Pergamon

Bioorganic & Medicinal Chemistry 7 (1999) 1539–1547

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis and Functional Characterization of Novel Derivatives Related to Oxotremorine and Oxotremorine-M

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Received 5 October 1998; accepted 28 December 1998

Abstract—Two subseries of nonquaternized (**5a**–**10a**) and quaternized derivatives (**5b**–**10b**) related to oxotremorine and oxotremorine-M were synthesized and tested. The agonist potency at the muscarinic receptor subtypes of the new compounds was estimated in three classical in vitro functional assays: M_1 rabbit vas deferens, M_2 guinea pig left atrium and M_3 guinea pig ileum. In addition, the occurrence of central muscarinic effects was evaluated as tremorigenic activity after intraperitoneal administration in mice. In in vitro tests a nonselective muscarinic activity was exhibited by all the derivatives with potencies values that, in some instances, surpassed those of the reference compounds (i.e. **8b**). Functional selectivity was evidenced only for the oxotremorine-like derivative **9a**, which behaved as a mixed M_3-agonist/M_1-antagonist ($pD_2 = 5.85$; $pA_2 = 4.76$, respectively). In in vivo tests non-quaternary compounds were able to evoke central muscarinic effects, with a potency order parallel to that observed in vitro.

Introduction

The muscarinic receptor population represents a class of cholinergic receptors abundant in the parasympathetic and in the central nervous systems, where they mediate both excitatory and inhibitory effects.[1] At present, the muscarinic receptor pharmacological classification distinguishes among M_1, M_2, M_3, and M_4 subtypes. A fifth subtype, termed m_5, has been predicted by molecular cloning studies, even though its physiological role is at present missing.[2] The characterization of these multiple muscarinic receptors came from the availability of ligands, primarily antagonists, able to selectively interact with one of the subtypes.[3–6] In the last decade a number of potent and selective muscarinic antagonists has been successfully designed.[7–11] However, current interest is also addressed to the research of agonists, since the development of new highly potent and subtype-selective muscarinic receptor agonists could provide both improved experimental tools and novel therapeutic agents, useful, for example, in the treatment of pain and Alzheimer's disease.[12,13]

In the course of previous studies on the structure–activity relationships of muscarinic ligands structurally related to natural muscarine **1**,[14–18] we synthesized the highly effective agonist azamuscarone **2** (Fig. 1), which proved to be as potent as the parent compound at guinea pig, atrial and ileal muscarinic sites.[14,15] More recently, we prepared derivatives **5a**, **6a**, and **6b** (Fig. 1) by incorporating the isoxazolidin-3-one moiety of **2** into the skeleton of the potent yet nonselective muscarinic agonists oxotremorine (**3**) and oxotremorine-M (**4**).[19] Compounds **5a**, **6a**,**b** displayed different binding affinities at brain and heart muscarinic subtypes.[19]

Based on these preliminary results, a larger set of oxotremorine/oxotremorine-M-like derivatives has been designed by modifying further both the nature of the heterocyclic ring and the attachment point of the acetylenic side chain. Accordingly, in addition to the isoxazolidin-3-one derivatives **5a**,**b**–**6a**,**b**, we prepared and tested the regioisomeric Δ^2-isoxazolinyl-ethers **7a**,**b**–**8a**,**b** and the related isoxazolyl-ethers **9a,b**–**10a,b** (Fig. 1).

Key words: Synthesis; oxotremorine; oxotremorine-M; muscarinic agonists; vas deferens; left atrium; ileum; tremorigenic activity.
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PII: S0968-0896(99)00107-8

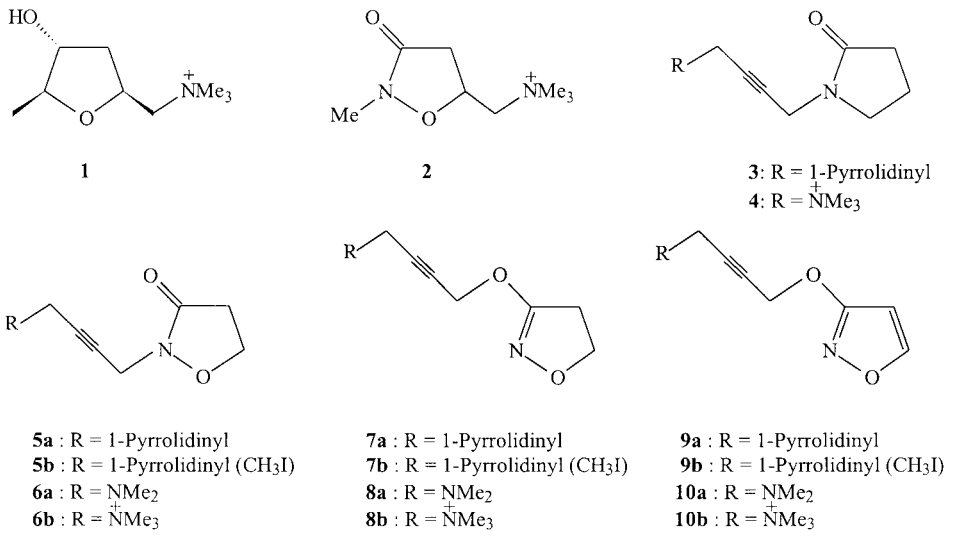
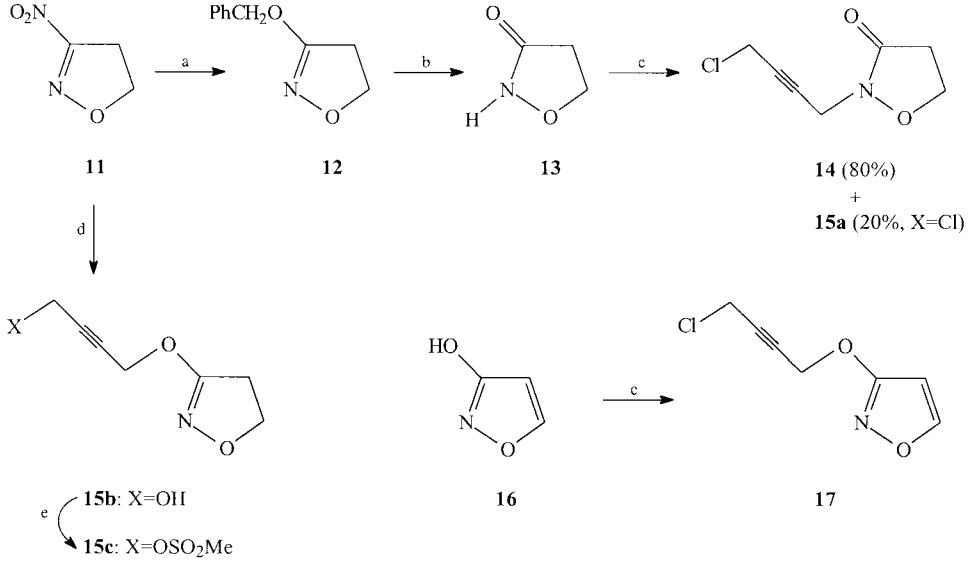


