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Substrate interaction with 5α-reductase enzyme: influence of the 17β-chain chirality in the mechanism of action of 4-azasteroid inhibitors

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Abstract

A series of steroidal compounds were synthesized in order to evaluate the possible influence of the configuration of a stereocenter in the 17β-side chain on the inhibitory activity on the enzyme 5α-reductase (5AR). For this purpose diastereomerically pure 4-azasteroids epimers at C-22 were prepared (compounds **1–11**) and tested as inhibitors of 5AR in 'in vitro' tests. The obtained data showed that in most cases the couples of epimers possess a significant difference in their biological activity. We also considered, for the tested molecules, a series of chemico-physical parameters in order to find a possible correlation with their biological activity. The findings allowed us to propose a model of the binding site of 5AR which comprises also, for 4-azasteroid inhibitors, the configurational aspect of the 17β-side chain.

Keywords: 4-Azasteroids; Testosterone 5α reductase; Inhibitors; Molecular modeling; Chirality; Biological activity

1. Introduction

The study of selective inhibitors of 5α-reductase (5AR), the enzyme which catalyses the NADPH-dependent reduction of testosterone to dihydrotestosterone (DHT) [1], is at present the focus of growing interest [2–4]. These molecules show therapeutic potential in the treatment of several diseases, whose pathogenic mechanism seems to be an abnormal formation of dihydrotestosterone (as probably occurs in benign prostatic hyperplasia, acne, hyrsutism and male pattern baldness) [5–7]. The recently discovered existence of two different genes encoding for two 5AR isoenzymes, types 1 and 2 [8], with different biochemical characteristics and tissue distribution [9], gave a further impulse to this research [10–12], since the possibility to discriminate between the two enzymes could allow the use of highly selective drugs with minimal side effects. The primary structure of these enzymes was determined after isolation and cloning of their cDNA's, but only indirect information is available on the three-dimensional structure of their binding sites. Data obtained from structure-activity relationship studies with several synthetic derivatives showing variable inhibitory activity on 5AR [13–14] are the only source of information so far. Further structure-activity data would provide considerable help to the drug designer. Several studies [2–7] are available on the relationship between the structure and the inhibitory activity of a class of steroidal compounds, the 4-azasteroids. From these studies it appears that, while the chemical nature and size of the functional groups in rings A and B were extensively analyzed (even if not completely defined), the effect of the size and the configuration of the 17β side chain on the activity of 4-azasteroids has not yet been clarified. In fact, the binding site of 5AR seems able to accept both 'small' and 'large' substituents to position 17β. In particular, groups of high steric hindrance, like adamantane [15] or the long aliphatic chain of cholesterol [16], can be inserted without loss of the inhibitory activity. We therefore decided to study the inhibitory effect of 4-azasteroids differently substituted at position 17β. We have focused our attention on amides of

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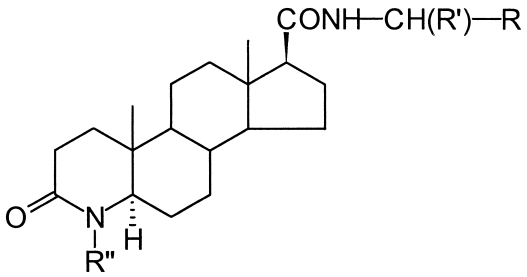


1.R=$PhCH_2$-;R'=$CONHCH_3$;R"=CH_3;(S)
2.R=$PhCH_2$-;R'=$CONHCH_3$;R"=CH_3;(R)
3.R=COOEt ;R'=$CH_2CH(CH_3)_2$;R"=CH_3;(R)
4.R=COOEt ;R'=$CH_2CH(CH_3)_2$;R"=CH_3;(S)
5.R=$PhCH_2$-;R'=$COOCH_3$;R"=H;(S)
6.R=$PhCH_2$-;R'=$COOCH_3$; R"=H;(R)
7.R=$CH_2CH(CH_3)_2$;R'=$CONHCH_3$;R"=CH_3;(R)
8.R=$CH_2CH(CH_3)_2$;R'=$CONHCH_3$;R"=CH_3;(S)
9.R =COOH; R' = R" = H
10.R=Ph;R' = R" = CH_3;(R)
11.R=Ph;R' = R" = CH_3;(S)

Fig. 1.

17β-carboxylic acid with different aminoacids (**1–9** in Fig. 1) and R- and S-phenyl ethyl amine (compounds **10–11** in Fig. 1). The above compounds show the common feature of possessing a stereogenic center in the alpha position (conventionally named C-22) to the 17β-carboxamide group. The presence of a stereogenic center in the substituents provides the opportunity of studying the effect of the configuration at this position on the activity of the drug. Literature data report no examples of such chiral discrimination: in fact even if a molecule with a chiral center at C-22 [17] has been described, only the mixture of two epimers was biologically tested.

2. Experimental

2.1. Materials and methods

Solvents and reagents were purchased from Fluka (Switzerland). Melting points were determined with a Büchi apparatus (model 535) and are uncorrected. ^{1}H NMR spectra were obtained in $CDCl_3$ on a Bruker AM-500 spectrometer at 303 K or, when indicated, on a Varian 360 L spectrometer with tetramethylsilane as internal standard. ^{1}H NMR spectra (500 MHz) were also used to check the diastereomeric purity of obtained products. IR spectra were recorded in chloroform solution on a Perkin Elmer 1600 Series FTIR instrument. MS spectra were recorded on a Hewlett-Packard 5988A instrument. Analytical TLC were performed on silica gel Merck 60 F254 plates and column chromatographies were carried out on silica gel Merck 60 (230–400 mesh). The MOLGEN-3 software [18] has been used for both the optimization of the molecular structures and the evaluation of physicochemical properties.

