Synthesis, Biological Evaluation and Molecular Docking of Avicequinone C Analogues as Potential Steroid 5α-Reductase Inhibitors

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Avicequinone C (5a), a furanonaphthoquinone isolated from the Thai mangrove Avicennia marina has been shown previously to have interesting steroid 5α-reductase type 1 inhibitory activity. In this study, a series of avicequinone C analogues containing furanonaphthoquinone with different degrees of saturation and substituents at the furan ring were synthesized. The resulting synthetic avicequinone C analogues (5a–f) along with some related compounds including 2,5-dihydroxy-1,4-benzoquinone (6) and natural naphthoquinones such as lawsone (7a) and lapachol (7b) were evaluated for their in vitro cell viability and steroid 5α-reductase type 1 inhibitory activities using the cultured cell line of human keratinocytes (HaCaT). This cell-based bioassay was performed based on a direct detection of the enzymatic product dihydrotestosterone (2) by using a non-radioactive high performance thin layer chromatography (HPTLC) method. Among the furanonaphthoquinones in this series, 5a having a propionic substituent at furan ring possessed approximately 22-fold more potent than the original isolated compound 5a. However, the compounds without furan motif such as 6, 7a and b could not inhibit the activity of steroid 5α-reductase. Molecular docking results of the in silico three-dimensional steroid 5α-reductase type 1-reduced nicotinamide adenine dinucleotide phosphate (NADPH) binary complex was performed via AutoDock Vina and it illustrated that the furanonaphthoquinone moiety and the substituent at furan ring might play a key role as pharmacophores for the steroid 5α-reductase inhibitory activity.

Key words furanonaphthoquinone; avicequinone C analogue; steroid 5α-reductase inhibitor; dihydrotestosterone; androgenic alopecia

Steroid 5α-reductase is a membrane bound enzyme in the oxido-reductase family, which controls the biological actions in steroid metabolism.1,2) Presently, three isozymes of this enzyme are known and their amino acid sequence similarity has been analyzed and compared.3) The steroid 5α-reductase type 1 is mainly found in the scalp, non-genital skin, sebaceous gland and liver, while steroid 5α-reductase type 2 is specifically located in the prostate gland, testis, epididymis and scrotum.4,5) Steroid 5α-reductase type 3, on the other hand, has been reported in benign and malignant tissues.5,6) Physiologically, the overexpression of steroid 5α-reductase, especially types 1 and 2, is believed to affect the balance between testosterone (I) and dihydrotestosterone (2), and such imbalance is implicated in androgenic disorders, including prostate cancer, hirsutism and androgenic alopecia.6)

Androgenic alopecia is described as a form of scalp-hair loss caused by decreasing the growth of hair, which may lead to baldness.8,9) This symptom can happen in both male and female, although the severity and frequency are greater in men than women and in some specific ethnic groups. The causes of androgenic alopecia are genetically related, causing some physical disorders that lead to the overproduction of 5α-dihydrotestosterone.10) The most common prescribed drugs for androgenic alopecia treatment are finasteride (3), which is a steroid 5α-reductase type 2 inhibitor,11,12) and dutasteride (4), which is an effective inhibitor to both 5α-reductase types 1 and 213–15) (Chart 1). Currently, there have been several reports on the discovery and development of new steroid 5α-reductase inhibitors for baldness treatment, including steroid and non-steroid analogues.9,16) However, most of the reported 5α-reductase inhibitors have not been designed specifically for the inhibition of steroid 5α-reductase at the catalytic site, due to the lack of a single X-ray crystal structure, which has hindered a mechanistic understanding and delayed the discovery and development of an effective inhibitor. Thus, several in silico structures of steroid 5α-reductase have recently been reported to use as a surrogate structure in the screening of potential inhibitors of this enzyme.17–19)

