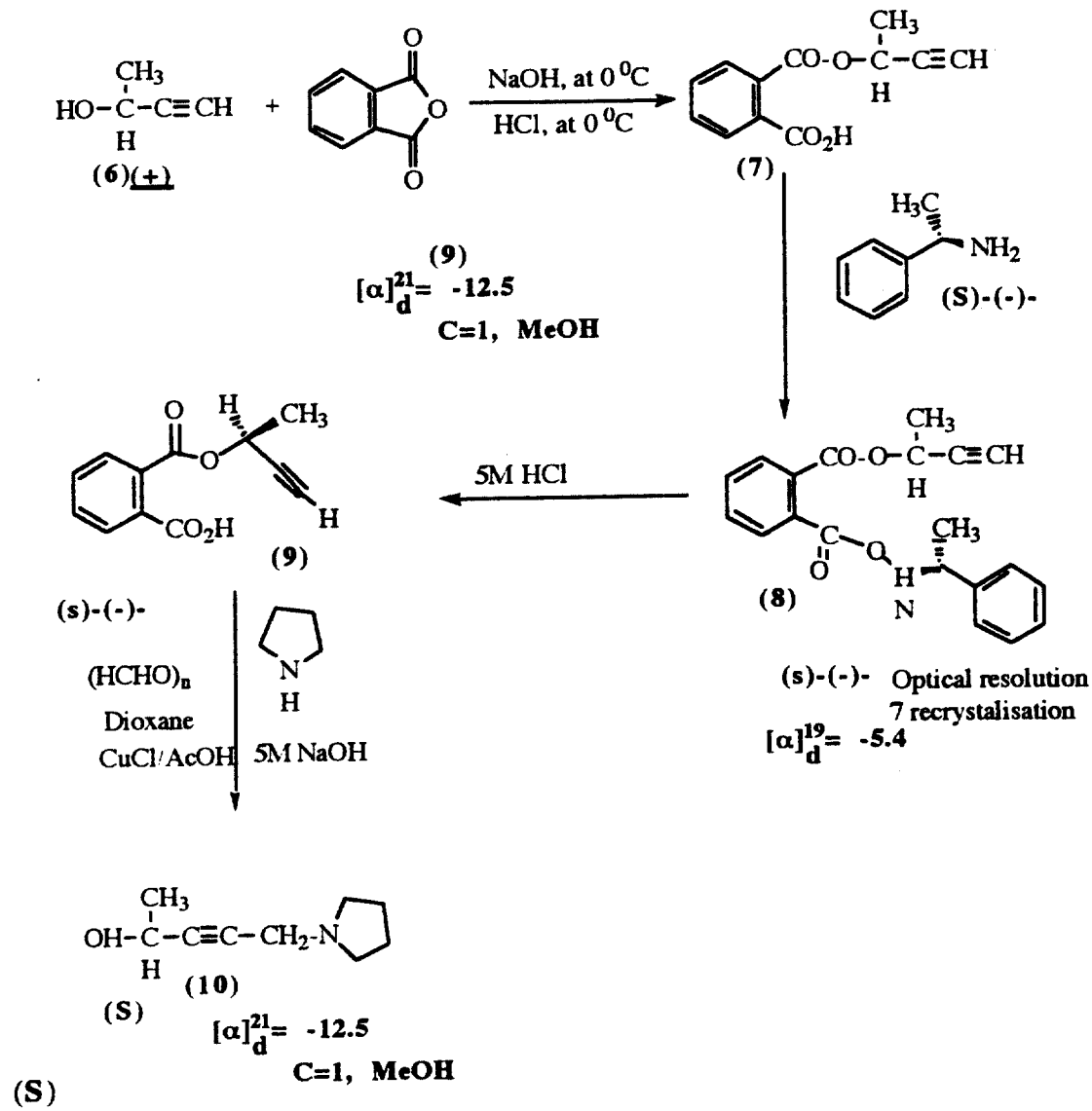

Figure 2. Synthesis of (*S*)-4-(pyrrolidino)-1-methyl-2-butyn-1-ol.