*2.2. Preparation of compounds **1–16***

Compounds **1–6, 9–11** were prepared by reaction of the thiopyridyl ester [3] **12** with the required amino derivatives, as reported in Scheme 1. Compounds **7** and **8** were prepared according to Scheme 2 starting from the carboxy-derivative **13**: intermediate **14** was prepared by reaction of acyl chloride derivative (obtained from **13** by reaction with oxalyl chloride) and the ethyl ester of the required R- or S-aminoacid. The epimers **14** were transformed in the corresponding secosteroids **15** by the use of $KMnO_4/NaIO_4$ [2]. The next step, the reaction with methylamine, leads to the lactamization of the A ring and a complete conversion of the ester moiety to methylamide. The unsaturated azasteroids **16** were then hydrogenated to obtain the desired compounds **7** and **8**.

*2.3. N-[(S)-phenylalanyl methyl amide]-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide (**1**)*

A suspension of S-phenylalanine hydrochloride (1.46 g, 7.282 mmol) in a mixture of dry THF (26 ml) and pyridine (0.6 ml) was vigorously stirred at room temperature. After 10 min (2-pyridyl)-3-oxo-4-aza-5α-androstane-17β-thiocarboxylate (**12**) (499 mg, 1.214 mmol) was added. The reaction mixture was maintained under stirring at room temperature for 18 h. After this period CH_2Cl_2 (5 ml) was added and the reaction was maintained for an additional 18 h under stirring at room temperature. The reaction was then heated for 4 h and the solvents evaporated under vacuum. The residue was dissolved in CH_2Cl_2, washed with an aqueous saturated $NaHCO_3$ solution, dried with Na_2SO_4, filtered and evaporated under vacuum to give a crude which was purified by column chromatography (basic aluminium oxide, CH_2Cl_2/CH_3OH = 98:2 v/v as eluant) affording pure **1** (0.55 g, 94%). For analytical purposes the product was crystallized from ethyl acetate: mp 225°C**;** ^{1}H NMR δ 0.60 (3 H, s, *CH*$_3$-18), 0.87 (3 H, s, *CH*$_3$-19), 2.44 (2 H, m, H-2), 2.69 (3 H, d, J = 4.91 Hz, C*H*$_3$-NH), 2.92 (3 H, s, C*H*$_3$-4), 2.97–3.05 (3 H, overlapped, H-5 and C*H*$_2$Ph), 4.57 (1 H, m, C*H*-NH), 5.62 (1 H, broad q, N*H*-CH_3), 5.98 (1 H, d, J = 7.7 Hz, N*H*-CH), 7.18–7.32 (5 H, m, arom); IR 1657, 1622, 1496 cm^{-1}; MS *m/z* 493 (M^+), 435, 332, 316, 288. Elemental analysis calculated for $C_{30}H_{43}O_3N_3$: theoretical C = 72.99%, H = 8.78%, *N* = 8.51%; found C = 72.97%, H = 8.76%, *N* = 8.50%.

The following amino acid derivatives were prepared with the same procedure: *N*-[(R)-phenylalanyl methyl amide]-

4-methyl-3-oxo-4-aza-5α-androstane-17-carboxamide (**2**): mp 139–141°C; ^{1}H NMR δ 0.47 (3 H, s, H-18), 0.86 (3 H, s, H-19), 2.44 (2 H, m, H-2), 2.71 (3 H, d, *J* = 4.9 Hz, C*H*$_3$-NH), 2.91 (3 H, s, C*H*$_3$-4), 3.01 (1 H, dd, *J* = 3.5, 12.6 Hz, H-5), 3.05 (2 H, d, *J* = 7.4 Hz, C*H*$_2$Ph), 4.68 (1 H, m, C*H*-NH), 5.93 (1 H, d, *J* = 7.7 Hz, N*H*-CH), 6.03 (1 H, broad q, N*H*-CH$_3$), 7.19–7.30 (5 H, arom); IR 1657, 1622, 1497 cm^{-1}; MS *m/z* 493 (M$^+$), 435, 332, 316, 288. Elemental analysis calculated for $C_{30}H_{43}O_3N_3$: theoretical C = 72.99%, H = 8.78%, *N* = 8.51%; found C = 73.01%, H = 8.75%, *N* = 8.47%.