Previously, it was reported that avicequinone C (5a), a furanonaphthoquinone, isolated from the methanolic extract of Avicennia marina, could inhibit the activity of steroid 5α-reductase type 1 at a micromolar scale (IC 50 = 38.8 µM). This finding was based on the use of a newly developed non-radioactive human hair dermal papilla cell-based assay, which directly detects dihydrotestosterone (DHT, 2), the enzymatic product, by high performance thin layer chromatography (HPTLC).20,21) Even though the steroid 5α-reductase inhibitory activity of 5a was not as potent as dutasteride (4), the discovery of a new chemical scaffold has provided a potential for the development of a new group of steroid 5α-reductase inhibitors. Thus, we hypothesized that compounds having a furanonaphthoquinone, similar to 5a and a more simplified structure, naphthoquinone, would be used as the potential steroid 5α-reductase inhibitors.
Michael-β-alkylation followed by methylation. The resulting furanonaphthoquinones (2-hydroxy-3-(3-methylbut-2-enyl)naphthalene-1,4-dione, 5a) along with natural naphthoquinones such as lawsone (2-hydroxy-1,4-naphthoquinone, 7a) and lapachol (2-hydroxy-3-(3-methylbut-2-enyl)naphthalene-1,4-dione, 7b) were employed to investigate the assumption about the importance of the furanonaphthoquinone moiety in the inhibition of steroid 5α-reductase type 1 (Chart 2). Avicequinone C and analogues (5a–f) were synthesized from lawsone (7a) by chemical transformations involving the two-step-one-pot Michael-O-alkylation followed by methylation. The resulting synthetic furanonaphthoquinone analogues 5a–f, quinone 6, and naphthoquinones 7a and b were then evaluated for their in vitro cytotoxicity and steroid 5α-reductase inhibitory activities using HaCaT, a human keratinocyte cell line. This cell line has been shown to be a source of testosterone (1) metabolism,22,23 and to have steroid 5α-reductase type 1 expression.24 Thus, the HaCaT cell line could serve as a general cell-based assay for the study of steroid 5α-reductase inhibition. The steroid 5α-reductase inhibition was measured based on the amount of dihydrotestosterone (2) produced, determined using HPTLC as previously described.20,21 Molecular docking using the homologous protein template of steroid 5α-reductase type 119) was performed via AutoDock Vina25) to predict the biomolecular interaction between the 5α-reductase-reduced nicotineamide adenine dinucleotide phosphate (NADPH) binary complex and the respective furanonaphthoquinones. Herein, an essential pharmacophore and inhibitory activity of a potential steroid 5α-reductase inhibitor are discussed.

Results and Discussion

The synthetic protocol involved the two-step transformations. First, the reaction of one-pot Michael-O-alkylation pertaining bromination and base-mediated cyclization was employed to construct a furan ring.26–28) Second, the resulting 2-acyl furanonaphthoquinone was subjected to methylation via Grignard’s reaction to form an isopropyl alcohol side chain (Chart 3). Under this strategy, lawsone (7a) was used as the starting material and was treated with an effective Michael acceptor, such as methyl vinyl ketone (8a), pen-1-en-3-one (8b), and methyl vinyl sulfone (8c) to obtain the desired furanonaphthoquinones 5a–f at a yield of 12–57%. During base-mediated cyclization, refluxing gave furans 5b and e, while heating at 60°C furnished 5c as the major product. For the preparation of 5f, initial optimizations were examined with methyl vinyl sulfone (8c), but only low yields were obtained. Next, the resulting furanonaphthoquinones 5b and e were reacted with methyl magnesium bromide to convert their acetyl group to isopropyl alcohol, which gave an acceptable yield. Under this condition, 5a was synthesized at a gram-scale, with a yield of up to 19% yield.

The desired avicequinone C (5a), 2,3-dihyronaphth[2,3-b]furan analogues (5c and d), and furanonaphthoquinone analogues (5b, e, and f) were characterized by standard spectroscopic analyses. Spectroscopic data of 5a, b, d, and f were matched with the reported natural furanonaphthoquinone structures of avicequinone C,20) napabucasin,29) (±)-stenocarpouquinone B,30) and avicequinone B,30) respectively.

Next, all synthetic avicequinone C analogues 5a–f, quinone 6 and naphthoquinones 7a and b were evaluated for their in vitro cytotoxicity and steroid 5α-reductase inhibitory activities using the HaCaT cell line. The results would verify the importance of the furanonaphthoquinone moiety, which could be a key pharmacophore for the inhibition of 5α-reductase activity.

