Eur. J. Med. Chem. 36 (2001) 195–202

PII: S0223-5234(00)01205-8/SCO

**Short Communication**

# Synthesis and neuromuscular blocking activity of 16β-piperidinosteroidal derivatives

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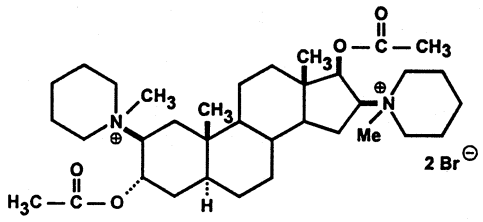
Received 8 March 2000; revised 29 November 2000; accepted 29 November 2000

**Abstract** – The synthesis and pharmacological profiles of some new steroidal mono- and bisquaternary ammonium derivatives have been described. The compounds featured have been conceptually derived structurally from two lead structures: pancuronium bromide **1** and chandonium iodide **2**. In vitro and in vivo neuromuscular blocking studies have indicated the monoquaternary compound **15** to be less active than the bisquaternary compounds **10** and **11**. The compound **11** has been found to be more active than *d*-tubocurarine. © 2001 Éditions scientifiques et médicales Elsevier SAS

**monoquaternary / bisquaternary / piperidine / chick biventer / anaesthetized cat / *d*-tubocurarine / carbachol / nondepolarizing / steroidal**

## 1. Introduction

The search for new neuromuscular blocking agents has been motivated by the long-time course of action and pronounced side effects of the compounds available in clinical practice [1]. Since the discovery of nondepolarizing neuromuscular blocking activity of pancuronium bromide **1** [2–4], tremendous numbers of steroidal [5–12] and nonsteroidal [1, 7, 13] neuromuscular blocking agents have been synthesized and their muscle relaxation properties examined. Shortly after the development of pancuronium **1**, azasteroidal compounds, in which the quaternary nitrogen atom is situated in the ring D of the steroidal skeleton, were synthesized and studied [5]. One of the compounds in this series, chandonium iodide **2**, is a powerful nondepolarizing neuromuscular blocking agent with short duration and rapid onset being only slightly less active than pancuronium **1** [14]. The general assumption between these structures and skeletal muscle relaxation is that the compounds must have two tertiary nitrogens

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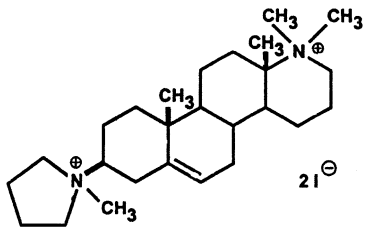


2

at appropriate distances (1.0–1.2 nm) of which one or both the nitrogens should be quaternized. As the interonium distance between two quaternary heads falls below 1.0 nm, the skeletal muscle relaxation

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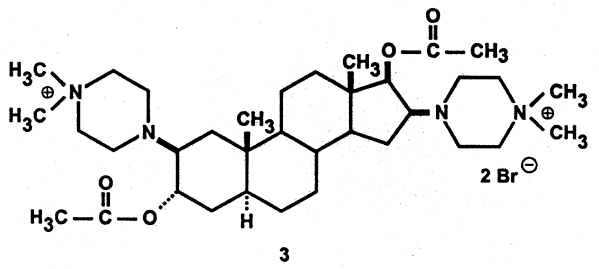
property diminishes and the ganglion blocking activity becomes prominent [15]. Pipecuronium bromide **3** is an example that does not follow this structure




**3**

activity parameter [16]. In the present study, our basic design concept was to combine structural features taken from pancuronium **1** and chandonium **2** [5], known to be essential for their nondepolarizing neuromuscular blocking activity, and to investigate the role of interonium distances between two quaternary heads at position 3 and 16 of the compounds. Furthermore, it was desired to verify the structural requirements in aminosteroids for skeletal muscle relaxation properties. In this paper, we present the synthesis and biological evaluation of the bisquaternary ammonium compounds **10** (DPJ-471) and **11** (DPJ-489) and monoquaternary compound **15** (DPJ-464).

## 2. Chemistry

To prepare mono- and bisquaternary compounds, 16α-bromo-17-oxo-5-androsten-3β-ol **4** was the starting material. It was prepared by a procedure reported in the literature [17–19]. 16-Piperidino functionality was introduced to obtain **5** by treating **4** with piperidine at refluxing temperature [18]. A one-proton double doublet at $\delta$ 3.12 in the NMR spectrum indicated the 16α-*H*. The assignment of the configuration at position 16 in compound **5** has been made on the basis of earlier reports [18, 19].