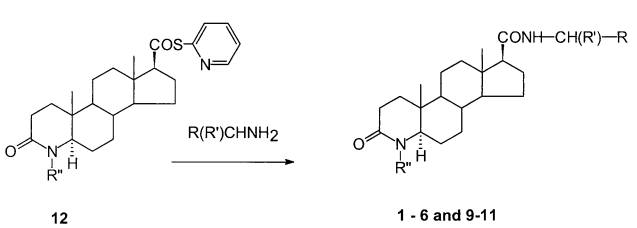
N-[(R)-leucyl ethyl ester]-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide (**3**) ^{1}H NMR δ 0.70 (3 H, s, H-18), 0.89 (3 H, s, H-19), 0.96 (3 H, d, *J* = *7* Hz, C*H*$_3$CH), 0.95 (3 H, d, *J* = *6* Hz, C*H*$_3$CH*)*, 1.27 (3 H, t, *J* = 7 Hz, C*H*$_3$CH$_2$), 2.40 (2 H, m, H-2), 2.89 (3 H, s, 4-C*H*$_3$), 3.0 (1 H, dd, *J* = 3.5, 12.6 Hz, H-5), 4.20 (2 H, q, *J* = 7 Hz, CH$_3$C*H*$_2$O), 4.65 (1 H, m, C*H*-NH), 5.67 (1 H, d, *J* = 8 Hz, N*H*-CH); IR 1732, 1672, 1622, 1503.5 cm^{-1}; MS *m/z* 474, 459, 429, 418, 401, 316, 288. Elemental analysis calculated for $C_{28}H_{46}O_4N_2$: theoretical C = 70.05%, H = 9.77%, *N* = 5.90%; found C = 70.01%, H = 9.75%, *N* = 5.84%. *N*-[(S)-leucyl ethyl ester] 4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide (**4**) mp 161–162°C; ^{1}H NMR δ 0.68 (3 H, s, H-18), 0.88 (3 H, s, H-19), 0.94 (3 H, d, *J* = 6.0 Hz, C*H*$_3$CH), 0.95 (3 H, d, *J* = 6.0 Hz, C*H*$_3$CH), 1.27 (3 H, t, *J* = 7.0 Hz, CH$_2$C*H*$_3$), 2.40 (2 H, m, H-2), 2.97 (3 H, s, 4-H), 3.02 (1 H, dd, *J* = 3.5, 12.6 Hz, H-5), 4.18 (2 H, m, CH$_3$C*H*$_2$O), 4.64 (1 H, m, C*H*-NH), 5.59 (1 H, d, *J* = 8.4 Hz, N*H*); IR 1732, 1671.5, 1621, 1503.5 cm^{-1}; MS *m/z* 474 (M$^+$), 459, 429, 418, 401, 316, 288. Elemental analysis calculated for $C_{28}H_{46}O_4N_2$: theoretical C = 70.05%, H = 9.77%, *N* = 5.90%; found C = 70.08%, H = 9.78%, *N* = 5.85%. *N*-[(S)-phenylalanyl methyl ester]-3-oxo-4-aza-5α-androstane-17β-carboxamide (**5**) mp 175–176°C; ^{1}H NMR δ 0.65 (3 H, s, H-18), 0.88 (3 H, s, H-19), 2.39 (2 H, m, H-2), 3.04 (1 H, dd, *J* = 4.2, 11.9 Hz, H-5), 3.07–3.17 (2 H, m, C*H*$_2$Ph), 3.72 (3 H, s, COOC*H*$_3$), 4.89 (1 H, m, *CH* (L)), 5.51 (1 H, s, 4-N*H*), 5.67 (1 H, d, *J* = 7.7 Hz, N*H*), 7.09 (2 H, m, arom), 7.27 (3 H, m, arom); IR 1740, 1657, 1499 cm^{-1}; MS *m/z* 480 (M$^+$), 465, 421, 318, 302, 274. Elemental analysis calculated for $C_{29}H_{40}O_4N_2$: theoretical C = 72.47%, H = 8.39%, *N* = 5.83%; found C = 72.42%, H = 8.35%, *N* = 5.80%.

N-[(R)-phenylalanyl methyl ester]-3-oxo-4-aza-5α-androstane-17β-carboxamide (**6**) mp 193°C; ^{1}H NMR δ 0.51 (3 H, s, H-18), 0.89 (3 H, s, H-19), 2.40 (2 H, m, H-2), 2.9–3.6 (2 H, m, H-5 and C*H*Ph), 3.17 (1 H, dd, *J* = 5.6, -14 Hz, C*H*-Ph), 3.73 (3 H, s, OC*H*$_3$), 4.93 (1 H, m, *CH* (D)), 5.48 (1 H, br s, 4-N*H*), 5.65 (1 H, d, *J* = 8.1 Hz, N*H*-CH), 7.13 (2 H, m, arom), 7.27 (3 H, m, arom); IR 1740, 1657, 1498 cm^{-1}; MS *m/z*. 480 (M$^+$), 421, 318, 302, 274. Elemental analysis calculated for $C_{29}H_{40}O_4N_2$: theoretical C = 72.47%, H = 8.39%, *N* = 5.83%; found C = 72.44%, H = 8.34%, *N* = 5.79%. *N*-Glycinyl-3-oxo-4-aza-5α-androstane-17β-carboxamide (**9**) mp > 280°C; ^{1}H NMR δ 0.69 (3 H, s, H-18), 0.90 (3 H, s, H-19), 2.40 (2 H, m, H-2), 3.05 (1 H, dd, *J* = 3.5, 12.6 Hz, H-5), 3.97 (2 H, m, COC*H*$_2$NH), 5.5 (1 H, s, 4-N*H*), 6.09 (1 H, broad t, N*H*-CH$_2$); IR 1684(sh), 1653.5 cm^{-1}; MS *m/z* 376 (M+), 375, 361, 358, 318, 302, 274. Elemental analysis calculated for $C_{21}H_{32}O_4N_2$: theoretical C = 66.99%, H = 8.57%, *N* = 7.44%; found C = 66.95%, H = 8.54%, *N* = 7.40%.