3.2. Functional experiments

As (*S*)-4-F-MePyMcN has been reported to be a partial agonist at M_1 receptors [5], it was tested on rabbit vas deferens preparations, which have presynaptic M_1 receptors that modulate release of the excitatory neurotransmitters, noradrenaline and ATP [7, 8]. Addition of McN-A-343 at concentrations above 0.1 μM reduced the twitch responses of the vas deferens to field stimulation without affecting postsynaptic sensitivity (*figure 5a*). (*S*)-4-F-MePyMcN also reduced responses to field stimulation at concentrations of 100 nM and above (*figure 5a*). The EC_{50} was about 10^{-5} M. However, at the highest concentration tested (10^{-4} M), (*S*)-4-F-MePyMcN also reduced postsynaptic sensitivity to ATP (to 27 ± 5% of control; *figure 5b*), noradrenaline (to 54 ± 9%; *figure 5c*) and KCl

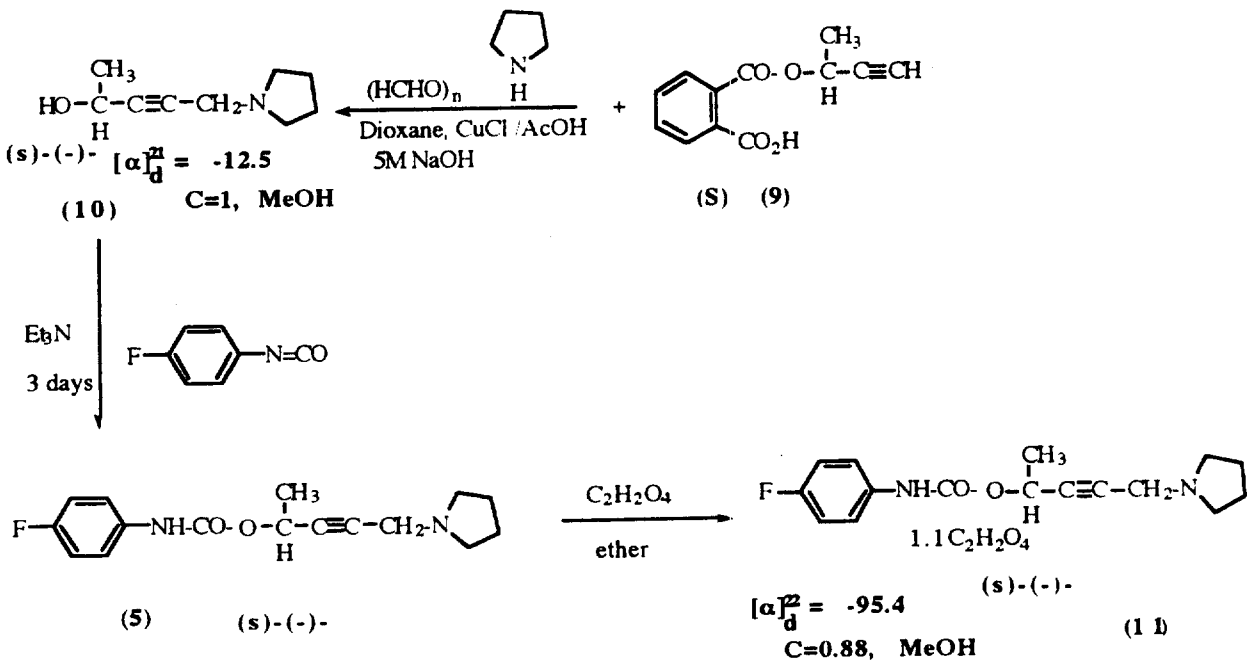

Figure 3. Synthesis of the oxalate salt of (*S*)-4-F-MePyMcN.

