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Bansal, R., Guleria, S., Thota, S., Bodhankar, S.L.,  
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A.L.**

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(2012) Steroids, 77 (6), pp. 621-629.**

# **Design, synthesis and evaluation of novel 16-imidazolyl substituted steroidal derivatives possessing potent diversified pharmacological properties**

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**Key words:** 16-Imidazolyl steroids; Aromatase inhibitors; Bis-quaternary steroids; Skeletal muscle relaxants; Anticholinesterases

## **Abbreviations:**

DDQ, 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone; SRB, Sulforhodamine B; DCC, dextran-coated charcoal; AChE, Acetylcholinesterase; AD, Alzheimer's disease

## Abstract

As a part of our investigations into the structural-activity relationship studies of a novel class of medicinally active 16-substituted steroids, several new 16-imidazolyl substituted steroidal derivatives have been synthesized and pharmacologically evaluated in the current study. The new steroidal analogues **5**, **6**, **8**, **9**, **11** and **12** exhibited moderate cytotoxic effects in sixty cancer cell lines derived from nine cancers types. The imidazolyl substituted steroidal derivatives **6** (DPJ-RG-1241) and **7** (RB-401) were obtained as the powerful inhibitors of aromatase with  $IC_{50} = 0.18 \mu\text{M}$  and  $IC_{50} = 0.168 \mu\text{M}$ , respectively, approximately 1.2 and 1.4 times more potent in comparison to standard drug exemestane. The bis-quaternary steroids **13** and **14** displayed potent skeletal muscle relaxant properties. An affinity constant of  $0.007 \mu\text{M}$  was observed for compound **14** on frog rectus abdominis muscle preparation and **13** displayed a very high anticholinesterase activity  $K_i = 25 \text{ nM}$ , approximately 115-fold higher in comparison to standard drug galanthamine ( $K_i = 2.9 \mu\text{M}$ ).

## 1. Introduction

Heterosteroids have been accredited with a great amount of attention over the years by medicinal chemists for drug discovery. The interesting structural and stereochemical features of the steroid nucleus provide additional fascination to the researchers and thereby alterations in the steroidal skeleton have been envisaged to discover new chemical entities with a potential to afford some promising drugs of the future. The incorporation of a heterocyclic ring or a heteroatom in the steroid backbone affects the chemical properties of a steroid and often results in useful alterations in its biological activities [1,2]. The products obtained by introduction of heteroatom in the steroid nucleus are called nuclear heterosteroids. When heteroatom(s) forms a part of fused ring system, attached group or a side chain of the steroid nucleus then the products are known as extranuclear heterosteroids. Heterosteroids encompass a wide range of compounds with varied biological activities, e.g., 5 $\alpha$ -reductase inhibitors like finasteride [2], GABA receptor antagonists, e.g., RU-5135 [2], aromatase inhibitors such as formestane and exemestane [3], anticancer agents like 2-methoxyestradiol [4] and neuromuscular junction blocking agents like pipecuronium [5].

16-Substituted steroids have shown diversified pharmacological activities and are of interest for a medicinal chemist to develop new molecules. Many potent steroidal derivatives with substitution at position 16 have been described in the literature [6,7]. Some interesting 16*E*-arylidensteroids have recently been reported from our laboratory as strong *in vitro* inhibitors of the growth of many types of human tumor cells [8-10]. These findings motivated us to continue the exploration of biological properties of 16-substituted steroidal derivatives by designing and synthesizing new analogues with suitable structural modifications. This paper embodies the synthesis, study and biological

evaluation of newer 16-imidazolyl substituted steroids for their cytotoxic, aromatase inhibitory, NMJ blocking and acetylcholinesterase inhibitory activities.

Development of hybrid structures, in which pharmacologically crucial structural elements from two molecules are combined to produce a non-identical twin drug, is a rational approach to obtain therapeutically useful molecules. The main focus of the current study is to design and develop synthetic strategies to produce new chemical hybrids of steroidal aromatase inhibitors such as formestane, exemestane and non-steroidal aromatase inhibitors, e.g., fadrozole (fig 1). These compounds may show high specificity and increased potency as aromatase inhibitors. In view of the significance ofazole moiety to inhibit P450 enzyme inhibitors including aromatase [11,12], we hypothesized that introducing imidazole moiety to the androstane nucleus might yield specific and potent P450 inhibitors. With this design, it may be possible to produce substrate like chemical entities, which not only interact with the steroid binding site of the enzyme, thus introducing high specificity, but also provide a ligand to the enzyme heme iron resulting in tight binding [13]. Literature survey further indicates that structural modifications in steroidal A and D ring brings out noticeable changes in aromatase inhibitory potential of steroidal molecules and provides potent, easily obtainable and structurally simple aromatase inhibitors [7,14,15]. Therefore, as performed with non-steroidal inhibitors [16-18], imidazolyl-substituted D-ring modified steroidal derivatives containing a suitably positioned heteroatom capable of binding to cytochrome P450 enzymes have been synthesized.

Neuromuscular junction blocking agents act on cholinergic receptors on the skeletal muscle endplate to produce muscle paralysis. These agents are clinically used to provide adequate skeletal neuromuscular relaxation during surgery, controlled respiration

and orthopedic manipulation. These agents are used to induce safer anesthesia, decrease the severity of muscle contraction during electroconvulsive treatment and may also be used in the management of tetanus, in various spastic disorders and in diagnosis of myasthenia gravis [2,5].

Quaternary ammonium steroids represent an important class of skeletal muscle relaxants [5]. These are non-depolarising type NMJ blockers and act as competitive antagonists at the acetylcholine receptors. The block can be reversed by acetylcholinesterase inhibitors due to increase in concentration of acetylcholine at the junction by inhibiting the enzyme acetylcholinesterase. Most of the acetylcholinesterase inhibitors are also quaternary ammonium compounds [19]. Interonium distance between the two quaternary centres in steroids is crucial for NMJ blocking activity. It has also been observed from our previous studies that a variation in the interonium distance, although increased, resulting from the built in flexibility about the single bond on the moieties linked to ring D of a bisquaternary steroid is not desirable for favourable NMJ blocking effects [19]. Pipecuronium bromide with an interonium distance of 16.07 Å produces less cardiovascular side effects in comparison to pancuronium bromide (11.00 Å) (fig 2) [20,21], however an increase in onset of action was observed. A value of about 10-14 Å is considered as optimal interonium distance for skeletal muscle relaxant effects. Taking a note of this observation, some biquaternary steroids with a little extended, but fixed interonium distance has also been prepared and evaluated for NMJ blocking and anticholinesterase activity in the current study.

## 2. Experimental section

### 2.1. General

Melting points were determined on a Veego melting point apparatus and are uncorrected. UV (wavelengths in nm) spectra were recorded on Lambda 15 and IR (wave numbers in  $\text{cm}^{-1}$ ) spectra on Perkin-Elmer spectrum RX 1, FT-IR spectrophotometer models using KBr pellets.  $^1\text{H}$  NMR spectra were recorded on Bruker AC-300F, 300MHz using deuterated-chloroform ( $\text{CDCl}_3$ ) or deuterated dimethylsulfoxide ( $\text{DMSO-}d_6$ ) containing tetramethylsilane as internal standard (chemical shifts in  $\delta$ , ppm). Elemental analyses were carried out on a Perkin-Elmer-2400 model CHN analyzer. Plates for TLC were prepared according to Stahl (E. Merck) using EtOAc as solvent (activated at  $110^\circ\text{C}$  for 30 min) and were visualized by exposure to iodine vapors. Anhydrous sodium sulphate was used as a drying agent. All solvents were distilled prior to use according to standard procedures. All target compounds possessed a purity of  $\geq 95\%$  as verified by elemental analyses by comparison with the theoretical values.

