



Research Article

Synthesis of Novel Androstane-N-Cyclohexyl-17-Carboxamides, and Their Effect on the 5 α -Reductase Isoform 2, the Androgen Receptor, and Androgen-Dependent Glands

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Article Info

Article History:

Received: 28 May 2021

Accepted: 26 November 2021

Published: 29 November 2021

Keywords:

-Androstane analogues
-Androgen-dependent
Afflictions
-Prostate gland

Abstract

Background: Benign Prostatic Hyperplasia (BPH), and Prostate Cancer (PCa) are androgen-dependent diseases. PCa is associated with excessive signalling of the androgen receptor (AR) due to the binding of 5 α -dihydrotestosterone (5 α -DHT) and testosterone (T). BPH is related to high levels of 5 α -DHT, biosynthesized from T by 5 α -reductase (5RD5A). The inhibition of 5RD5A and the blockage of AR are targets for their treatment. In this study, the synthesis and determination of biological activity of the new N-cyclohexyl-3 β -hydroxyandrost-5,16-diene-17-carboxamide (**6**), N-cyclohexyl-3-oxoandrost-4,6,16-triene-17-carboxamide (**7**), and N-cyclohexyl-3-oxoandrost-4,16-diene-17-carboxamide (**8**) were carried out to find new drugs to improve these afflictions.

Methods: The synthesis of **6** to **8** was confirmed by spectroscopic and spectrometric analyses. Competitive binding assays determined the affinity of **6** to **8** to the AR. The inhibitory activity of 5RD5A isoform 2 (5RD5A2) (IC₅₀) was established by the conversion of [³H]-T to [³H]-5 α -DHT and it was compared with finasteride (FIN). The pharmacological effect of **6** to **8** was determined on the weight of the prostate and seminal vesicles glands of castrated hamsters treated with T, and on the diameter size of their flank organs.

Results: Compounds **7** and **8** bound lightly (*ca.* 15 %) to AR. Comparing to FIN (IC₅₀ = 8.5 nM), **6** to **8** (IC₅₀ = 0.169, 0.105 and 0.155 nM, respectively) showed higher potency as inhibitors of 5RD5A2. Compound **6** decreased the prostate and seminal vesicles weight, as well as the hamsters' diameter flank organs. However, **7** only decreased the diameter of flank organs. Surprisingly, **8** increased these pharmacological parameters.

Conclusion: Androstane-17-carboxamide **6** is a 5RD5A2 inhibitor that reduces the weight of androgen-dependent glands such as the prostate, suggesting it could be a lead for new drugs to treat BPH and PCa.

Introduction

Androgens play a crucial role in regulating male phenotype. For example, androgens mature and maintain male's sexual organs. Testosterone (T) is the primary androgen produced by endocrine pathways involving the hypothalamus, pituitary, adrenal cortex, and testicles. T is a primary source of the testicular secretion.¹ Once T reaches its target cells in the prostate, three 5 α -reductase (SRD5A) isozymes (encoded by the SRD5A1, SRD5A2, and SRD5A3

genes), reduce T to 5 α -dihydrotestosterone (5 α -DHT).² T binds to the androgen receptor (AR) (a steroid hormone nuclear receptor that is a ligand-dependent transcription factor) to exert androgenic responses such as prostatic cell proliferation and growth of the prostate.³ This process is considered a mechanism of androgenic amplification because the produced 5 α -DHT is 2-5-fold higher in prostate cells and activates AR signalling 10-fold compared to T.^{2,3}

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Benign prostatic hyperplasia (BPH), and Prostate cancer (PCa) are public health problems in aged men related to prostatic cell proliferation. PCa is the sixth leading cause of cancer death among men worldwide.⁴ In 2020, 414259 new cases and 375304 deaths were associated with this type of cancer.⁴

BPH is one of the most common benign diseases in aged men, leading to a considerable number of surgeries resulting from the clinical manifestations attributed to prostate growth.⁵ BPH and PCa are androgen-dependent illnesses because BPH is related to high levels of 5 α -DHT. PCa is connected to both T and 5 α -DHT. The inhibition of 5RD5A is a strategic target to treat BPH.^{5,6} The blockage of AR is a strategic target for PCa treatment.³

Several steroidal and non-steroidal compounds have been reported as 5RD5A inhibitors, or antiandrogens. The steroidal compounds have attracted more attention as a template for the design of new drugs because they resemble the structure of the endogenous hormones. The 4-azasteroids finasteride and dutasteride are 5RD5A inhibitors approved by the FDA to treat BPH.⁶ They decrease intra-prostatic 5 α -DHT levels, reduce the risk of urinary retention and the need for surgery.² However, they show adverse events that restrict their use.^{5,6} The AR antagonists cyproterone acetate, and the non-steroidal flutamide and bicalutamide,³ are frequently used for inoperable PCa to prevent initial flare-up induced by the treatment with luteinizing hormone-releasing hormone agonists. Nevertheless, these antagonists also show adverse events. For these reasons, new and more potent drugs with potentially less adverse effects are necessary.

Previous studies on steroidal compounds^{7,8} led to the establishment of structural requirements for inhibiting 5RD5A. On the one hand, an electrophilic moiety at A or B rings such as an α,β - or $\alpha,\beta,\gamma,\delta$ -unsaturated carbonyl group, would act as a pharmacophore being reduced by 5RD5A. On the other hand, a bulky lipophilic group at C-17, such as an amide (as is seen in finasteride and dutasteride), would be an auxophore reinforcing the union of the inhibitor to a hydrophobic region at the catalytic site of the enzyme, conferring it also metabolic stability.^{7,8} According to Kurup *et al.*,⁷ a cyclohexyl group substituting the amide at C-17 would be beneficial to achieve this goal. Steroidal compounds should have a 3-oxo (3-keto) function to antagonize the AR. The role of this function is to establish electrostatic interactions at the ligand-binding domain (LBD).^{9,10}

This study is focused on the synthesis of N-cyclohexyl-17-carboxamides, having a steroidal androstane skeleton, and investigates their biological activity. These compounds either lack of, or have an α,β - or $\alpha,\beta,\gamma,\delta$ -unsaturated ketone as pharmacophore and a N-cyclohexyl amide group at C-17 as auxophore. Additionally, their lipophilicity and standard molar Gibbs free energy of transfer was calculated to establish relationships between their bioactivity and physicochemical properties that help understand their behaviour.