N-[(R)-α-methylbenzyl]-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide (**10**) ^{1}H NMR δ 0.68 (3 H, s, H-18), 0.84 (3 H, s, H-19), 1.45 (3 H, d, *J* = 7.0 Hz, C*H*$_3$-CH), 2.38 (2 H, m, H-2), 2.87 (3 H, s, C*H*$_3$N), 2.98 (1 H, dd, *J* = 3.5, 12.6 Hz, H-5), 5.12 (1 H, m, C*H*Ph), 5.57 (1 H, d, *J* = 8.1 Hz, NH), 7.19–7.33 (5 H, m, arom); IR 1660, 1621, 1496 cm^{-1}; MS *m/z* 436 (M$^+$), 421, 331, 317, 288. Elemental analysis calculated for $C_{28}H_{40}O_2N_2$: theoretical C = 77.02%, H = 9.23%, *N* = 6.42%; found C = 77.05%, H = 9.27%, *N* = 6.39%.

N-[(S)-α-methylbenzyl]-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide (**11**) mp 155°C; ^{1}H NMR δ 0.60 (3 H, s, H-18), 0.86 (3 H, s, H-19), 1.48 (3 H, d, *J* = 7.0 Hz, C*H*$_3$CH), 2.43 (2 H, m, H-2), 2.91 (3 H, s, C*H*$_3$N), 3.01 (1 H, dd, *J* = 3.5, 12.6 Hz, H-5), 5.15 (1 H, m, C*H*Ph), 5.49 (1 H, d, *J* = 7.7 Hz, N*H*), 7.2–7.3 (5 H, m, arom); IR 1659, 1621, 1495 cm^{-1}; MS *m/z* 436 (M$^+$), 421, 331, 317, 288. Elemental analysis calculated for $C_{28}H_{40}O_2N_2$: theoretical C = 77.02%, H = 9.23%, *N* = 6.42%; found C = 77.07%, H = 9.29%, *N* = 6.40%.

N-[(S)-leucyl ethyl ester]-androst-4-en-3-oxo-17β-carboxamide (**14**). A solution of androst-4-en-3-oxo-17β-carboxylic acid **(13)** (6.92 g, 21.9 mmol) in dry toluene (104 ml) and pyridine (2.43 ml) was cooled at 10°C and a solution of oxalyl chloride (2.822 ml) in toluene (11.66 ml) was added under vigorous stirring. The reaction was stirred at 10°C (1 h), then a suspension of S-leucine ethyl ester hydrochloride (20.85 g, 106.8 mmol) in toluene (104 ml) and pyridine (8.807 ml) was added. After heating at 40°C for 4 h, under vigorous stirring, the reaction mixture was cooled at room temperature and acidified to pH 2, filtered by suction and the precipitate washed with toluene. The collected filtrates were washed with aqueous saturated $NaHCO_3$ to neutrality, dried on Na_2SO_4, filtered and the solvents were evaporated under vacuum to give the crude product (14.6 g). After silica gel column chromatography (CH_2Cl_2/CH_3OH = 9:1 v/v), pure **14** was obtained (6.79 g, 66% yield). ^{1}H NMR (60 MHz) δ 0.75 (3 H, s, H-18), 1.15 (3 H, s, H-19), 0.9–2.8 (complex), 5.1 (1 H, s, exch, N*H*),



Scheme 1.

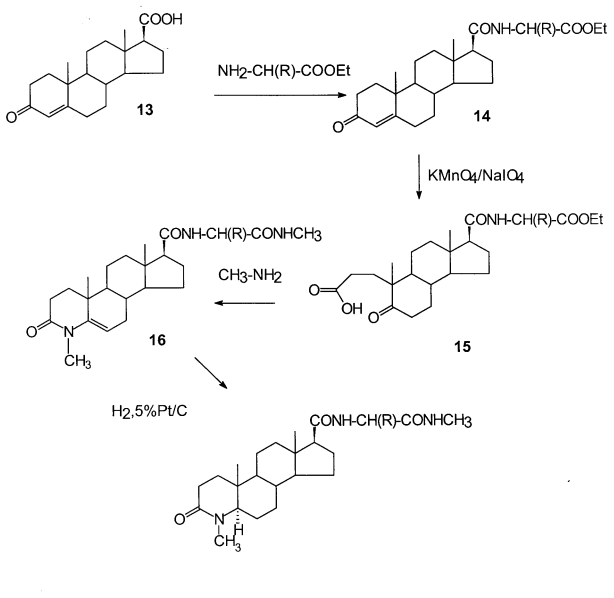
5.9 (1 H, s, H-4); IR 1735, 1670, 1503 cm^{-1}. Elemental analysis calculated for $C_{28}H_{43}O_4N$: theoretical C = 73.49%, H = 9.47%, *N* = 3.06%; found C = 73.47%, H = 9.45%, *N* = 3.01%.

*2.4. Preparation of secosteroid **15***

N-[(S)-leucyl ethyl ester]-androst-4-en-3-oxo-17β-carboxamide **(14)** (3.1 g, 6.78 mmol) was dissolved in *t*-butanol (41.34 ml) and an aqueous solution of Na_2CO_3 (1.1 g in 6.7 ml) was added. The reaction mixture was heated at 80°C and a hot aqueous solution (42 ml) of $KMnO_4$ (80 mg) and $NaIO_4$ (10.5 g) was added under stirring, during 45 min. After 1 h at reflux the mixture was cooled at room temperature, kept under stirring for 2 h, filtered through a celite pad, concentrated under vacuum to near 1/3 of initial volume. The solution obtained was cooled at 10°C and carefully acidified under vigorous stirring to pH = 2 with 6 N HCl. The precipitate was recovered by filtration, dissolved in CH_2Cl_2 and the solution was dried on Na_2SO_4. After evaporation under vacuum pure **15** (3 g, 93% yield) was obtained: ^{1}H NMR (60 MHz) δ 0.75 (3 H, s, H-18), 0.9–2.8 (complex), 5.4 (2 H, m, exchangeable); IR 1731, 1702.5, 1673, 1505 cm^{-1}; MS *m/z* 459, 432, 404, 372, 318. Elemental analysis calculated for $C_{27}H_{43}O_6N$: theoretical C = 67.90%, H = 9.07%, *N* = 2.93%; found C = 67.85%, H = 9.11%, *N* = 2.90%.

