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Synthesis and Biological Evaluation of 4-(4-(Alkyl- and Phenylaminocarbonyl)benzoyl)benzoic Acid Derivatives as Non-steroidal Inhibitors of Steroid 5α-Reductase Isozymes 1 and 2

The synthesis and biological evaluation of 4-(4-(alkyl- and phenylaminocarbonyl)-benzoyl)benzoic acids (**4a**–**4d**) as non-steroidal inhibitors of steroid 5α-reductase are described. The compounds were tested in vitro for inhibitory activity toward rat and human 5α-reductase isozymes 1 and 2 at a concentration of 10 μM. The most active inhibitor for the human type 2 isozyme was 4-(4-(phenylaminocarbonyl)benzoyl)benzoic acid, compound **4c** (IC_{50} = 0.82 μM).

Keywords: Non-steroidal 5α-reductase inhibitors; Substituted benzoylbenzoic acids; Rat and human steroid 5α-reductase isozymes 1 and 2; Benign prostatic hyperplasia; Prostate cancer

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Introduction

Dihydrotestosterone (DHT) is the most potent androgen, and is formed as a metabolite of the predominant circulating androgen testosterone (T). Steroid 5α-reductase catalyzes the conversion of T to DHT. Benign prostatic hyperplasia (BPH) is a disease resulting in urinary dysfunction which is assumed to be mediated by DHT [1]. The reduction of DHT concentration by inhibition of 5α-reductase can lead to a partial remission of BPH and thus can improve the disease. DHT is also implicated in the etiology of prostate cancer [2], and several disorders of the skin including acne [3], androgenic alopecia [4], and hirsutism [5]. Steroidal inhibitors have demonstrated clinical efficacy for the treatment of BPH, among them finasteride (Proscar®) [6] or epristeride [7] (Chart 1). However, unwanted side effects are likely to occur [8], as most steroidal drugs show such activities. On the other hand, non-steroidal inhibitors have not yet shown clinical efficacy for the treatment of BPH. The discovery of two isozymes (type 1 and type 2) and the determination of their tissue distribution have led to the prospect of developing more specific drugs [9]. An effective dual inhibitor of type 1 and type 2 human 5α-reductase may lower circulating DHT to a greater extent than finasteride and thus may show advantages in the treatment of BPH and other diseases that depend on DHT [10]. A class of benzophenone carboxylic acids with potent 5α-reductase type 2 isozyme specific inhibitory activity has been described [11] and we recently found that some biphenyl-4-carboxylic acid derivatives with alkyl- or arylaminocarbonyl substituents show fairly strong inhibitory activity against human 5α-reductase type 2, and moderate inhibitory activity toward human type 1 enzyme [12]. In order to find more potent inhibitors, we introduced those alkyl- or arylaminocarbonyl groups which are also present in the 17β-position of most steroidal inhibitors into the flexible benzophenone skeleton. In the following we describe the synthesis of compounds **4a**–**4d** and **6** (Chart 1) and the evaluation of their inhibitory activity toward human and rat 5α-reductase isozymes 1 and 2.

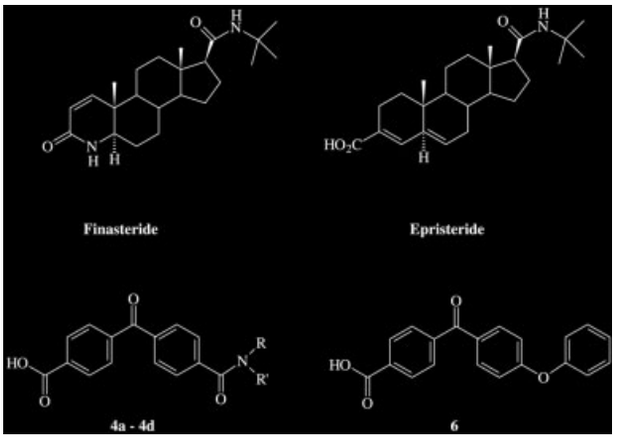

Chart 1. Inhibitors of 5α-reductase and title compounds.