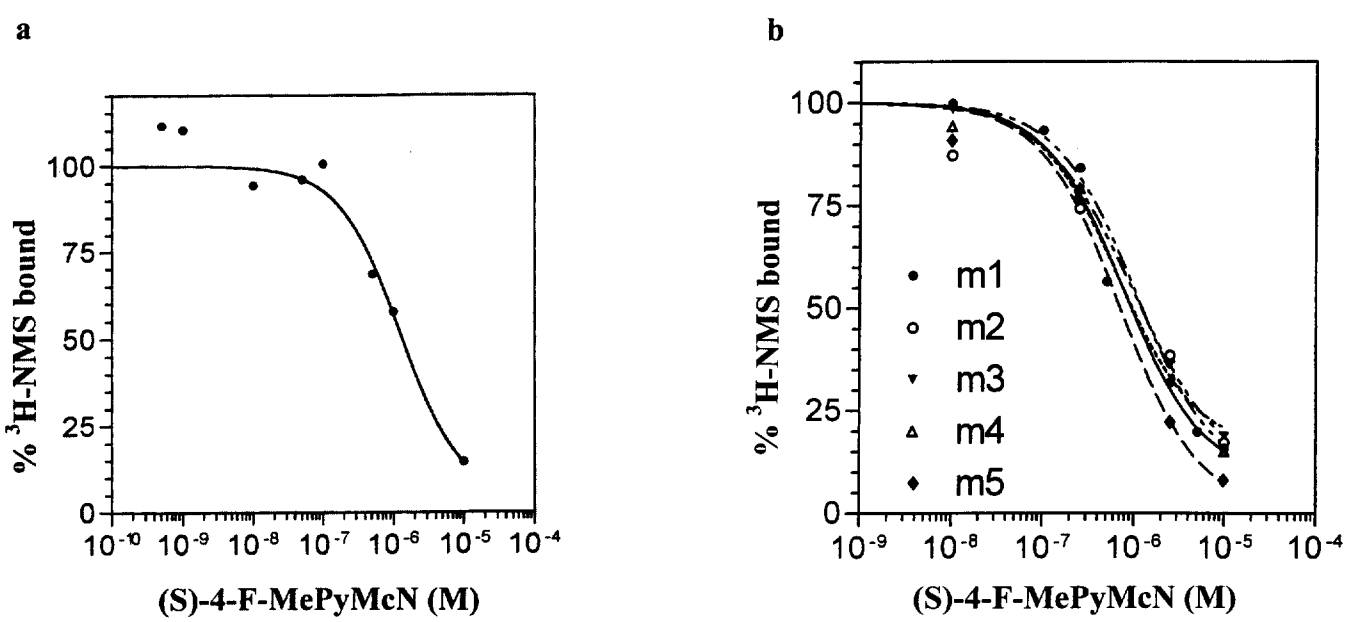

Figure 4. Competition binding experiments between ^{3}H-N-methylscopolamine and 4-F-MePyMcN. (a) Binding to synaptosomal membranes prepared from rat cerebral cortex. (b) Binding to membranes containing cloned human muscarinic receptor subtypes: m1 (solid circles), m2 (open circles), m3 (solid triangles), m4 (open triangles), and m5 (diamonds).

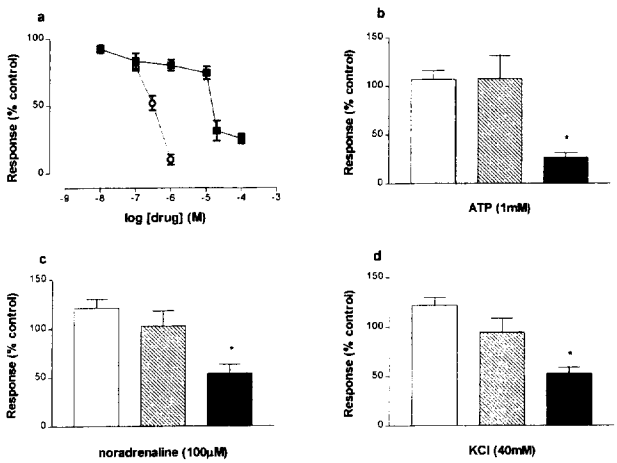

Figure 5. Effects of (*S*)-4-F-MePyMcN on the rabbit vas deferens preparation. (a) Effects of McNeil-A-343 (open circles) and (*S*)-4-F-MePyMcN (solid squares) on twitch responses to electrical stimulation. (b, c, d) Effect of McNeil-A-343 (1 μM) (open areas), (*S*)-4-F-MePyMcN (10 μM) (hatched areas), and (*S*)-4-F-MePyMcN (100 μM) (solid areas) on responses to submaximal concentrations of exogenously applied (b) ATP, (c) noradrenaline, and (d) KCl. $n = 4$ to 40. *: $p < 0.05$, using the Mann–Whitney U test. Bars show the standard error of the mean.