16 $\alpha$ -Bromo-17-oxo-5-androsten-3 $\beta$ -ol (**2**) was synthesized according to reported procedure [22].

*2.1.1. Preparation of 16 $\alpha$ / $\beta$ -bromo-4-androstene-3,17-dione (3)* 16 $\alpha$ -Bromo-17-oxo-5-androsten-3 $\beta$ -ol (**2**, 1 g, 2.72 mmol) was dissolved in a mixture of cyclohexanone (10 mL) and dry toluene (100 mL). Traces of moisture were removed by azeotropic distillation of toluene. The distillation was continued at a slow rate while adding a solution of aluminium isopropoxide (1 g) in dry toluene (20 mL) dropwise. The reaction mixture was refluxed for 1 h and allowed to stand at room temperature overnight. The slurry was filtered and the residue was washed with dry toluene. The combined filtrate

and the washings were steam distilled until the complete removal of organic solvents was affected. The solid obtained was filtered, washed with water, dried and crystallized from a mixture of acetone and *n*-hexane to obtain **3**. 60% yield, mp 128-130 °C, IR (KBr): 2930.0, 1748.5, 1667.2, 1612.1, 1453.5, 1228.6, 1017.9 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 5.76 ppm (s, 1H, 4-CH), 4.56 (d) and 4.12 (t) (4:6 area ratio, 1H, 16β-H and 16α-H), 1.22 (s, 3H, 19-CH<sub>3</sub>), 1.13 (s, 3H, 18-CH<sub>3</sub>).

*2.1.2. 16α/β-Bromoandrosta-1,4-diene-3,17-dione (4)* A mixture of 16α/β-bromo-4-androstene-3,17-dione (**3**, 0.25 g, 0.68 mmol), 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ, 0.5 g) was heated under reflux in dry dioxane (50 mL) for 50 h. The progress of the reaction was monitored by TLC. The reaction mixture was cooled at room temperature and filtered. Chloroform (50 mL) was added to the filtrate and washed with 1% aqueous solution of potassium hydroxide (2 x 25 mL) and then with water to neutrality. The organic layer was dried and removed under vacuum to yield an oily residue, which revealed two spots on TLC and were separated by fractional crystallization. The oily residue was refluxed with diethyl ether, filtered and filtrate was concentrated to afford crystals of 16α-isomer **4a** (30%). The residue left was crystallized from a mixture of petroleum ether and diethyl ether to obtain 16β-isomer **4b** (70%).

*16α-Bromoandrosta-1,4-diene-3,17-dione (4a)*

<sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.04 ppm (d, 1H, *J*<sub>cis</sub> = 10.15 Hz, 2-CH), 6.25 (d, 1H, *J*<sub>cis</sub> = 10.14 Hz, 1-CH), 6.10 (s, 1H, 4-CH), 4.54 (t, 1H, 16β-H), 1.26 (s, 3H, 19-CH<sub>3</sub>), 0.99 (s, 3H, 18-CH<sub>3</sub>).

*16 $\beta$ -Bromoandrosta-1,4-diene-3,17-dione (4b)*

<sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  7.04 ppm (d, 1H,  $J_{cis}$  = 10.15 Hz, 2-CH), 6.23 (d, 1H,  $J_{cis}$  = 10.23 Hz, 1-CH), 6.09 (s, 1H, 4-CH), 4.10 (t, 1H, 16 $\alpha$ -H), 1.26 (s, 3H, 19-CH<sub>3</sub>), 1.17 (s, 3H, 18-CH<sub>3</sub>).

*2.1.3. General procedure for the synthesis of compounds 5-7*

A finely triturated mixture of requisite bromo steroid **2-4** (1g, 2.74 mmol) and powdered imidazole (1.5g, 10 mmol) was heated at 130-135 °C for 1 h. The reaction mixture was cooled to room temperature and cold water was added to it. The solid obtained was filtered, washed with water, dried and crystallized from a mixture of acetone and *n*-hexane to afford **5-7**, respectively

*2.1.3a. 16 $\beta$ -(Imidazol-1-yl)-17-oxo-5-androsten-3 $\beta$ -ol (5) (DPJ-RG-1240)*

51% yield, mp 249-251 °C, IR (KBr): 3303.8, 2943.0, 1744.8, 1499.5, 1456.1, 1374.7, 1318.3, 1243.9, 1065.8, 1041.4 and 914.2 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  7.70 ppm (s, 1H, 2-CH, imidazole), 7.12 (s, 1H, 4-CH, imidazole), 6.95 (s, 1H, 5-CH, imidazole), 5.39 (d, 1H,  $J$  = 4.9 Hz, 6-CH), 4.47 (m, 1H, 16 $\alpha$ -H), 3.55 (m, 1H, 3 $\alpha$ -H), 1.07 (s, 3H, 19-CH<sub>3</sub>), 1.02 (s, 3H, 18-CH<sub>3</sub>). Calcd for C<sub>22</sub>H<sub>30</sub>N<sub>2</sub>O<sub>2</sub>: C, 74.54; H, 8.53; N, 7.90. Found: C, 74.29; H, 8.84; N, 7.73.

*2.1.3b. 16 $\beta$ -(Imidazol-1-yl)-4-androstene-3,17-dione (6) (DPJ-RG-1241)*

41% yield, mp 179-181 °C, IR (KBr): 2947.6, 1748.7, 1667.5, 1497.7, 1456.0, 1229.8, 1108.9, and 1045.0 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  7.66 ppm (s, 1H, 2-CH, imidazole), 7.09 (s, 1H, 4-CH, imidazole), 6.95 (s, 1H, 5-CH, imidazole), 5.76 (s, 1H, 4-CH), 4.46 (m, 1H,

16 $\alpha$ -H), 1.24 (s, 3H, 19-CH<sub>3</sub>), 1.06 (s, 3H, 18-CH<sub>3</sub>). Calcd for C<sub>22</sub>H<sub>28</sub>N<sub>2</sub>O<sub>2</sub>: C, 74.97; H, 8.01; N, 7.95. Found: C, 74.71; H, 8.28; N, 8.12.

2.1.3c. 16 $\beta$ -(Imidazol-1-yl)-androsta-1,4-diene-3,17-dione (**7**)

45% yield, mp 209-211 °C, IR (KBr): 2928.4, 1750.0, 1657.3, 1495.0, 1457.0, 1241.9, 1110.2, and 1046.7 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>+DMSO-*d*<sub>6</sub>):  $\delta$  7.54 ppm (s, 1H, 2-CH, imidazole), 7.03 (d, 1H, *J*<sub>cis</sub> = 12 Hz, 2-CH), 6.97 (s, 1H, 4-CH, imidazole), 6.92 (s, 1H, 5-CH, imidazole), 6.14 (d, 1H, *J*<sub>cis</sub> = 12.1 Hz, 1-CH), 5.99 (s, 1H, 4-CH), 4.58 (t, 1H, *J* = 8.36 Hz, 16 $\alpha$ -H), 1.23 (s, 3H, 19-CH<sub>3</sub>), 1.03 (s, 3H, 18-CH<sub>3</sub>). Calcd for C<sub>22</sub>H<sub>26</sub>N<sub>2</sub>O<sub>2</sub>: C, 75.40; H, 7.48; N, 7.99. Found: C, 75.21; H, 7.22; N, 8.02.