Synthesis

Monomethyl terephthalate was the precursor for the synthesis of the benzoylbenzoic acids **4a**–**4d** and **6**

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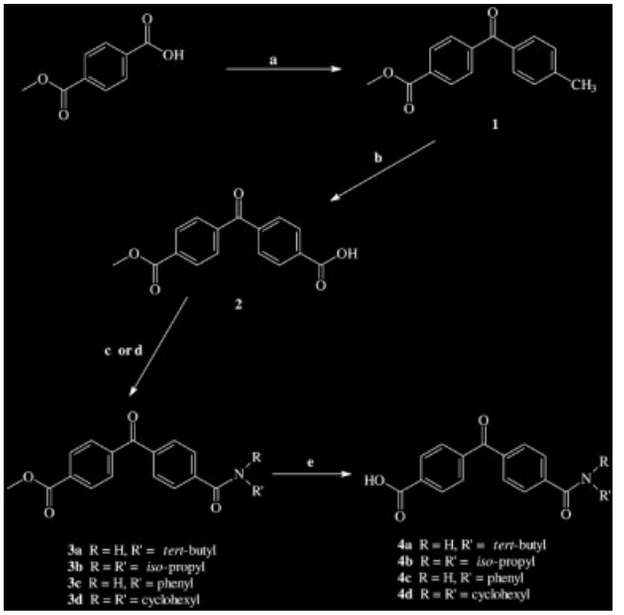
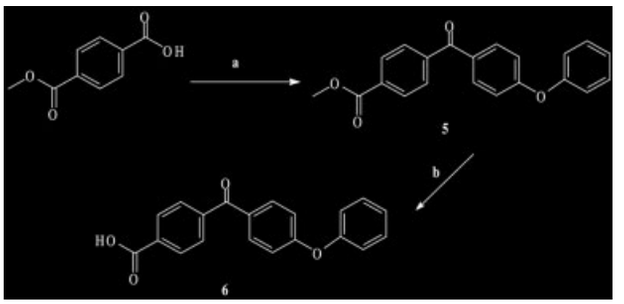


(a) i) $SOCl_2$ ii) $AlCl_3$, toluene, 25 °C;
(b) CrO_3, HOAc, H_2SO_4;
(c) 2-Chloro-1-methylpyridinium iodide, NEt_3, *tert*-butylamine, reflux;
(d) Oxalyl chloride, then amine, 25 °C;
(e) K_2CO_3, methanol – water (9/1), reflux;

Scheme 1. Synthesis of **4a**–**4d.**



a) i) $(COCl)_2$ ii) $AlCl_3$, diphenylether, CS_2, reflux;
(b) K_2CO_3, methanol – water (9/1), reflux

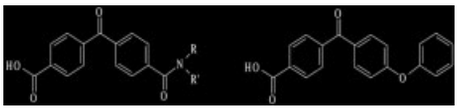
Scheme 2. Synthesis of **5** and **6.**

(Schemes 1 and 2). In analogy to the procedure of Schäfer et al. [13], Friedel-Crafts acylation of toluene with the acid chloride of monomethyl terephthalate using $AlCl_3$ as a catalyst afforded the benzoyl benzoic acid methyl ester derivative 1 in good yield. Oxidation of the methyl group to the corresponding carboxylic acid was achieved using a chromic oxide/sulfuric acid/acetic acid mixture to give the 4,4´-benzophenone dicarboxylic acid monomethyl ester **2**. The *tert*-butylamide derivative **3a** was synthesized in one step using the Mukaiyama reagent (2-chloro-1-methylpyridinium iodide) in the presence of triethyl-amine as acid scavenger [14] in refluxing acetonitrile. The same procedure delivered the amides **3b**–**3d**, but only in poor yields. To improve the overall efficiency, changing to a two-step procedure gave the corresponding amides **3b**–**3d** in appreciably higher yields: using oxalyl chloride to transform the acid **2** to the acid chloride and treating the crude reaction mixture with an excess of the appropriate amine. For the synthesis of compound **6** [11], we followed a new route. 4-(4-(Phenoxybenzoyl)benzoic acid methyl ester **5** was synthesized by Friedel-Crafts acylation of diphenyl ether with the acid chloride of monomethyl terephthalate in carbon disulfide using $AlCl_3$ as a catalyst in analogy to the two-step procedure of Kipper [15]. Saponification of the synthesized esters **3a**–**3d** and **5** using potassium carbonate in refluxing methanol-water afforded the corresponding carboxylic acids **4a**–**4d** and **6** in high yields.