(to 52 ± 6%; *figure 5d*), implying a nonspecific depressant effect on the smooth muscle preparation. At a concentration causing equivalent reduction in twitch response, McN-A-343 (1 μM) did not significantly depress responses to ATP, noradrenaline or KCl (*figure 5b–d*).

4. Conclusions

(*S*)-4-F-MePyMcN was confirmed as being able to bind to M_1, M_2 and M_3 receptors [5], and was also shown to be equally effective at binding to the m4 and m5 genetic subtypes of muscarinic receptor. The affinities of binding of the compound to the cloned m1, m2 and m3 receptors were similar to the values derived previously from functional pharmacological experiments in rabbit vas deferens (for M_1 receptors), guinea pig atria (for M_2 receptors), and guinea pig ileum preparations (for M_3 receptors) [5]. Although it was previously reported [5] that (*S*)-4-F-MePyMcN was a potent partial agonist at M_1 receptors because it reduced twitch responses of rabbit vas deferens preparations to field stimulation, at least some of this effect could be due to the compound's ability to block this preparation postsynaptically, as shown by the reduction in responses to ATP, noradrenaline and KCl. For this reason, (*S*)-4-F-MePyMcN may not be a suitable tool for studying the functions of M_1 muscarinic receptors.

5. Experimental protocols

5.1. Chemistry

5.1.1. Analysis

Melting points (m.p.) were determined using a Gallenkamp melting point apparatus and are uncorrected. Elemental analyses were carried out in the microanalytical laboratory at the University of Strathclyde, Glasgow. Infra-red (IR) spectra were obtained using a Mattson Galaxy FTIR spectrometer with samples as pressed potassium bromide (KBr) discs or as a liquid film (liq. film) on sodium chloride discs. The nature of the bands has been abbreviated as follows: (br) broad; (s) strong; (m) medium; (w) weak; (str) stretching; (def) deformation. ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra and ^{13}C nuclear magnetic resonance (^{13}C NMR) spectra were obtained in a variety of deuterated solvents using a Bruker WM-250 (250 MHz) or a Bruker AMX-400 (400 MHz) spectrometer. Chemical shifts are expressed in parts per million (ppm) as values. Multiplicities are abbreviated as: (s) singlet; (d) doublet; (dd) doublet of doublet; (t) triplet; (q) quartet; (m) multiplet. Coupling constants (*J*) are expressed in hertz (Hz).

Electron impact (EI) and Fast atom bombardment (FAB) mass spectra (MS) were obtained using a Jeol JMS-AX505HA mass spectrometer for both high resolution (high res.) and low resolution (low res.) measurements.

5.1.2. (+)-3-Butyn-2-ol-hydrogen phthalate 7

To a mixture of (+)-3-butyn-2-ol (28 g, 0.40 mol) and phthalic anhydride (89 g, 0.6 mol) was added ice-cold aqueous 10% NaOH (240 mL) in three portions, with swirling. The mixture was shaken for 5 min, rapidly filtered, and then acidified by gradual addition of ice-cold 5 N hydrochloric acid. The mixture was extracted with chloroform (4 × 100 mL), and the combined organic layers were filtered, dried over $MgSO_4$ and evaporated to give after crystallisation from hexane 63 g (73%).

M.p. 97–99 °C; literature [9] m.p. 96.5–98 °C.

5.1.3. (S)-3-Butyn-2-ol-hydrogen phthalate (S)-α-methylbenzylammonium salt 8

To (+)-3-butyn-2-ol-hydrogen phthalate (36 g, 0.0165 mol) stirring in acetone (500 mL) was added

(*S*)-(–)-α-methylbenzylamine (20 g, 0.0165 mol) $[\alpha]_D$ –39 (neat) over a 5 min period. The resulting semi crystalline mass was mixed thoroughly by shaking, then the mixture was heated at gentle reflux for 30 min, and then left at room temperature overnight. Seven recrystallisations of the precipitate and its mother liquors from acetone afforded 17 g (32%) of pure (*S*)-3-butyn-2-ol-hydrogen phathalate (*S*)-α-methylbenzylammonium salt.