Oppenauer oxidation of **5**, using a cyclohexanone-toluene system, afforded compound **6**. In the NMR spectrum, the vinylic proton appeared at $\delta$ 5.70 (s; 4-C*H*). Compound **7** was prepared by refluxing the 4-en-3-one derivative **6** with pyrrolidine in methanol. The NMR signals appeared at $\delta$ 4.76 (s; 1H, 4-C*H*) and 5.06 (m; 1H, 6-C*H*). The enamine **7** was subsequently reduced with sodium borohydride in methanol to give the 3β-pyrrolidino derivative **8**. The esterification of **8** with acetic anhydride in pyridine in a boiling water bath provided the desired ester **9** (*figure 1*). Its NMR spectrum exhibited a three-proton singlet at $\delta$ 2.13 (-OCOC$H_3$), a single-proton dd at $\delta$ 3.03 (16α-*H*, $J = 9$ Hz). IR spectrum showed a band at 1725 cm$^{-1}$ (C=O stretching).

The bisquaternary compounds **10** (DPJ-471) and **11** (DPJ-489) were prepared by treating **8** and **9** with methyl iodide in dichloromethane at room temperature (*figure 2*). The *N*-methyl protons of the bisquaternary compounds **10** and **11** appeared at $\delta$ 3.0 and 3.1 in the $^1$H-NMR spectrum, respectively.

Reduction of **5** with sodium borohydride and acetylation of **12** with acetic anhydride in pyridine yielded compounds **12** and **13**, respectively (*figure 3*). The NMR spectrum of 16β-piperidino-5-androstene-3β, 17β-diol **12** exhibited signals at $\delta$ 2.76 (dd; 1H, $J = 9$ Hz, 16α-*H*); 3.40 (d; 1H, $J = 9$ Hz, 17α-H) and 5.33 (m; 1H, 6-C*H*). In the ester **13**, the NMR signals appeared at $\delta$ 2.03 (s; 3H, 3β-OCOC$H_3$); 2.10 (s; 3H, 17β-OCOC$H_3$); 3.03 (dd; 1H, $J = 9$ Hz, 16α-*H*) and 4.80 (d; 1H, $J = 9$ Hz, 17α-*H*). Preparation of the monoquaternary compound **14** was accomplished by refluxing **5** in absolute ethanol with methyl iodide whereas compounds **15** and **16** were prepared by treating **12** and **13** with methyl iodide in dichloromethane at room temperature (*figure 4*). The 16α-*H* of compound **14** appeared at $\delta$ 4.89 in the $^1$H-NMR spectrum. The deshielding effect of quaternary nitrogen is the reason for downfield shift of 16α-*H*.

## 3. Neuromuscular blocking activity

The pharmacological activity of compounds **10**, **11** and **15** was examined on the isolated chick biventer cervicis muscle preparation, a preparation selected for its ability to elicit the potency of nicotinic antagonists and its important indicative information on mechanism of action. All three compounds reduced the twitch response to nerve stimulation. There was no evidence of any of the compounds producing contracture of the muscle, indicating a lack of depolarizing activity. Each compound was added to the preparation in a concentration sufficient to produce a 75–90% reduction of control twitch tension in ca. 30 min; the concentrations required to produce these effects are shown in *table I*, and compared to those for tubocurarine as a standard. The effects of all three test compounds were quickly reversed by washing