Results and discussion

The inhibitory activity of compounds **4a**–**4d**, **6** [11] and **finasteride** as a reference was determined in vitro at a concentration of 10 μM using human prostate homogenate (BPH tissue for type 2 isozyme) and the DU 145 cell line (for human type 1 enzyme) and rat prostate homogenates (pH 6.6, type 1; pH 5.5, type 2). In case of potent inhibition IC_{50} values were determined. As can be seen in Table 1, all non-steroidal compounds were less active than finasteride. For the human type 2 isozyme, compounds with an aliphatic (*tert*-butyl, di-*iso*-propyl, and dicyclohexyl) aminocarbonyl substituent displayed weak to moderate inhibitory activity (13–55%), while compound **4c** with a phenylaminocarbonyl substituent showed significantly higher inhibitory activity (86% inhibition; IC_{50} = 0.82 μM) indicating that an aromatic ring in this position is important for potency. However, the described compound **6** (IC_{50} = 0.053 μM) still is more potent (15.5 times). All compounds which are active in human type 2 isozyme are active in rat isozyme 2 as well. However, compounds **4c** and **6** do not reach the activity shown in the human enzyme. In the human type 1 isozyme (DU 145 cell line), all compounds were inactive or rather poor inhibitors, while in the case of rat prostatic homogenate isozyme type 1 compounds **4c** and **4d** displayed moderate activity (59% and 66% inhibition, respectively). It cannot be excluded that the weak inhibitory activity in intact cells (DU 145) is due to an insufficient permeability across the cell membrane. Further experiments regarding this issue are in progress. As a conclusion, we have presented in this paper a new class of substituted benzoylbenzoic acids as 5α-reductase inhibitors. One of these derivatives, compound **4c**, might be further optimized to become a lead for more potent non-steroidal 5α-reductase inhibitors. Further structural modifications in this class of compounds are ongoing.

Table 1. In vitro inhibition of human and rat steroid 5α-reductase isoenzymes type 1 and type 2 by compounds **4a**–**4d**, **6** and **finasteride.**



4a–**4d** **6**

Compd. No.	R, R´	Human % Inhibition at 10 μM, (IC_{50}, μM) Type 2[a,d,g]	Human % Inhibition at 10 μM, (IC_{50}, μM) Type 1[b,g]	RVP[c] % Inhibition at 10 μM, (IC_{50}, μM) Type 2[d,e,g]	RVP[c] % Inhibition at 10 μM, (IC_{50}, μM) Type 1[d,f,g]
4a	H, *tert*-butyl	45	n.i.	11	13
4b	di-*iso*-propyl	13	n.i.	5	n.i.
4c	H, phenyl	86 (0.82)	n.i.	68	59
4d	dicyclohexyl	55	10	69	66
6		82[h] (0.053)	n.i.	33	53
finasteride		(2–3 nM)	(0.043)	(0.011)	(0.01)

[a] Human prostate homogenate; 200–300 μg protein per incubation, pH 5.5.
[b] Human prostatic carcinoma (DU 145 cell line) expressing type 1 isozyme; Substrate ^{3}H-androstenedione 5 nM.
[c] Rat ventral prostate; 200–250 μg protein per incubation.
[d] Substrate 1β,2β-^{3}H-testosterone 210 nM.
[e] pH 5.5. [f] pH 6.6.
[g] Mean value; tests have been run in duplicate two times; standard deviation for IC_{50} values <20%; standard deviation for percent inhibition <10%. n.i. no inhibition (percent inhibition <5%).
[h] Percent inhibition at 0.4 μM.

Acknowledgements

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Experimental section

Solvents for reactions under anhydrous conditions were dried according to standard procedures. All reactions, except those involving water as a reagent, were conducted under nitrogen atmosphere. Melting points were determined on a Kofler microscope (Reichert, Austria) and are uncorrected. Mass spectra (EI, 70 eV) were measured on a HP G1800A GCD. ^{1}H-NMR spectra were measured on a Bruker AM-400 (400 MHz) and are consistent with the assigned structures. Chemical shifts are given in parts per million relative to TMS as an internal standard. IR spectra were measured as KBr discs on a Perkin-Elmer infrared spectrophotometer 398.

