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Synthesis and pharmacology of 1-(2-thioxopyrrolidino)-4-pyrrolidino-2-butyne, the thiolactam analogue of oxotremorine

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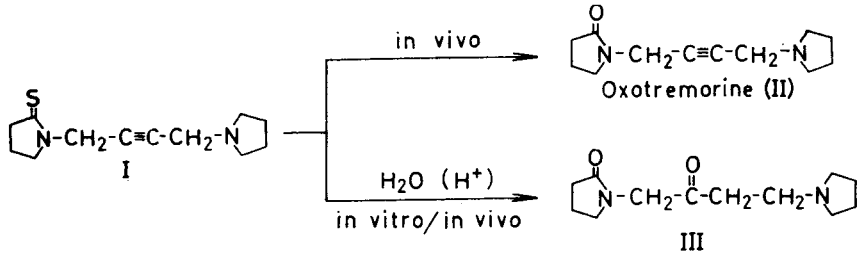
The thiolactam analogue of oxotremorine (I) has been prepared and examined for muscarinic activity in mice and on guinea-pig ileal preparations. It is inactive in itself, but is transformed *in vivo* to oxotremorine (II), which has been identified in the urine of mice. The urine also contains 1-(2-oxopyrrolidino)-4-pyrrolidino-2-butanone (III) which is formed non-enzymatically from I under acidic conditions, but does not display any acetylcholine-like properties *in vivo* or *in vitro*. Oxotremorine is not formed from I *in vitro*.

Oxotremorine [1-2(-oxopyrrolidino)-4-pyrrolidino-2-butyne] the principal active metabolite of tremorine, has specific muscarinic properties with about the same potency as acetylcholine (Cho, Haslett & Jenden, 1962; George, Haslett & Jenden, 1962). The carbonyl group apparently plays a vital role in its muscarinic activity, since tremorine is devoid of any comparable activity (Cho & others, 1962). Oxotremorine is relatively selective in producing central as opposed to peripheral effects, and this property is probably due to its favourable distribution to the brain (Karlén, Träskman & Sjöqvist, 1971). We have prepared the thiolactam analogue of oxotremorine (thioxotremorine) to see what effect substitution of sulphur for oxygen might have on the muscarinic potency of the resulting compound.

Thioether analogues of acetylcholine, acetyl-β-methylcholine (Renshaw, Dreisbach & others, 1938; Günther & Mautner, 1963), muscarine (Waser, 1961a) and muscarone (Waser, 1961a, b) in general were found to be less active than their oxygen counterparts. Thiocarbonyl analogues of acetylcholine-like compounds have not been thoroughly studied, although acetylthionocholine is reported to equal acetylcholine in its ability to depolarize *Electrophorus electricus* electroplax (Chu & Mautner, 1970). This type of activity appears to be more nicotinic than muscarinic, and is blocked by tubocurarine.

This paper describes the synthesis of thioxotremorine (I), its metabolic conversion to oxotremorine, and its non-enzymatic conversion under acidic conditions to 1-(2-oxopyrrolidino)-4-pyrrolidino-2-butanone (III, Scheme 1). The acetylcholine-like effects of these compounds have been examined in mice and guinea-pig ileal preparations.



Scheme 1

METHODS

Chemistry

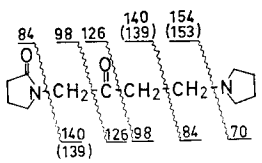
General. Melting points were determined with calibrated Anschütz thermometers and are uncorrected. Microanalyses were carried out in the laboratories of Dr. A. Bernhardt, Mühlham, West Germany. All compounds were analysed for C, H, N, S. The analytical results were within $\pm 0{\cdot}4\%$ of the theoretical value. Infrared spectra were recorded on a Perkin-Elmer 237 spectrophotometer, and nuclear magnetic resonance spectra on a Varian A60 spectrometer using $CDCl_3$ solutions. Chemical shifts are expressed in ppm (τ) with TMS as the internal standard. The coupling constants were of the expected order. Mass spectra were obtained with an LKB 9000 mass spectrometer using a direct probe heated to a suitable temperature. The ionizing energy was maintained at 70 eV, the accelerating energy at 3500 V and the temperature of the ion source was kept at 270°. Thin-layer chromatography was performed using Eastman chromatogram sheets coated with silica gel. The spots were made visible by spraying with Dragendorff reagent after developing the sheets in heptane–chloroform–ethanol–diethylamine (12:2:1:1). Gas liquid chromatography was with an Aerograph 1200 gas chromatograph equipped with a flame ionization detector. A silanized 6 ft. $\frac{1}{8}$ inch glass column was packed with 3% OV 17, 1% phenyldiethanolamine succinate on Gas-Chrom Q. The column temperature was 210°.