As the results shown in Table 1, 5a and its synthetic furanonaphthoquinone analogues (5b–f) exhibited an in vitro HaCaT cellular toxicity in the range of 2.66–155.79 μM. The most potent cytotoxic compound in this series was 5b
(LC₅₀=2.66 µM), which is a furanonaphthoquinone containing a conjugated structure with an acetyl group at the furan ring. We observed that 5b was 10-fold more cytotoxic than 5a (LC₅₀=28.06 µM), which contains an isopropanol substituent at the furan ring. Additionally, 5b showed a broadly similar cytotoxic level to 5c (LC₅₀=2.92 µM) and 5e (LC₅₀=3.94 µM), which have a 2,3-dihydrofuran ring and a one-carbon longer propionic group at furan ring, respectively. Without the substituent at furan ring, 5f (LC₅₀=25.09 µM) displayed equal HaCaT cellular toxicity to 5a. The loss of both the electron withdrawing group and conjugated system by having a 2,3-dihydrofuran moiety and isopropanol substituent, 5d appeared to tremendously reduce cytotoxicity of the compound. Interestingly, 5d showed the lowest cytotoxicity (LC₅₀=155.79 µM) among compounds in this series against HaCaT cell line. This observation was similar to the cytotoxic results of 6, 7a, and 7b; the quinone and naphthoquinones without the furan ring, which showed no toxicity at 200 µM against the HaCaT cell line. In this way, we speculated that furanonaphthoquinone moiety and the electron withdrawing substituent at the furan ring might play a role in the cell viability against the HaCaT cell line.

Next, avicequinone C and synthetic analogues (5a–f) along with synthetic precursor (7a) and the non-furanonaphthoquinone compounds (6 and 7b) were preliminary screened for their steroid 5α-reductase type 1 inhibitory activity using the HaCaT cells for steroid 5α-reductase type 1 expression to convert testosterone (1) to dihydrotestosterone (2) in conjugation with the non-radioactive HPTLC method for the detection of 2. In this work, the HaCaT cells were cultured and treated with all nine test compounds including 5a–f, 6, 7a, and 7b using the highest concentration, which no cell death more than half population was observed. Aqueous medium of each experiment was collected and partitioned with ethyl acetate for extracting the remaining 1, which was added as an enzyme substrate and the resulting 2, the steroid 5α-reductase product. The steroid 5α-reductase activity was then evaluated based upon the amount of 2 by HPTLC using a mixture of cyclohexane–ethyl acetate–triethylamine (1.5:1:0.1, v/v/v) as the mobile phase. Then, 1 and 2 were visualized under 366 nm on a phosphoric acid staining TLC plate. We found that the furanonaphthoquinone analogues (5a–f) enabled to inhibit the activity of steroid 5α-reductase type 1 (Fig. 1).

Furthermore, the steroid 5α-reductase type 1 inhibitory activity of furanonaphthoquinones 5a–f, along with quinone 6, synthetic precursor 7a (lawsone), and natural naphthoquinone 7b (lapachol) were evaluated for their IC₅₀ profiles. HaCaT cells were treated with various non-cytotoxic concentrations of 5a–f, 6, 7a, and 7b that were chosen based on cytotoxicity obtained at 24 h (Table 1). As shown in Fig. 2 and Table 2, furanonaphthoquinones 5a–f exhibited the inhibitory activity in the range of 0.20–9.63 µM. It was found that both furanonaphthoquinones 5a and f displayed similar degree of inhibition (IC₅₀=4.45 and 4.26 µM, respectively). The less cytotoxic 5d; the 2,3-dihydrofuran analog having isopropanol substituent resemble to 5a, showed 2-fold less inhibitory activity toward steroid 5α-reductase type 1 (IC₅₀=9.63 µM), relative to 5a. The group of highly toxic analogues 5e and e, which contained the ketone substituents adjacent to 2,3-dihydrofuran and furan moieties possessed strong steroid 5α-reductase type 1 inhibitory profile, approximately 4- and 22-fold more inhibitory activity (IC₅₀=1.21 and 0.20 µM), respectively, relative to 5a. The steroid 5α-reductase inhibitory activity of the most potent cytotoxic 5b could not be examined due to the death of...
HaCaT cell line. Thus, the cell-free enzyme activity assay will be required for further study of 5b. Interestingly, the non-furanonaphthoquinones 6, 7a, and 7b were found to be incapable to inhibit the steroid 5α-reductase activity. According to the results, it seems that compounds with furanonaphthoquinone such as 5a–f showed a promising 5α-reductase inhibitory activity, which could potentially serve as a new 5α-reductase inhibitor that will be further studied and developed for the treatment of androgenic disorder such as prostate cancer, benign prostatic hyperplasia, acne, hirsutism, and androgenic alopecia.2)