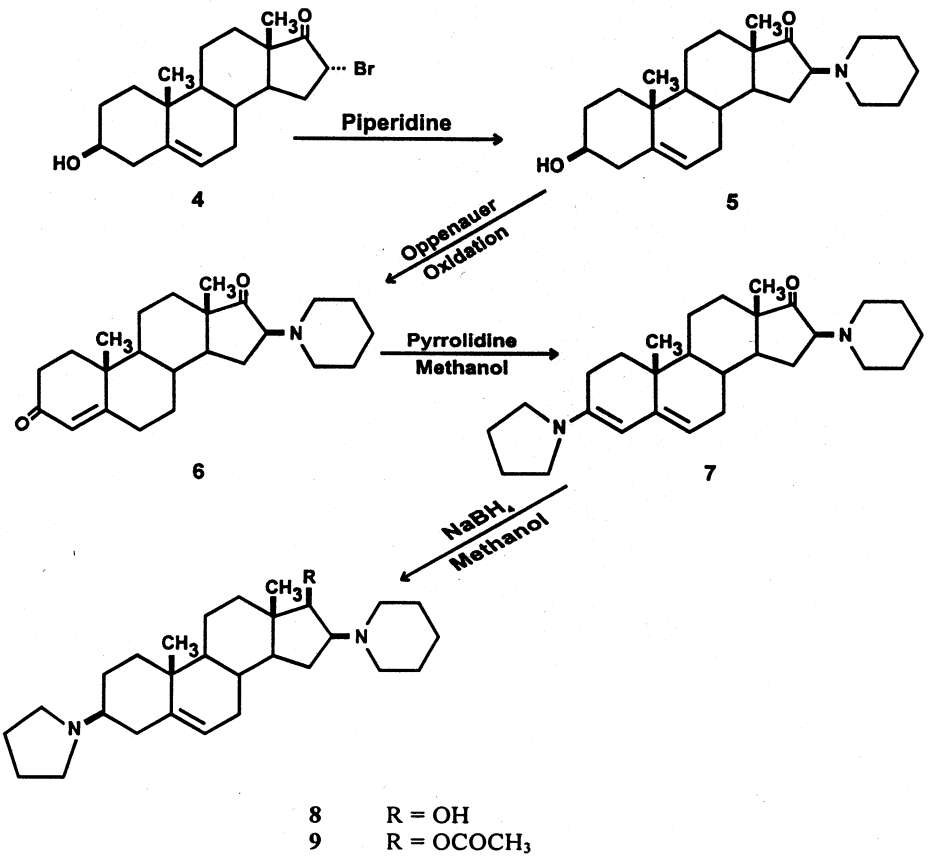



**Figure 1.**

from the tissue bath. However, if left in the bath and challanged with the anticholinesterase drug neostigmine (0.5 μM), only the effects of compound **11** were reversed (to 65% control twitch tension) thus indicating competitive antagonism. This conclusion was confirmed by the finding that although increasing concentrations of compound **11** produced a parallel rightwards shift of carbachol-induced dose–response curves, yielding a slope value of close to unity in Arunslakshana and Schild plots (*table II*). In contrast, the effect of compound **10** on twitches was not reversed at all by neostigmine and that of compound **15** reversed to only about 20% of control twitch tension. The lack of reversibility of the two weaker compounds of the three tested suggests a mechanism other than reversible competitive antagonism of nicotinic receptors. These observations for compounds **10** and **15** suggest a form of noncompetitive action, perhaps at the level of endplate ion channel.

In the two bisquaternary compounds in the series, there was a 13-fold drop in potency with the change from 17-acetoxy **11** to 17-hydroxy **10** showing the crucial importance of the acetoxy moiety in combination with the 16-quaternary nitrogen for binding to the nicotinic receptor. Removal of the A-ring quaternary group from **10** produced only a modest drop in

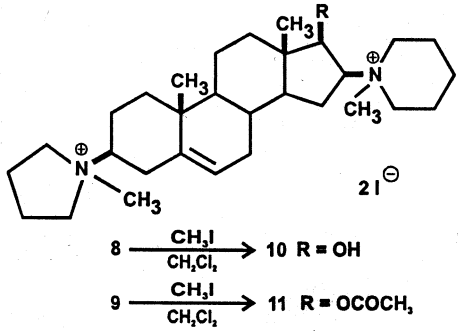



**Figure 2.**

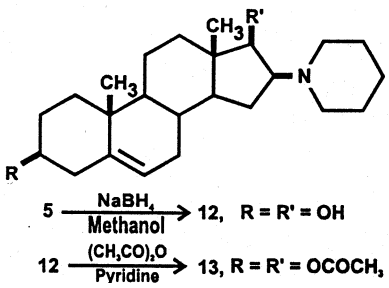



**Figure 3.**

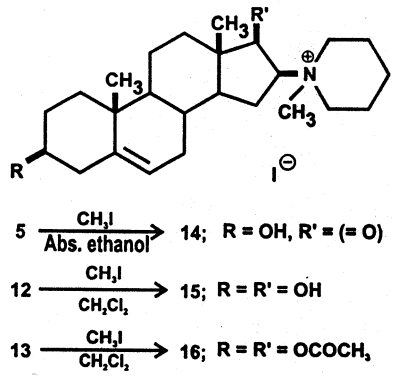



**Figure 4.**

potency (1.05–14-fold), again confirming the importance of the D-ring substituents.