4-(4-Methylbenzoyl)benzoic acid methyl ester (**1**)

The title compound was synthesized in analogy to the two-step procedure of Schäfer et al. [13] starting from monomethyl terephthalate. Mp 127-28 °C (Literature [13], 122–124 °C). IR: $\nu_{max.}$ = 2960, 1720, 1645, 1610, 1280, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ = 2.45 (s, 3H, Ar-CH_3), 3.96 (s, 3H, OCH_3), 7.29 & 7.71 (d, 4H, *J* = 8.2 Hz, Ar-H), 7.81 & 8.13 (d, 4H, *J* = 8.4 Hz, Ar-H).

4,4´-Benzophenone dicarboxylic acid monomethyl ester (**2**)

To a precooled mixture of **1** (254 mg, 1.00 mmol) and glacial acetic acid (1.90 mL), sulfuric acid (0.15 mL) was added slowly with stirring. The reaction mixture was cooled to 5 °C and chromic oxide (0.30 g, 3.00 mmol) was added in small portions at such a rate that the temperature did not rise above 10 °C. The reaction mixture was stirred at 5–10 °C for 2 hours, then at room temperature overnight. The reaction mixture was poured onto ice and filtered. The crude acid was purified by recrystallization from ethyl acetate-methanol to yield 210 mg (74%) of the title compound as white crystals. Mp 284–285 °C. IR: $\nu_{max.}$ = 3000, 1730, 1700, 1680, 1650, 1290, 1110, 940, 880 cm^{-1}. ^{1}H-NMR (d_6-DMSO): δ = 3.91 (s, 3H, OCH_3), 7.85 & 7.87 (d, 4H, *J* = 8.4 Hz, Ar-H), 8.10 & 8.12 (d, 4H, *J* = 8.0 Hz, Ar-H), 13.26 (s, 1H, OH).

4-(4-(tert-Butylaminocarbonyl)benzoyl)benzoic acid methyl ester (**3a**)

A mixture of 2 (284 mg, 1.00 mmol), 2-chloro-1-methylpyridinium iodide (260 mg, 1.00 mmol), triethylamine (200 mg, 2.00 mmol) and *tert*-butylamine (90.0 mg, 1.20 mmol) in an-

hydrous acetonitrile (30 mL) was refluxed for 2 hours. After cooling, dichloromethane (50 mL) was added and the organic layer was washed once with hydrochloric acid (10%), then with a saturated solution of sodium bicarbonate, water and brine. The organic layer was dried over $MgSO_4$, filtered and evaporated under reduced pressure to give 200 mg (59%) of **3a**. Recrystallization from hexane-ethyl acetate yielded white crystals. Mp 161–162 °C. IR: $\nu_{max.}$ = 3340, 2980, 1720, 1660, 1640, 1540, 1285, 1100, 880, 725 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ = 1.50 (s, 9H, $C(CH_3)_3$), 3.97 (s, 3H, OCH_3), 5.98 (s, 1H, NH), 7.81–7.83 (m, 6H, Ar-H), 8.15 (d, 2H, *J* = 8.4 Hz, Ar-H). MS (153 °C): *m*/*z* = 339 (M^+, 44%), 267 (100%), 284 (44%).

General procedure for the synthesis of **3b**–**3d**

4-(4-(Diisopropylaminocarbonyl)benzoyl)benzoic acid methyl ester (**3b**)