*2.4.1. N-[(S)-leucyl methylamide]-4-methyl-3-oxo-4-aza-4-androstene-17β-carboxamide (**16**)*

Compound **15** (5 g, 10.47 mmol) was dissolved under stirring in ethylene glycol (50 ml), at room temperature. After cooling to −10°C methylamine (26 ml) was added and the solution was slowly heated to reach 180°C, and kept at this temperature for 20 min. The reaction mixture was then cooled to room temperature and acidified with 3N HCl. The precipitate was recovered by filtration, dried under vacuum at 80°C and used without further purification (4.25 g) in the next step. For analytical purposes a sample has been purified by silica gel column chromatography (1:80 w/w; CH_2Cl_2/CH_3OH = 8:2 v/v as eluant); MS *m/z* 457 (M^+), 442, 412, 401, 384, 299, 271. Elemental analysis calculated for $C_{27}H_{43}O_3N_3$: theoretical C = 70.86%, H = 9.47%, *N* = 9.18%; found C = 70.80%, H = 9.41%, *N* = 9.12%



Scheme 2.

*2.4.2. N-[(S)-leucyl methylamide]-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide (**8**)*

The crude compound **16** (1.4 g, 3.06 mmol) was dissolved in acetic acid (20 ml) and hydrogenated on 5% Pt/C (1.4 g) at 3 atm, 75°C during 6 h. The solvent was evaporated under vacuum after filtration through a celite pad to give a residue which was dissolved in CH_2Cl_2 and washed with an aqueous solution of $NaHCO_3$ to neutrality. The solution was then dried on Na_2SO_4, and after filtration evaporated to dryness affording a crude. After purification by silica gel column chromatography (1:100 = w/w, CH_2Cl_2/CH_3OH = 95:5 v/v as eluant), pure **8** was obtained (1.2 g, 2.61 mM, 85% yield), mp 225–226°C; ^{1}H NMR δ 0.61 (3 H, s, H-18), 0.84 (3 H, s, H-19), 0.92 (3 H, d, *J* = 5.9 Hz, C*H*$_3$CH), 0.94 (3 H, d, *J* = 6.3 Hz, C*H*$_3$CH), 2.40 (2 H, m, H-2), 2.74 (3 H, d, *J* = 4.9 Hz, C*H*$_3$NH), 2.89 (3 H, s, NC*H*$_3$), 3.01 (1 H, dd, *J* = 3.5, 12.6 Hz, H-5), 4.45 (1H, m, C*H* (L)), 5.90 (1 H, d, *J* = 8 Hz, N*H*-CH), 6.65 (1 H, m, N*H*-CH$_3$); IR 1669 (sh), 1654, 1622, 1502 cm^{-1}; MS *m/z* 460 (M+1), 459 (M^+), 444, 429, 401, 331, 316. Elemental analysis calculated for $C_{27}H_{45}O_3N_3$: theoretical C = 70.55%, H = 9.87%, *N* = 9.14%; found C = 70.60%, H = 9.82%, *N* = 9.10%.

Analogously was prepared *N*-[(R)-leucyl methylamide]-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide (**7**) mp 150°C; ^{1}H NMR δ 0.68 (3 H, s, H-18), 0.87 (3 H, s, H-19), 0.92 (3 H, d, *J* = 5.9 Hz, C*H*$_3$CH), 0.94 (3 H, d, *J* = 6.3 Hz, C*H*$_3$CH), 2.42 (2 H, m, H-2), 2.75 (3 H, 17β d, *J* = 4.2 Hz, C*H*$_3$NH), 2.90 (3 H, s, N-C*H*$_3$), 3.01 (1 H, dd, *J* = 3.5, 12.6 Hz, H-5), 4.50 (1 H, m, C*H* (D)), 5.97 (1 H, d, *J* = 8.4, N*H*-CH), 6.69 (1 H, broad q, N*H*-CH$_3$); IR 1663, 1622, 1505 cm^{-1}; MS *m/z* 460 (M+1), 459 (M^+), 429, 444, 401, 316. Elemental analysis calculated for $C_{27}H_{45}O_3N_3$: theoretical C = 70.55%, H = 9.87%, *N* = 9.14%; found C = 70.58%, H = 9.85%, *N* = 9.11%.

3. Biological activity

3.1. Pharmacological testing

At the moment the unique compound of this class of molecules entered into therapy is represented by Finasteride

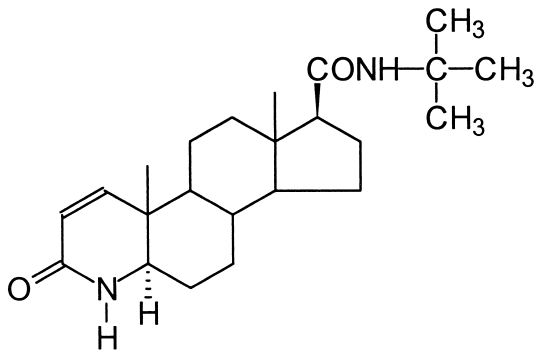


Fig. 2. Finasteride.