Figure 1.

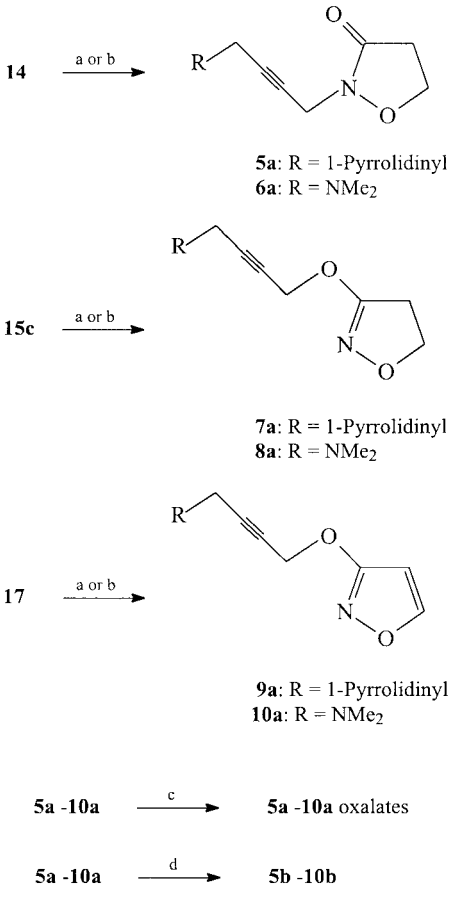
This paper deals with the synthesis of derivatives **5a,b**–**10a,b** and the evaluation of their pharmacological profile by classical in vitro functional assays, in order to estimate their agonist potency at muscarinic M_1, M_2, and M_3 receptor subtypes. In addition, we investigated the ability of the compounds under study to produce tremor in mice after ip administration. Such a symptom can be taken as a measure of the activity of the compounds at central muscarinic receptors.[20]

Chemistry

Target compounds were synthesized along the reaction sequences reported in Schemes 1 and 2. Isoxazolidin-3-one **13** was conveniently prepared by converting known 3-nitro-Δ^2-isoxazoline **11**[21] into the corresponding 3-benzyloxy derivative **12**, followed by catalytic hydrogenolysis (Scheme 1). Electrophilic addition of 1,4-dichloro-2-butyne to **13**, carried out in refluxing acetone in the presence of K_2CO_3, produced a 4:1 mixture of regioisomers **14** and **15a** (Scheme 1). The *N*- and *O*-alkynyl derivatives **14** and **15a** were easily separated by column chromatography (42% overall yield). Their structure was assigned by taking into account the difference between the ^{1}H NMR chemical shift value of the CH_2 protons attached to the heteroatom in the side chain (4.34 δ for **14** and 4.82 δ for **15a**).[22] The high regioselectivity of the alkylation step hampered the application of this reaction sequence to the preparation of the Δ^2-isoxazoline derivatives **7a,b** and **8a,b**. Alternatively, by refluxing **11**, 2-butyne-1,4-diol and sodium hydride in THF, primary alcohol **15b** was isolated in 51% yield. Such an intermediate was then transformed



Scheme 1. (a) $C_6H_5CH_2OLi$/DMSO; (b) H_2–Pd/C, 5%; (c) 1,4-dichloro-2-butyne, K_2CO_3/acetone; (d) 2-butyne-1,4-diol, NaH/THF; (e) MsCl, NEt_3/CH_2Cl_2.



Scheme 2. (a) Pyrrolidine, DMF; (b) NMe_2, DMF; (c) $C_2H_2O_4$, ether; (d) CH_3I, ether.

into the corresponding mesylate **15c** by a standard procedure (Scheme 1). The preparation of intermediate isoxazolyl-ether **17** (Scheme 1) was achieved through the reaction of 3-hydroxyisoxazole **16**[23] with 1,4-dichloro-2-butyne, according to the procedure previously applied to **13**.

The desired final compounds were synthesized by treatment of the suitable intermediate (**14**, **15c** or **17**) with a DMF (or methanol) solution of pyrrolidine (dimethylamine) at room temperature (Scheme 2). 1-Pyrrolidinoalkynyl (**5a**, **7a**, **9a**) or dimethylaminoalkynyl (**6a**, **8a**, **10a**) derivatives were then converted into the corresponding oxalates, or quaternized with iodomethane to yield the trimethylammonium salts **5b**–**10b**.