1-*Propargyl-2-thioxopyrrolidine.* Finely powdered P_2S_5 (10 g, 0·05 mol) was added to a stirred solution of 1-propargyl-2-pyrrolidone (11 g, 0·09 mol) (British Patent, 1965) in dry toluene (150 ml). The stirred mixture was left at 30–40° for 15 h. The mixture was filtered off and the filter cake was washed repeatedly with hot toluene. The combined filtrate and washings were evaporated *in vacuo*. The product crystallized upon standing and was purified by extraction of the solidified mass with boiling light petroleum (40–60°), m.p. 46–47°. Yield 5·6 g (45%). Infrared: ν_{max} (KBr) 3300 (≡CH), 2120 (C≡C), 1500 (N–C=S), 1130 (C=S) cm^{-1}; nmr: doublet at τ 5·37 (2H, side-chain, C_1, $J_{1-3} = 2{\cdot}6$ Hz, propargylic coupling, triplet at 6·14 (2H, thiolactam ring, C_5), triplet at 6·97 (2H, thiolactam ring, C_3), triplet at 7·65 (1H, side-chain, C_3), interfering with a quintet, centred at 7·91 (2H, thiolactam ring, C_4). (C_7H_9NS) C, H, N, S.

1-(2-*Thioxopyrrolidino*)-4-*pyrrolidino*-2-*butyne* (*I*). A mixture of 1-propargyl-2-thioxopyrrolidine (3·0 g, 0·022 mol), paraformaldehyde (0·75 g, 0·025 mol), pyrrolidine (1·6 g, 0·023 mol) and CuCl2 (0·06 g) in peroxide-free dioxane (10 ml) was heated at 70° for 30 min. After cooling, H_2O (100 ml) was added. The mixture was acidified with 1M HCl, extracted as fast as possible with Et_2O (50 ml) and made

alkaline with 1M Na_2CO_3. The temperature was kept as low as possible under the acidic conditions. The aqueous phase was extracted with $CHCl_3$ (5 × 50 ml), the extract dried (K_2CO_3) and the solvent removed *in vacuo*. The residue was dissolved in a small volume of Et_2O, and chromatographed on an Al_2O_3-column with Et_2O. The fractions lacking infrared absorption at 1660 cm^{-1} (C=O) were collected and pooled. After evaporation of the solvent and treatment with cold light petroleum, the product crystallized. Recrystallization from a mixture of ether and light petroleum gave the pure product, m.p. 30–31°. Yield 1·8 g (37%). Infared: ν_{max} (KBr) 1500 (N–C=S), 1130 (C=S) cm^{-1}; nmr: singlet at τ 5·41 (2H, side-chain, C_1), triplet at 6·17 (2H, thiolactam ring, C_5), singlet at 6·60 (2H, side-chain, C_4), triplet at 6·99 (2H, thiolactam ring, C_3), 2 multiplets at 7·23–8·61 (10H, thiolactam ring, C_4 and pyrrolidine ring, C_{2-5}). ($C_{12}H_{18}N_2S$) C, H, N, S.