Materials and Methods

Materials

Reagents and solvents for the synthesis of compounds were analytical grades. For biological activity assays, [1,2,6,7-³H]-testosterone ([³H]-T) (95 Ci/mmol), and [17 α -methyl-³H]-mibolerone ([³H]-MIB) (70–87 Ci/mmol) (Perkin Elmer Life and Analytical Sciences) were used. [³H]-T was purified by high performance thin layer chromatography (HPTLC) on Keisegel 60 F₂₅₄ plates (Merck) eluting in the solvents recommended by the manufacturer. Radio inert testosterone (T), 5 α -dihydrotestosterone (5 α -DHT), and mibolerone (MIB) (Steraloids) as well as NADPH (Sigma Chemical Co.), triamcinolone (Sigma-Aldrich), hydroxyapatite (Bio-Rad), and Ultima Gold scintillation liquid (Packard*) were also used. Finasteride (FIN) was extracted from Proscar® (Merck, Sharp & Dohm), as Trapani *et al.*¹¹ indicated.

Melting points were determined on a Büchi SMP-20 apparatus and are uncorrected. UV spectra were recorded in MeOH using a Shimadzu UV1700 spectrophotometer. IR spectra were recorded on a Perkin-Elmer FT-IR RX1 spectrometer in KBr. 1 and 2D NMR spectra were acquired on a Bruker Avance 600 or 400 MHz spectrometer at 600 or 400 MHz (¹H), or 125 or 100 MHz (¹³C) in 1,4-dioxane-d₈ (99.0 %) or CDCl₃ (99.9 %), and TMS as internal standard. MestRE-C@v 4.8.6.0 program (trial version) processed the FIDs. FAB⁺-MS was recorded on a DFS (Double Focus Sector) Thermo-Electron spectrometer, bombarding the sample with caesium ions (50 °C) in a 3-nitrobenzyl alcohol matrix; EI-MS on DFS Thermo-Electron mass spectrometer with direct insertion probe and heating rate 100 °C/min. ESI-MS was recorded on an MS-2010 mass spectrometer (Shimadzu) by direct insertion, and the probe was operated in positive scan mode: CDL, 300 °C; block at 240 °C; flow gas (N₂) at 1.5 L/min; CDL voltage, 150.0 kV; Q array voltage RF 150 V; detector voltage, 1.5 kV; and scan range m/z 100 - 800. Open column chromatography (CC) was carried out on Si-gel 60 (0.069 - 0.200 mm, Merck*). Thin layer chromatography (TLC) was conducted on Si-gel 60 F₂₅₄ coated aluminium backed foils (Merck*), and the spots were visualized by UV light or staining with CoCl₂ (1 %, dissolved in H₂SO₄, 2 N) and heating (110 °C).

Synthesis of compounds

Synthesis of 3 β -hydroxyandrosta-5,16-diene-17-carboxylic acid (2).

Compound **2** was synthesized according to Zhu *et al.*¹² with some modifications. A solution of sodium hydroxide (1.46 g, 36.5 mmol) in water (12.5 mL) was cooled *ca.* -5 °C, then bromine (1.5 g, 9.5 mmol, 0.5 mL) was added dropwise, stirring, and cooling *ca.* 0 °C. The resulting mixture was diluted with cold dioxane (8.3 mL) and kept *ca.* 0 °C under darkness until required.¹²

A solution of 16-dehydropregnenolone acetate (**1**) (100 mg, 0.28 mmol) in dioxane (5.0 mL) and water (1.1 mL) was cooled between 8 to 10 °C. Cold sodium hypobromite solution (10.0 mL) was added dropwise, and the resulting

mixture was stirred *ca.* 18 h between 8 to 10 °C. The remaining sodium hypobromite in the reaction mixture was destroyed with sodium hyposulfite (10.0 %, 10 mL). The mixture was refluxed 15 min, and while still hot, it was acidified (pH 5 - 6) with HCl (5 %, *ca.* 1.0 mL). The formed precipitate was filtrated on the *vacuum*, washed with water, and dried. The solid was recrystallized from an *n*-hex:EtOAc (7:3) mixture to afford **2** (53 mg; 60 % yield) as a white powder; mp: 255 - 257 °C; IR (KBr) ν_{\max} 3450 (O-H), 2965 (C-H), 2934 (C-H), 2864 (C-H), 1693 (C=O), 1606 (C=C), 1041 (C-O) cm^{-1} . $^1\text{H-NMR}$ (400 MHz, 1,4-dioxane-*d*8) δ ppm 1.43 (3H, *s*, H-18), 1.56 (3H, *s*, H-19), 5.82 (1H, *d*, $^3J = 4.4$ Hz, H-6 vinylic), 7.21 (1H, *s*, H-16 vinylic), 11.36 (1H, *s*, -COOH). $^{13}\text{C-NMR}$ (100 MHz, 1,4-dioxane-*d*8) δ ppm 15.9 (C-18), 19.3 (C-19), 71.3 (C-3), 120.8 (C-6 vinylic), 142.5 (C-5), 143.6 (C-16 vinylic), 147.4 (C-17), 165.6 (-COOH). EI-MS m/z : 316 (M^+), 298 ($\text{M}^+ - \text{H}_2\text{O}$).

Synthesis of 3 β -acetoxyandrosta-5,16-diene-17-carboxylic acid (**3**)

Compound **3** was synthesized according to López-Lezama *et al.*¹³ Acetic anhydride (1.2 mL) was added to a solution of 3 β -hydroxyandrosta-5,16-diene-17-carboxylic acid (**2**) (100 mg, 0.316 mmol) in pyridine (0.6 mL), letting stirring overnight at room temperature. Water (20.0 mL) was added, and the formed precipitate was filtrated on the *vacuum* and recrystallized from glacial acetic acid to afford **3** (91 mg; 80 % yield). White powder; mp: 240 - 244 °C; IR (KBr) ν_{\max} 3434 (O-H), 2938 (C-H), 2896 (C-H), 1734 (C=O), 1680 (C=O), 1603 (C=C), 1254 (C-O), 1035 (C-O) cm^{-1} . $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ ppm 0.96 (3H, *s*, H-18), 1.06 (3H, *s*, H-19), 2.03 (3H, *s*, CH_3COO), 3.72 (1H, *m*, H-3), 5.38 (1H, *d*, $^3J = 4.3$ Hz, H-6 vinylic), 6.93 (1H, *s*, H-16 vinylic). $^{13}\text{C-NMR}$ (100 MHz, CDCl_3) δ ppm 15.7 (C-18), 19.2 (C-19), 21.4 (CH_3COO), 73.8 (C-3), 121.8 (C-6), 140.2 (C-5), 145.8 (C-16), 148.9 (C-17), 166.8 (C-20), 170.5 (CH_2COO). FAB⁺-MS m/z : 359 [$\text{M} + \text{H}$]⁺, 316 [$\text{M}^+ - \text{CH}_2 = \text{C} = \text{O}$], 298 [$\text{M}^+ - \text{CH}_3\text{COOH}$].