Results and Discussion

The pharmacological evaluation of the agonist potency and selectivity of compounds **5**–**10** towards M_1, M_2, and M_3 muscarinic receptors was performed in vitro on three suitable preparations. Rabbit electrically-stimulated vas deferens, guinea pig electrically-driven left atrium and guinea pig ileum were used, respectively, since in each preparation the functional response is primarily due to the activation of a single receptor population subtype.[24–26] Oxotremorine and oxotremorine-M were adopted as reference compounds and the results on the first functionally selective muscarinic agonist McN-A-343 were also taken into account. Further experiments were performed in vivo on mice to evaluate the ability of the new muscarinic agonists to produce central cholinergic effects, through the detection of their tremorigenic activity after peripheral administration.

The conventional muscarinic agonists oxotremorine and oxotremorine-M, the prototype molecules of the two subseries of compounds assayed (**5a,b**, **7a,b**, **9a,b** and **6a,b**, **8a,b**, **10a,b**, respectively), exhibited a good potency in stimulating vas deferens (M_1), atrial (M_2) and ileal (M_3) receptors, although with a low selectivity (Table 1). Within the oxotremorine-like derivatives **5a,b**, **7a,b**, **9a,b**, the only isosteric replacement of the methylene group in the pyrrolidin-2-one ring with oxygen gave a compound (**5a**) with a M_3 potency superimposable to that of the prototype compound, along with a slightly higher ileum/vas deferens selectivity. On the contrary, the corresponding trimethylammonium derivative **5b** showed an overall decrease in agonist potency at all the muscarinic receptor subtypes.

In the same subset of compounds, on passing from isoxazolidin-3-ones (**5a,b**) to Δ^2-isoxazoline (**7a,b**) and isoxazole (**9a,b**) derivatives, different pharmacological profiles emerged. Between Δ^2-isoxazoline derivatives **7a** and **7b**, only **7b** showed an increased potency when compared to the corresponding regioisomer **5b**, whereas lower or comparable potency was observed when the results of the isoxazole congeners **9a** and **9b** were taken into account. Interestingly enough, **9a** and **9b** were able to discriminate the muscarinic receptor subtypes. Methiodide **9b** showed a M_1/M_2 potency ratio of about 30, whereas the non quaternized analogue **9a** exhibited different efficacy at M_1, M_2, and M_3 muscarinic receptors. This latter compound was in fact able to fully induce M_3-mediated ileal contractions ($pD_2 = 5.85 \pm 0.05$), while showing only a partial M_2-related inhibition of the inotropism in electrically-driven left atrium ($pD_2 = 6.31 \pm 0.01$, $\alpha = 0.5$) (Fig. 2). As expected for a partial agonist, in this preparation **9a** antagonized oxotremorine concentration–response curves ($pK_B = 5.22 \pm 0.21$).

Finally, in electrically-stimulated vas deferens, **9a**, up to 0.1 mM, did not produce any M_1-dependent inhibition of twitch response but, on the contrary, it acted as a competitive antagonist in inhibiting McN-A-343 induced responses ($pA_2 = 4.76 \pm 0.9$, Fig. 3). The profile of this oxotremorine-like agonist clearly differed from that of oxotremorine, since **9a** was a mixed muscarinic agonist/antagonist. It behaved as a full M_3 agonist, a partial agonist at M_2 receptors and a competitive antagonist at M_1 receptors (intrinsic activity equal to 1, 0.5 and 0, respectively). Based on these results, such a compound can be classified as a functionally selective M_3 agonist, according to the criteria postulated for McN-A-343.[27]

As far as the oxotremorine-M-like derivatives **6a,b**, **8a,b**, **10a,b** are taken into account, isoxazolidin-3-one

Table 1. In vitro potencies (pD_2 values) of oxotremorine (Oxo), oxotremorine-M (Oxo-M) and **5a**,**b**–**10a**,**b** at muscarinic receptor subtypes and in vivo tremorigenic activity in mice (ED_{50} values) (data for the muscarinic agoinst McN-A-343 are also included)[a]

Compd	Electrically-stimulated rabbit vas deferens (M_1-receptor)	Electrically-driven guinea pig left atrium (M_2-receptor)	Guinea pig ileum (M_3-receptor)	Tremor ED_{50} (mg mg^{-1} ip)
Oxo	7.72 ± 0.20	8.41 ± 0.06	7.98 ± 0.09	0.58
5a	7.31 ± 0.11	7.73 ± 0.10	7.98 ± 0.12	2.73
5b	6.27 ± 0.02	6.95 ± 0.07	6.89 ± 0.09	> 4.0
7a	6.76 ± 0.16	7.00 ± 0.07	7.41 ± 0.03	1.58
7b	8.99 ± 0.01	8.30 ± 0.03	8.80 ± 0.03	> 4.0
9a	4.76 ± 0.09[b]	6.31 ± 0.01[c]	5.85 ± 0.05	> 30.0
9b	5.68 ± 0.07	7.20 ± 0.07	7.01 ± 0.07	> 4.0
Oxo-M	7.85 ± 0.08	7.94 ± 0.03	8.40 ± 0.03	> 4.0[d]
6a	6.82 ± 0.17	6.62 ± 0.07	8.01 ± 0.07	3.68
6b	8.16 ± 0.12	8.36 ± 0.05	8.63 ± 0.09	> 4.0[d]
8a	7.77 ± 0.17	7.94 ± 0.09	8.23 ± 0.06	0.26
8b	9.87 ± 0.07	10.1 ± 0.13	9.78 ± 0.10	> 0.1[d]
10a	6.47 ± 0.11	6.66 ± 0.06	6.37 ± 0.08	13.74
10b	8.47 ± 0.08	8.54 ± 0.03	8.34 ± 0.11	> 4.0[d]
McN-A-343	6.45 ± 0.09	Inactive	5.48 ± 0.03[e]	> 20.0

[a] Mean values ± SEM are given for each parameter.
[b] pA_2 value versus McN-A-343 response.
[c] Intrinsic activity (α) is equal to 0.5 (otherwise $\alpha = 1$).
[d] Lethal effects were observed.
[e] The value was taken from ref 33, where the conventional M_3-blockers unsurmountably antagonized McN-A-343 response.

trimethylammonium derivative **6b** showed a pharmacological profile quite superimposable to that of the parent compound, whereas a drop in the agonist potency was found for the corresponding non quaternized analogue **6a**. The data obtained for **6b** confirm its full potent muscarinic agonism, as already anticipated by radioligand binding assays.[19] Conversely, the predicted relevant M_2/M_1 selectivity[19] has not been corroborated by the present functional results.