2.1.4. 16 $\beta$ -(Imidazol-1-yl)-5-androstene-3 $\beta$ ,17 $\beta$ -diol (**8**) (DPJ-RG-1317) To a stirred suspension of 16 $\beta$ -(imidazol-1-yl)-17-oxo-5-androsten-3 $\beta$ -ol (**5**, 1 g, 2.82 mmol) in methanol (100 mL) at room temperature, sodium borohydride (1.5 g) was added in small fractions over a period of 2 h. The reaction mixture was further stirred for 4 h. Solvent was removed under reduced pressure and cold water was added. The precipitate obtained was filtered, washed with water, dried and crystallized from methanol to afford **8**. 74% yield, mp 285-287° C, IR (KBr): 3259.0, 2930.9, 1501.4, 1459.5, 1365.1, 1235.8, 1152.7, 1083.2 and 955.1 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  7.59 ppm (s, 1H, 2-CH, imidazole), 7.04 (s, 1H, 4-CH, imidazole), 6.97 (s, 1H, 5-CH, imidazole), 5.32 (d, 1H, *J* = 4.9 Hz, 6-CH), 4.59 (t, 1H, 16 $\alpha$ -H), 3.79 (d, 1H, 17 $\alpha$ -H), 3.43 (m, 1H, 3 $\alpha$ -H), 1.03 (s, 3H, 19-CH<sub>3</sub>), 0.89 (s, 3H, 18-CH<sub>3</sub>), Calcd for C<sub>22</sub>H<sub>32</sub>N<sub>2</sub>O<sub>2</sub>: C, 74.12; H, 9.05; N, 7.86. Found: C, 74.39; H, 9.32; N, 8.02.

*2.1.5. 16 $\beta$ -(Imidazol-1-yl)-3 $\beta$ -pyrrolidino-5-androsten-17 $\beta$ -ol (11) (DPJ-RG-1319).*

Pyrrolidine (1 mL) was added to a refluxing solution of 16 $\beta$ -(imidazol-1-yl)-4-androstene-3,17-dione (**6**, 1 g, 2.84 mmol) in methanol (20 mL). The reaction mixture was further refluxed for 1 h and chilled on ice. The crystalline material **10** obtained was filtered, washed with methanol and immediately used for further reaction.

To a stirred suspension of above obtained 16 $\beta$ -(imidazol-1-yl)-3-pyrrolidino-3,5-androstadiene-17-one (**10**) in methanol (100 mL) at room temperature, sodium borohydride (1 g) was added in small amounts over a period of 2 h at room temperature and stirring was further continued for 4 h. Solvent was removed under reduced pressure and iced water was added. The precipitate obtained was filtered, washed with water, dried and crystallized from acetone to furnish **11**. 41% yield, mp 265-267 ° C, IR (KBr): 3379.7, 2939.1, 1661.7, 1453.1, 1234.0 and 1085.6 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>+DMSO-*d*<sub>6</sub>):  $\delta$  7.52 ppm (s, 1H, 2-CH, imidazole), 7.06 (s, 1H, 4-CH, imidazole), 7.00 (s, 1H, 5-CH, imidazole), 5.37 (m, 1H, 6-CH), 4.62 (t, 1H, *J* = 9.1 Hz, 16 $\alpha$ -H), 3.81 (d, 1H, *J* = 9.26 Hz, 17 $\alpha$ -H), 2.77 (brs, 4H, -N-(CH<sub>2</sub>)<sub>2</sub>, pyrrolidine), 1.05 (s, 3H, 19-CH<sub>3</sub>), 0.89 (s, 3H, 18-CH<sub>3</sub>). Calcd for C<sub>26</sub>H<sub>39</sub>N<sub>3</sub>O: C, 76.24; H, 9.60; N, 10.26. Found: C, 76.59; H, 10.01; N, 10.51.

*2.1.6. General procedure for the synthesis of compounds 9 and 12*

A mixture of compound **8** or **11** (2.80 mmol), acetic anhydride (2 ml) and dry pyridine (2 ml) was heated in a steam bath for 2 h. The reaction contents were then poured into cold water and basified with liquid ammonia. The precipitate obtained was filtered, washed with water, dried and crystallized from acetone to afford **9** and **12**, respectively.

*2.1.6a. 16 $\beta$ -(Imidazol-1-yl)-5-androstene-3 $\beta$ ,17 $\beta$ -diol diacetate (9) (DPJ-RG-1318)*

64% yield, mp 197-199 °C, IR (KBr): 2937.5, 1732.9, 1494.1, 1435.5, 1371.8, 1250.1, 1072.9, 1037.7 and 906.0 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  7.48 ppm (s, 1H, 2-CH, imidazole), 7.02 (s, 1H, 4-CH, imidazole), 6.89 (s, 1H, 5-CH, imidazole), 5.39 (d, 1H, *J* = 4.89 Hz, 6-CH), 4.79 (m, 2H, 16 $\alpha$ -H and 17 $\alpha$ -H), 4.61 (m, 1H, 3 $\alpha$ -H), 2.04 (s, 3H, 3 $\beta$ -OCOCH<sub>3</sub>), 1.74 (s, 3H, 17 $\beta$ -OCOCH<sub>3</sub>), 1.07 (s, 3H, 19-CH<sub>3</sub>), 1.02 (s, 3H, 18-CH<sub>3</sub>). Calcd for C<sub>26</sub>H<sub>36</sub>N<sub>2</sub>O<sub>4</sub>: C, 70.88; H, 8.24; N, 6.36. Found: C, 70.59; H, 8.45; N, 6.49.

*2.1.6b. 16 $\beta$ -(Imidazol-1-yl)-3 $\beta$ -pyrrolidino-5-androsten-17 $\beta$ -yl acetate (12) (DPJ-RG-1320)*

45% yield, mp 220-222 °C, IR (KBr): 2937.9, 2791.6, 1735.9, 1491.9, 1455.5, 1372.9, 1239.3, 1139.4, 1073.8, 1037.9 and 897.1 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.42 ppm (s, 1H, 2-CH, imidazole), 7.00 (s, 1H, 4-CH, imidazole), 6.88 (s, 1H, 5-CH, imidazole), 5.34 (d, 1H, *J* = 4.42 Hz, 6-CH), 4.78 (m, 2H, 17 $\alpha$ -H and 16 $\alpha$ -H), 2.63 (brs, 4H, -N-(CH<sub>2</sub>)<sub>2</sub>, pyrrolidine), 1.73 (s, 3H, -OCOCH<sub>3</sub>), 1.05 (s, 3H, 19-CH<sub>3</sub>), 1.02 (s, 3H, 18-CH<sub>3</sub>). Calcd for C<sub>28</sub>H<sub>41</sub>N<sub>3</sub>O<sub>2</sub>: C, 74.46; H, 9.15; N, 9.30. Found: C, 74.71; H, 9.39; N, 9.11.

*2.1.7. General procedure for the synthesis of compounds 13 and 14*

Methyl iodide (2.0 ml) was added to a solution of compound **11** or **12** (0.15 g, mmol) in dichloromethane (20.0 ml). The reaction mixture was left at room temperature for 7 days and stirred intermittently. The solvent was removed under reduced pressure and the residue was treated with dry solvent ether to remove impurities. The solid obtained was dried and crystallized from dry diethylether in case of **13** and from a mixture of dry methanol-acetone for **14**.

2.1.7a. *16β-(1-Imidazolyl)-3β-pyrrolidino-5-androsten-17β-ol dimethiodide (13)*

90% yield, mp 270-272 ° C, IR (KBr): 3485, 2938, 1605, 1447, 1267 and 1085 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>-DMSO-*d*<sub>6</sub>): 9.19 ppm (s, 1H, 2-CH, imidazole), 7.58 (s, 1H, 5-CH imidazole), 7.66 (s, 1H, 4-CH imidazole), 5.52 (d, 1H, *J* = 4 Hz, 6-CH), 5.24 (d, 1H, *J* = 5 Hz, 17α-*H*), 4.83 (t, 1H, 16α-*H*, *J* = 9 Hz), 3.96 (s, 3H, N<sup>⊕</sup>-CH<sub>3</sub>, imidazole), 3.55 (m, 4H, N<sup>⊕</sup>-(CH<sub>2</sub>)<sub>2</sub>, pyrrolidine), 2.98 (s, 3H, N<sup>⊕</sup>-CH<sub>3</sub>, pyrrolidine), 1.08 (s, 3H, 19-CH<sub>3</sub>), 0.81 (s, 3H, 18-CH<sub>3</sub>). Calcd for C<sub>28</sub>H<sub>45</sub>N<sub>3</sub>OI<sub>2</sub>: C, 48.49; H, 6.54; N, 6.06. Found: C, 48.84; H, 6.40; N, 6.15.