Synthesis of N-cyclohexyl-3 β -acetoxyandrosta-5,16-diene-17-carboxamide (**5**)

To a solution of 3 β -acetoxyandrosta-5,16-diene-17-carboxylic acid (**3**) (100 mg, 0.28 mmol) in CH_2Cl_2 under a stream of dry nitrogen, SOCl_2 (387 μL) was added and stirred 6 h at room temperature to form **4**. Cyclohexylamine (780 μL) was added dropwise, forming a precipitate and gas (HCl) trapped on a sodium hydroxide trap. The precipitate was dissolved in CH_2Cl_2 (10.0 mL), poured into a separating funnel, and washed with brine (1 %, 3 x 10.0 mL), and water (3 x 10.0 mL). The combined organic phases were dried over anhydrous sodium sulphate, and the solvent was removed by *vacuum* affording a yellow solid which was recrystallized from *n*-hex:EtOAc (1:1) to give **5** (86 mg; 70 % yield). White powder; mp: 154 - 156 °C; IR (KBr) ν_{\max} 3317 (N-H), 2934 (C-H), 2855 (C-H), 1734 (C=O), 1638 (N-C=O), 1526 (N-H, δ), 1246 (C-O),

1033 (C-O) cm^{-1} . $^1\text{H-NMR}$ (600 MHz, CDCl_3) δ ppm 1.01 (3H, *s*, H-18), 1.14 (3H, *s*, H-19), 2.04 (3H, *s*, CH_3COO), 3.78 (1H, *m*, H-3), 5.98 (1H, *d*, $^3J = 6.1$, H-6 vinylic), 6.27 (1H, *s*, H-16 vinylic), 6.74 (1H, *br s*, NHCO). $^{13}\text{C-NMR}$ (150 MHz, CDCl_3) δ ppm 16.4 (C-18), 19.3 (C-19), 21.5 (CH_3COO), 73.8 (C-3), 122.1 (C-6), 134.8 (C-5), 140.2 (C-16), 151.0 (C-17), 165.1 (C-20), 170.6 (CH_2COO). FAB⁺-MS m/z : 440 [$\text{M} + \text{H}$]⁺.

Synthesis of N-cyclohexyl-3 β -hydroxyandrosta-5,16-diene-17-carboxamide (**6**)

A solution of N-cyclohexyl-3 β -acetoxyandrosta-5,16-diene-17-carboxamide (**5**) (100 mg, 0.25 mmol) in MeOH (20.0 mL) was heated at 40 °C until dissolved. NaOH (2 %, 7.0 mL) was added, and the mixture was refluxed 20 min while stirring. Water (20.0 mL) was added, and the precipitated reaction product was filtrated on the *vacuum* and washed with water affording a white solid. The solid was purified by CC eluting with *n*-hex:EtOAc (7:3) mixture, and recrystallization from *n*-hex:EtOAc (1:1) giving **6** (72 mg; 80 % yield) as white crystals; mp: 186 - 188 °C; IR (KBr) ν_{\max} 3433 (O-H), 3306 (N-H), 2928 (C-H), 2855 (C-H), 1637 (N-C=O), 1542 (N-H, δ) cm^{-1} . $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ ppm 1.00 (3H, *s*, H-18), 1.13 (3H, *s*, H-19), 3.78 (1H, *m*, H-3), 5.97 (1 H, *d*, $^3J = 4.4$, H-6 vinylic), 6.26 (1 H, *s*, H-16 vinylic), 6.71 (1H, *br s*, NHCO). $^{13}\text{C-NMR}$ (100 MHz, CDCl_3) δ ppm 16.6 (C-18), 19.5 (C-19), 71.9 (C-3), 121.3 (C-6 vinylic), 135.0 (C-5), 141.4 (C-16 vinylic), 151.0 (C-17), 165.2 (C-20). FAB⁺-MS m/z : 399 [$\text{M} + 2\text{H}$]²⁺.

Synthesis of N-cyclohexyl-3-oxoandrosta-4,6,16-triene-17-carboxamide (**7**)

A mixture of N-cyclohexyl-3 β -hydroxyandrosta-5,16-diene-17-carboxamide (**6**) (100 mg, 0.25 mmol), Li_2CO_3 (308 mg, 4.17 mmol), LiBr (206 mg, 2.37 mmol) and DMF (1.4 mL) was stirred 15 min, and heated *ca.* 80 °C. A bromine (0.3 mL) solution in dioxane (8.3 mL) was added dropwise (880 μL), and the mixture was refluxed *ca.* 80 °C, 3 h. Once the reaction was cooled at room temperature, and filtered on the *vacuum*, a solution of sodium bicarbonate and sodium hyposulfite (0.02 % each salt, 12.0 mL) was added to the filtrate provoking the appearance of a yellow precipitate. This precipitate was filtrated on the *vacuum* and washed with water. The solid was purified by preparative TLC eluting with *n*-hex:EtOAc (6:4) mixture affording **7** (69 mg; 70 % yield). White powder; mp: 175 - 178 °C; UV λ_{\max} (MeOH) nm: 282; IR (KBr) ν_{\max} 3356 (N-H), 2933 (C-H), 2855 (C-H), 1653 (C=O), 1646 (N-C=O), 1618 (C=C), 1523 (N-H, δ) cm^{-1} . $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ ppm 1.02 (3H, *s*, H-18), 1.15 (3H, *s*, H-19), 5.70 (1H, *s*, H-4 vinylic), 5.98 (1H, *d*, $^3J = 4.0$, H-6 vinylic), 6.15 (1H, *s*, H-16 vinylic), 6.26 (1H, *dd*, $^3J = 3.2, 1.7$, H-7 vinylic). $^{13}\text{C-NMR}$ (300 MHz, CDCl_3) δ ppm 16.3 (C-18), 16.4 (C-19), 123.9 (C-4 vinylic), 128.4 (C-6 vinylic), 133.6 (C-7 vinylic), 140.3 (C-16 vinylic), 151.0 (C-17), 163.5 (C-5), 164.8 (C-20), 199.5 (C-3). (+) ESI-MS m/z : 394.15 [$\text{M} + \text{H}$]⁺.