The introduction of the Δ^2-isoxazolinyl-ether moiety gave either compound **8a**, which was almost equiactive, or the related methiodide **8b**, which was significantly more potent than the reference agonist. In particular, **8b** proved to be the most potent, albeit non selective, muscarinic agonist in the present study, since its potency (pD_2 from 9.78 to 10.1) was about 25- to 150-fold higher than that estimated for oxotremorine-M. A parallel trend was observed by considering the results on the related isoxazole derivatives, since **10a** was about 100-fold less potent than the corresponding trimethylammonium salt **10b**, which retained (or increased) the potency values found for oxotremorine-M.

On the whole, the introduction of a cationic trimethylammonium head brought about a remarkable increase of in vitro agonist potency at muscarinic receptors.

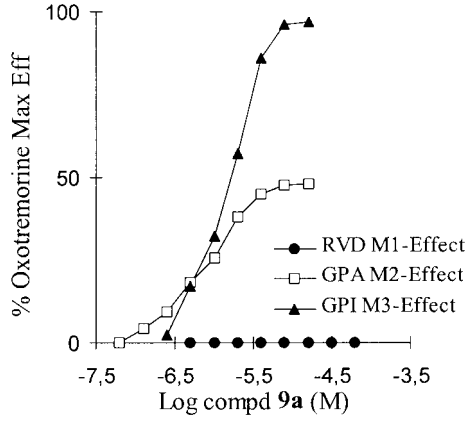


Figure 2. Concentration–response curves of **9a** on (RVD) rabbit vas deferens twitch responses, (GPA) electrically-driven guinea pig left atrium inotropism and (GPI) guinea pig ileum resting tone. Lack of M_1 agonism (●), M_2 partial agonism (□) and M_3 full agonism (▲) are expressed as percentage of the maximum response to oxotremorine in the different preparations. Each point is the mean of five experiments and vertical lines indicate SEM.

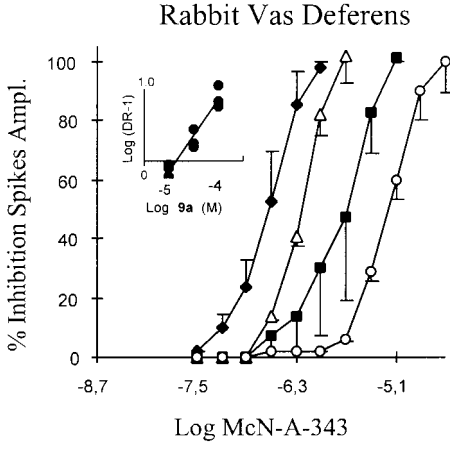


Figure 3. Concentration–response curves to McN-A-343 in electrically-stimulated rabbit vas deferens, in the absence (◆) and presence of 10 μM (△), 30 μM (■) and 100 μM (○) **9a**. Points are means with SEM ($n = 3$). Inset: Schild plot for the antagonism of McN-A-343-induced inhibitory effects by **9a**. Points are individual observations; the slope of the regression line (1.15 ± 0.16) is not significantly different from unity ($P > 0.05$).

The sole exception is represented by the pair **5a**/**5b**, **5b** being less active than the non quaternized counterpart **5a**, which is actually the closest structural analogue of oxotremorine within the compounds under study. All the methiodides were devoid of subtype selectivity except **9b**, which displayed a certain ability to discriminate the M_2 receptor subtype.

In in vivo studies on central muscarinic activity, oxotremorine-M as well as all the trimethylammonium agonists **5b**–**10b** failed, as expected, to produce tremor when given up to 4 mg kg^{-1} ip in mice, although they produced peripheral cholinergic effects (Table 1). In detail, following the administration of oxotremorine-M and dimethylamino methiodides **6b**, **8b**, and **10b**, lethal effects were observed, probably due to cardiovascular and respiratory effects caused by peripheral muscarinic receptors overstimulation. On the other hand, pyrrolidino methiodides **5b**, **7b**, and **9b** evoked only *para*-sympathomimetic responses, such as salivation (data not reported). The non quaternized compounds **5a**, **6a**, **7a**, **8a**, and **10a** caused a cholinergic-mediated tremor, exhibiting a potency order, in the stimulation of central muscarinic receptors, similar to that observed in isolated tissue preparations.

The low muscarinic potency detected for **9a** in in vitro tests was consistent with in vivo experiments, where it failed to induce both central (tremor) and peripheral (salivation) effects, even when administered up to high doses (30 mg kg^{-1} ip). No salivation was produced also by high doses of the non-tremorigenic muscarinic agonist McN-A-343, which shared with **9a** a poor M_3 activity at guinea pig ileum subtype.

