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MEDICINAL CHEMISTRY RESEARCH

SYNTHESIS AND NEUROMUSCULAR BLOCKING ACTIVITY OF 16β-PIPERIDINO EPIANDROSTERONE DERIVATIVES

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Abstract. This study reports the synthesis of steroidal quaternary ammonium compounds **11** and **12**, with quaternary nitrogen at position 3 and 16 of the steroid nucleus in 5α-epiandrosterone series; along with their neuromuscular blocking activity using chick biventer cervicis muscle preparation. The compound **12** was found to be five times more potent than **11** in reducing twitch response to nerve stimulations, indicating the importance of extended interonium distances and 17-acetoxy function for potent antagonist activity.

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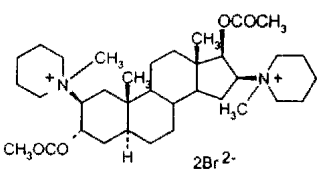
Introduction

Neuromuscular blocking agents interrupt the transmission of motor nerve impulses at the skeletal neuromuscular junction. On the basis of distinct electrophysiological differences in their mechanism of action, they are classified either as depolarizing agents e.g. suxamethonium [1] or as non depolarizing agents e.g. pancuronium **1** [2, 3], chandonium **2** [4], atracurium [5] etc.

The first indication that useful muscle relaxant activity might be found among compounds with a steroidal nucleus and quaternary nitrogen center came from the observation that the steroid malouetine from *Maloueta bequaertiana* showed the same potency as *d*-tubocurarine[6]. The steroid molecule provides a rigid skeleton into which the other groups can be incorporated. There are several mono- and bisquaternary steroidal compounds (e.g. **1, 2** and vecuronium[7]) that antagonize the nicotinic actions of acetylcholine both at neuromuscular junction and at autonomic ganglia [8, 9]. The general assumption for active bis-



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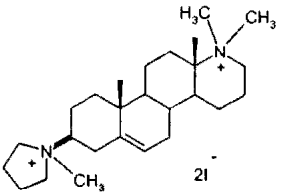

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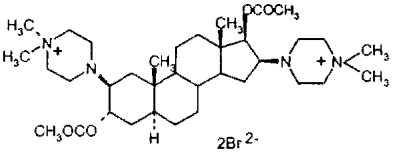
quaternary heterosteroidal neuromuscular blocking agents is that that the distance between the two quaternary nitrogen heads should be between 1.0-1.2 nm [10]. However, in the androstane series 2,16-dipiperazinium compound, pipecuronium bromide **3** [11] an analogue of pancuronium bromide does not follow this parameter [12] and is an extremely potent neuromuscular blocker. The lack of an intact acetylcholine like fragment in pipecuronium



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bromide has not compromised its potency but rather increased it, presumably due to increased interonium distance [13]. The present work, which is an extension of our earlier work[14], was thus envisaged to see the effect of extended interonium distance on neuromuscular blocking activity by substituting pyrrolidino moiety at position 3 and piperidino moiety at position 16 of steroid nucleus in 5α-epiandrosterone series.

