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Novel Ferrocenic Steroidal Drug Derivatives and Their Bioactivities

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Seven novel ferrocenic derivatives, compounds **1**−**7**, were synthesized from steroidal drugs by Aldol condensation reaction. The derivatives were purified by chromatography, and their structures were determined on the basis of HR-ESI-MS and two-dimensional NMR spectroscopy. The purity of all derivatives was more than 95%. Compounds **1**−**5** demonstrated anti-proliferative activity on HeLa cell line by SRB assay more than their parent compounds. All seven derivatives showed anti-oxidative activities evaluated by DPPH scavenging and metal ion chelating, while their parent compounds gave no activity. Compound **1** indicated the most potent anti-proliferative activity similar to doxorubicin, with the GI_{50} at 0.223 ± 0.014 μg/mL. Compounds **6** and **7** demonstrated similar potent in vivo anti-inflammatory to their parent compounds (prednisolone and hydrocortisone) at 80.99 ± 13.5 and 68.24 ± 10.4% edema inhibition, respectively. This study has suggested that the novel compound **1** was the most potential derivative that can be further developed for cancer treatment.

1. Introduction

Steroidal androgens, estrogens, and glucocorticoids have been used as medicines both in the original and derivative forms. As known, the main purpose of derivatization of the compounds is to improve their pharmacodynamic and phamacokinetic properties. Androgens and estrogens (e.g., testosterone and progesterone) are sex hormones that can interact with the androgenic and estrogenic receptors and mediate pharmacological effects by the slow genomic mechanism through nuclear receptors as well as by the fast nongenomic mechanism through membrane-associated receptors and signaling cascades.[1] For glucocorticoid steroids (e.g., prednisolone and hydrocortisone), they have been developed for clinical application since the early 1950s. The derivatives of androgens, estrogens, and glucocorticoids have been continuously developed in order to increase potency and reduce side effects.[2] Ferrocene [$Fe(C_2H_5)$] is an organometallic and multielectron transfer compound, which can be used as a precursor for the preparation of organometallic complexes, gasoline additives to prevent "knocking" in motors, anion and cation sensors,[3−5] DNA biosensors,[6] and mediators of electron transfer between active sites of oxidoreductases enzyme.[7] It is also used as catalysts for the oxidative organic synthesis, molecular chemistry, water treatment, and photolytic chemistry.[8,9] Several pharmacological activities of ferrocene derivatives have been reported. The introduction of metal in the compounds may lead to changes in solubility, stability, drug−receptor interaction, and drug−ligand binding affinity.[10,11] The anti-proliferation effects on hormone-dependent and independent breast cancer cell lines were increased when tamoxifen, an anticancer drug was derivatized to hydroxyferrocifens.[11] This was due to the increase of relative binding affinity (RBA[a]) owing to superior lipophilicity and lengthening chain of the tamoxifen derivative.[11] Ferroquine, a modified ferrocenic form of chloroquine, showed an increase in antimalarial activity.[12] Ferrocenic aminohydroxynaphthoquinones also exhibited antiplasmodial activity.[13] The ferrocenyl complexes of testosterone and dihydrotestosterone (DHT) substituted at the C-17 position of the steroid skeleton with an ethynyl substituent grafted with various sandwich or semisandwich organometallic units [ferrocenyl, (η5-C_5H_4)-$Re(CO)_3$, (η5-C_5H_4)-$Mn(CO)_3$, (η6-C_6H_5)-$Cr(CO)_3$] and of 3β-androstanediol substituted at C-16 and C-17 by a ferrocenyl vinyl and a ferrocenyl ethynyl unit, respectively, have been shown to have strong anti-proliferative activity on prostate cancer cells.[14] However, the biological activities of the seven novel ferrocenic derivatives of steroidal sex hormones and anti-inflammatory drugs from this present study have never been performed. In this study, seven novel ferrocenic steroidal drug derivatives (compounds **1**−**7**) synthesized from five steroidal sex hormone and two steroidal anti-inflammatory drugs were investigated for their biological activities including anti-proliferation in HeLa cell lines, in vivo anti-inflammatory activity by hind paw edema in rats, anti-oxidation assays including DPPH