To rational the structure–activity relationship of furanonaphthoquinone analogues (5a–f) toward steroid 5α-reductase type 1, the docking studies were performed. The in silico three-dimensional (3D) 5α-reductase type 1-NADPH binary complex was prepared based on the previously reported strategy.19) The binding pattern and intermolecular interaction of all furanonaphthoquinone analogues (5a–f) were predicted (Fig. 3). The furanonaphthoquinones 5a–e were resided closely adjacent to NADPH and likely interacted with Tyr95 of the in silico 5α-reductase type 1 as well as NADPH. Note that the oxygen atom of the substituent at the furan ring was in correspondence to the reported result of 4.19) The binding site of furanonaphthoquinone 5f to the 5α-reductase type 1-NADPH binary complex was slightly different from the others; however its oxygen atom on quinone motif was able to form a hydrogen bond with Tyr95. The molecular docking results emphasized the importance of the substituents at furan ring and quinone moieties; however the structure–activity relationship might not be intensively concluded here. The further in vitro study is required to identify the inhibitory mechanism of furanonaphthoquinone toward steroid 5α-reductase inhibition.

Table 2. Steroid 5α-Reductase Inhibitory Activity (IC50) via HaCaT Cell-Based Assay at 48 h of Avicequinone C Analogues (5a–f), Quinone 6, and Naphthoquinones 7a and 7b

<table>
<thead>
<tr>
<th>Compound</th>
<th>5α-Reductase inhibitory activity IC50±S.D. (µM)</th>
<th>Compound</th>
<th>5α-Reductase inhibitory activity IC50±S.D. (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a</td>
<td>4.45±0.42</td>
<td>5f</td>
<td>4.26±0.38</td>
</tr>
<tr>
<td>5b</td>
<td>n.d. b)</td>
<td>6</td>
<td>&gt;200</td>
</tr>
<tr>
<td>5c</td>
<td>1.21±0.12</td>
<td>7a</td>
<td>&gt;200</td>
</tr>
<tr>
<td>5d</td>
<td>9.63±0.78</td>
<td>7b</td>
<td>&gt;200</td>
</tr>
<tr>
<td>5e</td>
<td>0.20±0.03</td>
<td></td>
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a) Dutasteride (20 µM) was employed as a control and provided complete inhibition. b) n.d. refers to not determined.

**Conclusion**

In conclusion, the synthesis of six furanonaphthoquinone analogues (5a–f) was achieved. All synthetic furanonaphthoquinones (5a–f), the commercial available quinone 6, naphthoquinone 7a, employed as a synthetic starting material in this work, and the known natural naphthoquinone 7b were examined for their anti-proliferative and steroid 5α-reductase
type 1 inhibitory activity against the HaCaT cell line. The steroid 5α-reductase type 1 inhibitory activity was measured based on the production of 5α-dihydrotosterone (2) via the non-radioactive HPTLC procedure. The series of furanonaphthoquinones (5a–f) showed interesting cellular toxicity and enabled to inhibit the activity of steroid 5α-reductase type 1 of the HaCaT cell line with the improved inhibitory activity, relative to avicequinone C (5a); the natural furanonaphthoquinone isolated from Avicennia marina. To our surprise, 5e, the most cytotoxic compound among all synthetic furanonaphthoquinones having a propionic substituent, exhibited approximately 22-fold more potent toward the steroid 5α-reductase inhibitory activity, comparing to 5a. Considering the results between the in vitro steroid 5α-reductase inhibitory activity and molecular docking of the in silico 3D steroid 5α-reductase type 1-NADPH binary complex, we concluded that the furanonaphthoquinone moiety and the substituent at furan ring are the potential pharmacophores for the steroid 5α-reductase inhibitory activity. New chemical feature for 5α-reductase inhibitor displaying a stronger 5α-reductase inhibition than 5a was discovered. Further studies in molecular dynamics along with in vitro experiments could provide insight information regarding the biological mechanisms toward the steroid 5α-reductase metabolism. Finally, a new series of furanonaphthoquinone will be developed as the promising steroid 5α-reductase inhibitors for the treatment of androgenic disorders such as androgenic alopecia.