Only compound **11** was considered to have the appropriate mechanism and potency to warrant further testing. This was confined to a single anaesthesized cat experiment, using identical techniques to those used to evaluate neuromuscular blockers such as vecuronium and rocuronium whose values are shown for comparison with compound **11** (*table III*). Compound **11** produced a reduction of twitch tension in the tibialis anterior and soleus muscles induced by stimulation of the sciatic nerve. No pre-block twitch augmentation was observed, again indicating a lack of depolarizing activity. The onset of action was in the rapid category, being the same as that of the rapid-onset rocuronium and shorter than that of the medium-onset vecuronium. Both the recovery and duration times of the block were long. The compound had no effect on the contractions of the pre-ganglionically-stimulated nictitating membrane, indicating a lack of ganglion blocking activity. There was no effect on general arterial blood pressure, but the compound produced a moderate reduction of the bradycardial response of the heart rate to vagal stimulation, indicating a probable block of cardiac muscarinic receptors; such an effect is observed with pancuronium and is associated with tachycardia in man. The effect of compound **11** in the cat is shown in *figure 5*.

**Table I.** The neuromuscular blocking potency (75–90% twitch block in the chick biventer cervicis muscle, μm) of the quaternary androstene compounds with tubocurarine as control drug.

| Compound | Concentration for 75–95% inhibition (μM) | n[b] | Relative potency[a] |
|---|---|---|---|
| *d*-Tubocurarine | $1.0 \pm 0.13$ | 6 | 1 |
| **15** (DPJ-464) | $2.45 \pm 0.44$ | 6 | 0.4 |
| **10** (DPJ-471) | $2.32 \pm 0.16$ | 5 | 0.43 |
| **11** (DPJ-489) | $0.18 \pm 0.01$ | 4 | 5.5 |

[a] Indicates the relative potency to tubocurarine as $1 \times 10^{-6}$ M = 1
[b] n, number of experiments.

**Table II.** Compared $pA_2$ value of compound **11** (DPJ-489) in isolated chick biventer cervicis nerve muscle preparation with tubocurarine as control.

| Compound | $pA_2$ (mean ± S.E.M.) | Slope (mean ± S.E.M.)[a] | $K_D$ (mean ± S.E.M.) μM | n[b] |
|---|---|---|---|---|
| *d*-Tubocurarine | $6.0 \pm 0.13$ | $1.1 \pm 0.03$ | $1.1 \pm 0.5$ | 8 |
| **11** (DPJ-489) | $7.6 \pm 0.25$ | $1.04 \pm 0.07$ | $0.025 \pm 0.02$ | 4 |

[a] S.E.M., standard error of mean.
[b] n, number of experiments.

**Table III.** Effective dose and course values for neuromuscular effects of the compound **11** (DPJ-489) investigated in an anaesthetized cat. Values obtained in identical experiments are included for vecuronium and rocuronium [20].

| Compound | Dose to produce 80–95% twitch block (soleus) (μg $kg^{-1}$) | Onset time injection to max. block (min) | Duration: injection to 90% recovery (min) | Recovery: 25–75% twitch-height recovery (min) |
|---|---|---|---|---|
| Vecuronium | 38 | 5.0 | 17.4 | 5.2 |
| Rocuronium | 311 | 2.9 | 13.7 | 5.2 |
| **11** (DPJ-489) | 150 | 2.9 | 26 | 9.9 |