1-(2-*Oxopyrrolidino*)-4-*pyrrolidino*-2-*butanone* (*III*). 1-(2-Thioxopyrrolidino)-4-pyrrolidino-2-butyne (0·80 g, 0·0036 mol) was dissolved in 1M HCl (15 ml) and left at room temperature for 24 h. The solution was made alkaline with 1M Na_2CO_3 to pH 8, extracted with $CHCl_3$ (3 × 20 ml). The $CHCl_3$ extract was dried (Na_2SO_4) and the solvent removed *in vacuo*. The semisolid residue was extracted with hot light petroleum from which the product was obtained as white needles. Recrystallization from ether or light petroleum gave the pure product, 0·55 g (68%), m.p. 45–46°. (The base could be precipitated as the oxalate salt and recrystallized from ethanol - acetone, m.p. 140-142°). Infrared: ν_{max} (KBr) 1680 (C=O, lactam), 1730 (C=O, ketone) cm^{-1}; nmr: Singlet at τ 5·85 (2H, side-chain, C_1), triplet at 6·57 (2H, lactam ring, C_5), multiplets at 7·00–8·50 (16H). MS: Prominent peaks at *m/e* 224 (M^+), 153, 139, 126, 98, 84 (base peak), and 70. ($C_{12}H_{20}N_2O_2$) C, H, N.



Pharmacology

Tremor was measured using an electronic device described by Silverman & Jenden (1970). Acetylcholine-like activity *in vitro* was assessed on guinea-pig ileal strips in oxygenated Krebs solution at 37° Contractions were recorded isotonically at 1 g tension, using a Collins displacement transducer and potentiometric recorder (Karlén, Lindeke & others, 1970). Antagonism to acetylcholine was measured in a series of cumulative dose-response curves to acetylcholine with and without the test compound at concentrations increasing in the ratio 1:3:10:30. The preparation was allowed to equilibrate for 30 min before the acetylcholine dose-response curve was obtained.

Characterization of oxotremorine in urine from mice

Compound I (10 mg/kg, i.p.) was administered to groups of five mice kept in metabolic cages and the urine was collected for the following 20 h.

Gas liquid and thin-layer chromatography. The urine (2 ml) was made alkaline with 2M ammonium citrate in 7·5M ammonium hydroxide (2 ml) and extracted twice with $CHCl_3$ (1 ml). The pooled $CHCl_3$ extracts were then extracted with 0·5M citric acid (0·6 ml). The $CHCl_3$ phase was discarded, and ammonium citrate

hydroxide buffer (0·6 ml) (see above) and $CHCl_3$ (0·1 ml) were added. After extraction and centrifuging, 5–10 μl of the $CHCl_3$ phase was aspirated and injected into the gas chromatograph. For t.l.c., 0·1 ml of the $CHCl_3$ extract was concentrated to about 20 μl.

Bioassay. The urine was diluted with an equal volume of 0·1M phosphate buffer of pH 7·5. The buffered urine was extracted twice with half the volume of $CHCl_3$; the extracts were collected and extracted once with half the volume of 0·1N HCl. After adjusting the pH to 7·4 the extract was assayed on guinea-pig ileal strips using oxotremorine as reference agonist.