2.1.7b. *16β-(1-Imidazolyl)-3β-pyrrolidino-5-androsten-17β-yl acetate dimethiodide (14)*

78% yield, mp 220-222 ° C, IR (KBr): 3442, 2942, 1732, 1624, 1241 and 1032 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>-DMSO-*d*<sub>6</sub>): 9.57 ppm (s, 1H, 2-CH, imidazole), 7.62 (s, 1H, 4-CH, imidazole), 7.47 (s, 1H, 5-CH, imidazole), 5.52 (d, 1H, 6-CH), 5.15 (t, 1H, 16α-*H*), 5.08 (d, 1H, 17α-*H*), 4.04 (s, 3H, N<sup>⊕</sup>-CH<sub>3</sub>, imidazole), 3.54 (s, 4H, N<sup>⊕</sup>-(CH<sub>2</sub>)<sub>2</sub>, pyrrolidine), 3.02 (s, 3H, N<sup>⊕</sup>-CH<sub>3</sub>, pyrrolidine), 1.89 (s, 3H, 17β-OCOCH<sub>3</sub>), 1.09 (s, 3H, 19-CH<sub>3</sub>), 0.98 (s, 3H, 18-CH<sub>3</sub>). Calcd for C<sub>30</sub>H<sub>47</sub>N<sub>3</sub>O<sub>2</sub>I<sub>2</sub>: C, 48.99; H, 6.44; N, 5.71. Found: C, 49.25; H, 6.48; N, 5.63.

## 2.2. Biological activity

### 2.2.1. Antineoplastic activity

The synthesized compounds were screened at National Cancer Institute, Bethesda, USA for *in vitro* antineoplastic activity.

#### 60-Cell line assay

The compounds **5**, **6**, **8**, **9**, **11** and **12** were selected by Drug Synthesis and Chemistry Branch, National Cancer Institute, based in general, on the basis of degree of novelty of the structure and computer modeling techniques for anticancer screening. These compounds were assayed *in vitro* against a panel consisting of 60 human tumor cell lines, derived from nine cancer types (leukemia, lung, colon, CNS, melanoma, ovarian, renal, prostate and breast cancers), using five concentrations at 10-fold dilutions, the highest being  $10^{-4}$  M. A 48 h continuous drug protocol was used and a sulforhodamine B (SRB) protein assay was used to estimate the cell viability or growth [23,24]. Mean log dose response parameters such as GI50 (drug concentration resulting in a 50% reduction in the net protein increase), TGI (drug concentration for total growth inhibition) and LC50 (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) were calculated. Two standard drugs, meaning that their activities against the cell lines are well documented, were tested against each cell line: NSC 19893 (5-Fluorouracil) and NSC 123127 (Adriamycin).

### *2.2.2. Aromatase inhibitory activity*

#### *2.2.2a Preparation of aromatase*

The enzyme was obtained from the microsomal fraction of freshly delivered human term placental tissue according to the procedure of Thompson and Siiteri [25]. The isolated microsomes were suspended in the minimum volume of phosphate buffer (0.05 M, pH 7.4, 20% glycerol). Additionally DTT (dithiothreitol, 10 mM) and EDTA (1 mM) were added to protect the enzyme from degradation. The enzyme preparation was stored at -70 °C as described. No loss of activity was observed within four months.

#### *2.2.2b. Inhibition of aromatase in vitro*

The assay was performed similar to the described methods [26-28] monitoring enzyme activity by measuring the  $^3\text{H}_2\text{O}$  formed from [ $1\beta\text{-}^3\text{H}$ ]androstenedione during aromatization. Each incubation tube contained 15 nM [ $1\beta\text{-}^3\text{H}$ ]androstenedione (0.08  $\mu\text{Ci}$ ), 485 nM unlabeled androstenedione, 2 mM NADP, 20 mM glucose-6-phosphate, 0.4 units of glucose-6-phosphate-dehydrogenase and inhibitor (in at least three different concentrations for determining the  $\text{IC}_{50}$  value) in phosphate buffer (0.05 M, pH 7.4). The test compounds were dissolved in DMSO and diluted with buffer. The final DMSO concentration in the control and inhibitor incubation was 2 %. Each tube was preincubated for 5 min at 30°C in a water bath. Microsomal protein was added to start the reaction (0.1 mg). The total volume for each incubation was 0.2 ml. The reaction was terminated by the addition of 200  $\mu\text{l}$  of a cold 1 mM  $\text{HgCl}_2$  solution. After addition of 200  $\mu\text{l}$  of an aqueous dextran-coated charcoal (DCC) suspension (2 %), the vials were shaken for 20 min and centrifuged at 1500 x g for 5 min to separate the charcoal-absorbed steroids. The supernatant was assayed for  $^3\text{H}_2\text{O}$  by counting in a scintillation mixture using a LKB-Wallac  $\beta$ -counter. The calculation of the  $\text{IC}_{50}$  values was performed by plotting the percent inhibition versus the concentration of inhibitor on a semi-log plot. From this the molar concentration causing 50% inhibition was calculated.

#### *2.2.2c. Irreversible inhibition of aromatase*

The assay was performed similar to the test procedure described above. A preincubation of the aromatase containing microsomes was performed along with a regenerating system (2 mM NADP, 20 mM glucose-6-phosphate, 0.4 units of glucose-6-phosphate-dehydrogenase) and inhibitor in phosphate buffer (0.05 M, pH 7.4) for 30 min at 30 °C. The test compounds were dissolved in DMSO and diluted with buffer. The final DMSO concentration in the control and inhibitor incubation was 2

%). After preincubation an aqueous dextran coated charcoal (DCC) suspension (2 %) (Sigma, St. Louis, MO) was added followed by a shaking step for 20 min at 4 °C. After full-speed centrifugation 250 µl of the supernatant were supplemented with 50 µl of regenerating system and 50 µl substrate (30 nM [ $1\beta$ - $^3\text{H}$ ]androstenedione (0.2 µCi), 185 nM unlabeled androstenedione) to start the enzymatic reaction at 30 °C. After several time points (8, 16, 24 min) 100 µl of the sample were stopped by the addition of 100 µl of a cold 1 mM HgCl<sub>2</sub> solution. After addition of 100 µl of Norit A (2 %) (Serva, Heidelberg, Germany), the vials were shaken for 20 min and centrifuged at 1500 x g for 5 min to separate the charcoal-absorbed steroids. The supernatant was assayed for  $^3\text{H}_2\text{O}$  by counting in a scintillation mixture using a  $\beta$ counter. Exemestane was used as a positive control that irreversibly binds to aromatase, as a negative control (not binding irreversibly) aminoglutethimide was used. The inhibition values after the three different incubation times were related to the DMSO control. The compounds well as the two reference compounds aminoglutethimide and exemestane were tested for irreversible inhibition of aromatase in a concentration around their IC<sub>50</sub> values.

### 2.2.3. Neuromuscular blocking activity

Quaternary ammonium steroids **13** and **14** were screened for *in vitro* neuromuscular blocking activity using paralysis in chicks [29], Rabbit head drop method U.S.P.1975 [30] and isolated frog rectus abdominis muscle preparation [31].