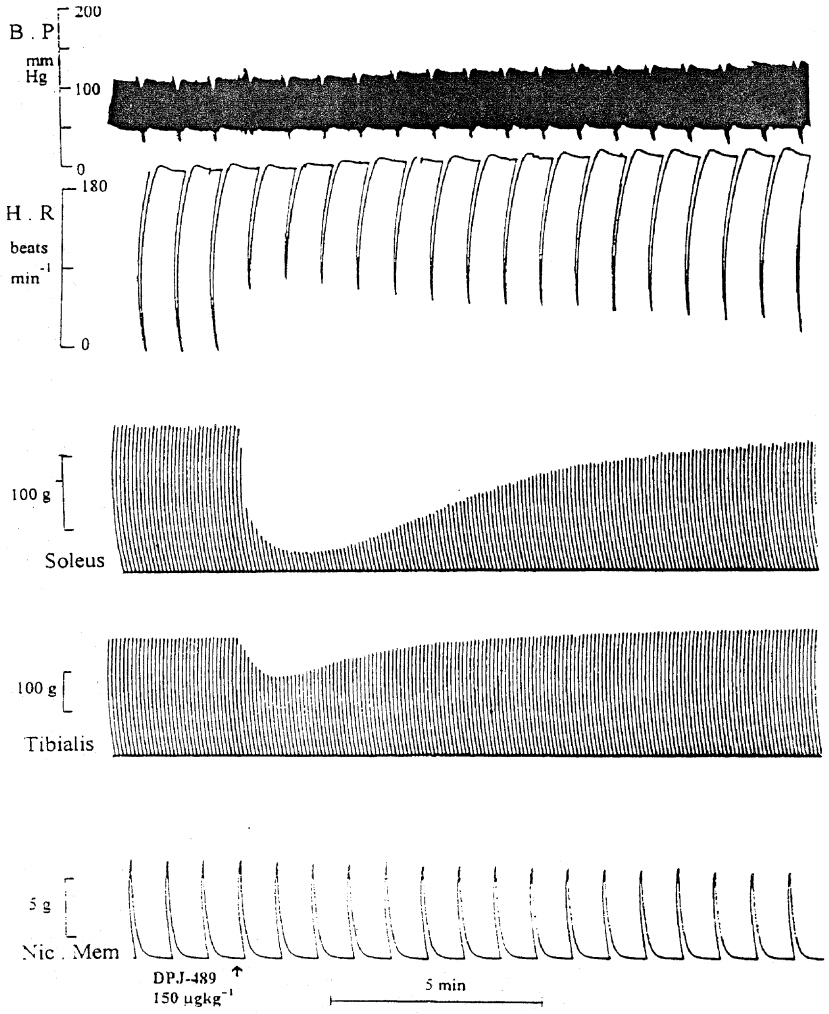



**Figure 5.** Chloralose-anesthetized cat. Effect of DPJ-489 (150 μg $kg^{-1}$) on blood pressure (B.P.), heart rate (H.R.), response of the nictitating membrane (Nic. Mem) to preganglionic stimulation and responses of the soleus and tibialis anterior muscles to indirect stimulation at 0.1 Hz.

## 4. Experimental protocols

### 4.1. 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 $\delta$, 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 (TLC) and by elemental analysis (C, H, N). Elemental analyses were carried out on a Perkin-Elmer-2400. Mass spectra were recorded on a V6-11-250 J 70S. Anhydrous sodium sulphate was used as a drying agent. UV spectra were recorded in methanol ($\lambda_{max}$ in nm). IR spectra were obtained with potassium bromide pellets ($V_{max}$ in cm$^{-1}$).

#### 4.1.1. 16β-Piperidino-4-androstene-3,17-dione **6**

17-Oxo-16β-piperidino-5-androsten-3β-ol **5** (2.0 g, 5.38 mmol) was dissolved in a mixture of cyclohexanone (20 mL, 92.97 mmol) and dry toluene (200 mL) and the solution was subjected to azeotropic distillation to remove traces of moisture. The distillation was continued at a slow rate, during dropwise addition of a solution of aluminium isopropoxide (2.0 g, 9.79 mmol) in dry toluene (20 mL). The reaction mixture was refluxed for 4 h and allowed to stand at room temperature for 12 h. The slurry was filtered and filtrate was steam-distilled until the complete removal of organic solvents was effected. The residual aqueous suspension was allowed to stand at room temperature and extracted with chloroform (4×50 mL). The combined chloroform extract was washed with water, dried and the solvent removed to obtain a semisolid residue **6** which could not be crystallized and was used as such for further reaction; $UV_{max}$(MeOH): 239.2 nm; IR (KBr): 2910, 1725, 1660, 1600, 1430, 1220 and 855 cm$^{-1}$; $^1$H-NMR ($CDCl_3$): $\delta$ 0.90 (s; 3H, 18-C$H_3$); 1.26 (s; 3H, 19-C$H_3$); 2.30–2.90 (m; 4H, *N*-methylenes of piperidino function); 3.16 (dd; 1H, 16α-C*H*) and 5.70 (s; 1H, 4-C*H*).