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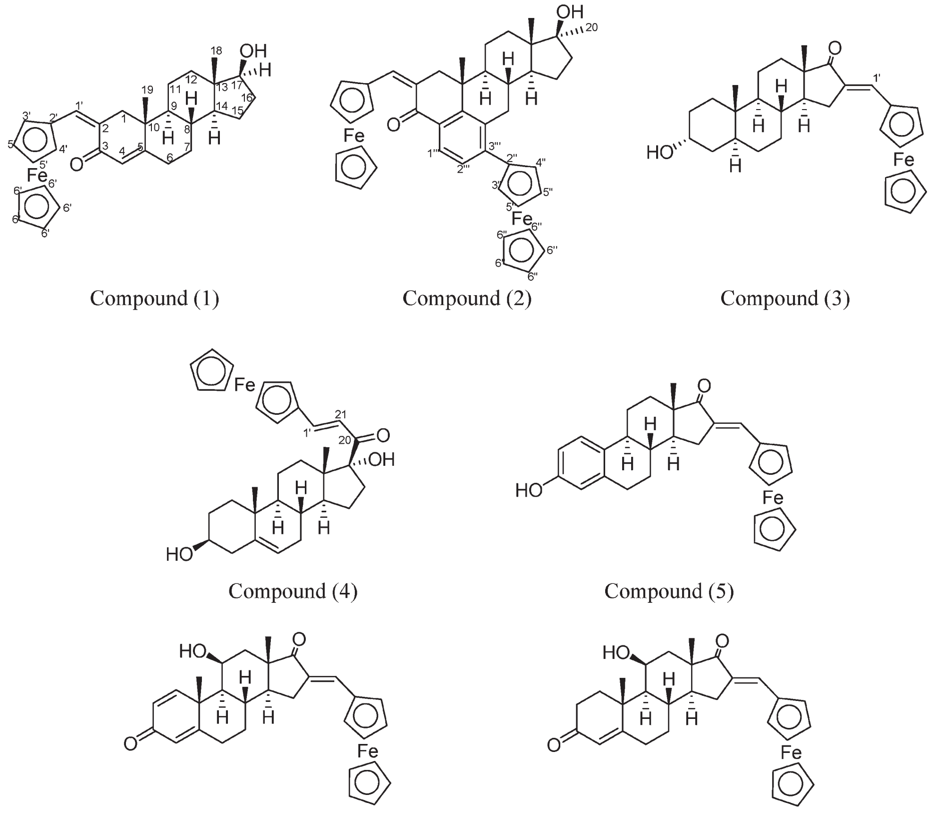
[a] Abbreviations: RBA, relative binding protein; DPPH, 1,1-diphenyl-2-picryl-hydrazil; $CDCl_3$, deuterium chloroform; SRB, suforhodamine B; DMEM, Dulbecco's Modified Eagle Medium.



Compound (1) Compound (2) Compound (3)

Compound (4) Compound (5)

Compound (6) Compound (7)

Figure 1. Chemical structures of the novel ferrocenic steroidal drug derivatives; 2-ferrocenylidene-17β-hydroxy androst-4-en-3-one (compound **1**), 2-ferrocenylidene-3‴-ferrocenic benz[4,5,6]-androst-4-en-3-one (compound **2**), 16-ferrocenylidene-3α-hydroxy androstan-17-one (compound **3**), 21-ferrocenylidene-3β,17β-dihydroxy pregn-5-en-18-one (compound **4**), 16-ferrocenylidene-3-hydroxy estra-1,3,5-trien-17-one (compound **5**), 16-ferrocenylidene-17β-hydroxy androst-1,4-diene-3,17-dione (compound **6**), and 16-ferrocenylidene-11β-hydroxy androst-4-ene-3,17-dione (compound **7**).

scavenging, and ferrous metal ion chelating in order to preliminary investigate for their therapeutic uses.

2. Results and Discussion

2.1. Synthesis of Ferrocenic Steroidal Drug Derivatives. Aldol condensation reaction is an organic reaction in which an enolate ion reacts with a carbonyl compound to form a β-hydroxy aldehyde or β-hydroxy ketone, followed by dehydration to give a conjugate enone. This reaction provides a good C–C bond forming in many compounds. The synthesized ferrocenic steroidal drug derivatives have been purified and were in crystalline form, which were chemical stable at room temperature (25 ± 2 °C) for 3 months since no chemical degradation of these compounds was observed (data not shown). These compounds have been identified, and their chemical structures have been elucidated. The chemical structures of seven novel ferrocenic steroidal derivatives, compounds **1**–**7** were shown in Figure.1. They were synthesized from their parent compounds, which were testosterone (1), methyltestosterone (2), androsterone (3), 17α-hydroxy pregnenolone (4), estrone (5), prednisolone (6), and hydrocortisone (7), respectively. The seven ferrocenic steroidal drug derivatives **1**–**7** are novel compounds, and their chemical structures (Figure 1) were determined based on HR-ESI-MS and two-dimensional NMR spectroscopy (refer to Section 4.2 for the assigned HR-ESI-MS, and ^{1}H and ^{13}C NMR data). The molecular modifications of these novel compounds may have different biological activities from their parent compounds.

2.2. Anti-proliferative Activity. Table 1 showed the anti-prolifertive activity in HeLa cell lines by SRB assay of five novel ferrocenic steroidal sex hormone derivatives, compounds **1**–**5**, in comparing to the standard anticancer drug, doxorubicin. All derivatives showed dose dependent relationships with more activity than their parent compounds. Compounds **1** and **2**, which were the ferrocenic derivative of testosterone and methyl testosterone, respectively, indicated the same activity at the GI_{50} values of 0.223 ± 0.014 and 0.271 ± 0.273 μg/mL as doxorubicin (0.250 ± 0.017 μg/mL). Testosterone and methyltestosterone gave about half activity of doxorubicin. Compounds **4** and **5** gave about half activity of doxorubicin while their parent compounds showed almost no activity. The ferrocenic derivatization of the hormones may facilitate the exchange of electron and be oxidized to ferrocenium species in the electronoxidation process, thereby enhancing the anti-proliferative activity.[19,20] This has suggested that the ferrocenic modification of these steroidal sex hormones can increase this activity, which will be certainly beneficial for several anticancer treatment. As known, the growth of some hormone sensitive tumors including breast and prostate cancer can be inhibited by providing or blocking of certain hormone including estrogen and testosterone, respectively.