Conclusion

The overall data of the present investigation evidenced that the new oxotremorine/oxotremorine-M-like analogues retained variable muscarinic agonist activity with respect to the parent agonists. The most interesting results, in terms of potency or selectivity, were achieved when the pyrrolidin-2-one ring was replaced either by the Δ^2-isoxazoline or the isoxazole moieties, with a concomitant shift of the acetylenic side chain. The first modification produced trimethylammonium derivative **8b**, which showed an unusual high potency in stimulating muscarinic receptors. This compound can be included in the group of highly powerful muscarinic agonists, potentially useful as a new experimental tool. On the other hand, the introduction of the isoxazole nucleus (i.e. a heteroaromatic ring) gave rise to **9a** and **9b** that, quite peculiarly, discriminated muscarinic receptor subtypes. While **9b** preferentially activated M_2 atrial receptors, the formal oxotremorine analogue **9a** behaved as a functionally selective M_3 muscarinic agonist, and may represent a reference compound for the design of new muscarinic agonists with enhanced selectivity, addressed towards M_3 receptors. Indeed, the availability of selective M_3 muscarinic agonists may be advantageous in the therapy of atonic conditions of the gastrointestinal/urogenital tract.[28]

Experimental

Material and methods

^{1}H NMR spectra were recorded with a Bruker AC-E 200 (200 mHz) spectrometer in $CDCl_3$ solutions (unless otherwise indicated); chemical shifts (δ) are expressed in ppm and coupling constants (*J*) in Hertz. TLC analyses were performed on commercial silica gel 60 F_{254} aluminum sheets; spots were further evidenced by spraying with a dilute alkaline potassium permanganate solution. Melting points were determined with a Büchi Mod.B 540 apparatus and are uncorrected. Liquid compounds were characterized by the oven temperature for Kugelrohr distillations. Microanalyses (C, H, N) of new compounds agreed with the theoretical value ±0.4%. 3-Nitro-Δ^2-isoxazoline **11** and 3-hydroxyisoxazole **16** were prepared according to published procedures.[21,23] Oxotremorine sesquifumarate and oxotremorine-M were purchased from Sigma.

Synthesis of 2-(4-chloro-2-butynyl)-isoxazolidin-3-one 14. A. To a solution of 63 mL (65.77 g, 0.608 mol) of benzyl alcohol in DMSO (300 mL) were added dropwise 59 mL of butyllithium 1.6 M in hexane at 0°C under stirring. To the resulting suspension, maintained under stirring for additional 30 min, a solution of **11**[21] (4.80 g, 41.30 mmol) in DMSO (20 mL) was added at room temperature. After stirring overnight, the reaction mixture was poured into 400 mL ice-water, then extracted with 5×100 mL portions of ether. The pooled organic extracts were dried over anhydrous sodium sulphate and concentrated at reduced pressure. The crude reaction mixture was first distilled at 100°C/10 mm Hg, then submitted to silica gel column chromatography (eluant: 20% ethyl acetate/petroleum ether) to yield 4.46 g (61%) of the desired benzyl ether.

3-Benzyloxy-Δ^2-isoxazoline **12**: light-yellow liquid, bp 120–125°C/0.5 mm Hg; R_f 0.31 (15% ethyl acetate/cyclohexane); ^{1}H NMR 3.01 (t, 2, H-4, J=9.5), 4.43 (t, 2, H-5, J=9.5), 5.16 (s, 2, CH_2Ph), 7.39 (bs, 5, arom.).

B. A solution of 4.20 g (23.73 mmol) of **12** in 50 mL MeOH was hydrogenated at atmospheric pressure over 5% Pd/C (450 mg). After absorption of one equivalent of hydrogen, the catalyst was removed by suction filtration over Celite and the solvent was evaporated under vacuum to give a crystalline residue of **13** (1.98 g, 96% yield). Isoxazolidin-3-one **13**: mp 71–71.5°C (colorless prisms from 50% ethyl acetate/petroleum ether); R_f 0.16 (50% ethyl acetate/cyclohexane); ^{1}H NMR 2.76 (t, 2, H-4, J=8.2), 4.42 (t, 2, H-5, J=8.2), 9.21 (bs, 1, NH, exchanges with D_2O).

C. To a solution of **13** (1.80 g, 20.69 mmol) in acetone (20 mL) was added potassium carbonate (5.72 g, 41.38 mmol). The suspension was heated at reflux for 1 h, then cooled at room temperature. 1,4-Dichloro-2-butyne (12.13 mL, 0.124 mol) was hence added and, after refluxing for 24 h, the crude reaction mixture was poured into water (100 mL) and extracted with dichloromethane (3×50 mL). After the usual work up, the dark brown residue was chromatographed on silica gel

(eluant: 40% ethyl acetate/petroleum ether) to give regioisomers **14** (1.22 g, 34% yield) and **15a** (0.280 g, 7.8% yield).

2-(4-Chloro-2-butynyl)-isoxazolidin-3-one **14**: mp 73.5–74.5°C (pale-yellow prisms from 10% ethyl acetate/petroleum ether); R_f 0.19 (30% ethyl acetate/cyclohexane); ^{1}H NMR 2.78 (t, 2, H-4, J=8.1), 4.14 (t, 2, CH_2Cl, J=1.8), 4.34 (t, 2, CH_2N, J=1.8), 4.38 (t, 2, H-5, J=8.1). Anal. $C_7H_8ClNO_2$ (C, H, N).

3-(4-Chloro-2-butynyl)oxy-Δ^2-isoxazoline **15a**: colorless liquid, bp 115–120°C/0.5 mm Hg; R_f 0.61 (30% ethyl acetate/cyclohexane); ^{1}H NMR 3.0 (t, 2, H-4, J=9.6), 4.18 (t, 2, CH_2Cl, J=1.8), 4.43 (t, 2, H-5, J=9.6), 4.82 (t, 2, O−CH_2−C≡, J=1.8). Anal. $C_7H_8ClNO_2$ (C, H, N).

Synthesis of 3-(4-hydroxy-2-butynyl)oxy-Δ^2-isoxazoline mesylate 15c. A. To a stirred solution of 2-butyne-1,4-diol (11.13 g, 0.129 mol) in THF (120 mL) was added portionwise sodium hydride (3.57 g, 0.149 mol) under an inert atmosphere. After stirring for 30 min at room temperature, a solution of **11** (5.0 g, 43.10 mmol) in THF (40 mL) was added dropwise, and the reaction mixture was heated at reflux for 24 h. After careful addition of water (70 mL), the crude reaction was concentrated at reduced pressure and extracted with ethyl acetate (6×50 mL). After the usual work up, the residue was submitted to column chromatography (eluant: 50% petroleum ether/ethyl acetate) to afford 3.39 g (51%) of the desired unsaturated primary alcohol.