#### 2.2.3a. Paralysis in chicks

Paralysis in chicks was carried out as per the procedure described by Buttle and Zaimis [29]. Female layer chicks weighing between 20-25 gm were divided into 8 groups. Each group consisted of six chicks. Quaternary ammonium steroid **13** and **14** and d-

tubocurarine were administered at various doses intravenously in neck vein. The onset and duration of action of action was recorded. The end point was considered when the chick was unable to stand on its legs and showed a typical contracture with flaccid paralysis. The mean onset and duration of action against dose of drug with mean standard deviation was calculated.

### *2.2.3b. Rabbit head drop method*

Rabbit head drop method was carried out as per USP-1975 [30]. New Zealand white rabbits weighing between 1.5-3 kg and of either sex were used. The rabbits were divided in two equal groups of at least four rabbits each. They were suitably restraint in prone position with the head free, taking precautions to prevent struggling. The standard drug was filled in a small bore burette which was joined with flexible polythene tube to a hypodermic needle (21 gauge) inserted in the marginal ear vein. The drug solution was administered at the rate of 0.1 ml every 15 seconds into each rabbit until head drop occurred. The end point was complete when rabbit was unable to raise and hold its head in response to a light tap on the back. The number of doses injected was recorded. The procedure was repeated in rabbits of both the groups. On the following day crossover was completed. Potency and confidence interval were calculated according to the method described in USP-1975

### *2.2.3c. Effect on isolated frog rectus abdominis muscle preparation*

Frog rectus abdominis muscle preparation was carried out as described [31]. Standard procedure for setting up isolated tissue was used. Responses to various concentrations of acetylcholine were recorded. Frog ringer solution present in the reservoir was replaced by

modified frog ringer solution containing test drug and responses to various concentrations to acetylcholine were recorded. Concentrations of acetylcholine were increased four times to study the nature of antagonism. Mean height of contractions was measured and a log dose response curve was plotted.

#### *2.2.4. Anticholinesterase activity*

The compound **13** was also tested for *in vitro* AChE inhibitory activity for its ability to inhibit the activity of electric eel acetylcholinesterase by modified Ellman method [32].

### **3. Results and discussion**

#### *3.1. Chemistry*

The synthetic routes to the preparation of various new steroidal derivatives have been outlined in schemes 1 and 2. Bromination of **1** was carried out using cupric bromide in dry methanol and dry benzene to afford 16 $\alpha$ -bromo-17-oxo-androsten-3 $\beta$ -ol (**2**) [22]. The configuration at position 16 has been assigned alpha in accordance with the earlier reports, which states that 16 $\beta$ - and 16 $\alpha$ -protons in 16-bromosteroids resonates at  $\delta$  4.46 and 4.14 ppm, respectively [22,33]. The proton NMR spectrum of compound **2** exhibited a triplet at  $\delta$  4.55 ppm (16 $\beta$ -H). Oppenauer oxidation of **2** using aluminium isopropoxide-cyclohexanone-toluene system resulted into formation of a mixture of 16 $\alpha$ -bromo (16 $\beta$ -H at 4.56, 40%) and 16 $\beta$ -bromo (16 $\alpha$ -H at 4.12, 60%) isomers of  $\alpha,\beta$ -unsaturated ketosteroid compound **3**. Such epimerization of 16 $\alpha$ -bromo-17-oxosteroids to the 16 $\beta$ -isomer in alkaline medium has previously been reported [33]. To allow irreversible binding of steroid to the aromatase enzyme, double bond was inserted between C<sub>1</sub> and C<sub>2</sub>

by oxidation of **3** using 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) in refluxing dry dioxane. This afforded a mixture of 16 $\alpha$ -bromoandrost-1,4-diene-3,17-dione (**4a**, 30%) and 16 $\beta$ -bromoandrost-1,4-diene-3,17-dione (**4b**, 70 %), which were separated using fractional crystallization. A little variability in the ratio of 16 $\alpha$ - and 16 $\beta$ -bromo isomers in compound **4** in comparison to compound **3** may be attributed to the crystallization process. The synthesis of **4** could also be achieved by direct DDQ oxidation of 3-hydroxy derivative **2**, but it took 4 days to complete the reaction for the formation of **4**. The target 16-imidazolyl substituted steroids **5-7** were obtained by thermal fusion of corresponding 16-bromosteroids **2-4** with powdered imidazole. Only 16 $\beta$ -imidazolyl derivative could be isolated by crystallization. The configuration of imidazolyl moiety at C<sub>16</sub> has been assigned  $\beta$  as is evident from <sup>1</sup>H NMR spectral data.

Repeated efforts to carry out Oppenauer oxidation of 3-hydroxy-5-ene derivative **5** for the synthesis of imidazolyl substituted 3-keto-4-ene steroid **6**, as well as DDQ reduction of 3-keto-4-ene steroid **6** to prepare target compound **7** remained unsuccessful. Therefore synthetic route, where fusion with imidazole is the last step, was adopted for the synthesis of target compounds as shown in scheme 1. To improve the yield, microwave assisted synthesis [34] was also performed for synthesizing highly potent aromatase inhibitor **7** by fusion of bromo product **4** with imidazole. Although this method resulted in reduced reaction time from 30 min (conventional) to 15 min (microwave technique) but no significant change in yield was observed.

To study structure activity relationship in this particular series of compounds, further modifications of steroidal nucleus were carried out. Treatment of 16 $\beta$ -imidazolyl steroid **5** with sodium borohydride in methanol at room temperature afforded 3 $\beta$ ,17 $\beta$ -diol

**8**, which upon subsequent acetylation with acetic anhydride and dry pyridine in a steam bath yielded 3 $\beta$ ,17 $\beta$ -diacetoxy derivative **9**.

To create two bistertiary nitrogens with a central steroid nucleus, 16 $\beta$ -(imidazol-1-yl)-4-androstene-3,17-dione (**6**) was heated under reflux with freshly distilled pyrrolidine in methanol to furnish the unstable dienamine **10**, which upon immediate reduction with sodium borohydride in methanol at room temperature afforded 3 $\beta$ -pyrrolidinyl derivative **11** as shown in scheme 2. Further acetylation of **11** using acetic anhydride and dry pyridine in a steam bath yielded 16 $\beta$ -(imidazol-1-yl)-3 $\beta$ -pyrrolidino-5-androsten-17 $\beta$ -yl acetate (**12**).

Bisquaternary ammonium steroids **13** and **14** were prepared by treating the bistertiary amines **11** and **12** with methyl iodide in dichloromethane at room temperature. 2-CH of imidazole resonated characteristically downfield at  $\delta$  9.91 ppm indicating the quaternization of imidazolyl nitrogen. <sup>1</sup>H-NMR signals for protons of quaternary pyrrolidino methyl at  $\delta$  ~2.98 and quaternary imidazolyl methyl at 4 ppm were observed. The interonium distances of the quaternary compounds were calculated using drying models, which indicated a value of 12.5 Å.

### 3.2. Biological activity

Newly synthesized 16-imidazolyl substituted steroidal derivatives **5**, **6**, **8**, **9**, **11** and **12**, selected for screening by NCI, exhibited antiproliferative effects in sixty cancer cell lines. Mean inhibitory concentrations ranging from 10-100  $\mu$ M were observed for various response parameters such as GI50, TGI and LC50 in the 60-cell line assay of various compounds. In general, it was observed that 16-azolyl group in steroids represents a reasonable pharmacophore for cytotoxic activity. Introduction of a pyrrolidine group at 3

position of 16-azolyl steroid skeleton did not bring any significant change in cytotoxicity profile of the compounds.