**Experimental**

**Materials** Reactions were performed in oven-dried glassware and magnetically stirred under an inert atmosphere using a syringe tube equipped with an argon or nitrogen balloon. Room temperature was 25°C. Commercial solvents and reagents were used as received. Anhydrous solvents were dried over 4Å molecular sieves. All reactions were monitored by TLC using aluminium silica gel 60 F254 (Merck). IR spectra were measured on a Fourier transform (FT)-IR spectrometer, PerkinElmer, Inc. (U.S.A.) 1H- and 13C-NMR spectra were obtained on a Bruker Avance DPX-300 FT-NMR spectrometer. 1H-NMR chemical shifts (δ) and coupling constants (J) were given in ppm and Hz, respectively. Deuterated chloroform (CDCl3) served as an internal standard for both 1H- and 13C-NMR spectra at 7.26 and 77.0 ppm, respectively. Mass spectra were recorded on a microTOF Bruker Daltonics mass spectrometer.

**Synthesis of Avicequinone C (5a)** To a stirred solution of methyl vinyl ketone (8a) (2.5 equiv, 7.18 mmol, 0.6 mL) in 6 mL of pentane was added a solution of bromine (2.6 equiv, 7.46 mmol, 0.3 mL) in 3 mL of pentane at −15°C (NaCl mixed ice bath). The reaction was stirred for 10 min. Then, all volatiles were evaporated under reduced pressure to yield the crude 1,2-dibromobutan-2-one, which was further used in the next step without purification. Next, lawsone (7a) (1 equiv, 2.87 mmol, 500 mg) was weighed into a 50-mL oven-dried round-bottomed flask and dissolved in 15 mL of tetrahydrofurran (THF), followed by the addition of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (3.7 equiv, 10.62 mmol, 1.6 mL), giving a red solution. The reaction was stirred at 0°C (ice bath) under a nitrogen atmosphere for 20 min. A freshly prepared solution of 1,2-dibromobutan-2-one in 3 mL of THF was added and the ice bath was immediately removed. The reaction mixture was gradually warmed up and was then heated to reflux for 5 h. Another portion of DBU (0.52 equiv, 1.49 mmol, 0.2 mL) was added and the reaction
mixture was continuously refluxed and stirred for another 3 h. The reaction was then quenched by the addition of saturated aqueous ammonium chloride and extracted with chloroform (25 mL×3 times). The organic extracts were combined, washed with water and saturated sodium chloride, dried over anhydrous Na2SO4, filtered and evaporated under reduced pressure to obtain the crude product. Purification of the crude reaction by silica gel column chromatography eluting with dichloromethane–hexanes (4:1, v/v) provided 2-acetylnaphtho[2,3-b]furan-4,9-dione (5b) as a yellow powder at a yield of 420 mg (57%). 1H-NMR (CDCl3, 300 MHz) δ: 8.26 (1H, m, 5-H), 8.25 (1H, m, 8-H), 7.81 (1H, m, 6-H), 7.81 (1H, m, 7-H), 7.61 (1H, s, 3-H), 2.67 (3H, s, 11-H); 13C-NMR (CDCl3, 100 MHz) δ: 187.5 (C-10), 179.7 (C-4), 173.9 (C-9), 155.5 (C-9a), 152.9 (C-2), 134.4 (C-6), 134.3 (C-7), 131.1 (C-8a), 132.6 (C-4a), 130.7 (C-3a), 127.4 (C-8), 127.2 (C-5), 112.3 (C-3), 26.8 (C-11); IR (KBr) 3532, 3388, 3178, 3121, 2986, 2926, 1682, 1665, 1586, 1476, 1376, 1291, 958, 721 cm−1; HR-MS-ESI m/z 277.0464 ([M+Na]+, calculated for C15H14O4Na2+ 277.0471).