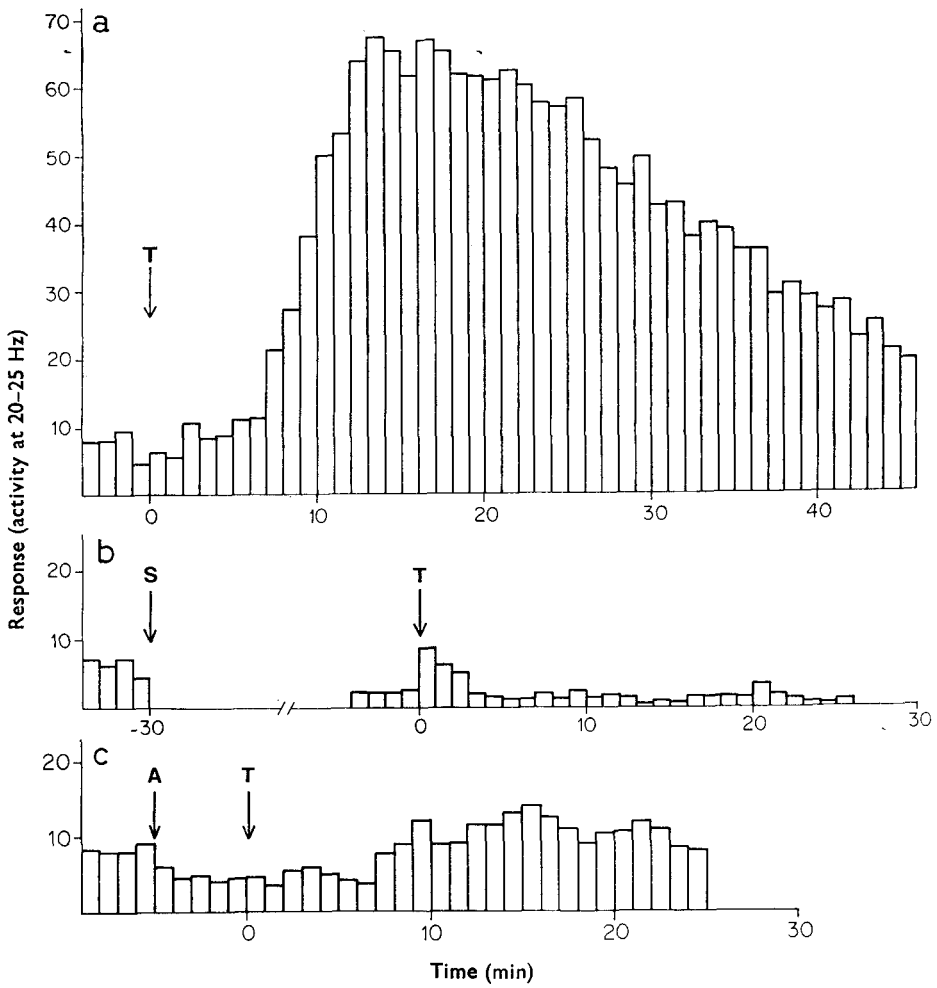


FIG. 1. Tremor recording after thioxotremorine given intraperitoneally. a. The data are means of 8 mice. The mean maximal response (5 animals) to oxotremorine (0·4 mg/kg, i.p.) was 50–55 units and occurred 5–12 min after the injection.

b. Recording after pretreatment with atropine sulphate. The data are means of 5 mice. c. After pretreatment with SKF 525 A. The data are means of 5 mice. The mean maximal response (5 animals) to oxotremorine (0·4 mg/kg, i.p.) after pretreatment with SKF 525 A (25 mg/kg. i.p. for 30 min) was 60–70 units and occurred 5–10 min after the injection.
A. Atropine sulphate 1mg/kg. S. SKF 525-A 25mg/kg. T. Thioxotremorine 10 mg/kg.

RESULTS

Pharmacology

Compound I produced tremor, rigidity, salivation, and other signs of central and peripheral muscarinic effects in mice after doses from 2 mg/kg (i.p.). After 10 mg/kg some of the mice died from signs of hypoxia and cardiovascular collapse. There was a time lage of 7–8 min after the administration of I before the tremor developed (Fig. 1a) compared to less than 1 min after an equiactive dose of oxotremorine (Cho & others, 1962), which suggested that the compound was biotransformed before the effects were seen. This was supported by the observation that intraperitoneal injection was much more effective than intravenous injection of the same dose. Furthermore, pretreatment with SKF 525A, an inhibitor of microsomal hydroxylating enzymes, completely abolishes the effects produced by I (Fig. 1b). The tremor could be fully blocked by atropine sulphate (1 mg/kg, i.p.) (Fig. 1c), demonstrating the muscarinic nature of the induced effects. However, atropine methyl bromide (1 mg/kg, i.p.) administered 15 min before I (10 mg/kg, i.p.) blocked the peripheral muscarinic effects, e.g. salivation and lacrimation, but not the tremor. These *in vivo* observations suggest that I is transformed in the liver to oxotremorine or another centrally active muscarinic agent.

Compound I did not produce contraction of guinea-pig isolated ileal preparations and is thus not itself active. But a urine extract from mice treated with I caused contraction of the ileum, which could be blocked by atropine (10^{-6}M). Acetylcholine blocking activity of I was sought on the ileum, but could not be detected in concentrations up to $2{\cdot}5 \times 10^{-3}$M.

Compound III did not produce any acetylcholine-like effects *in vivo*, when administered intraperitoneally or intravenously to mice in doses up to 25 mg/kg, nor did it stimulate the guinea-pig ileum *in vitro* at concentrations up to $2{\cdot}5 \times 10^{-3}$M.