To a suspension of **2** (568 mg, 2.00 mmol) in anhydrous dichloromethane (20 mL) and a few drops of anhydrous dimethylformamide, oxalyl chloride (0.19 mL, 2.20 mmol) was added carefully. After the effervescence had ceased, the reaction mixture was stirred for further 2 h at room temperature. The solvent and unreacted oxalyl chloride were evaporated under reduced pressure. The residual acid chloride was dissolved in anhydrous dichloromethane (10 mL) and added dropwise to a solution of di-*iso*-propylamine (510 mg, 5.00 mmol) in dichloromethane (20 mL). The mixture was stirred for 1 h at ambient temperature. Ethyl acetate (50 mL) was added and the organic layer was washed successively with hydrochloric acid (10%), saturated sodium bicarbonate solution, water and brine. The organic layer was dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the solid obtained was recrystallized from hexane-ethyl acetate to give 400 mg (54%) of **3b** as white crystals. Mp 153–154 °C. IR: $\nu_{max.}$ = 2945, 1710, 1640, 1620, 1430, 1270, 1100, 920, 840 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ = 1.17 & 1.59 (2 br s, 12H, $CH(CH_3)_2$), 3.97 (s, 3H, OCH_3), 7.43 & 7.82 (d, 4H, *J* = 8.0 Hz, Ar-H), 7.84 & 8.16 (d, 4H, *J* = 8.0 Hz, Ar-H), MS (145 °C): *m*/*z* = 367 (M^+, 19%), 267 (100%), 324 (45%).

4-(4-(Phenylaminocarbonyl)benzoyl)benzoic acid methyl ester (**3c**)

Yield: 53%. Mp 242–243 °C. IR: $\nu_{max.}$ = 3280, 1730, 1650, 1600, 1540, 1445, 1280, 910, 830 cm^{-1}. ^{1}H-NMR (d_6-DMSO): δ = 3.91 (s, 3H, OCH_3), 7.11–7.80 (m, 5H, Ar-H), 7.87–7.89 (m, 4H, Ar-H), 8.12 & 8.14 (d, 4H, *J* = 8.4 Hz, Ar-H), 10.43 (s, 1H, NH). MS (207 °C): *m*/*z* = 359 (M^+, 61%), 267 (100%).

4-(4-(Dicyclohexylaminocarbonyl)benzoyl)benzoic acid methyl ester (**3d**)

Yield: 54%. Mp 151–152 °C. IR: $\nu_{max.}$ = 2930, 2840, 1720, 1650, 1600, 1430, 1280, 1100, 710 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ = 1.02–1.27 (br d, 6H, cyclohexyl-H), 1.60–1.83 (m, 12H, cyclohexyl-H), 2.62 (br s, 2H, cyclohexyl-H), 3.05 (s, 1H, cyclohexyl-H), 3.29 (s, 1H, cyclohexyl-H), 3.97 (s, 3H, OCH_3), 7.41 & 8.16 (d, 4H, *J* = 8.0 Hz, Ar-H), 7.82 & 7.85 (d, 4H, *J* = 8.4 Hz, Ar-H).

4-(4-(Phenoxybenzoyl)benzoic acid methyl ester (**5**)

To a suspension of monomethyl terephthalate (910 mg, 5.00 mmol) in anhydrous dichloromethane (30 mL) and few drops of anhydrous dimethylformamide, oxalyl chloride (0.47 mL, 5.50 mmol) was added. The mixture was stirred for 2 h at room temperature. The solvent was evaporated under reduced pressure. The crude acid chloride was dissolved in anhydrous carbon disulfide (30 mL) and the resulting solution was added dropwise to a mixture of diphenyl ether (850 mg, 5.00 mmol) and $AlCl_3$ (1.67 g, 12.5 mmol) in anhydrous carbon disulfide (50 mL) at ambient temperature. The mixture was refluxed for 1 h, and then poured onto ice. The product was extracted with chloroform (4 × 50 mL). The combined organic layers were washed with water and brine and dried over $MgSO_4$. After evaporation of the solvent the residual solid was purified by recrystallization from hexane-ethyl acetate. Yield 1.20 g (72%) of white crystals. Mp 167–168 °C. IR: $\nu_{max.}$ = 2920, 1715, 1635, 1600, 1580, 1480, 1430, 1300, 1250, 1160, 1100, 930, 840, 750, 690 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ = 3.96 (s, 3H, OCH_3), 7.03 (d, 2H, *J* = 8.8 Hz, Ar-H), 7.08–7.42 (m, 5H, Ar-H), 7.79–7.82 (m, 4H, Ar-H), 8.13 (d, 2H, *J* = 8.4 Hz, Ar-H).