3-(4-Hydroxy-2-butynyl)oxy-Δ^2-isoxazoline **15b**: colorless liquid, bp 180–185°C/0.5 mm Hg, R_f 0.35 (40% cyclohexane/ethyl acetate); ^{1}H NMR 1.68 (bs, 1, OH), 3.01 (t, 2, H-4, J=9.5), 4.34 (t, 2, CH_2OH, J=1.8), 4.42 (t, 2, H-5, J=9.5), 4.80 (t, 2, O−CH_2−C≡, J=1.8). Anal. $C_7H_9NO_3$ (C, H, N).

B. To a solution of **15b** (3.23 g, 20.97 mmol) and triethylamine (4.35 mL, 31.20 mmol) in dichloromethane (80 mL) a solution of methanesulfonyl chloride (2.42 mL, 31.20 mmol) in dichloromethane (20 mL) was added dropwise at 0°C. After stirring for 5 h at room temperature, 1 N HCl (50 mL) was added, the phases were separated and the aqueous layer was extracted with dichloromethane (3×30 mL). After the usual work up, the residue was column chromatographed (eluant: 50% petroleum ether/ethyl acetate) to give 3.65 g (75%) of ester **15c**.

3-(4-Hydroxy-2-butynyl)oxy-Δ^2-isoxazoline mesylate **15c**: mp 49–50°C (colorless prisms from 30% ethyl acetate/petroleum ether); R_f 0.43 (40% cyclohexane/ethyl acetate); ^{1}H NMR 3.01 (t, 2, H-4, J=9.4), 3.17 (s, 3, CH_3), 4.43 (t, 2, H-5, J=9.4), 4.83 (bs, 2, O−CH_2−C≡), 4.94 (bs, 2, CH_2OSO_2). Anal. $C_8H_{11}NO_5S$ (C, H, N).

Synthesis of 3-(4-chloro-2-butynyl)oxyisoxazole 17. The title compound was prepared in 36% yield from **16**,[23] following the procedure described above for the synthesis of **14** and increasing the overall refluxing time to 48 h.

3-(4-Chloro-2-butynyl)oxyisoxazole **17**: colorless liquid, bp 125–130°C/0.5 mm Hg; R_f 0.32 (10% ethyl acetate/cyclohexane); ^{1}H NMR 4.20 (s, 2, CH_2Cl), 4.94 (s, 2, OCH_2C≡), 6.01 (d, 1, H-4, J=1.8), 8.16 (d, 1, H-5, J=1.8). Anal. $C_7H_6ClNO_2$ (C, H, N).

General procedure for the synthesis of derivatives 5a,b and 10a,b. The following protocol is representative.

A. A stirred solution of **14** (1.0 g, 5.76 mmol) in DMF (20 mL) was reacted at room temperature with pyrrolidine (952 μL, 11.52 mmol). After completion of the conversion (about 3 h), the crude reaction mixture was distilled at 50°C/0.5 mmHg and the residue, acidified by addition of 30 mL 2 N HCl, was treated with ether (3×10 mL). The residual aqueous phase was made alkaline by portionwise addition of solid K_2CO_3 and extracted with dichloromethane (4×20 mL).The pooled organic extracts were dried over anhydrous sodium sulphate, the solvent was evaporated under vacuum and the oily residue was purified by silica gel column chromatography (eluant: 2–5% methanol/dichloromethane) to afford 0.616 g (51% yield) of the desired tertiary amine.

2-[4-(1-Pyrrolidino)-2-butynyl]-isoxazolidin-3-one **5a**: colorless oil, bp 140–145°C/0.5 mm Hg; R_f 0.39 (10% methanol/chloroform); ^{1}H NMR 1.80 (m, 4), 2.60 (m, 4), 2.77 (t, 2, H-4, J=8.1), 3.42 (t, 2, NCH_2−C≡, J=1.9), 4.31 (t, 2, ≡C−CH_2N−O, J=1.9), 4.36 (t, 2, H-5, J=8.1).

B. To a solution of **5a** (0.370 g, 1.78 mmol) in MeOH (5 mL) was added a solution of anhydrous oxalic acid (0.320 g, 3.56 mmol) in ether (20 mL). The corresponding sesquioxalate precipitated quantitatively on standing. **5a** ×1.5 $C_2H_2O_4$: mp 123–124°C (colorless prisms from 2-propanol). Anal. $C_{14}H_{19}N_2O_8$ (C, H, N).

C. An ethereal solution of **5a** was treated with a fivefold excess of methyl iodide at room temperature. The corresponding trimethylammonium derivative precipitated quantitatively as a thick colorless oil, which did not crystallize in different solvent mixtures.

2-[4-(1-Pyrrolidino)-2-butynyl]-isoxazolidin-3-one methiodide **5b**: ^{1}H NMR 2.32 (m, 4), 2.82 (t, 2, H-4, J=8.0), 3.46 (s, 3, NMe), 3.86 (m, 2), 4.02 (m, 2), 4.37 (t, 2, ≡C−CH_2N−O, J=1.9), 4.41 (t, 2, H-5, J=8.0), 4.82 (t, 2, MeNCH_2−C≡, J=1.9). Anal. $C_{12}H_{19}IN_2O_2$ (C, H, N).

Dimethylamino derivative **6a** was synthesized through reaction of **14** with dimethylamine, with a procedure similar to that applied for the preparation of **5a**. The desired compound was obtained in 65% yield after column chromatography (eluant: 5% methanol/dichloromethane).