Formation of 1-(2-*oxopyrrolidino*)-4-*pyrrolidino*-2-*butanone from compound I*

The thiolactam analogue (I) of oxotremorine is unstable under acidic conditions and is transformed to III. At pH 5·5 and below, H_2S was liberated after only a few minutes and could be characterized by precipitation with Cu^{2+}. Thin-layer chromatography of the water phase gave 3 spots with R_F 0·47 (compound I), 0·17 (compound III), and one at the origin (not identified), in a small amount. Oxotremorine (R_F 0·37) was not formed under these conditions. After 24 h, compound I had disappeared in the chromatogram and the main spot was the one with R_F 0·17. The spot at the origin was very faint. The experiment was repeated in a preparative scale in 1M HCl. The main transformation product is formed by hydration of the alkyne group and the resulting carbonyl function can appear in either position 2 or 3. The assignment of the structure as 1-(2-oxopyrrolidino)-4-pyrrolidino-2-butanone rests on infrared, mass spectrometric and nmr data. The appearance of *m/e* 222 (M^+) and of mass fragments *m/e* 153, 139, 126, 98, 84 and 70 upon electron impact is in good agreement with the proposed structure. The absence of *m/e* 112 excludes position 3 as carrying the carbonyl function. The nmr chemical shift of C_1 (τ 5·85) is close to that of C_1 in *N*-acetonyl-2-pyrrolidone (τ 5·88), but differs from that of C_1 in *N*-acetonylpyrrolidine (τ 6·76). These data taken together strongly suggest that the proposed structure is correct. Details of this chemical transformation and of the elucidation of this structure will be published separately.

Identification of oxotremorine in urine of mice treated with compound I

A chloroform extract subjected to thin-layer chromatography showed three spots with R_F 0·37 (oxotremorine), 0·17 (compound III), and 0·05 (not identified). Compound I (R_F 0·47) was not detected in the urine extract, presumably because of its instability below pH 5·5. Gas chromatography of a urine chloroform extract gave one peak with the same retention time as oxotremorine. Neither I nor III could be recovered by gas chromatography on the column used.

Quantitative estimation of oxotremorine in urine from mice

The urine content of oxotremorine formed from Compound I was determined both by bioassay and gas chromatography. Urine was collected for 20 h after the injection of I (10 mg/kg, i.p.). In two experiments the concentration of oxotremorine in pooled urine from five mice was 2 μg/ml (7·5 ml urine) and 3·3 μg/ml (6·9 ml urine), respectively by bioassay. A third experiment in which gas chromatography was used, gave the value 8 μg/ml (2·5 ml urine). These results indicate that at least 2% of the thioxotremorine is metabolized to oxotremorine.

DISCUSSION

Thioxotremorine itself is seen to be inactive as a muscarinic agent. The central and peripheral muscarinic effects after thioxotremorine may be adequately explained by its metabolic transformation to oxotremorine, which can be recovered from the urine in the amount of about 2% of the dose of thioxotremorine. The dioxo derivative III is also found in the urine extract but is pharmacologically inert. It has been shown to be formed non-enzymatically from thioxotremorine under acidic conditions, and its presence in the urine extract could be due to this; however, the metabolic conversion of thioxotremorine to III has not been ruled out.

The metabolic desulphuration of thioxotremorine to oxotremorine is not unexpected, and similar reactions are known to occur with thiopentone, thiouracil (Spector & Shideman, 1959) and perathion (Davison, 1955). Its non-enzymatic conversion to III is more surprising and the mechanism of this reaction is being examined further.

Bebbington, Brimblecombe & Shakeshaft (1966) concluded from a study of some oxotremorine analogues that compounds in which there is a large single bond character in the carbonyl group are likely to be more active. Since the thiocarbonyl bond has much more single bond character than a corresponding carbonyl (Lee & Kumler, 1962), the muscarinic inactivity of thioxotremorine conflicts with this generalization. This conflict might be rationalized in terms of the greater covalent radius of sulphur compared to oxygen, its diminished hydrogen bonding ability, or the greater basicity of the lactam oxygen relative to the thiolactam sulphur (Edward & Stollar, 1963). Metabolic lability appears to be ruled out as an explanation by the finding that thioxotremorine *per se* is inert *in vitro* as well as *in vivo*.

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