Synthesis of N-cyclohexyl-3-oxoandrosta-4,16-diene-17-carboxamide (8)

A solution of N-cyclohexyl-3 β -hydroxyandrosta-5,16-diene-17-carboxamide (**6**) (100 mg, 0.25 mmol), N-methyl-4-piperidone (360 μ L) and toluene (10.0 mL) was heated under reflux using a Dean-Stark trap. After removed 10.0 mL of distillate, Al(O-*i*-Pr)₃ was added (94 mg, 0.46 mmol), and the mixture was refluxed 5 h under a stream of dry nitrogen. The solvent was evaporated under the *vacuum*, and water (5.0 mL) and EtOAc (10.0 mL) were added, keeping stirring 1 h. The aqueous layer was separated from this mixture, and it was washed with EtOAc (3 x 10.0 mL). The combined EtOAc phases were successively washed with HCl (5 %, 3 x 10.0 mL), NaHCO₃ (10 %, 3 x 10.0 mL), and water (3 x 10.0 mL), and dried over anhydrous sodium sulphate. Once the solvent was removed under *vacuum*, a yellow solid was produced, purified by preparative TLC eluting with *n*-hex:EtOAc (6:4) affording **8** (70 mg; 70 % yield). White powder; mp: 165 - 168 °C; UV λ_{\max} (MeOH) nm: 239; IR (KBr) ν_{\max} 3320 (N-H), 2918 (C-H), 2855 (C-H), 1648 (C=O), 1637 (N-C=O), 1528 (N-H, δ) cm⁻¹. ¹H-NMR (400 MHz, CDCl₃) δ ppm 1.00 (3H, s, H-18), 1.04 (3H, s, H-19), 5.73 (1H, s, H-4 vinylic), 6.23 (1H, s, H-16 vinylic), 6.76 (1H, br s, -NH). ¹³C-NMR (100 MHz, CDCl₃) δ ppm 16.4 (C-18), 17.2 (C-19), 124.0 (C-4 vinylic), 134.2 (C-16 vinylic), 150.9 (C-17), 164.9 (C-20), 170.3 (C-5), 199.5 (C-3). (+) ESI-MS *m/z*: 396.20 [M+H]⁺.

Determination of the antiandrogenic activity and inhibition of 5 α -reductase *in vitro*

Animals and tissues

Adult Wistar rats (8 months old; *ca.* 500 g) were used to isolate cytosol to perform binding assays to the androgen receptor. Adult male golden hamsters (2.5 months old; 150 to 200 g) were used to evaluate the *in vivo* effect on the weight of the prostate, and seminal vesicles as well as the size of flank organs. Animals were bred at the Animal Care Facility at the Universidad Autónoma Metropolitana-Xochimilco, and they were housed under controlled temperature 22 °C, and humidity 45 to 55 %, as well as light and dark cycles of 12 hours. Food and water were provided *ad libitum*.

Human prostate was obtained from a man who deceased of diabetes, and renal insufficiency at General Hospital, Mexico City. This tissue was used as a source of 5RD5A. Once the prostate was extirpated, it was rinsed with NaCl (150 mM), chilled in ice-cold, and stored (- 20 °C) until its use.

Isolation of cytosol from rat prostate as a source of androgen receptor

The isolation of cytosol from rat prostate was carried out as described by Bratoeff *et al.*¹⁴ All procedures were performed on a bed of chopped ice at 0 °C, unless otherwise is specified. Rat prostates were homogenized by a tissue homogenizer using TEMD buffer (1 volume) containing protease inhibitors (2 mM PMSE, 10 μ g antipain/mL,

5 mM leupeptin). Then, they were centrifuged 60 min (140000 \times g) in an SW 60 Ti rotor (Beckman Instruments). The final protein concentration (6 mg protein/200 μ L) of the supernatant containing cytosol was quantified by Bradford's method. Cytosol was stored at - 70 °C until its use.

Androgen receptor binding assays

The affinity of the androstane N-cyclohexyl-17-carboxamides and synthesis intermediates to bind to androgen receptor was carried out by an *in vitro* competitive binding assay between the radioactive ligand [³H]-MIB and the test compounds, according to Bratoeff *et al.*¹⁴ Tubes containing [³H]-MIB (1 nM) with the addition of MIB or androstane analogues (10⁻¹⁰ to 10⁻⁷ M), dissolved in acetone or ethanol), or without them were prepared. Additionally, triamcinolone (200 nM, dissolved in ethanol) was added to avoid the binding of MIB to glucocorticoid or progesterone receptors. Once the solvent of these samples was evaporated by *vacuum*, aliquots of cytosol (5 mg protein/200 μ L) plus TEMD buffer containing protease inhibitors (pH 8; 300 μ L) were added, and the resultant mixture was incubated at 4 °C, 18 h. Hydroxyapatite let separate bound MIB to AR from free [³H]-MIB and as result of this process, an ethanol fraction was obtained. Scintillation liquid (10.0 mL) was added to ethanol fraction (0.8 mL), and its radioactivity was counted in a Tri-Carb 2100 TR scintillation counter (Packard Instruments).

The concentration of each androstane compound necessary to displaced [³H]-MIB from AR at the fifth percentage (IC₅₀) was determined from graphs of concentration *vs* the percentage of binding.