#### 4.1.2. 16β-Piperidino-3-pyrrolidino-3,5-androstadien-17-one **7**

Freshly distilled pyrrolidine (9 mL, 107.82 mmol) was added dropwise to a refluxing solution of 16β-piperidino-4-androstene-3,17-dione **6** (1.5 g, 4.06 mmol) in methanol (15 mL). Refluxing was further continued for 15 min and the solution was concentrated to induce crystallization. The crystalline material was filtered, washed with methanol and dried to obtain **7**. Yield: 1.50 g (87.21%); m.p.: 165–170 °C; $UV_{max}$ (MeOH): 274 nm (log∈4.36); IR (KBr): 2920, 2825, 1725, 1580, 1390, 1340, 1165, 1025 and 818 cm$^{-1}$; $^1$H-NMR ($CDCl_3$): $\delta$ 0.93 (s; 3H, 18-C$H_3$); 1.03 (s; 3H, 19-C$H_3$); 2.96 (dd; 1H, 16α-C*H*); 3.10 (m; 4H, *N*-methylenes of pyrrolidino function); 4.76 (s; 1H, 4-C*H*) and 5.06 (m; 1H, 6-C*H*). Anal. for $C_{28}H_{42}N_2O$: C, 79.57; H, 10.02; N, 6.63. Found: C, 79.41; H, 10.01; N, 6.56.

#### 4.1.3. 16β-Piperidino-3β-pyrrolidino-5-androsten-17β-ol **8**

Sodium borohydride (1.0 g, 26.43 mmol) was added to a stirred suspension of 16β-piperidino-3-pyrrolidino-3,5-androstadien-17-one **7** (1.0 g, 2.37 mmol) in methanol (150 mL) in small quantities at room temperature. Stirring was continued for 2 h, the reaction mixture was poured into ice-cold water (800 mL) and the aqueous suspension was extracted with chloroform (4× 100 mL). The combined chloroform extract was washed with water, dried and solvent removed under reduced pressure to give a solid residue which was crystallized from acetone to afford **8**. Yield: 0.60 g (59.46%); m.p.: 168–170 °C; IR (KBr): 2930, 2870, 1440, 1340, 1125, 1070 and 885 cm$^{-1}$; $^1$H-NMR ($CDCl_3$): $\delta$ 0.70 (s; 3H, 18-C$H_3$); 1.03 (s; 3H, 19-C$H_3$); 2.30–2.60 (m; 8H, *N*-methylenes of piperidino and pyrrolidino functions); 2.83 (dd; 1H, $J = 9$ Hz, 16α-C*H*); 3.26 (d; 1H, $J = 9$ Hz, 17α-C*H*) and 5.23 (m; 1H, 6-C*H*); $[\alpha]_D^{22}$: 22.39° (C 0.046 g 100 mL$^{-1}$, $CHCl_3$); MS: $m/z$: 427 [M$^+$]. Anal. for $C_{28}H_{46}N_2O$: C, 78.82; H, 10.87; N, 6.57. Found: C, 78.60; H, 10.83; N, 6.39.

#### 4.1.4. 16β-Piperidino-3β-pyrrolidino-5-androsten-17β-yl acetate **9**

16β-Piperidino-3β-pyrrolidino-5-androsten-17β-ol **8** (0.5 g, 1.17 mmol) was heated in a mixture of pyridine (0.25 mL, 3.09 mmol) and acetic anhydride (0.5 mL, 5.30 mmol) for 1 h on steam bath. The reaction mixture was poured into ice-cold water basified with ammonia and the precipitated product was filtered, washed with water and dried. The product obtained was crystallized from acetone to give **9**. Yield: 0.30 g (54.64%); m.p.: 215 °C; IR (KBr): 2925, 2745, 1725, 1365, 1232, 1135 and 1025 cm$^{-1}$; $^1$H-NMR ($CDCl_3$): $\delta$ 0.90 (s; 3H, 18-C$H_3$); 1.03 (s; 3H, 19-C$H_3$); 2.13 (s; 3H, -OCOC$H_3$); 2.16–2.76 (dd; 8H, *N*-methylenes of piperidino and pyrrolidino functions); 3.03 (q; 1H, $J = 9$ Hz, 16α-C*H*);

4.83 (d; 1H, $J = 9$ Hz, 17α-C*H*) and 5.30 (m; 1H, 6-C*H*). Anal. for $C_{30}H_{48}N_2O_2$: C, 76.87; H, 10.32; N, 5.98. Found: C, 76.51, H, 9.92; N, 6.17.