(Fig. 2) [19]; for this reason we have utilized this molecule as reference standard for the pharmacological tests. The inhibitory effect of the compounds synthesized as above on the 5AR activity has been tested in the homogenate of rat prostate at neutral pH, comparing the molecules' IC_{50}s (Table 1) with that of Finasteride (prepared according published procedure [3]).

In these assay conditions the activity measured is mostly that of type 1 isozyme which has a wide range of pH optimum (6–8.5), while type 2 isozyme has a narrow acidic pH optimum (around 5.0) and it is almost inactive at pH 7. Metabolic studies at acidic pH have not been effected since it is almost impossible to have a clear separation of the two enzymatic activities changing the incubation medium pH, at least in the rat prostate. The two recombinant enzymatic isoforms expressed in a suitable yeast expression system [20] will be used in future experiments.

The 5α-reductase activity has been assayed radioenzymatically as routinely done in our laboratory. The transformation of testosterone into its main 5α-reduced metabolites (dihydrotestosterone or DHT and 5α-androstane-3α,17β-diol or 3α-diol) has been evaluated after the in vitro incubation of [^{14}C]testosterone with an aliquot of normal adult rat prostate fresh homogenate. Briefly the method is the following: aliquots of an homogenate of the rat ventral prostate, freshly dissected from an adult animal (Crl: CD®RR Charles River Italia S.p.A., 200–300 g of body weight), containing about 100 μg of protein (measured according the method of Bradford [21]) are incubated with increasing amounts of the test substance (initially in the range of 10^{-5}–10^{-8} M; lower concentrations have been included for some very active products in subsequent tests) or without any inhibitor. Each point is evaluated in duplicate. The incubation medium is a Krebs-Ringer buffer solution (250 μl-pH 7.1) containing a NADPH generating system (NADPH, disodium salt (Boehringer Mannheim) 3.32×10^{-3} M; glucose 6-phosphate, disodium salt (Boehringer Mannheim) 11.76×10^{-2} M and glucose 6-phosphate dehydrogenase from yeast grade 1 (Boehringer Mannheim), 3.5×10^{-2} U.I. or 1 mg/ml) and [^{14}C]testosterone 3.2×10^{-6} M (specific activity $\approx$ 56.9 mCi/mM, Amersham England). The incubation is carried out at 37°C in a Dubnoff metabolic shaker under a stream of O_2/CO_2 98:2 (v:v), for 2 h. At the end of the incubation the reaction is stopped by freezing the samples to −20°C. Tritium labeled DHT and 3α-diol (about 5000 dpm each) are added to each sample in order to evaluate the recoveries. The metabolites formed are extracted twice with diethyl ether, non-radioactive steroids (40 μg/100 ml) are added to each sample in order to aid visualization of the steroid on the TLC plates. Samples are then separated on a thin layer silica gel plate (Merck 60 F_{254}, DC) eluting three times with a mixture of dichloromethane-diethyl ether (11:1 v/v). DHT and 3α-diol spots are identified with iodine vapors, scraped off and the radioactivity counted in a Packard 1600 CA liquid scintillation spectrometer. Quench corrected dpm of the isotope are obtained by a calibration standard curve. The identification of the metabolites has been performed, when the method was validated by recrystallization to constant ^{3}H/^{14}C ratio. The 5α-reductase activity is expressed as pg of steroids (DHT+3α-diol) formed in the incubation time per mg of protein. The enzymatic activity in all the assays has been normalized to the finasteride, which was included in each experiment. The intraassay variation was 10–30% and the

Table 1
5α-Reductase in vitro inhibitory activities; values are expressed in relation to finasteride

Compound	Configuration[a]	(**a**) group	(**b**) group	Relative activity
1	S	-CH_2Ph	-$CONHCH_3$	0.00
2	R	-$CONHCH_3$	-CH_2Ph	5.25
3	R	-COOEt	-$CH_2CH(CH_3)_2$	0.60
4	S	-$CH_2CH(CH_3)_2$	-COOEt	3.84
5	S	-CH_2Ph	-$COOCH_3$	0.15
6	R	-$COOCH_3$	-CH_2Ph	3.88
7	R	-$CONHCH_3$	-$CH_2CH(CH_3)_2$	1.60
8	S	-$CH_2CH(CH_3)_2$	-$CONHCH_3$	1.51
9	-	-H	-H	0.00
10	R	-Ph	-CH_3	16.60
11	S	-CH_3	-Ph	56.60
Finasteride	-			1.00

[a] Related to the stereogenic center at C-22.

interassay variation 30–50%. The curves obtained are statistically evaluated and IC_{50} calculated by the program ALLFIT developed by D. Rodbard and colleagues, NIH, USA in the Macintosh version prepared by Vincenzo Guardabasso and Giovanni Angeli, Biomathematics and Applied Informatic Research Unit, Consorzio Mario Negri Sud.