Isolation of 5 α -reductase isoform 2 from human prostate

The isolation of 5 α -reductase isoform 2 (5RD5A2) from human prostate was carried out as described by Bratoeff *et al.*¹⁴ with some modifications. All procedures were performed at 4 °C. The frozen prostatic tissue was thawed on ice and minced in buffer A (2 volumes; 20 mM sodium phosphate pH 6.5, supplemented with sucrose (0.32 M), and dithiothreitol (0.1 mM)). The tissue homogenate was centrifuged (1500 \times g; 60 min) in an SW 60 Ti rotor, and the resulting pellets were re-suspended in buffer A and stored (- 70 °C) until use. This suspension was the source of 5RD5A2, and its concentration (5 mg protein/mL) was quantified by Bradford's method.¹⁵

Inhibitory effect of androstane N-cyclohexyl-17-carboxamides and synthesis intermediates on 5 α -reductase isoform 2

The inhibitory activity of androstane N-cyclohexyl-17-carboxamides **6** to **8** and synthesis intermediates **2**, **3**, and **5** on the 5RD5A2 was established by quantifying the reduction of [³H]-T to [³H]-5- α -DHT, as described by Bratoeff *et al.*¹⁴ Tubes containing [³H]-T and six different concentrations (10⁻¹¹ to 10⁻³ M) of test compounds (androstane analogues or FIN, dissolved in DMSO) were

incubated in a reaction medium (1.0 mL final volume, dithiothreitol (1 mM), phosphate buffer (40 mM) pH 6.5, and NADPH (2 mM)). The reaction was started by adding the human enzymatic fraction (134 µg of protein) letting incubate 60 min at 37.5 °C and it was stopped by adding dichloromethane (1.0 mL). This procedure without the addition of the enzyme was performed as a control. Each reaction mixture was shaken 1 min (Type 16700 mixer, Barnstead Thermoline) to extract the produced [³H]-DHT, and after separation of phases, the dichloromethane phase was collected to an individual tube. The aqueous phase was washed with fresh dichloromethane (4 x 1.0 mL), and after pool organic phases, their solvent was evaporated to dryness by a nitrogen current. The residue was dissolved in methanol (50 µL) and spotted on a HPTLC plate along with T and 5-α-DHT as standards. The plates were developed in chloroform:acetone (9:1) mixture (3 times) and air-dried. 5-α-DHT was visualized staining with phosphomolybdic acid and T, by UV light (254 nm). The radioactivity of those zones in the plate with a retention factor (R_f) identical to the standards were scanned and quantified as [³H]-T or [³H]-5-α-DHT (Bioscanner AR2000, Bioscan). The percentage of produced [³H]-5-α-DHT was calculated considering the total radioactivity of the plate. The same procedure was performed for control incubations.

Six different concentrations (10^{-11} - 10^{-3} M) of test compounds were evaluated in duplicate to calculate the concentration that inhibits 50 % of the enzyme's activity (IC_{50}). From curves plotting the evaluated concentration vs. response, IC_{50} was calculated.

Anti-androgenic in vivo effect of androstane N-cyclohexyl-17-carboxamides on prostate, seminal vesicles, and flank organs of male hamsters

The *in vivo* effect of androstane N-cyclohexyl-17-carboxamides **6** to **8** on the weight of the prostate and seminal vesicles, and on the size of flank organs of castrated-T treated hamsters was determined as described by Cabeza *et al.*¹⁶ Castrations were carried out under isoflurane anaesthesia 30 days prior to the assays. The castrated hamsters were randomly separated in groups of four; three of them were the controls and they were dosed subcutaneously (sc) with sesame oil (200 µL, vehicle) (group 1), T (1 mg/kg body weight (bw)) dissolved in vehicle (group 2), and T (1 mg/kg bw) + finasteride (FIN) (1 mg /kg bw) dissolved in vehicle (group 3), respectively. From the fourth to the sixth group, the test compounds (2 mg/kg bw dissolved in vehicle) + T (1 mg /kg bw) were dosed daily (6 d) sc. After the last administration (24 h), the hamsters were euthanized using CO₂ and the diameter of their flank organs was measured. Then, their prostates, and seminal vesicles were dissected and weighed. The weights of these organs were expressed as mg of organ per 100 g of body weight.

Statistical analysis

The data were analysed by one-way analysis of variance

(ANOVA), and Tukey's test for means comparison by using Real Statistics Resource Pack software (release 7.2).¹⁷

Determination of lipophilicity and permeability of androstane N-cyclohexyl-17-carboxamides and synthesis intermediates

The lipophilicity and permeability of the androstane N-cyclohexyl-17-carboxamides **5** to **8**, and synthesis intermediates **2** and **3** was determined as previously reported by Valencia-Islas *et al.*¹⁸ calculating their partition coefficient (P) and standard molar Gibbs free energies of transfer from water to *n*-octanol ($\Delta_t G^\circ$). Molinspiration® interactive Log P software (<http://www.molinspiration.com/cgi-in/properties>) let to calculate P. In turn, $\Delta_t G^\circ$ at 298.15 K was calculated using P values according to:

$$\Delta_t G^\circ = -RT \ln P$$

Where R: ideal molar gas constant (8.314 J mol⁻¹ K⁻¹), T: absolute temperature in kelvin.

Results and Discussion

PCa and BPH are public health problems in aged men related to prostatic cell proliferation caused by androgens.^{3,5} Mainly, BPH is related to high levels of 5α-DHT, which is biosynthesized from T by 5RD5A2, and it binds with more affinity to the AR than T.⁵ PCa is related to binding T and 5α-DHT to the AR and its excessive signalling, leading to cell proliferation and prostatic growth.³ The inhibition of 5RD5A2 and the blockage of AR are strategic targets for treating these illnesses.^{3,5,6}

This study identified some androstane N-cyclohexyl-17-carboxamides as antiandrogens or inhibitors of the 5RD5A2 with the potential to be new bioactive entities to treat androgen-dependent prostatic diseases. The lipophilicity of **6** to **8** and their Gibbs-free energy allowed us to understand the relationship with their biological properties.

The androstane carboxamides: N-cyclohexyl-3β-hydroxyandrosta-5,16-diene-17-carboxamide (**6**), N-cyclohexyl-3-oxoandrosta-4,6,16-triene-17-carboxamide (**7**), and N-cyclohexyl-3-oxoandrosta-4,16-diene-17-carboxamide (**8**) (Figure 1) were designed as potential 5RD5A2 inhibitors. These compounds either lack (as **6**), or have an α,β- or α,β,γ,δ-unsaturated ketone (as **7** and **8**, respectively) as pharmacophore. Besides, all of them bear an N-cyclohexyl amide group at C-17 as an auxophore. To our knowledge, compounds **5**, **7**, and **8** are described here for the first time. We have previously reported the synthesis of compound **6**.¹³ Additionally, the synthesis intermediates **2**, **3**, and **5** were also evaluated to establish possible structure-activity relationships.