2-[4-(Dimethylamino)-2-butynyl]-isoxazolidin-3-one **6a**: colorless liquid, bp 130–135°C/0.5 mm Hg; R_f 0.40 (10% methanol/chloroform); ^{1}H NMR 2.38 (s, 6, NMe_2), 2.78 (t, 2, H-4, J=8.1), 3.37 (t, 2, CH_2NMe_2, J=1.9), 4.33 (t, 2, C≡CH_2N−O, J=1.9), 4.38 (t, 2, H-5, J=8.1).

6a×$C_2H_2O_4$: mp 130.5–131.5°C (colorless prisms from 2-propanol). Anal. $C_{11}H_{16}N_2O_6$ (C, H, N). 2-[4-(Dimethylamino)-2-butynyl]-isoxazolidin-3-one methiodide **6b**: mp 182.5–183.5°C, dec. (colorless prisms from 2-propanol); ^{1}H NMR (DMSO-d_6) 2.81 (t, 2, H-4, J=8.0), 3.15 (s, 9, NMe_3), 4.37 (t, 2, H-5, J=8.0), 4.42 (bs, 2, C≡CH_2N−O), 4.49 (bs, 2, CH_2NMe_3). Anal. $C_{10}H_{17}IN_2O_2$ (C, H, N).

Compound **7a** was prepared according to the procedure described for **5a**, by using mesylate **15c** as the substrate and methanol (instead of DMF) as the solvent. In this instance the reaction time was increased to 24 h. Tertiary base **7a** was obtained as a pure compound in 64% yield, after solvent extraction and distillation at reduced pressure.

3-[4-(1-Pyrrolidino)-2-butynyl]oxy-Δ^2-isoxazoline **7a**: pale-yellow liquid, bp 150–155°C/0.5 mm Hg; R_f 0.45 (10% methanol/chloroform); ^{1}H NMR 1.81 (m, 4), 2.62 (m, 4), 3.0 (t, 2, H-4, J=9.5), 3.48 (t, 2, NCH_2C≡, J=1.9), 4.41 (t, 2, H-5, J=9.5), 4.80 (t, 2, OCH_2 C≡, J=1.9).

7a×$C_2H_2O_4$: mp 118.5–119.5°C (colorless prisms from 2-propanol). Anal. $C_{13}H_{18}N_2O_6$ (C, H, N).

3-[4-(1-Pyrrolidino)-2-butynyl]oxy-Δ^2-isoxazoline methiodide **7b**: mp 101–102°C (colorless powder from 2-propanol). ^{1}H NMR 2.34 (m, 4), 3.02 (t, 2, H-4, J=9.5), 3.46 (s, 3, NMe), 3.87 (m, 2), 4.07 (m, 2), 4.45 (t, 2, H-5, J=9.5), 4.83 (bs, 2, OCH_2C≡) 4.94 (bs, 2, CH_3NCH_2). Anal. $C_{12}H_{19}IN_2O_2$ (C, H, N).

Dimethylamino derivative **8a** was synthesized through reaction of **15c** with a methanol solution of dimethylamine. The desired tertiary base was obtained in 69% after the extraction procedure, followed by distillation at reduced pressure.

3-[4-(Dimethylamino)-2-butynyl]oxy-Δ^2-isoxazoline **8a**: colorless liquid, bp 125–130°C/0.5 mm Hg; R_f 0.49 (10% methanol/chloroform); ^{1}H NMR 2.32 (s, 6, NMe_2), 3.01 (t, 2, H-4, J=9.7), 3.37 (t, 2, CH_2NMe_2, J=2.2), 4.42 (t, 2, H-5, J=9.7), 4.81 (t, 2, OCH_2C≡, J=2.2).

8a×$C_2H_2O_4$: mp 132.5–133.5°C (colorless prisms from 2-propanol). Anal. $C_{11}H_{16}N_2O_6$ (C, H, N). 3-[4-(Dimethylamino)-2-butynyl]oxy-Δ^2-isoxazoline methiodide **8b**: mp 211.5–212.5°C, dec. (colorless prisms from 2-propanol); ^{1}H NMR (DMSO-d_6) 3.07 (t, 2, H-4, J=9.8), 3.17 (s, 9, NMe_3), 4.37 (t, 2, H-5, J=9.8), 4.49 (bs, 2, OCH_2C≡), 4.99 (bs, 2, CH_2NMe_3). Anal. $C_{10}H_{17}IN_2O_2$ (C, H, N).

Isoxazole **9a** was prepared, similarly to Δ^2-isoxazoline **7a**, by reacting intermediate **17** with a methanol solution of pyrrolidine at room temperature. The desired pyrrolidino derivative was obtained in 82% after column chromatography of the extracted reaction mixture (eluant: 5% methanol/dichloromethane), followed by distillation under vacuum.

3-[4-(1-Pyrrolidino)-2-butynyl]oxyisoxazole **9a**: colorless liquid, bp 135–140°C/0.5 mm Hg; R_f 0.48 (10% methanol/chloroform); ^{1}H NMR 1.80 (m, 4), 2.61 (m, 4), 3.48 (t, 2, ≡CCH_2N, J=2.1), 4.92 (t, 2, OCH_2C≡, J=2.1), 6.01 (d, 1, H-4, J=1.9), 8.15 (d, 1, H-5, J=1.9).

9a×$C_2H_2O_4$: mp 79.5–80.5°C (colorless prisms from 10% methanol/petroleum ether). Anal. $C_{13}H_{16}N_2O_6$ (C, H, N).

3-[4-(1-Pyrrolidino)-2-butynyl]oxyisoxazole methiodide **9b**: mp 92–93.5°C (colorless powder from 20% acetone/ethyl acetate); ^{1}H NMR 2.32 (m, 4), 3.44 (s, 3, NMe), 3.87 (m, 2), 4.03 (m, 2), 4.89 (bs, 2, OCH_2C≡), 4.98 (bs, 2, ≡CCH_2N), 6.03 (d, 1, H-4, J=1.9), 8.19 (d, 1, H-5, J=1.9). Anal. $C_{12}H_{17}IN_2O_2$ (C, H, N).