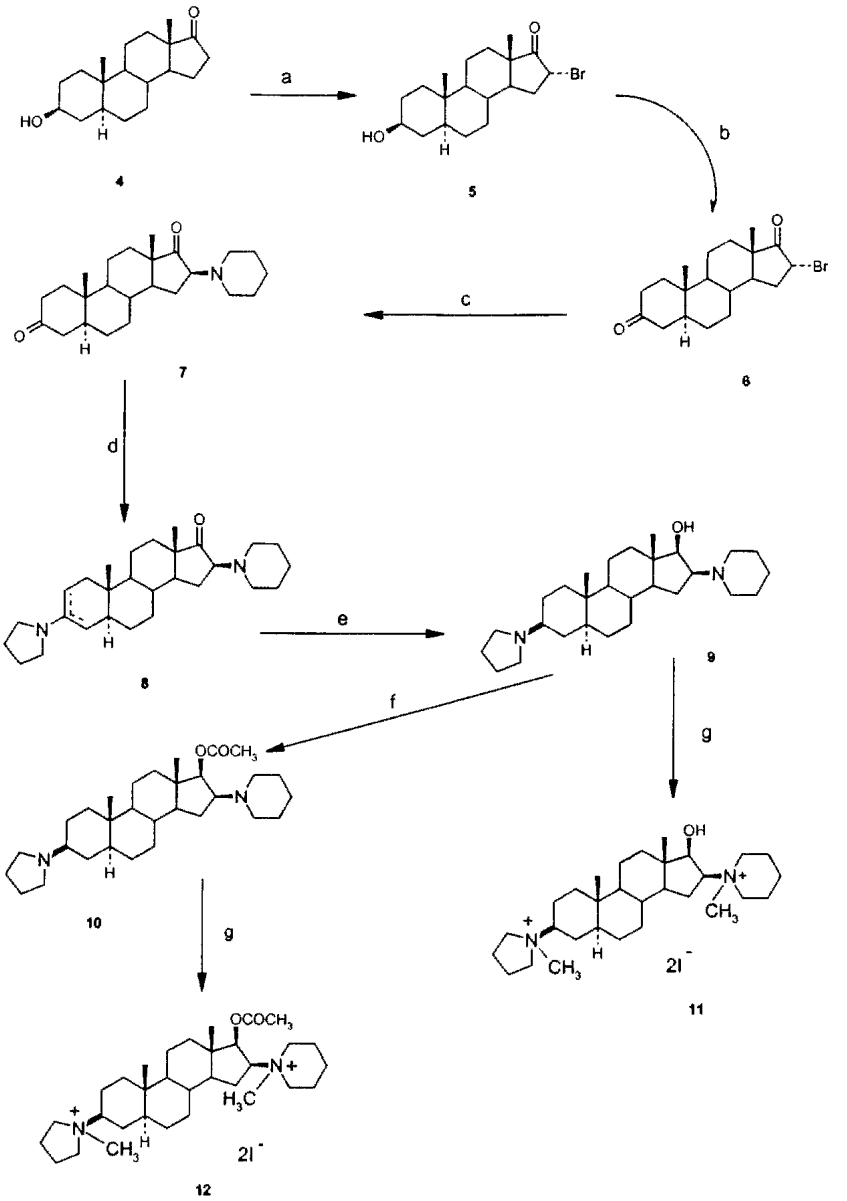
Chemistry

To prepare bisquaternary compounds, 16α-bromo-17-oxo-5α-androstan-3β-ol **5** was used as the starting material, which was prepared from 5α-epiandrosterone **4** [15]. The proton NMR spectrum of **5** exhibited a multiplet at δ 3.62 (3α-*H*) and a triplet at 4.52 (16β-C*H*) ppm. The Jones' oxidation of **5** with chromium trioxide afforded **6**. 16-Piperidino functionality was introduced to obtain **7** by treating the oxidized product **6** with piperidine at refluxing temperature [16]. The NMR spectrum indicated a double doublet at δ 3.10 for 16α-*H*. The assignment of configuration at position 16 has been made on the basis of earlier reports [15, 17].

The enamine **8** was prepared by refluxing **7** with pyrrolidine for 15 min and it was subsequently reduced with sodium borohydride in methanol to give **9.** The ^{1}H-NMR spectrum showed the *N*-methylenes of pyrrolidine and piperidine functionalities at δ 2.52 (br) and 2.62 (singlet) respectively and the 17α-*H* appeared as a singlet at δ 3.38. The acetylation of **9** with acetic anhydride in dry pyridine yielded **10,** which exhibited a singlet for three protons at δ 2.09 (OCOC*H*$_3$) in nuclear magnetic resonance spectrum. The IR spectrum showed a band at 1730 cm^{-1} for C=O stretching.

The bisquaternary compounds **11** and **12** were prepared by treating **9** and **10** respectively, with methyl iodide in dichloromethane at room temperature. The NMR spectrum showed *N*-

methyl protons of pyrrolidine and piperidine for compound **11** at δ 2.99 and 3.30 respectively. The same protons in **12** appeared at δ 2.94 and 3.26 respectively in the NMR spectrum (Scheme1).