4. Results and discussion

The data reported in Table 1 show that inhibition obtained in vitro with this series of derivatives is significantly different for the two epimers of compounds **1–6** and **10–11,** while the glycine derivative (**9**) shows no inhibitory activity and compounds **7** and **8** exhibit the same activity. Although our screening is restricted to a small number of compounds, the results obtained on the couples of epimers (**1–6** and **10–11)** show that the configuration of the stereogenic center in the 17β-side chain can be related to different levels of pharmacological activity. The above data indicate that the inhibition of 5AR is influenced not only by the nature of the group bound to the 17β-carbamoyl group but also by its stereochemistry. The diastereoselectivity should therefore be taken into account in designing molecules with a stereocenter in 17β-side chain, in order to optimize the interaction with the binding site of 5AR. As compounds **2, 4, 6, 11** show a higher relative activity compared to **1, 3, 5** and **10,** the former can be defined as eutomers (more biologically active stereoisomers) and the latter as distomers (less active isomers), according to Lehmann et al. [22]. The above different biologic effect would not be detectable if a mixture of both stereoisomeric forms had been used. On the basis of the obtained results we have attempted to develop a coherent model to elucidate some conformational aspects of the binding site of 5AR. In our model, the side-chain dependent portion of the binding site of 5AR which would accommodate the 17β chain of 4-azasteroids, consists of 2 cavities, **A** and **B,** in which the (**a**) and (**b**) substituents at the stereocenter in position 22 are to be lodged (Fig. 3).

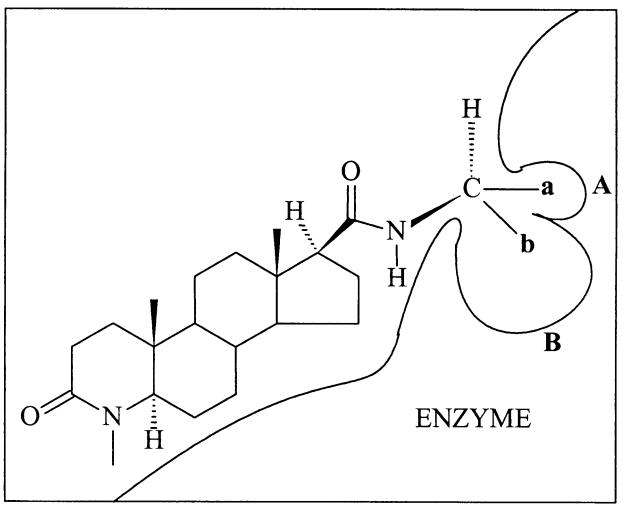


Fig. 3.

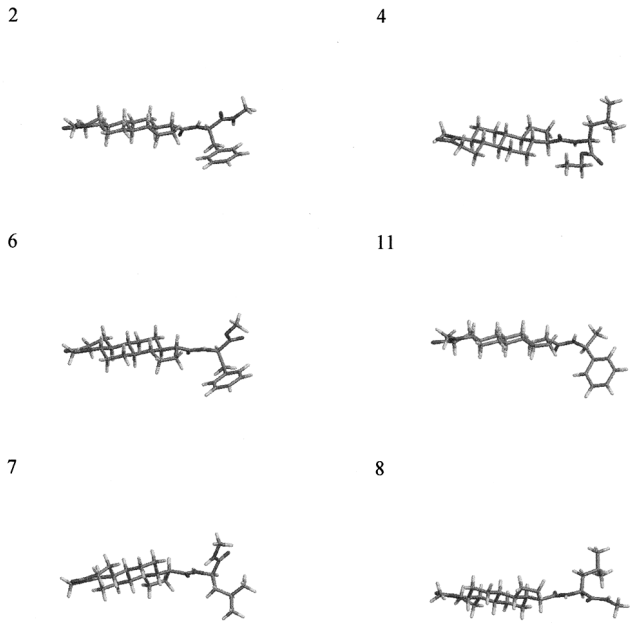


Fig. 4. Optimized geometries for the more active 4-azasteroid epimers **2**, **4**, **6** and **11** in Table 1. Both **7** and **8** diastereoisomers have been reported, being of nearly equal activity. Molecules have been projected with the larger group downward and the smaller upward, according to the scheme of Fig. 3.

Tables 2, 3 and 4 show a number of chemico-physical parameters for substituents (**a**) and (**b**) for all the molecules tested. We were hoping to be able to relate the inhibitory effect of a molecule in an epimeric pair (e.g. **1** and **2, 3** and **4**) to some of the chemico-physical parameters we had considered. The parameters were calculated on optimized molecular structures. The MOLGEN-3 software [23] was used for both the optimization of the structures and the evaluation of the properties.

Fig. 4 shows the optimized molecular structures for more active epimers **2, 4, 6, 11** and for the nearly equally active couple of epimers **7** and **8**. Table 2 shows values of log P

Table 2
Lipophilicity of substituents at chiral C-22: calculated molar refractivity (MR) and logP for tested compounds

Compound	Relative activity	$logP_{(a)}$	$logP_{(b)}$	$MR_{(a)}$	$MR_{(b)}$
1	0	1.88	−0.35	30.99	13.67
2	5.25	−0.35	1.88	13.67	30.99
3	0.60	0.66	1.44	16.51	20.67
4	3.84	1.44	0.66	20.67	16.51
5	0.15	1.88	0.32	30.99	11.71
6	3.88	0.32	1.88	11.71	30.99
7	1.60	−0.35	1.44	13.67	20.67
8	1.51	1.44	−0.35	20.67	13.67
10	16.6	1.69	0.21	24.88	6.84
11	56.6	0.21	1.69	6.84	24.88

Table 3
Spatial parameters of substituents at chiral C-22: $v_{(a)}$ or $v_{(b)}$ = calculated van der Waals volumes and $d_{H(a)}$ or $_{(b)}$ = calculated distance (Å) between the chiral carbon atom and the farther H of the groups (**a**) or (**b**) of tested compounds