Synthesis of compounds

According to Figure 1, the synthesis of compounds started from 16-dehydropregnenolone acetate (**1**) and the carboxylic acid **2** was synthesized by the bromoform reaction (Br₂/NaOH) on **1** (60 % yield). Under these reaction conditions, the acetate group's hydrolysis at

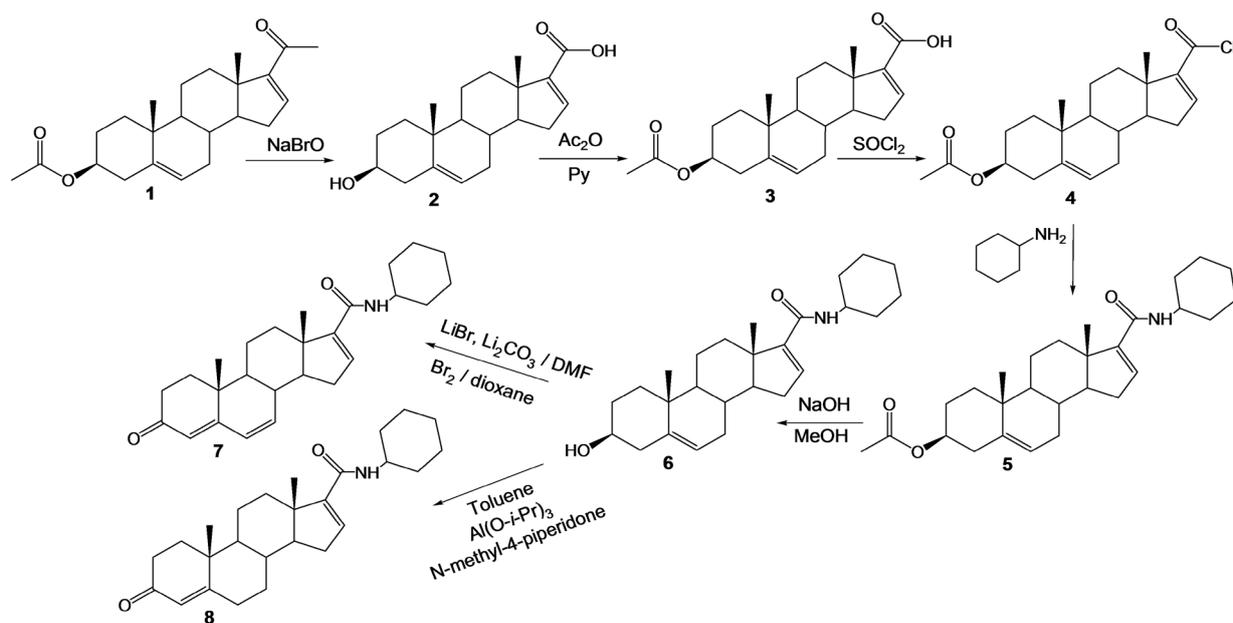


Figure 1. Synthetic route for the preparation of the androstane-N-cyclohexyl-17-carboxamides **6** to **8**.

C-3 was carried out, producing an alcohol, which was protected by acetylation giving compound **3** (80 % yield). The treatment of the carboxylic acid at C-17 of **3** with thionyl chloride (SOCl_2) in CH_2Cl_2 resulted in the *in-situ* formation of the acyl chloride **4**, which was reacted with cyclohexylamine to afford the amide **5** (70 % yield) that had the auxophoric group.

The basic hydrolysis (NaOH/MeOH) of the acetate group at C-3 of **5** led to the alcohol **6** (80 % yield), which was oxidized by two different methods; 1) Shapiro oxidation¹⁹ with bromine and $\text{LiBr}/\text{Li}_2\text{CO}_3$ as bases, afforded compound **7** (70 % yield) having an $\alpha,\beta,\gamma,\delta$ -unsaturated ketone (4,5-diene-3-one) as pharmacophore and, 2) Rupert Viktor Oppenauer oxidation²⁰ using N-methyl-4-piperidone as a hydride acceptor and $\text{Al}(\text{O}-i\text{-Pr})_3$ as a base afforded compound **8** (70 % yield) having an α,β -unsaturated ketone (4-ene-3-one) as pharmacophore. The structures of **5** to **8** were confirmed by spectroscopic and spectrometric analyses.

According to its molecular mass, the FAB^+ -MS spectrum of compound **5** had a *pseudo*-molecular ion $[\text{M}+\text{H}]^+$ at m/z 440 according to its molecular mass. The ^1H -NMR spectrum revealed a characteristic singlet for the amide proton at C-17 (δ_{H} 6.74 ppm). The ^{13}C -NMR showed the carbonyl amide signal group (δ_{C} 165.1 ppm). By the cross-peaks observed at its HSQC spectrum, the double bonds at C-6 (δ_{H} 5.99 / δ_{C} 129.1) and C-16 (δ_{H} 6.25 / δ_{C} 134.5) were established.

The (+) ESI-MS spectra of compounds **7** and **8** had *pseudo*-molecular ions $[\text{M}+\text{H}]^+$ at m/z 394.15 and 396.20, respectively, according to their molecular mass. IR spectra showed the disappearance of the alcohol band at C-3 and the emergence of a band at *ca.* 1647 cm^{-1} for a conjugated ketone to a double bond. The ^1H and ^{13}C NMR spectra of **7**, revealed the formation of the $\alpha,\beta,\gamma,\delta$ -unsaturated moiety

by signals corresponding to the double bonds at C-4 (δ_{H} 5.70 / δ_{C} 123.9), C-5 (δ_{C} 163.5), C-6 (δ_{H} 5.98 / δ_{C} 128.4) and C-7 (δ_{H} 6.26 / δ_{C} 133.6).

The ^1H and ^{13}C -NMR spectra of **8** confirmed the formation of the α,β -unsaturated moiety by the emergence of the vinylic hydrogen at H-4 (δ_{H} 5.73 / δ_{C} 124.0 ppm) and the disappearance of the signal corresponding to the vinyl hydrogen at H-6 (δ_{H} 6.97 / δ_{C} 121.3 ppm).

The ^{13}C -NMR spectra of **7** and **8** revealed the emergence of a signal for a ketone carbonyl (δ_{C} 199.5 ppm) instead of the characteristic carbon bonded to an oxygen atom by a single bond *ca.* δ_{C} 71.9 ppm.