*4.1.5. 16β-Piperidino-3β-pyrrolidino-5-androsten-17β-ol dimethiodide* **10** *(DPJ-471)*

Methyl iodide (4 mL, 64.25 mmol) was added to a solution of 16β-piperidino-3β-pyrrolidino-5-androsten-17β-ol **8** (0.30 g, 0.703 mmol) in dichloromethane (20 mL) and allowed to stand for 5 days at room temperature with occasional stirring. The reaction mixture was concentrated to dryness and crystallized from acetone. The crystalline material was filtered, washed and dried to give **10**. Yield: 0.33 g (66.13%); m.p.: 300 °C; IR (KBr): 3215, 1445, 1145, 1025 and 920 $cm^{-1}$; $^1$H-NMR ($CDCl_3$-DMSO-$d_6$): $\delta$ 2.99 (s; 3H, N-C$H_3$ of pyrrolidino moiety) and 3.11 (s; 3H, N-C$H_3$ of piperidino moiety). Anal. for $C_{30}H_{52}N_2OI_2$: C, 50.71; H, 7.38; N, 3.94. Found: C, 50.52; H, 7.32; N, 3.82.

*4.1.6. 16β-Piperidino-3β-pyrrolidino-5-androsten-17β-yl acetate dimethiodide* **11** *(DPJ-489)*

Quaternization of 16β-piperidino-3β-pyrrolidino-5-androsten-17β-yl acetate **9** (0.30 g, 0.64 mmol) in dichloromethane was done as above to afford **11**. Yield: 0.20 g (41.49%). IR (KBr): 1735, 1440 and 1210 $cm^{-1}$; $^1$H-NMR ($CDCl_3$-DMSO-$d_6$): $\delta$ 2.21 (s; 3H, 17β-OCOC$H_3$); 3.00 (s; 3H, *N*-C$H_3$ of pyrrolidino moiety) and 3.19 (s; 3H, *N*-C$H_3$ of piperidino moiety). Anal. for $C_{32}H_{54}N_2O_2I_2$: C, 51.07; H, 7.23, N, 3.72. Found: C, 51.12; H, 7.23; N, 3.61.

*4.1.7. 16β-Piperidino-5-androstene-3β,17β-diol diacetate* **13**

16β-Piperidino-5-androstene-3β, 17β-diol **12** (1.0 g, 2.68 mmol) was acetylated as in compound **9** to afford **13**. Yield: 0.8 g (65.04%); m.p.: 208–210 °C; IR (KBr): 2910, 1722, 1435, 1352, 1235 and 1030 $cm^{-1}$; $^1$H-NMR ($CDCl_3$): $\delta$ 0.82 (s; 3H, 18-C$H_3$); 1.03 (s; 3H, 19-C$H_3$); 2.03 (s; 3H, 3β-OCOC$H_3$); 2.10 (s; 3H, 17β-OCOC$H_3$); 2.38–2.54 (m; 4H, *N*-methylenes of piperidino function); 3.03 (dd; 1H, $J = 9$ Hz, 16α-C*H*); 4.60 (m; 1H, 3α-C*H*); 4.80 (d; 1H, $J = 9$ Hz, 17α-C*H*) and 5.30 (d; 1H, 6-C*H*). Anal. for $C_{28}H_{43}NO_4$: C, 73.48; H, 9.47; N, 3.06. Found: C, 73.21; H, 9.21; N, 3.13.

*4.1.8. 17-Oxo-16β-piperidino-5-androsten-3β-ol methiodide* **14** *(DPJ-458)*

Quaternization of 17-oxo-16β-piperidino-5-androsten-3β-ol **5** (0.5 g, 1.34 mmol) was done in absolute ethanol to afford **14**. Yield: 0.5 g (72.4%); m.p.: 248–250 °C; IR (KBr): 3400, 1730, 1460 and 1045 $cm^{-1}$; $^1$H-NMR ($CDCl_3$): $\delta$ 3.18 (s; 3H, *N*-C$H_3$ of piperidino moiety); 4.89 (dd; 1H, $J = 9$ Hz, 16α-C*H*). Anal. for $C_{25}H_{40}NO_2I$: C, 58.47; H, 7.85, N, 2.73. Found: C, 58.39; H, 7.95, N, 2.35.

*4.1.9. 16β-Piperidino-5-androstene-3β, 17β-diol methiodide* **15** *(DPJ-464)*

Quaternization of 16β-piperidino-5-androstene-3β,17β-diol **12** (0.5 g, 1.34 mmol) was done in dichloromethane to afford **15**. Yield: 0.45 g (65.03%); m.p.: 218–220 °C; IR (KBr): 3300, 2910, 1445, 1270, 1140 and 1045 $cm^{-1}$; $^1$H-NMR ($CDCl_3$): $\delta$ 3.09 (s; 3H, *N*-C$H_3$ of piperidino moiety) and 4.60 (dd; 1H, $J = 9$ Hz, 16α-C*H*). Anal. for $C_{25}H_{42}NO_2I$: C, 58.24; H, 8.21; N, 2.72. Found: C, 58.41; H, 8.04; N, 2.37.