Scheme-1: Synthetic procedure of compounds. Reagents and conditions: (a) Cupric bromide-benzene-dehydrated methanol/ reflux; (b) chromium trioxide-acetic acid, room temperature ; (c) piperidine, reflux; (d) pyrrolidine, methanol/reflux; (e) sodium borohydride-methanol, room temperature; (f) acetic anhydride- pyridine, reflux; (g) methyl iodide/dichloromethane, room temperature.

Biological Activity

The neuromuscular blocking activity of compounds **10** and **11** was examined in vitro on the isolated chick biventer cervicis muscle preparation, a preparation selected for its ability to elicit the potency of nicotinic antagonists and reveal indicative information on mechanism of action.

Results and Discussion

Compounds **11** and **12** were tested on chick biventer cervicis preparations for their ability to reduce twitch responses to nerve stimulation. Both produced time- and concentration-dependent block of twitch responses at concentrations of 1 μM and above. Compound **12** was about five times more potent than compound **11**: 1 μM of **12** caused complete twitch blockade in 9 min, whereas 5 μM of **11** completely blocked twitches in 10 min (Fig. 1). The block of twitches was completely reversed by washout of the compounds. Addition of the anticholinesterase neostigmine (1 μM) before complete twitch blockade led to a rapid recovery of twitch height in the presence of both **11** and **12.**

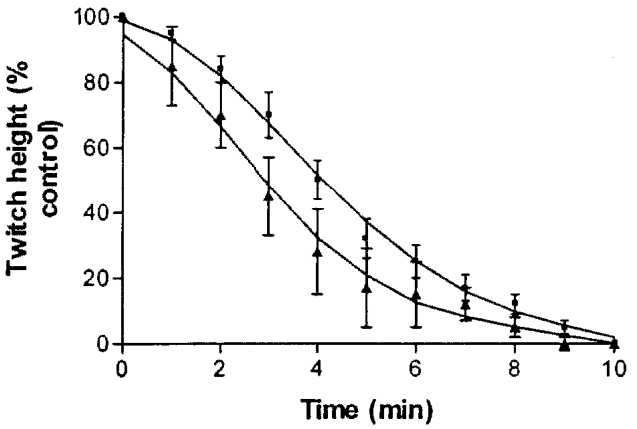


Fig. 1: Time course of the effects of **11** (5 μM, closed squares) and **12** (1 μM, closed triangles) on the twitch response of chick biventer cervicis preparations to nerve stimulation. Each point represents the mean ± S.E.M of 4 experiments.

Both compounds blocked twitches by binding to acetylcholine receptors because they reduced muscle contractures induced by the cholinomimetic agonist carbachol. From an analysis of the concentration-response curves to carbachol in the presence and absence of test compounds, affinity constants for binding to the receptors were estimated to be 0.9 ± 0.1 μM for **11** and 0.2 ± 0.03 μM for **12** (means ± SD).

Although the twitch block induced by **11** and **12** in the chick biventer cervicis preparations was reversed by an anticholinesterase, the compounds themselves inhibited acetylcholinesterase activity. When tested against enzyme from electric eel, the K_i s for **11** and **12** were 0.62 and 0.98 μM, respectively.

The bisonium compounds **11** and **12** reduced the twitch response to nerve stimulation by reversible binding to acetylcholine receptors. The effects of both the test compounds were completely reversed by washing from the tissue bath or by addition of the anticholinesterase drug, neostigmine, suggesting that the neuromuscular blocking action was predominantly due to a competitive antagonism of the post-junctional nicotinic acetylcholine receptors. Although the twitch block was reversed by neostigmine, both the compounds themselves inhibited acetylcholinesterase activity in a concentration-dependent manner.

There was a five fold drop in binding affinity for nicotinic cholinoceptors with the change from 17-acetoxy **12** to 17-hydroxy **11**, indicating , the replacement of 17-hydroxy group with 17-acetoxy in quaternary ammonium compounds led to a marked increase in the potency, which may be attributed to the increased bulkiness and increased lipophilicity of the molecule. These results are in agreement with our previous studies on similar types of quaternary ammonium compounds [14].