Affinity of androstane N-cyclohexyl-17-carboxamides and synthesis intermediates to bind the androgen receptor

The affinity of the androstane N-cyclohexyl-17-carboxamides **6** to **8** and synthesis intermediates **2**, **3**, and **5** to bind to the AR is shown in Figure 2.

This activity was determined on the cytosolic fraction of a rat prostate. A hamster prostate was not used because it

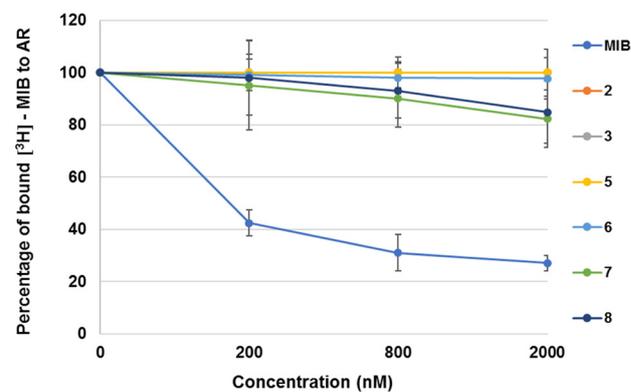


Figure 2. Bind of androstane-N-cyclohexyl-17-carboxamides **6** to **8**, and synthesis intermediates **2**, **3**, and **5** to the androgen receptor.

offers less cytosol and there is not difference in the binding of MIB between both sources of the AR. Unlabelled MIB competed with [³H]-MIB to bind the AR. As the concentration of MIB increased, the percentage of bound [³H]-MIB decreased. Compounds **2**, **3**, **5**, and **6** did not displace [³H]-MIB from AR and they did not bind to it. Interestingly, **7** and **8** (at 2000 nM) lightly displaced it (17.8 and 15.2 %, respectively) showing light affinity by AR. Comparing the structure and activity of compounds **7** and **8** vs. MIB (Figure 3), it can be inferred that the entity 4-ene-3-one or α,β -unsaturated ketone (present on **7**, **8**, MIB, and T) is an essential structural characteristic that allows the binding to AR. As is stated, such entity should be reduced into a 3-keto-5 α -steroid (as is observed in 5 α -DHT) to be more affine to bind AR.^{9,10}

Inhibitory effect of androstane N-cyclohexyl-17-carboxamides and synthesis intermediates of 5 α -reductase isoform 2

The human isoform 2 (5RD5A2) was evaluated in this study. 5 α -reductases are differently expressed in human and play diverse roles, being the isoform 2 the most abundant on the prostate and the better characterized in androgen-dependent disorders.²¹ The inhibitory effect of the androstane N-cyclohexyl-17-carboxamides **6** to **8** and synthesis intermediates **2**, **3** and **5** on 5RD5A2 is shown on Table 1 as IC₅₀.

Considering the inhibitory activity of 5RD5A2 shown by finasteride (FIN) (IC₅₀ = 8.5 nM), the compounds **2**, **3** and **5** (IC₅₀ = 1100, 467 and > 1 x 10⁵ nM, respectively) were inactive, whilst **6** to **8** (IC₅₀ = 0.169, 0.105 and 0.155 nM, respectively) were more potent inhibitors. Comparing the inhibitory activity and structure of compound **2** vs. **6**,

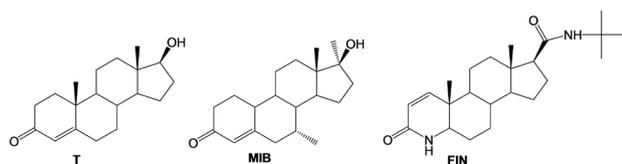


Figure 3. Structure of testosterone (T), mibolerone (MIB), and finasteride (FIN).

Table 1. Effect of compounds **2** to **8** on the activity of 5RD5A2 along with their partition coefficient and standard molar Gibbs free energy of transfer.

Compound	IC ₅₀ (nM)	Partition coefficient (log P)	Gibbs free energy of transfer $\Delta_t G^\circ$ (kJ mol ⁻¹)
FIN	8.5 ± 0.3	4.00	-22.8
2	1100.0 ± 2.0	3.51	-20.0
3	467.0 ± 2.1	4.21	-24.0
5	ND ^a	5.98	-34.1
6	0.169 ± 0.043	5.27	-30.1
7	0.105 ± 0.056	4.58	-26.1
8	0.155 ± 0.030	5.09	-29.1

^aNot determined

the addition of the N-cyclohexyl amide moiety at C-17 improves the inhibitory activity on 5RD5A2, confirming the need for lipophilic and bulky groups on such position.^{7,8} Collating the structure and the inhibitory activity of compound **6**, vs. **7** and **8**, the oxidation of the 3 β -hydroxyl group to a ketone carbonyl, along with its subsequent conjugation with a double bond (on A or A and B-rings), notably increases the inhibitory activity confirming the pharmacophoric group in these molecules.^{7,8} The more significant conjugation on the pharmacophore moiety, the greater the inhibitory activity, as it is observed when collate the structure and inhibitory activity of **7** (IC₅₀ = 0.105 nM, having an $\alpha,\beta,\gamma,\delta$ -unsaturated ketone) vs. **8** (IC₅₀ = 0.155 nM, having an α,β -unsaturated ketone). By comparing the structure and inhibitory activity of FIN vs. **8** (both compounds having an α,β -unsaturated ketone on A ring), it can be inferred that the 4-ene-3-one moiety (present on **8**) is a better pharmacophore to be reduced by the enzyme than the 1-ene-3-one, present on FIN.

Anti-androgenic in vivo effect of androstane N-cyclohexyl-17-carboxamides on the prostate and seminal vesicles of male hamsters

Since androstane N-cyclohexyl-17-carboxamides **6** to **8** were more potent than FIN to inhibit 5RD5A2 activity, *in vivo* experiments were conducted. These experiments displayed antiandrogenic effect on the androgen-dependent glands such as prostate, seminal vesicles and flank organs.^{16,22} Because log P values of **6** and **8** were higher than 5.0, they violated log P Lipinski's oral permeation criteria. Therefore, we administrated the compounds by subcutaneous injections in our pharmacological experiments.