Tertiary base **10a** was prepared from **17** by addition of a DMF solution of a twofold excess of dimethylamine at room temperature. After distillation at 50°C/0.5 mm Hg followed by acid/alkali extraction, the residue was chromatographed (eluant: 10% methanol/dichloromethane) to give **10a** in 78% yield.

3-[4-(Dimethylamino)-2-butynyl]oxyisoxazole **10a**: colorless liquid, bp 115–120°C/0.5 mm Hg, R_f 0.47 (10% methanol/chloroform); ^{1}H NMR 2.31 (s, 6, NMe_2), 3.33 (t, 2, CH_2NMe_2, J=2.0), 4.97 (t, 2, OCH_2C≡, J=2.0), 6.01 (d, 1, H-4, J=1.9), 8.17 (d, 1, H-5, J=1.9).

10a×$C_2H_2O_4$: mp 124.5–125.5°C (colorless prisms from 10% methanol/petroleum ether). Anal. $C_{11}H_{14}N_2O_6$ (C, H, N).

3-[4-(Dimethylamino)-2-butynyl]oxyisoxazole methiodide **10b**: mp 150–151°C, dec. (colorless prisms from 10% methanol/petroleum ether); ^{1}H NMR (DMSO-d_6) 3.13 (s, 9, NMe_3), 4.48 (bs, 2, OCH_2C≡), 5.14 (bs, 2, CH_2NMe_3), 6.48 (d, 1, H-4, J=2.0), 8.78 (d, 1, H-5, J=2.0). Anal. $C_{10}H_{15}IN_2O_2$ (C, H, N).

Pharmacology

Male albino guinea pigs (250–350 g), rabbits (3–3.5 kg) and mice (25–35 g) (Morini S. Polo Italy) were used. The tissues for in vitro experiments were dissected from guinea pigs and rabbits starved 24 h before the experiments and euthanized by CO_2 inhalation.

In vitro experiments

Rabbit vas deferens. According to Eltze,[24] the two prostatic portions of the organs (1.5 cm) were carefully removed and dissected free of the connective tissue. The preparations were set up in 10 mL organ baths containing the modified Krebs buffer solution (mM composition: NaCl 134, KCl 3.4, $CaCl_2$ 2.8, KH_2PO_4 1.3, $NaHCO_3$ 16, $MgSO_4$ 0.6, glucose 7.7) maintained at 31°C and aerated with 95% O_2–5% CO_2. The tissues were preloaded with 0.75 g and were left to equilibrate for 30 min before starting the electrical field stimulation (0.05 Hz, 0.5 ms, supramaximal voltage 40 V; LACE Elettronica Mod. ES3, Ospedaletto PI-Italy). Neurogenic

contractions were measured isometrically (Basile Mod. 7080 Italy) and recorded on a two-channel recorder (Basile Mod.7070). Throughout the experiments 1 μM yohimbine was added to the buffer solution to block α_2-adrenoceptors.

Guinea pig left atrium. Heart was rapidly dissected and right and left atria were separated out. As described by Eglen et al.,[25] left atrium was suspended under 0.5 g tension in a 20 mL organ bath and modified Krebs–Henseleit buffer solution (mM composition: NaCl 118.9, KCl 4.6, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4.7H_2O$ 1.2, glucose 11.1) gassed with carbogen (95% O_2–5% CO_2) at 32°C, served as bath fluid. The tissue was electrically-paced by rectangular submaximal impulses (2 Hz, 5 ms, 5 V) by using platinum electrodes. After a 30 min stabilization period, inotropic responses were measured as changes in isometric tension.

Guinea pig ileum. Segments of the terminal portion of the ileum (2 cm in length) were transferred into a 10 mL organ bath filled with Krebs–Henseleit solution (composition see above) gassed with carbogen at 37°C and loaded with a tension of 1 g. The preparation was allowed to equilibrate for 30 min before inducing isometric contractile responses.

Protocols. In all the experiments complete concentration–response curves to the compounds under study were obtained in a cumulative fashion by increasing the concentration of the agonists by 0.3 log units (in the range from 0.03 nM to 100 μM) following the method described by Van Rossum.[29] Oxotremorine and oxotremorine-M were used as reference drugs and McN-A-343 as a conventional M_1 agonist. Derivative **9a** was also investigated as antagonist against oxotremorine in guinea pig atria and McN-A-343 in rabbit vas deferens (in the range 10–100 μM).

The apparent potency of the agonists was expressed as their pD_2 value (−log of the molar concentration which gives 50% of the maximum response) calculated by linear regression using the least squares method. Intrinsic activity (α) was determined by comparing the maximum response to the reference drugs with that to the test compounds. In the antagonism studies, the pK_B values were calculated as indicated by Furchgott[30] and pA_2 values were obtained by Schild analysis.[31] All the data are presented as means ± SEM ($n = 8$) and significant differences were assessed by use of Student's t test, with a value of $P < 0.05$ being considered significant.

In vivo experiments. All the experiments were performed between 9 and 12 a.m. and the drugs, dissolved in saline, were given intraperitoneally at different doses to groups of eight mice in a volume of 1 mL/100 g body weight. The animals had access to food and water ad libitum before the experiment. Following drugs or saline administration, tremor was scored as present or not present during a period of 10 min. ED_{50} values (i.e. the doses required to produce tremor in 50% of the animals) were calculated by means of probit analysis.[32]

Acknowledgements

We thank G. Domenichini for his excellent technical assistance. Financial support from MURST (Ministero della Ricerca Scientifica e Tecnologica), Rome is gratefully acknowledged.

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