The saturation of 5, 6 double bond and lack of an intact acetylcholine like fragment (which was thought to be essential for neuromuscular blocking activity of some quaternary ammonium compounds) in compounds **11** and **12** has not compromised its potency, presumably due to increased interonium distances.

Conclusion

The present study reconfirms the importance of cationic nitrogen heads in the steroidal molecule for neuromuscular blocking action. The saturation of double bond at position-5 in steroidal ring and increase in interonium distance between two nitrogen heads retained the neuromuscular blocking activity. In the two bisquaternary compounds in the series, there was a 5-fold drop in potency with the change from 17-acetoxy **12** to 17-hydroxy **11** showing the crucial importance of the acetoxy moiety in combination with 16-quaternary nitrogen for binding to the nicotinic receptor. The results re-emphasized the importance of acetoxy moiety in compound with 16-quaternary nitrogen for binding to nicotinic receptor.

Experimental Protocol

Chemistry

Melting points reported are uncorrected. ^{1}H-NMR spectra were recorded on AC-300F, 300 MHz, Varian EM-390, 90 MHz and EM-360, 60 MHz NMR instruments using tetramethylsilane (TMS) as the internal standard (chemical shifts in δ, ppm). IR and UV spectra were recorded on Perkin-Elmer 882 and Lamda 15 spectrophotometer models respectively. The purity of the compounds was established by thin layer chromatography and by elemental analyses (C, H, N). Elemental analyses were carried out on a Perkin-Elmer-2400. Mass spectra were recorded on a V6-11-250J70S instrument. Anhydrous sodium sulphate was used as a drying agent and iodine vapours as developing agent. Ultraviolet spectra were recorded in methanol. IR spectra were obtained with potassium bromide pellets (ν_{max} in cm^{-1}).

16α-Bromo-17-oxo-5α-androstan-3β-ol (5)

17-Oxo-5α-androstan-3β-ol (epiandrosterone) (**4,** 1.0 g, 3.45 mmol) was dissolved in a mixture of dehydrated methanol (50 mL) and dry benzene (50 mL). Cupric bromide (3.5 g) was added to the solution and the reaction mixture was refluxed for 4 h. The hot reaction mixture was filtered; the solvent was reduced to half the volume and diluted with benzene (50 mL). To this mixture cold distilled water (500 mL) was added. The aqueous layer was extracted with benzene (3 x 50 mL). The combined organic layer was washed with distilled water, dried and solvent was

removed under reduced pressure to obtain white coloured product, which was crystallized from methanol to obtain **5** (2 g, 78.60%), m.p. 162-166°C. IR: 3440 (O-H), 1740 (C=O); ^{1}H-NMR ($CDCl_3$): δ 0.84 (s, 3H, 18-C*H*$_3$), 0.90 (s, 3H, 19-C*H*$_3$), 3.62 (m, 1H, 3α-*H*) and 4.52 (t, 1H, 16β-C*H*) ppm.

16α-Bromo-5α-androstane-3,17-dione (6)

A solution of chromium trioxide 0.2 g (dissolved in 1 mL of 80% acetic acid) was added to a solution of 16α-bromo-17-oxo-5-androstan-3β-ol (**5**, 2 g, 5.37 mmol) in dry dichloromethane (10 mL) and glacial acetic acid (10 mL) while stirring at room temperature for 1.5 h. The reaction mixture was then poured into crushed ice. The compound was extracted with dichloromethane (3 x 50 mL), washed the organic layer was washed with water and dried. The solvent was removed under reduced pressure and the product obtained was crystallized from methanol to give **6** (1.30 g, 65.40%), m.p. 195-200°C. IR: 1750 (C=O) and 1705 (C=O); ^{1}H-NMR ($CDCl_3$): δ 0.93 (s, 3H, 18-C*H*$_3$), 1.04 (s, 3H, 19-C*H*$_3$) and 4.53 (dd, 1H, 16β-C*H*) ppm. Anal. for $C_{19}H_{27}O_2Br$: C, 62.12 ; H, 7.41. Found: C, 62.43; H, 7.07.