Synthesis of 5e

The propionyl containing furanonaphthoquinone 5e was prepared by following the synthesis of 5b. Pen-1-en-3-one (8b) was employed as the Michael-O-alkylation electrophile to give 2-propionylnaphtho[2,3-b]furan-4,9-dione (5e) as a yellow powder at a yield of 259 mg (54%). 1H-NMR (CDCl3, 300 MHz) δ: 8.26 (1H, m, 5-H), 8.25 (1H, m, 8-H), 7.81 (1H, m, 6-H), 7.81 (1H, m, 7-H), 7.61 (1H, s, 3-H), 3.06 (2H, q, J=7.6, 14.7 Hz, 11-H), 1.26 (3H, t, J=7.2 Hz, 12-H); 13C-NMR (CDCl3, 100 MHz) δ: 190.8 (C-4), 179.8 (C-4), 173.9 (C-9), 155.4 (C-9a), 152.7 (C-2), 134.4 (C-7), 134.3 (C-6), 131.1 (C-8a), 132.6 (C-4a), 130.7 (C-3a), 127.3 (C-5), 127.3 (C-8), 112.0 (C-3), 32.6 (C-11), 7.4 (C-12); IR (KBr) 3385, 3115, 2978, 2987, 1698, 1672, 1579, 1567, 1354, 1219, 958, 721 cm−1; HR-MS-ESI m/z 277.0464 ([M+Na]+, calculated for C15H15O4Na+ 277.0471).

Synthesis of 5f

Methyl vinyl sulphone (8c) (1.0 equiv, 4.71 mmol, 500 mg) was weighted into a 50-mL oven-dried round-bottomed flask and dissolved in dichloromethane (10 mL). The reaction mixture was stirred at room temperature under an argon atmosphere. Bromine (1.5 equiv, 7.07 mmol, 0.2 mL) was slowly added into the reaction mixture giving a dark orange solution. The reaction mixture was heated to reflux for 6 h. The reaction mixture was then concentrated to yield a sticky residue, dissolved in THF (20 mL) and cooled at 0°C in an ice-bath under an argon atmosphere. DBU (1.5 equiv, 7.07 mmol, 1.1 mL) was slowly added dropwise over 20 min. The reaction mixture was stirred for 30 min at 0°C in an ice-bath under an argon atmosphere. Lawsone (7a) (1.0 equiv, 4.71 mmol, 820.2 mg) was added and another portion of DBU (1.5 equiv, 7.07 mmol, 1.1 mL) was slowly added dropwise over 20 min. The reaction mixture was stirred for 30 min at 0°C in an ice-bath under an argon atmosphere.
naptho[2,3-b]furan-4,9-dione (5f) as a pale yellow solid at a yield of 69 mg (12%). 1H-NMR (CDCl 3, 300 MHz) δ: 8.22 (1H, m, 5-H), 8.22 (1H, m, 8-H), 7.77 (1H, m, 6-H), 7.77 (1H, m, 7-H), 7.77 (1H, d, J=1.5 Hz, 2-H), 7.01 (1H, d, J=1.5 Hz, 3-H); 13C-NMR (CDCl 3, 100 MHz) δ: 180.6 (C-4), 173.6 (C-9), 152.7 (C-9a), 148.6 (C-2), 132.5 (C-4a), 134.0 (C-7), 133.9 (C-6), 133.2 (C-8a), 130.5 (C-3a), 127.1 (C-8), 127.0 (C-5), 108.7 (C-3); IR (KBr) 3142, 2853, 1683, 1585, 1566, 1478, 1365, 1206, 1182, 952, 714 cm −1; HR-MS-ESI m/z 221.0212 [(M+Na)+, calcd for C 7 17 H 18 O 4 Na]+.

Molecular Docking

The 3D predicted structures of 5α-reductase type 1 was achieved by following the reported protocol. 32) The homology modeling strategies involved automated homologous protein template searching by SWISS-MODEL. The isoprenylcysteine carboxyl methyltransferase (ICMT, PDB code: 4A2N) was automatically selected as a homologous protein template for building the in silico 3D structure of 5α-reductase type 1. Ramachandran plot for standard in silico structure evaluation, active site identification by docking study of finasteride (3) and dutasteride (4) using AutoDock Vina, 25) transmembrane site prediction via Discovery Studio 2.5, and docking of the reported 5α-reductase inhibitors to confirmed the active site and protein-ligand interactions. The 3D structures of all studied compounds were generated by the Open Babel software. 33) Each compound was then docked into the resulting in silico 5α-reductase type 1-NADPH binary complex with 100 independent runs using AutoDock Vina. 25) Then, each ligand was docked into the resulting in silico 5α-reductase type 1-NADPH binary complex with the grid box near the position of NADPH (center x=0.487, center y=36.43, and center z=96.802) and the grid box size is of 38×18×16 Å 3. The docking calculations of 5α-reductase type 1 were performed with 100 runs and the average results were analyzed.