General procedure for the synthesis of carboxylic acids **4a**–**4d** *and* **6**

A mixture of the ester **3a**–**3d** (1.00 mmol) and K_2CO_3 (3.00 mmol) in methanol/water (9:1) was refluxed for 1 h. The solution was cooled, diluted with water and acidified with hydrochloric acid (10%) to pH 5. The precipitated acid was filtered, washed thoroughly with water and dried. The crude product was purified by recrystallization from the solvent indicated in the individual procedures.

4-(4-(tert-Butylaminocarbonyl)benzoyl)benzoic acid (**4a**)

Recrystallized from ethyl acetate, yield: 92%. Mp 232–233 °C. IR: $\nu_{max.}$ = 3320, 3040, 2960, 1680, 1650, 1540, 1410, 1260, 1210, 1100, 930, 870, 800, 720 cm^{-1}. ^{1}H-NMR (d_6-DMSO): δ = 1.40 (s, 9H, $C(CH_3)_3$), 7.79 & 7.83 (d, 4H, *J* = 8.4 Hz, Ar-H), 7.95 & 8.11 (d, 4H, *J* = 8.0 Hz, Ar-H), 7.99 (s, 1H, NH). MS (188 °C): *m*/*z* = 325 (M^+, 40%), 253 (100%), 270 (41%).

4-(4-(Diisopropylaminocarbonyl)benzoyl)benzoic acid (**4b**)

Recrystallized from hexane-ethyl acetate, yield: 89%. Mp 229–30 °C. IR: $\nu_{max.}$ = 3465, 2940, 2900, 1710, 1680, 1650, 1590, 1450, 1340, 1270, 920, 830 cm^{-1}. ^{1}H-NMR (d_6-DMSO): δ = 1.12 (br s, 6H, $CH(CH_3)_2$), 1.45 (br s, 6H, $CH(CH_3)_2$), 3.63 (m, 2H, $CH(CH_3)_2$), 7.47 & 7.80 (d, 4H, *J* = 8.4 Hz, Ar-H), 7.85 & 8.11 (d, 4H, *J* = 8.2 Hz, Ar-H). MS (151 °C): *m*/*z* = 353 (M^+, 21%), 253 (100%), 310 (49%).

4-(4-(Phenylaminocarbonyl)benzoyl)benzoic acid (**4c**)

Recrystallized from ethyl acetate, yield: 90%. Mp 297–298 °C. IR: $\nu_{max.}$ = 3280, 3050, 2900, 1710, 1680, 1650, 1590, 1450, 1340, 1270, 920, 830 cm^{-1}. ^{1}H-NMR (d_6-DMSO): δ = 7.11–7.81 (m, 5H, Ar-H), 7.87 & 8.13 (d, 4H, *J* = 8.4 Hz, Ar-H), 7.89 & 8.12 (d, 4H, *J* = 8.4 Hz, Ar-H), 10.48 (s, 1H, NH). MS (270 °C): *m*/*z* = 345 (M^+, 73%), 253 (100%), 149 (53%), 254 (47%).

4-(4-(Dicyclohexylaminocarbonyl)benzoyl)benzoic acid (**4d**)

Recrystallized from hexane-ethyl acetate, yield: 95%. Mp 256–257 °C. IR: $\nu_{max.}$ = 2900, 2820, 1700, 1650, 1580, 1490, 1250, 920, 850 cm^{-1}. ^{1}H-NMR (d_6-DMSO): δ = 1.07–1.70 (m, 18H, cyclohexyl-H), 3.16–3.27 (br d, 4H, cyclohexyl-H), 7.44 & 7.80 (d, 4H, *J* = 8.2 Hz, Ar-H), 7.84 & 8.11 (d, 4H, *J* = 8.4 Hz, Ar-H). MS (177 °C): *m*/*z* = 433 (M^+, 14%), 253 (100%), 149 (34%).

4-(4-Phenoxybenzoyl)benzoic acid (**6**)

Recrystallized from ethyl acetate, yield 270 mg (85%). Mp 232–33 °C. IR: $\nu_{max.}$ = 3020, 1680, 1630, 1580, 1480, 1250, 920, 840, 740 cm^{-1}. ^{1}H-NMR (d_6-DMSO): δ = 7.09 & 7.80 (d, 4H, *J* = 8.8 Hz, Ar-H), 7.16–7.50 (m, 5H, Ar-H), 7.76 & 8.06 (d, 4H, *J* = 8.2 Hz, Ar-H).