M.p. 138–139 °C (literature: m.p. 138.5–139 °C). ^{1}H NMR (400 MHz) (DMSO-d_6) 7.30–7.72 (m, 10 ArH); 5.50 (dq, J = 6.7 and 2.0 Hz, C1–H); 4.33 (q, J = 6.8 Hz, benzylic *CH*); 3.51(d, J = 2.2 Hz, C3–H); 1.48 (d, J = 7.0 Hz, Ar CH–*CH*$_3$), 1.38 (d, J = 6.8 Hz, C1–*CH*$_3$). Optical rotation $[\alpha]_D$ at 19 °C = –5.4 (c = 1, C_2H_5OH; literature [6], $[\alpha]_D$ at 22 °C = –5.5, c = 0.88, C_2H_5OH).

5.1.4. (S)-4-(Pyrrolidino)-1-methyl-2-butyn-1-ol ***10***

Aqueous hydrochloric acid (5 M, 160 mL) was added to a solution of (*S*)-3-butyn-2-ol-hydrogen phathalate (*S*)-α-methylbenzylammonium salt (8.8 g, 26 mmol) in water (160 mL). The resulting solution was stirred at room temperature for 5 min, then extracted with diethyl ether (4 × 200 mL), and the combined organic layers were dried over $MgSO_4$. The solution was filtered and the solvent was removed under reduced pressure to leave a pale brown oil of (*S*)-3-butyn-2-ol-hydrogen phthalate (TLC R_f on silica = 0.22 [chloroform–methanol 10%]). This oil was dissolved in dioxane (250 mL), and paraformaldehyde (827 mg, 28 mmol), cuprous chloride (205 mg, 2.1 mmol), glacial acetic acid (2 mL) and pyrrolidine (3 g, 42 mmol) were successively added to the solution. The reaction flask was sealed and the mixture was heated at 55 °C (oil bath temperature) for 4 h. The dioxane was removed under reduced pressure, and water (90 mL), and 5 molar solution of NaOH (90 mL) were added to the residue. The resulting mixture was stirred at room temperature for 3 h. Diethyl ether (3000 mL) was added to the alkaline solution and the layers were separated. Additional extraction of the aqueous layer with diethyl ether (4 × 100 mL), was followed by drying over $MgSO_4$. The solution was filtered and the solvent was removed under reduced pressure at 30 °C to give an oil. This oil was purified by column chromatography on silica gel with the eluent [chloroform-methanol 5%]. This reaction gave 2.7 g (65%) of (*S*)-4-(pyrrolidino)-1-methyl-2-butyn-1-ol.

Part of the base was converted into the oxalate salt, and recrystallised from acetone–ether.

M.p. 116–117 °C. ^{1}H NMR (400 MHz) (Me-OD) 4.37 (q, J = 6.8 Hz, *CH*); 3.98 (s, CH_2–N); 3.17 (t, $(CH_2)_2$–N), 1.97 (t, $(CH_2)_2$); 1.27 (d, J = 4.0 Hz, CH_3). Optical rotation $[\alpha]_D$ at 21 °C = –12.4 (c = 1, C_2H_5OH).

5.1.5. (S)-4-(Pyrrolidino)-1-methyl-2-butynyl-N-(4-fluorophenyl) carbamate oxalate [11] and (S)-4-F-MePyMcN