The aromatase inhibitory activity data of the newly synthesized steroids is presented in table 1. Of these compounds, 16 $\beta$ -(imidazol-1-yl)-4-androstene-3,17-dione (**6**) ( $IC_{50} = 0.180 \mu M$ ) and 16 $\beta$ -(imidazol-1-yl)-androsta-1,4-diene-3,17-dione (**7**) ( $IC_{50} = 0.168 \mu M$ ) exhibited strong inhibition of the enzyme.

16-Bromo compounds **3** ( $IC_{50} = 2.65 \mu M$ ) and **4** ( $IC_{50} = 13.2 \mu M$ ) exhibited moderate inhibition of the enzyme. 17-keto-3-hydroxy imidazolyl substituted derivative **5** with  $IC_{50} = 3.3 \mu M$  displayed substantial binding with aromatase enzyme as compared to dihydroxy **8** and diacetoxy steroid **9**, which produced only 46% inhibition at 36  $\mu M$  indicating the importance of oxidation in D ring for enzyme binding. 16-Imidazolyl steroids **6** and **7** were found to be approximately 160 and 170 times more potent in comparison to aminoglutethimide and 1.2 and 1.4 times more potent in assessment to standard drug exemestane ( $IC_{50} = 230 \text{ nM}$ ), respectively. The exchange of the bromine by an imidazole ring resulted into potent inhibition of the aromatase enzyme. Substitution of pyrrolidine at 3 position in compound **11** resulted in loss of binding affinity for the enzyme, however introduction of 17-acetoxy as in **12** improved the activity profile. It is assumed that the observed variation in aromatase inhibitory profile of these derivatives might have resulted due to changes in three dimensional attachments of the compounds with the enzyme. As is evident from irreversible binding studies (table 2), steroidal derivatives except bromo substituted 1,4-diene **4** are acting as competitive inhibitors that compete with substrate androstenedione for noncovalent binding to the active site of the enzyme. It is also anticipated that imidazole group containing steroids interfere with

steroid hydroxylations by the binding of the sterically available N with the heme Fe (III) iron of cytochrome P<sub>450</sub>. Of all only compound **4** is behaving as an irreversible inhibitor although to a lesser extent in comparison to exemestane (table 2). Despite the structural similarity of compounds **4**, **7** and exemestane with respect to ring A, it seems that the nitrogen of the imidazolyl-substituted compound **7** is able to complex the heme-iron of the enzyme leading to a different binding mode than the bromo-substituted compound **4**, which is probably binding like exemestane. The present findings are potentially useful for understanding the spatial and electronic nature of the binding site of aromatase as well as for developing effective steroidal aromatase inhibitors.

Both the bisquaternary steroidal salts **13** and **14**, when given intravenously produced flaccid paralysis in chicks. The mean onset of action for **13** at 0.125 mg/kg was 5.55 min and mean duration of action was 29.52 min. 17-Acetoxy bisquaternary steroid **14** exhibited quicker onset of action of 2.20 min and a longer duration of action of 42 min at the same dose. The minimum lethal dose observed for **13** and **14** was 0.5 mg/kg. d-Tubocurarine at a dose of 1 mg/kg exhibited flaccid paralysis. The mean onset of action of action for d-tubocurarine (2 mg/kg) was 2.33 min and mean duration of action was 40 min. The minimum lethal dose observed for d-tubocurarine was 2 mg/kg.

Rabbit head drop method has been used to demonstrate the potency of neuromuscular blocking agents. This method has been accepted by the USP-1975 and the method for calculation of potency and confidence interval should not exceed 0.08. Administration of **13** and **14** produced complete head drop. The potency of **13** was found to be 41.25 times than that of d-tubocurarine and confidence interval was found to be 0.600 which lies well within the limits of USP-1975 specification. The potency of **14** was

found to be 149.26 times than that of d-tubocurarine and confidence interval was found to be 0.054, which lies well within the limits of USP-1975 specification.

In case of frog rectus abdominis muscle preparation, acetylcholine at 1.7, 3.4, 6.8 and 13.6  $\mu\text{M}$  produced concentration dependent contractions. Administration of both **13** and **14** resulted in relaxation of muscle. Responses of the muscle to acetylcholine were reduced in presence of **13** (0.68  $\mu\text{M}$ ) and **14** (0.0047  $\mu\text{M}$ ) as shown in table 3. Parallel shift to the right indicated competitive antagonism as depicted in fig 3. From the shift of acetylcholine dose response curves, the affinity constant of the compounds are calculated as 0.14  $\mu\text{M}$  for **13** and 0.007  $\mu\text{M}$  for 17-acetoxy bisquaternary salt **14**. Compound **13** is not only acting as an antagonist at acetylcholine receptors but is also inhibiting acetylcholinesterase activity at low concentrations. When subjected to the anticholinesterase assay, a high inhibitory activity ( $K_i = 25 \text{ nM}$ ), which was approximately 115-fold more in comparison to galanthamine ( $K_i = 2.9 \mu\text{M}$ ) was revealed. Thus, there are two functionally opposite effects: reduction of neuromuscular transmission because of the block of acetylcholine receptors and augmentation of transmission because of the reduced metabolism of the released acetylcholine. In contrast, clinically used NMJ blockers pancuronium ( $K_i = 7.4$ ) and pipecuronium ( $K_i = 5.9$ ), displayed anticholinesterase activity at much higher concentrations. The bisquaternary steroids **13** and **14** with a fixed interonium distance of 12.5 Å are displaying very high NMJ blocking activity in comparison to our previously reported [19] NMJ blockers possessing a degree of variation in interonium distance ranging from 11-17 Å. However exhibition of anticholinesterase activity in such low concentrations by these compounds discouraged us from further exploration of the current steroidal salts as NMJ blockers inspite of highly potent skeletal muscle relaxant properties. Still they may

be considered as good candidates as anticholinesterases. These may particularly be useful for the treatment of Alzheimer's disease (AD), which is the commonest form of degenerative dementia. Current treatments of AD provide symptomatic benefits but have not been shown to alter the underlying progression of the disease. With enhanced lipophilicity due to steroidal skeleton, higher penetration through the blood brain barrier and consequently better therapeutic effects may be expected [35].

#### **4. Conclusions**

The new steroidal analogues exhibited moderate cytotoxic effects in sixty cancer cell lines derived from nine cancers types. Introduction of an imidazole ring at 16-position along with increased unsaturation in ring-A of steroid nucleus improved the aromatase inhibitory activity in 3,17-diketo steroids **5** and **6**. A fixed interonium distance of around 12.5 Å in 3,17-amino substituted bis-quaternary steroids resulted in potent skeletal muscle relaxant properties, although higher anticholinesterase activity was also observed at lower concentrations.

It is concluded that 16-imidazolyl steroids represent an imperative pharmacophore for the development of medicinally active molecules. Suitable structural exploration of the steroid skeleton might lead to formation of molecules with potent cytotoxic, anti-aromatase, NMJ-blocking and acetylcholinesterase inhibitory properties. The present series represents a new class of steroidal derivatives of potential medicinal significance.

#### **Acknowledgements**

The authors are thankful to Department of Science and Technology and Council for Scientific and Industrial Research, India for providing financial assistance. The authors

express appreciation to National Cancer Institute, Bethesda, Maryland, USA for evaluation of compounds for antineoplastic activity. The generous supply of steroids by Cipla Ltd., India is gratefully acknowledged.