In Vitro Cell Viability 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay

HaCaT cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotic–antimycotic solution at 37°C in humidified atmosphere of 5% CO 2 in a T-75 flask. The cells were grown until about 80% confluency, and then subcultured. Cells were seeded at an initial cell density of 5000 cells/well onto 96-well plates and allowed to adhere overnight. After 24 h, the cells were individually treated with a series of synthetic avicequineone C and analogues (5a–f), 2,5-dihydroxy-1,4-benzoquinone (6), and natural naphthoquinones including lawsone (7a) and lapachol (7b). Dutasteride (4) was used as a positive control. Each test compound was prepared as the serial concentrations in DMEM containing 0.1% (v/v) dimethyl sulfoxide (DMSO). DMEM was used as a negative control. Cells were incubated at 37°C in atmosphere of 5% CO 2 for 24 h. Cell viability was measured by the MTT assay. 34) In the presence of viable cells, cells were incubated with 100 μL of a solution of MTT in DMEM at a concentration of 0.5 mg/mL. The tests plates were incubated at 37°C under 5% CO 2 atmosphere for 3 h. Then, all liquid medium were removed. The resulting formazan crystals were dissolved in DMSO (100 μL). The absorbance was measured at 540 nm using a VICTOR3 multi-label plate reader (PerkinElmer, Inc.). Experiments with triplicate data were performed to obtain mean cell viability. The mean±standard deviations (S.D.) were obtained from three independent experiments. The IC 50 values were calculated according to the cell viability ratios.

HaCaT-Based Steroid 5α-Reductase Inhibitory Assay

HaCaT cells were seeded at a cell density of 2×10 4 cells/well onto 6-well plates, and allowed to adhere for 24 h. Testosterone (1, substrate) and 5α-dihydrotestosterone (2, product) were employed as the internal standard and enzyme–activity control, respectively. Dutasteride (4) at 20 μM was used as a positive control. The overall volume of each well was 2 mL. DMEM (1 mL) was added into each standard and control wells. A solution of testosterone (1, substrate) at 50 μM (1 mL) in DMEM containing 1% (v/v) DMSO was added in each test well. The final concentration of 1 was 25 μM. Cells were treated with a serial solution at non-cytotoxicity of each test compound that was dissolved in DMEM containing 1% (v/v) DMSO (1 mL). Each test was repeated in triplicate. After incubating in humidified atmosphere of CO 2 at 37°C for 48 h, the cell culture medium was collected and the remaining viable cells were determined by MTT assay as previously explained. The aqueous medium was extracted with ethyl acetate (1.5 mL), vigorously shaken, centrifuged at 10000 rpm for 5 min, and the 1 mL of ethyl acetate layer was kept. The extraction was repeated two times. The resulting ethyl acetate extracts that consisted of the remaining 1 and the resulting 2 were combined and air dried at room temperature. The crude extract was then reconstituted with 20 μL of methanol and 8 μL of the resulting methanol solution was spotted onto a TLC silica gel 60 F254 aluminum plate using TLC sample loader (CAMAG Linomat 5, Switzerland). The TLC plate was developed using a mixture of cyclohexane–ethyl acetate–tri-methylamine (1.5:1:0.1, v/v/v) as a mobile phase. Then, the developed TLC plate was stained with a solution of 42.5% phosphoric acid in ethanol and heated at 120°C for 20 min for visualization. Visual detection of 5α-dihydrotestosterone (2) at 366 nm was captured using the TLC scanner (CAMAG TLC Scanner 3, Switzerland). The band intensity of 2 was measured by the Image J software and the inhibitory activity was calculated according to the inhibition ratios. In order to avoid false positive result, the cell viability of the treated cells was determined. The test compound at concentration showing more than 50% cell viability was further considered for IC 50 calculation. The IC 50 values were calculated according to the inhibition ratios. The mean±S.D. were obtained from three independent experiments.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Materials

The online version of this article contains supplementary materials. See supplementary.
materials for $^1$H- and $^{13}$C-NMR spectra and HPTLC chromatograms of 5a–f.

References