*4.2. 16β-Piperidino-5-androstene-3β,17β-diol diacetate methiodide* **16** *(DPJ-465)*

Quaternization of 16β-piperidino-5-androstene-3β,17β-diol diacetate **13** (0.8 g, 1.75 mmol) was done in dichloromethane to afford **16**. Yield: 0.63 g (60.1%); m.p.: 290–292 °C; IR (KBr): 2900, 2840, 1722, 1430, 1352, 1230 and 1010 $cm^{-1}$; $^1$H-NMR ($CDCl_3$): $\delta$ 2.02 (s; 3H, 3β-OCOC$H_3$); 2.22 (s; 3H, 17β-OCOC$H_3$); 3.41 (s; 3H, *N*-C$H_3$ of piperidino moiety) and 4.73 (dd; 1H, $J = 9$ Hz, 16α-C*H*). Anal. for $C_{29}H_{46}NO_4I$: C, 58.09; H, 7.33; N, 2.34. Found: C, 58.13; H, 7.83; N, 2.31.

## 5. Pharmacological methods

### *5.1. Chick biventer cervicis muscle preparation*

Biventer cervicis nerve-muscle preparations [21] were dissected from young chickens (3–10 days) and mounted in a 10 mL tissue bath maintained at 32 °C and containing Krebs solution of the following composition (mmol $L^{-1}$): NaCl, 118; KCl, 5; $CaCl_2$, 2.5; $NaHCO_3$, 30; $KH_2PO_4$, 1; $MgSO_4$, 1; glucose, 11 and pH 7.4 when aspirated with 5% carbon dioxide in oxygen. The motor nerves were stimulated at a frequency of 0.1 Hz with rectangular pulses of 0.2 ms duration and a voltage greater than that required to produce maximal twitches. Tension responses were recorded by Grass FT03C force displacement transducers.

In some experiments dose–response curves were constructed by adding increasing concentrations of the ago-

nist carbachol until maximal responses were obtained. Carbacol dose–response curves were constructed in the absence and the presence of three concentrations of the test compounds. Affinity constants ($K_D$ values) were calculated from plots of log (dose ratio-1) against molar concentration of antagonist [22]. Slope values from the plots were used to assess competitive antagonism or otherwise.

### 5.2. Anaesthetized cat

A cat was anaesthetized with a mixture of α-chloralose 80 mg/kg$^{-1}$ and pentobarbitone 5 mg/kg$^{-1}$ injected i.p. Lungs were ventilated with air at a rate of 26 b.p.m. at a tidal volume of approximately 13 mL kg$^{-1}$, adjusted to maintain arterial pH at 7.37–7.47.

The right hind limb was immobilized and the contractile responses of the tibialis anterior and soleus muscles to single shock stimulation of the sciatic nerve were recorded. The sciatic nerve is stimulated at a rate of 0.1 Hz using rectangular pulses of 0.2 ms duration and of length greater than that required to produce maximal twitch. This stimulation frequency was chosen to allow comparison of the results with test compounds with those for compounds reported previously in the literature. Contractions of the nictitating membrane were evoked in response to preganglionic stimulation of the cervical sympathetic nerve with trains (frequency 5 Hz, duration 10 s) of strength sufficient to produce maximal concentrations of nictitating membrane.

Arterial pressure was recorded from the carotid artery using a Statham PC45 pressure transducer. The arterial pressure pulse triggered a cardiachograph to display a heart rate. Both vagus nerves were ligated and, at 100 s intervals, the right vagus nerve was stimulated at a frequency of 2–5 Hz and with pulses of 0.5 ms duration and strength greater than that required to produce a maximal reduction in heart rate. Contractile responses of muscle were recorded using Grass FTO3Cand FT10 displacement transducers. All responses were displayed on a Grass model 5 ink oscillograph.

## Acknowledgements

We are pleased to acknowledge the financial support of the project by the Council of Scientific and Industrial Research, New Delhi, and Panjab University for providing the research facilities. The authors express appreciation to Panacea Biotech Limited, New Delhi for financial support for this investigation, and Cipla, Bombay for the supply of DHA acetate.

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