16β-Piperidino-5α-androstane-3,17-dione (7)

16α-Bromo-5α-androstane-3,17-dione (**6**, 1.0 g, 2.70 mmol) was refluxed with piperidine (15 mL) for 45 min. After removal of majority of piperidine under reduced pressure the product was precipitated with ice-cold water. Liquid ammonia was added and the precipitate was filtered and dried to afford **7** (1.0 g, 98.86%), which was used immediately for next step. IR: 1740 (C=O) and 1705 (C=O); ^{1}H-NMR ($CDCl_3$): δ 0.87 (s, 3H, 18-C*H*$_3$), 1.07 (s, 3H, 19-C*H*$_3$), 2.8 (br, 4H, *N*-methylenes of piperidine function) and 3.10 (dd, 1H, 16α-C*H*) ppm.

16β-Piperidino-3-pyrrolidino-5α-androst-2-en-17-one (8)

Freshly distilled pyrrolidine (2.0 mL) was added to a refluxing solution of 16β-piperidino-5α-androstane-3,17-dione (**7**, 1.0 g, 2.79 mmol) in methanol (15 mL). Refluxing was continued for 15 min and reaction mixture was concentrated to half its volume and cooled in ice bath. The solvent was removed under reduced pressure to yield the semi-solid enamine **8**, which was used for further reaction without crystallization. UV_{max} (MeOH): 340.0 nm; IR:1730 (C=O) and 1600 (C=C); ^{1}H-NMR ($CDCl_3$): δ 0.88 (s, 3H, 18-C*H*$_3$), 1.04

(s, 3H, 19-C*H*$_3$), 2.74 (br, 4H, *N*-methylenes of 3-pyrrolidine function), 2.85 (br, 4H, *N*-methylenes of 16-piperdine function) and 3.09 (dd, 1H, 16α-C*H*) ppm.

16β-Piperidino-3β-pyrrolidino-5α-androstan-17β-ol (9)

Sodium borohydride (1.0 g) was added in small quantities to a stirring solution of 16β-piperidino-3-pyrrolidino-5α-androst-2-en-17-one (**8**, 1.0 g, 2.68 mmol) in methanol (50 mL) at room temperature. After 4 h and the excess of solvent was removed by vacuum distillation. The concentrated reaction mixture was poured into ice-cold water and allowed to stand overnight. The precipitate was then filtered, washed with distilled water, dried and crystallized from acetone to afford **9** (0.70 g, 69.34%), m.p. 174-180°C. IR: 3400 (O-H); ^{1}H-NMR ($CDCl_3$): δ 0.66 (s, 3H, 18-C*H*$_3$), 0.82 (s, 3H, 19-C*H*$_3$), 2.52 (br, 4H, *N*-methylenes of pyrrolidine function), 2.62 (s, 4H, *N*-methylenes of piperidine function), 2.75 (dd, 1H, 16α-C*H*) and 3.38 (d, 1H, 17α-C*H*) ppm. Anal. for $C_{28}H_{48}N_2O$: C, 78.45; H, 11.29; N, 6.54. Found: C; 78.45; H, 11.72; N, 6.69.

16β-Piperidino-3β-pyrrolidino-5α-androstan-17β-yl acetate (10)

A mixture of 16β-piperidino-3β-pyrrolidino-5α-androstan-17β-ol (**9**, 0.5 g, 1.33 mmol) in acetic anhydride (2.0 mL) and dry pyridine (0.2 mL) was heated on a steam bath for 2.5 h. The reaction mixture was poured onto crushed ice and basified with strong potassium hydroxide solution (10% w/v). The precipitated material was filtered, dried and crystallized from acetone to yield **10** (0.25g, 45.53%), m.p. 180-184°C. IR:1730 (C=O) and 1230 (C-O-C); ^{1}H-NMR ($CDCl_3$): δ 0.79 (s, 6H, 18-C*H*$_3$ and 19-C*H*$_3$), 2.09 (s, 3H, 17-OCOC*H*$_3$), 2.39-2.56 (m, 8H, 4 protons each of *N*-methylenes of pyrrolidine and piperidine functions), 3.03 (m, 1H, 16α-*H*) and 4.77 (d, 1H, 17α-*H*) ppm. MS: m/z (relative intensity): 470 [M^+]. Anal. for $C_{30}H_{50}N_2O_2$: C, 76.54; H, 10.71; N, 5.95. Found: C; 76.24; H, 11.11; N, 6.27.