Figure 4 shows the effect of the androstane N-cyclohexyl-17-carboxamides **6** to **8** on the weight of the prostate and seminal vesicles of castrated male hamsters treated with T. Whereas castration reduced the weight of these glands, T-treatment increased them. Animals dosed with T along with FIN or T plus compound **6** significantly ($p < 0.05$) reduced the weight of both glands showing antiandrogenic effect. Animals dosed with T plus **7** reduced these organs'

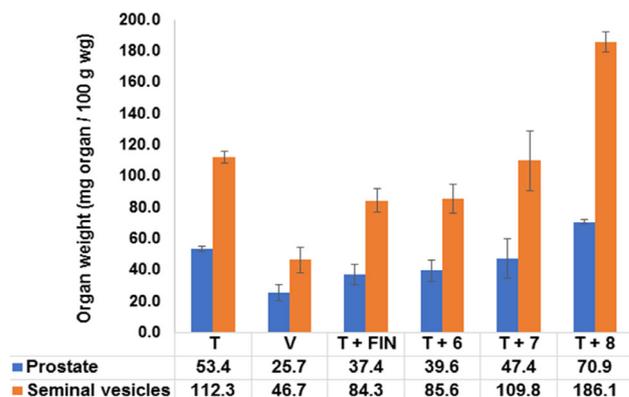


Figure 4. *In vivo* antiandrogenic effect of androstane-N-cyclohexyl-17-carboxamides **6** to **8** on the weight of the prostate and seminal vesicles of male hamsters.

weight; but this reduction was not significant ($p > 0.05$). The animals dosed with T along with **8** had the highest weights on these organs showing an androgenic effect.

In vivo antiandrogenic effect of androstane N-cyclohexyl-17-carboxamides on the diameter size of pigmented flank organs of male hamsters

Figure 5 shows the effect of androstane N-cyclohexyl-17-carboxamides **6** to **8** on the pigmented spot's diameter size of the flank organs of castrated male hamsters. The castrated animals treated with vehicle (V) displayed small flank organs, whereas T-treatment increased their size. Flank organs' diameter size was compared with those of the animals treated with T plus/or FIN, **6**, **7** and **8**. FIN, **6**, and **7** significantly ($p < 0.05$) reduced the diameter of these glands showing antiandrogenic effect. Compound **8** did not change this parameter showing light androgenic effect. This androgenic effect of **8** could be explained considering this compound binds to the AR as the competitive binding indicated, and it is an agonist of this receptor.

Determination of lipophilicity and permeability of androstane N-cyclohexyl-17-carboxamides and their synthesis intermediates

The calculated log P values for androstane N-cyclohexyl-17-carboxamides **6** to **8** and their synthesis intermediates **2**, **3**, and **5** (Table 1) indicated that they are more akin to the lipid phase than the aqueous one; therefore, they are lipophilic. Additionally, their calculated values of Gibbs free energy of transfer ($\Delta_i G^\circ$) were negative (Table 1), indicating that their passage through biological membranes is thermodynamically propitious and spontaneously occurs by a passive diffusion mechanism. Because log P and $\Delta_i G^\circ$ values indicated that compounds **6** to **8** are lipophilic, and that thermodynamically can diffuse through biological membranes, these compounds should not present permeability problems into the body. However, steroid **7** did not affect the prostate and seminal vesicles, but it was active to inhibit the 5RD5A2 in the *in vitro* assays. These data indicate that the pharmacokinetic processes are affecting compound **7** bioavailability before it reaches the androgen-dependent tissues.

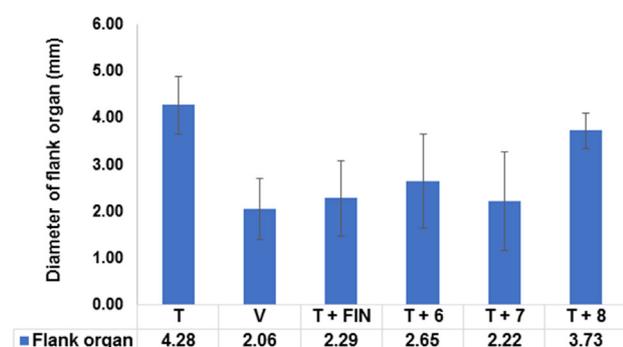


Figure 5. *In vivo* antiandrogenic effect of the androstane-N-cyclohexyl-17-carboxamides **6** to **8** on the diameter size of pigmented flank organs of male hamsters.

Conclusion

The N-cyclohexyl-3 β -hydroxyandrost-5,16-diene-17-carboxamide (**6**) is an antiandrogen that decreases the weight of androgen-dependent glands such as the prostate and seminal vesicles and reduces the hamsters' diameter flank organs. Since **6** inhibited 5RD5A2 *in vitro*, and it did not bind to AR, its pharmacological effect can be explained by the inhibition of this enzyme. Although the N-cyclohexyl-3-oxoandrost-4,6,16-triene-17-carboxamide (**7**) and the N-cyclohexyl-3-oxoandrost-4,16-diene-17-carboxamide (**8**) also inhibited *in vitro* 5RD5A2, they also lightly bound to AR. This explains why **7** shows a low antiandrogenic effect and **8** lacks it. Compound **6** could be a lead for new drugs to treat androgen-dependent illness.

Ethics Issues

Protocols working with animals were approved by the Institutional Care and Use Committee of the Universidad Autónoma Metropolitana-Xochimilco (UAM-X) under the regulation given by the standard NOM-062-ZOO 1999 (Approved number: CICUAL, UAM-X, Protocol 2), and the Universidad Nacional de Colombia (Act number 03 of August 26, 2010). The protocol involving the human prostate was approved by The Ethical Committee of the General Hospital in Mexico City (Approved number CI/06/102).

Acknowledgments

This study was supported by grants from Consejo Nacional de Ciencia y Tecnología (CONACYT project number 165049) and from UAM-CONACYT Scholarship Program for Research Support Stay. This research was also supported by a grant from the Dirección de Investigación de Sede Bogotá (DIB project number 15267) at Universidad Nacional de Colombia.

Author Contributions

JCLL synthesized the compounds and performed the pharmacological assays. YH and AS performed the pharmacological assays. NAV, JLR and MC conceived the study, designed the experiments, carried out data interpretation, and wrote the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors report no conflicts of interest.

Supplementary Data

Supporting information contains spectral data of **2** to **8** which is available on the journal's web site along with the published article.

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