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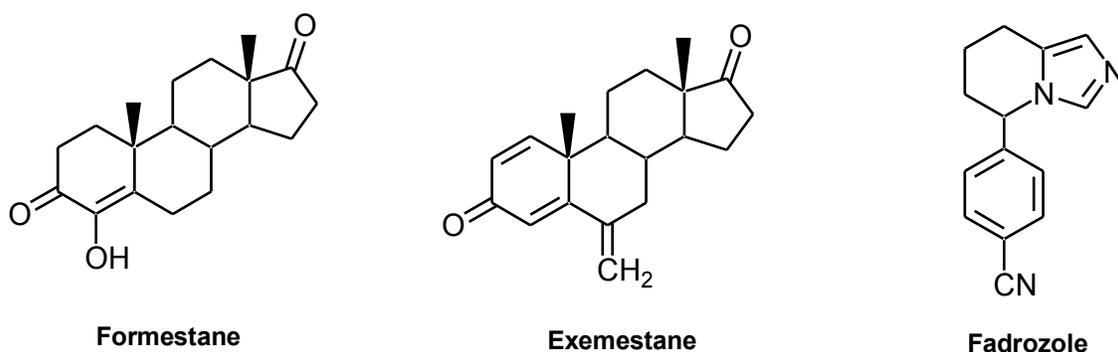


Fig 1. Structures of steroidal and non-steroidal aromatase inhibitors used in the clinical practice

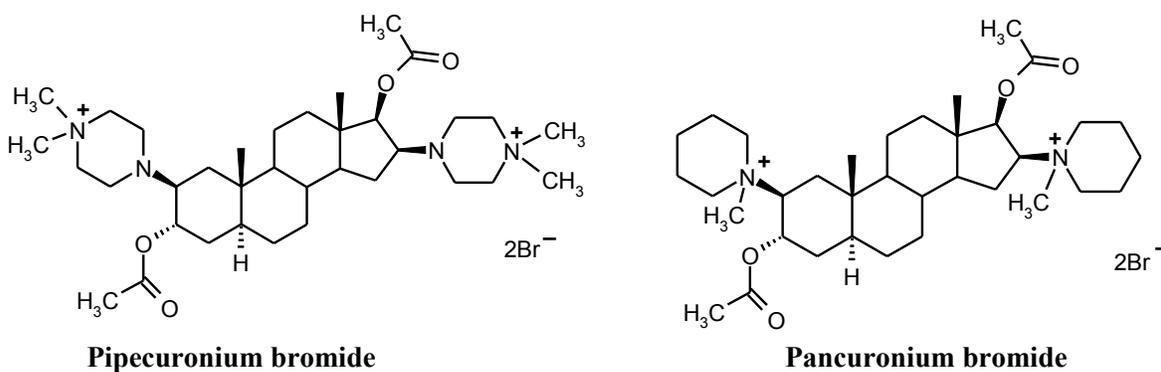
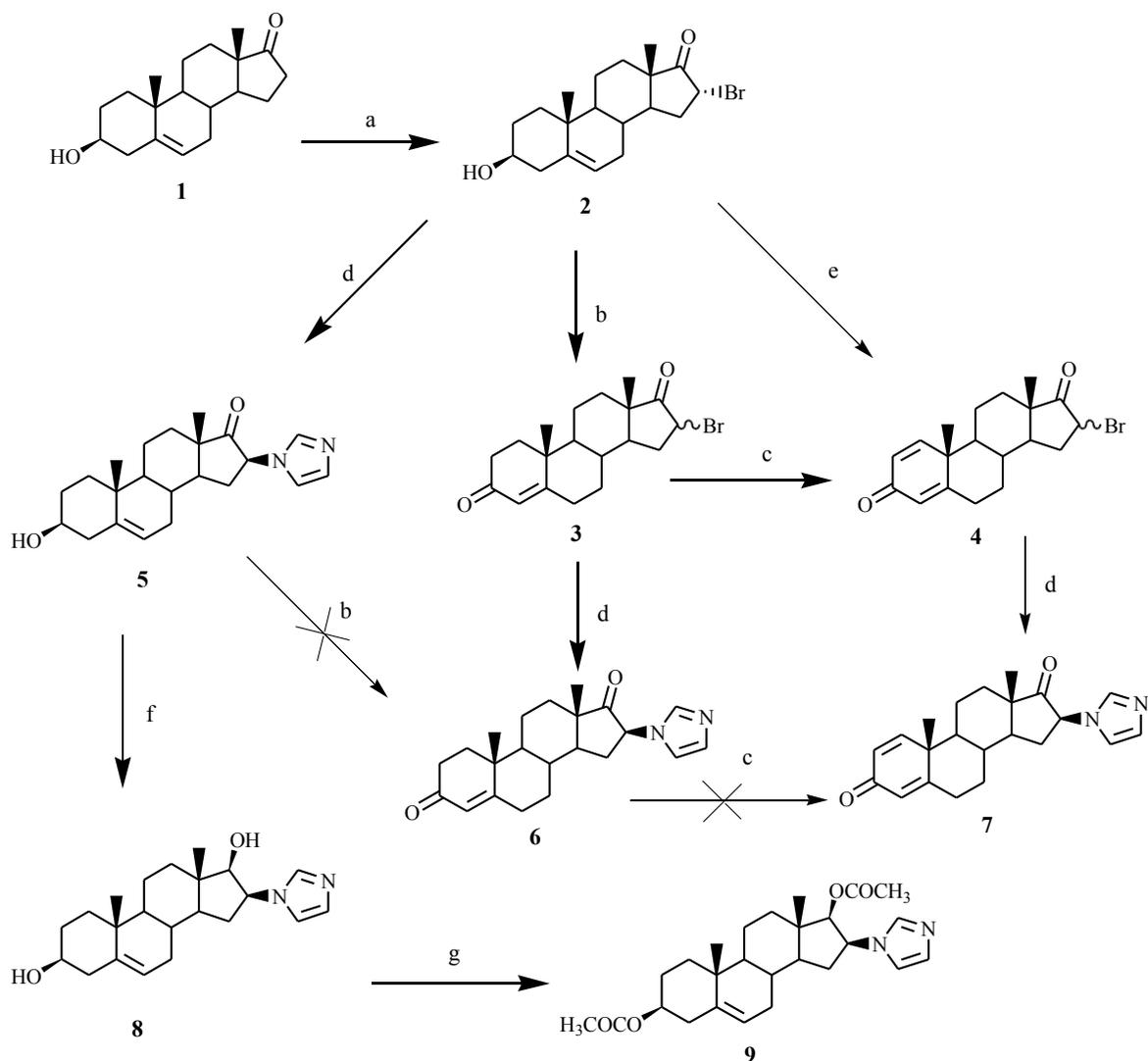