Compound	Relative activity	$v_{(b)}$	$v_{(a)}$	$d_{H(b)}$	$d_{H(a)}$
1	0	34.4	57.1	4.21	6.14
2	2.25	56.5	34.3	6.16	4.56
3	0.60	36.3	42.4	4.80	5.71
4	3.84	41.3	39.3	5.68	4.78
5	0.15	33.3	57.1	4.38	6.15
6	3.88	57.0	32.9	6.17	4.38
7	1.60	41.9	33.8	4.81	4.57
8	1.51	33.1	42.1	4.21	4.80
10	16.6	14.2	47.8	2.19	5.44
11	56.6	48.2	14.9	5.45	2.20

(partition coefficient in the system *n*-octanol/water) and molar refractivity [24] of the molecules studied. These values are measures of the lipophilicity of substituent groups (**a**) and (**b**). The data show that there is no correlation between inhibitory activity and lipophilicity. Table 3 lists spatial parameters v and d_H (van der Waals volumes [25] and the distance between the stereocenter and the furthest hydrogen in the substituents (**a**) and (**b**)). Substituent (**b**) is longer than (**a**) between 20% and 150% for pairs **1** and **2, 3** and **4, 5** and **6, 10** and **11.** While in the case of van der Waals volumes v there is not a direct correlation with the inhibitory activity levels, such correlation exists in the case of d_H values. This suggests that what is important for a significant inhibitory effect is not the right 'bulk,' but the proper 'length': to be a good inhibitor, a molecule must have a 'long' (**b**) substituent, able to fit into the **B** cavity (Fig. 3). In the opposite configuration, (**b** 'short' and **a** 'long'), the molecule would not fit well in the binding site, and the inhibitory effect would be much lower or not existent. This is borne out by the results obtained for pair **7** and **8,** where similar d_H values correspond to very close inhibitory activities. As the substituents are of very similar lengths, there is no preferential spatial arrangement of (**a**) and (**b**) in the **A** and **B** cavities, respectively. Table 1 shows that the **A** cavity can accept substituents as long as a phenyl group (d_H 5.44 Å, compound **10,** relative activity 16.6), but not as a benzyl group (d_H 6.15 Å, compounds **1** and **5,** relative activities 0 and 0.15, respectively). In Table 4 are listed other spatial parameters (volumes and areas accessible to the solvent), also showing a direct correlation with the inhibitory activity of the members of the epimeric pairs. A good correlation also appears to exist between the activity and the degree of compactness (Wiener and Randic topological indices [18]). For each pair, the more active epimer is the one that possesses the more extended, less compact, (**b**) substituent, in agreement with the previously discussed correlation to other parameters. Of the two indices, the Randic index is the more suitable, as it appears to have definite values for the greatest majority of the substituents.

In conclusion, our model's predictions, as borne out by the data obtained, are the following:

the binding site of 5AR (type 1 isozyme) consists of two cavities which are meant to accommodate the two substituents bound to C-22 stereocenter, at the α position of the β carbamoyl group in the 4-azasteroid molecules (Fig. 1)

these cavities have different sizes, but their lipophilic character is not well defined. Cavity **A** is smaller than cavity **B,** and the two are arranged as shown in Fig. 3. The size and shape of cavity **A** are such as to accommodate a phenyl group (5.4 Å long), but not a benzyl group (6.15 Å long)

if the arrangement around the C-22 stereocenter is such that the 'short' (**a**) group fits in the 'small' **A** cavity (and, conversely, the long (**b**) group fits in the 'large' **B** cavity), the interaction between the inhibitor and the enzyme is strong enough to produce an inhibitory effect

for the other epimer of the pair, where the spatial orientation is the opposite, the 'long' (**a**) group cannot fit well into the 'small' **A** cavity. The enzyme-inhibitor interac-

Table 4
Other spatial parameters of substituents at chiral C-22: $V_{sol\ (a)\ or\ (b)}$ and $S_{sol\ (a)\ or\ (b)}$ = calculated volumes ($Å^3$) and areas ($Å^2$) accessible to the solvent. Calculated Wiener and Randic indices as measures of molecular compactness

Compound	Relative activity	$V_{sol\ (b)}$	$V_{sol\ (a)}$	$S_{sol\ (b)}$	$S_{sol\ (a)}$	Wiener (**b**)	Wiener (**a**)	Randic (**b**)	Randic (**a**)
1	0	14.1	21.2	30.1	44.5	u.d.	u.d.	1.914	3.394
2	2.25	19.6	14.4	41.1	29.1	10	10	3.394	1.914
3	0.60	12.3	15.9	29.8	35.1	9	20	1.732	2.414
4	3.84	14.3	12.6	31.3	29.4	20	9	2.414	1.732
5	0.15	13.5	20.1	28.5	43.9	10	u.d.	1.914	3.394
6	3.88	19.8	13.5	42.0	28.1	u.d.	10	3.394	1.914
7	1.60	14.3	13.5	33.5	29.1	9	10	1.732	1.914
8	1.51	12.6	14.0	27.0	32.8	10	9	1.914	1.732
10	16.6	6.00	18.6	13.8	38.8	u.d.	u.d.	u.d.	3
11	56.6	19.0	6.40	39.1	14.9	u.d.	u.d.	3	u.d.

tion and the resulting relative activity are therefore reduced

when the (**a**) and (**b**) substituents are of equal length the corresponding level of inhibition is almost the same: either group can fit equally well into cavity **A** or **B**

Further research is needed, aimed primarily at obtaining a better definition of the 'large' cavity, and at identifying clearly the possible stereospecific effect of the spatial features of the substituents on isozyme 2 as well as on isozyme 1.

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