Enzyme inhibition tests

Preparation of tissue

Rat prostatic enzyme was prepared according to the method of Liang et al. [16] with slight modifications [17]. Male rats were killed and prostates were taken within 5 min and put in ice cold 0.9% NaCl solution. All the following operations were performed at 0–4 °C. The prostates were dissected free from fat and connective tissue, cut into pieces and weighed. Per 1 g of tissue, 3 mL of 20 mM phosphate buffer, pH 6.5, containing 0.32 mM sucrose, and 1 mM DTT were added. The tissue was homogenized by 10-s strokes at 20,500 rpm of an ultraturrax (IKA) in 60-s intervals, filtered through cheesecloth, and centrifuged for 60 min at 105,000 *g*. The pellet obtained was resuspended in phosphate buffer. Centrifugation was repeated, the final pellet resuspended in a minimum volume of phosphate buffer and stored in 300 µL portions at –70 °C. The 105,000 *g* pellet contains nuclei, mitochondria, and microsomes and is referred to as the enzyme preparation. The protein content was determined and was in the range of 15–25 mg/mL. Human prostatic tissue from BPH patients was processed in the same way using citrate buffer, pH 5.5.

Incubation procedure

The assay was performed as described [16] with modifications [17]. All values were run in duplicate. The incubation was carried out for 30 min at 37 °C in a total volume of 250 µL. In the case of rat enzyme preparation phosphate buffer (40 mM, pH 6.6 for type 1 and pH 5.5 for type 2) and in the case of human enzyme preparation citrate buffer (40 mM, pH 5.5) was used. The incubation mixture contained approximately 250 µg rat protein (200–300 µg human protein), 200 µM NADPH (human enzyme, 100 µM NADPH), 0.21 µM T including 100 nCi [1β,2β-^{3}H]T and 2% DMSO with or without test compound (10 µM). Above 60% inhibition, three concentrations were chosen for the determination of IC_{50} values. The reaction was started by adding the prostatic enzyme preparation and terminated by addition of 50 µL NaOH (10 M). The steroids were extracted with diethyl ether (500 µL) by shaking for 10 min. Subsequent centrifugation was performed for 10 min at 4000 rpm. The water layer was frozen and the ether layer was decanted in fresh tubes and evaporated to dryness.

DU 145-assay (human type 1 inhibition)

The assay was performed according to the procedure of Guarna et al. [18] with modifications [19,20]. The inhibitory potency of the compounds was determined by monitoring the conversion of the tritiated substrate androstenedione (5 nM) to androstanedione during an incubation period of 5 h. A day before the experiment, DU 145 cells were seeded in a 24-multiwell-plate at a density of 170,000 cells/well and allowed to become adherent overnight. Compounds to be tested were dissolved in DMSO and 5 µL of each was added to the cells in a final volume of 0.5 mL complete medium. Inhibitors were screened at a concentration of 10 µM. As control of conversion (typically about 35% under these conditions) served a triplicate of wells without inhibitors and as positive control for inhibition finasteride (80, 60, 40, 20 nM) was used. After the 5 h incubation period in 5% CO_2 at 37 °C, the medium samples were extracted twice with 1 mL diethyl ether and the steroids were separated by HPLC. Results are expressed as amount of formed androstanedione as percentage of control values.

HPLC procedure

Steroid separation was performed [17] similarly to the method of Cook et al. [21]. The steroids were dissolved in 50 µL methanol and 25 µL was injected into the computer-controlled HPLC system, which was checked before using labelled reference controls. Radioactivity was measured with a Berthold LB 506C monitor, using methanol/water (55/45, *w*/*w*) for T and DHT with a flow of 0.4 mL/min and an additive flow of 1.0 mL for scintillator; baseline separation of T and DHT was achieved within 20 min. For the steroids androstenedione and dihydroandrostenedione methanol/water (50/50, *w*/*w*) was used.

Calculation procedure

The amount of DHT formed was calculated (% DHT). The zero value was subtracted from the control (*cv*) and inhibition (*iv*) values (cv_{corr} and iv_{corr}). Inhibition (*I*) was calculated using the following equation: $\% I = (1 - iv_{corr}/cv_{corr})100$.

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