A solution of 4-fluoro isocyanate (2.15 g, 15.68 mmol) in tetrahydrofuran (15 mL) was added to an ice-cold stirred solution of (*S*)-4-(pyrrolidino)-1-methyl-2-butyn-1-ol (1.6 g, 10.45 mmol) in tetrahydrofuran (30 mL). Triethylamine (0.5 g, 4.95 mmol) was added to the reaction mixture. The ice bath was removed after 1 h, and the mixture was stirred at room temperature for 8 h. Then more 4-fluorophenyl isocyanate (0.5 g) and triethyl amine (150 mg) were added and the mixture was stirred at room temperature for 20 h. The tetrahydrofuran was removed under reduced pressure to leave a semi-solid mass. Hydrochloric acid 1 molar (30 mL) was added to the residue, and the mixture was extracted with diethyl ether (4 × 200 mL). The aqueous layer was made alkaline with solid sodium hydrogen carbonate to pH = 8; and the mixture was extracted with diethyl ether (5 × 150 mL). The combined organic layers were dried over $MgSO_4$, and the solvent was removed under reduced pressure to leave a brown oil.

This oil was purified by column chromatography on silica gel with the eluent (dichloromethane–methanol 5%) to give 1.5 g (50%) of pure free base.

The free base was converted into the oxalate salt and 1.7 g, (86%) of (*S*)-4-(pyrrolidino)-1-methyl-2-butynyl-N-(4-fluorophenyl) carbamate–oxalate was obtained after two recrystallisations from acetone–diethyl ether.

M.p. 102–104 °C. Infrared (Nujol, liquid film) 3189–3235 cm^{-1} (N–H, str); 1719 cm^{-1} (C=O), 1617 cm^{-1} (C=C); 779–695 cm^{-1} (subst. benzene). ^{1}H NMR (400 MHz) (DMSO-d_6) 10.06 (s, NH); 7.21–7.38 (m, 3 Ar-H); 6.83 (dt 1h, Ar-H); 5.47 (q, J = 4 Hz, C3–H); 3.98 (s, CH_2–N), 3.07 (s, 4H 2 × CH_2–N); 1.87 (s, 4H 2 × CH_2); 1.52 (d, J = 6.8 Hz, CH_3). Mass spectroscopy EI *m/z* 290. Microanalysis: Found C 56.84; H 6.64; N 7.31. Calculated for $C_{16}H_{19}FN_2O_2$: 1 $C_2H_2O_4$ C 56.83; H 5.56; N 7.36. Optical rotation $[\alpha]_D$ at 22 °C = –5.4 (c = 0.8, MeOH). HPLC indicated one spot 100% RT = 16.76 eluent chloroform–methanol.

5.2. Pharmacology protocols

Radioligand binding assays with ^{3}H-NMS were carried out using 0.5 mL aliquots of membranes containing 0.1 mg protein mL^{-1} of synaptosomal membranes prepared from rat cerebral cortex, or 0.02 mg protein mL^{-1} of membranes containing cloned muscarinic AChR subtypes (Biosignal, Montreal) in 50 mM Tris-HCl, 10 mM

$MgCl_2$, 1 mM EDTA buffer, pH 7.4. Parallel samples were incubated with 10 μM atropine to determine the non-specific binding. The reaction was terminated by filtering through Whatman GF/B filters. The filters were dried at 80 °C and placed in vials containing 3 mL 2.5-diphenyloxazole/xylene (5 g L^{-1}) as scintillation fluid. The experiments to construct the inhibition curves were performed in triplicate with aliquots of membranes, by incubating a fixed concentration of the radioligand ^{3}H-NMS (about 0.5 nM), with different concentrations of (*S*)-4-F-MePyMcN for 60 min at 25 °C. Details are given by Kornisiuk et al. [10].

5.3. Rabbit vas deferens

Vas deferens muscles were removed from New Zealand white rabbits (2.0–2.5 kg) that had been killed by injection of thiopentone. Each vasa was cut into prostatic and epididymal portions. Two preparations were suspended in 10 mL tissue baths in Krebs–Henseleit solution at 33 °C and pH 7.3. Resting tension was 1.0 g weight. The solution contained 1 mM $CaCl_2$ in order to enhance the sensitivity of the preparation to compounds acting at M_1 receptors [11]. Idazoxan (1 μM) was added to the solution to block presynaptic α_2-adrenoceptors, in preference to yohimbine [12]. Contractions were obtained to field stimulation at 0.05 Hz with 0.5 s trains of 0.5 ms pulses at 10 Hz.

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