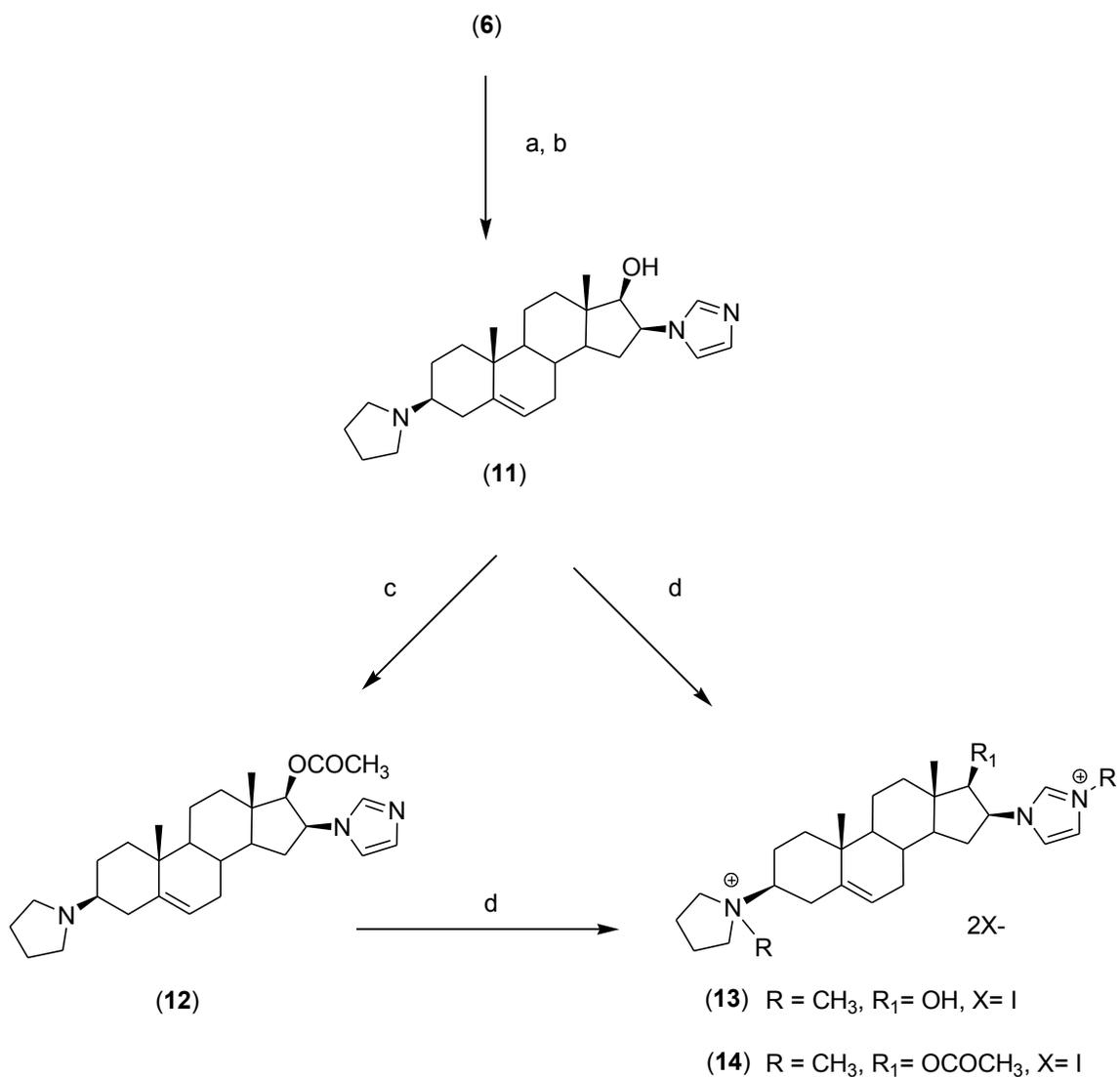
Fig 2. Structures of neuromuscular junction blockers used in the clinical practice

**Scheme 1.** Synthesis of compounds **2-9**



Reagents and conditions: (a) Cupric bromide, Dry methanol and Dry benzene; (b) Cyclohexanone, Toluene, Aluminum isopropoxide; (c) DDQ, Dioxane; 50 h; (d) Thermal fusion, imidazole; (e) DDQ, Dioxane; 4 days; (f) NaBH<sub>4</sub>/Methanol; (g) Acetic anhydride in dry pyridine

**Scheme 2.** Synthesis of compounds 10-14



Reagents and conditions: (a) Pyrrolidine/methanol; (b) NaBH<sub>4</sub> /methanol; (c) Acetic anhydride in dry pyridine; (d) CH<sub>3</sub>I, Dichloromethane

Table 1. Aromatase inhibitory data of various compounds

S. No.	COMPOUND	Inhibition on CYP 19 <sup>a</sup> IC <sub>50</sub> (μM)	RP <sup>b</sup>	RP <sup>c</sup>

1	3	2.65	11	0.09
2	4	13.20	2	0.02
3	5	3.30	9	0.07
4	6 (DPJ-RG-1241)	0.18	160	1.27
5	7 (RB-401)	0.16	170	1.43
6	8	no inhibition at 36 $\mu$ M		
7	9	53% inhibition at 36 $\mu$ M		
8	11	40 % inhibition at 36 $\mu$ M		
9	12	4.90	6	0.05

<sup>a</sup> [1 $\beta$ -<sup>3</sup>H ] androstenedione

<sup>b</sup> Relative Potency = Relative to aminoglutethimide (RP = 1; IC<sub>50</sub> = 28.5  $\mu$ M).

<sup>c</sup> Relative Potency = Relative to exemestane (RP = 1; IC<sub>50</sub> = 0.23  $\mu$ M).

**Table 2.** Irreversible aromatase inhibition of various compounds

S. No.	COMPOUND	Inhibition of aromatase after incubation for irreversible binding <sup>a</sup>	
		Inhibitor	% inhibition

		concentration [ $\mu\text{M}$ ]	
<b>1</b>	<b>3</b>	2.5	n. i. <sup>b</sup>
<b>2</b>	<b>4</b>	13	27.6 $\pm$ 3.6
<b>3</b>	<b>5</b>	3	n. i. <sup>b</sup>
<b>4</b>	<b>6 (DPJ-RG-1241)</b>	0.2	n. i. <sup>b</sup>
<b>5</b>	<b>7 (RB-401)</b>	0.2	n. i. <sup>b</sup>
	Exemestane	0.2	45.9 $\pm$ 6.8
	Aminoglutethimide	30	n. i. <sup>b</sup>

<sup>a</sup>[1 $\beta$ -<sup>3</sup>H] androstenedione

<sup>b</sup>n. i.: inhibition  $\leq$  10 %

**Table 3.** NMJ blocking activity of compounds **13** and **14** on frog rectus abdominis muscle preparation

Acetylcholine	Response mm $\pm$ S.D	
Dose ( $\mu\text{M}$ )	Normal tissue response	In presence of compound <b>13</b> (0.68 $\mu\text{M}$ )
1.7	13 $\pm$ 4.92	00 $\pm$ 0.00

3.4	$15 \pm 5.17$	$01 \pm 1.2$
6.8	$24 \pm 7.80$	$04 \pm 3.71$
13.6	$32 \pm 9.31$	$13 \pm 4.79$
27.2		$24 \pm 3.91$
	Normal tissue response	In presence of compound <b>14</b> ( $0.0047 \mu\text{M}$ )
1.7	$09 \pm 0.94$	$05 \pm 2.16$
3.4	$16 \pm 3.29$	$12 \pm 33.29$
6.8	$29 \pm 3.74$	$22 \pm 4.784$
13.6	$41 \pm 6.16$	$36 \pm 6.54$

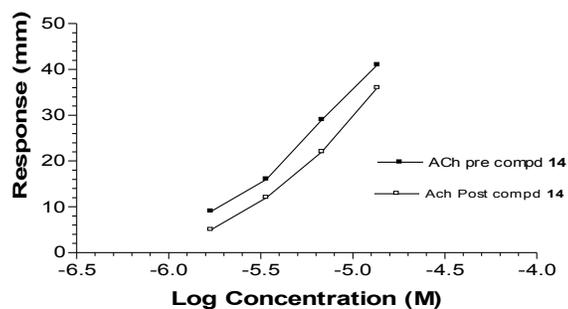
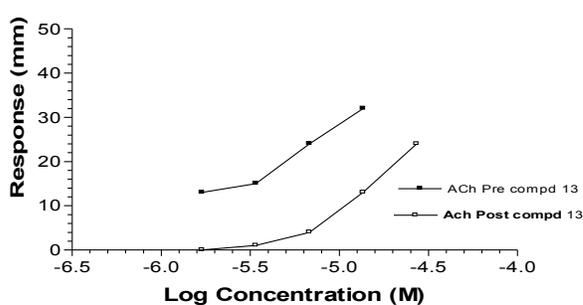


Fig 3. Effects of bisquaternary steroids **13** and **14** on isolated frog rectus abdominis muscle preparation