16β-Piperidino-3β-pyrrolidino-5α-androstan-17β-ol dimethiodide (11)

Methyl iodide (1.0 mL) was added to a solution of 16β-piperidino-3β-pyrrolidino-5α-androstan-17β-ol (**9**, 0.25 g, 0.67 mmol) in dry dichloromethane (20 mL) and allowed to stand at room temperature for 7 days. The solvent was removed by distilling under reduced pressure and treated with dry ether. Ether was

decanted and the solid crystalline compound **11** was collected (0.20 g, 48.13%), m.p. 280-285°C (decomp).IR: 3340 (O-H);^{1}H-NMR ($CDCl_3$+DMSO-d_6): δ 0.84 (s, 3H, 18-CH_3), 0.86 (s, 3H, 19-CH_3), 2.99 (s, 3H, $\overset{\oplus}{N}$-CH_3 of pyrrolidine), 3.69-3.89 (m, 8H, 4 protons each of *N*-methylenes of pyrrolidine and piperidine functions), 3.30 (s, 3H, $\overset{\oplus}{N}$-CH_3 of piperidine), 4.21 (d, 1H, 17α-*H*) and 4.34 (dd, 1H, 16α-*H*) ppm. Anal. for $C_{30}H_{54}N_2OI_2$: C, 50.56; H, 7.64; N, 3.93. Found: C; 50.21; H, 7.92; N, 4.17.

16β-Piperidino-3β-pyrrolidino-5α-androstan-17β-yl acetate dimethiodide (12)

Methyl iodide (1.0 mL) was added to a solution of 16β-piperidino-3β-pyrrolidino-5α-androstan-17β-yl acetate (**10**, 0.25 g, 0.58 mmol) in dry dichloromethane (50 mL) and allowed to stand at room temperature for 7 days. The solvent was removed under reduced pressure. The compound so obtained was treated with dry ether and consecutively with dry acetone to afford **12** (0.18 g, 44.91%), m.p. 265-270°C (decomp). IR: 2940, 1740 and 1240 ^{1}H-NMR ($CDCl_3$+DMSO-d_6): δ 0.83 (s, 3H, 18-CH_3), 0.86 (s, 3H, 19-CH_3), 2.22 (s, 3H, -OCOCH_3), 2.94 (br, 3H, $\overset{\oplus}{N}$-CH_3 of pyrrolidine function), 3.26 (br, 3H, $\overset{\oplus}{N}$-CH_3 of piperidine function), 3.57 (m, 4H, *N*-methylenes of pyrrolidine function) and 4.6 (m, 4H, *N*-methylenes of piperidine function) ppm. Anal. for $C_{32}H_{56}N_2O_2I_2$: C, 50.93; H, 7.48; N, 3.71. Found: C, 50.62; H, 7.30; N, 3.89.

Pharmacological Methods

Chick biventer cervicis nerve-muscle preparation

The chick biventer cervicis nerve-muscle preparations were set up as described previously [18]. Muscles were removed from 4-10 day-old chicks killed by exposure to CO_2. Preparations were mounted in pairs with a resting tension of approximately 1.0 g in 10 mL tissue baths containing Krebs-Henseleit solution. The solution was maintained at 34°C and bubbled with 95% O_2 and 5% CO_2. The muscles were stimulated via their motor nerves at 0.1 Hz with pulses of 0.2 msec duration and a voltage greater than that required for maximal contractions. Responses to carbachol were obtained in the absence of nerve stimulation; test compounds were replaced in the tissue bath after each washout of carbachol.

Acetylcholinesterase determination

The effects of compounds on the activity of acetylcholinesterase from electric eel were monitored at room temperature by the method of Ellman *et al.* [19] adapted to 96-well plates. Each assay was repeated four times.

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