2.3. Anti-oxidative Activities. In Table 2, compounds **1**–**7** showed DPPH radical scavenging activity (SC_{50}) of

Table 1. Comparison of Anti-proliferative Activity (GI_{50}) of the Ferrocenic Sex Steroidal Hormone Derivatives (Compounds **1**–**5**) and Their Parent Compounds (1–5) on HeLa Cell Lines by SRB Assay

sample code	compd names	GI_{50} (μg/mL)	folds of doxorubicin
compd **1**	2-ferrocenylidene-17β-hydroxy androst-4-en-3-one	0.223 ± 0.014	1.121
(1)	testosterone	0.750 ± 0.071	0.333
compd **2**	2-ferrocenylidene-$3'''$-ferocenic benz[4,5,6]-androst-4-en-3-one	0.271 ± 0.273	0.922
(2)	methyltestosterone	0.619 ± 0.029	0.404
compd **3**	16-ferrocenylidene-3α-hydroxy androstan-17-one	1.720 ± 0.346	0.145
(3)	androsterone	2.700 ± 0.073	0.093
compd **4**	21-ferrocenylidene-3β,17β-dihydroxy pregn-5-en-18-one	0.405 ± 0.054	0.617
(4)	17-hydroxy pregnenolone	1.830 ± 0.093	0.137
compd **5**	16-ferrocenylidene-3-hydroxy estra-1,3,5-trien-17-one	0.505 ± 0.128	0.495
(5)	estrone	3.410 ± 0.435	0.073
	standard anticancer drug: doxorubicin	0.250 ± 0.017	1.000

Table 2. Comparison of Anti-oxidative Activities [DPPH Scavenging (SC_{50}) and Ferrous Metal Ion Chelating (MC_{50})] of Seven Novel Ferrocenic Steroidal Drug Derivatives (Compounds **1**–**7**) and Their Parent Compounds

sample code	compd names	SC_{50} (mg/mL)	folds of vitamin C	MC_{50} (mg/mL)	folds of EDTA
compd **1**	2-ferrocenylidene-17β-hydroxy androst-4-en-3-one	0.243 ± 0.010	0.083	0.160 ± 0.002	0.188
(1)	testosterone	ND[a]		ND	
compd **2**	2-ferrocenylidene-$3'''$-ferocenic benz[4,5,6]-androst-4-en-3-one	0.359 ± 0.020	0.055	0.320 ± 0.010	0.094
(2)	methyltestosterone	ND		ND	
compd **3**	16-ferrocenylidene-3α-hydroxy androstan-17-one	6.890 ± 0.190	0.003	2.030 ± 0.230	0.015
(3)	androsterone	ND		ND	
compd **4**	21-ferrocenylidene-3β,17β-dihydroxy pregn-5-en-18-one	4.240 ± 0.320	0.005	4.520 ± 0.340	0.007
(4)	17-hydroxy pregnenolone	ND		ND	
compd **5**	16-ferrocenylidene-3-hydroxy estra-1,3,5-trien-17-one	4.740 ± 0.070	0.004	2.860 ± 0.170	0.010
(5)	estrone	ND		ND	
compd **5**	16-ferrocenic-11β-hydroxy androsta-1,4-diene-3,17-dione	0.760 ± 0.030	0.276	0.610 ± 0.070	0.049
(6)	prednisolone	ND		ND	
compd **7**	16-ferrocenic-11β-hydroxy androst-4-ene-3,17-dione	0.950 ± 0.060	0.021	0.980 ± 0.010	0.031
(7)	hydrocortisone	ND		ND	
	ferrocenic carboxaldehyde	0.120 ± 0.010	0.167	0.140 ± 0.010	0.214
	vitamin C	0.021 ± 0.002	1.000		
	EDTA			0.030 ± 0.001	1.000

[a] ND = not determined.

0.243 ± 0.010, 0.359 ± 0.020, 6.890 ± 0.190, 4.240 ± 0.320, 4.740 ± 0.070, 0.760 ± 0.030, and 0.950 ± 0.060 mg/mL and the ferrous metal ion chelating activity (MC_{50}) of 0.160 ± 0.002, 0.320 ± 0.010, 2.030 ± 0.230, 4.520 ± 0.340, 2.860 ± 0.170, 0.610 ± 0.070, and 0.980 ± 0.010 mg/mL. However, their parent compounds showed no scavenging and chelating activity, respectively. Compound **1** gave the highest scavenging activity but lower than ferrocenic carboxaldehyde and vitamin C of about 2 and 12 times, respectively. All derivatives had lower chelating activity than the standard EDTA. However, compound **1** indicated almost the same chelating activity as ferrocenic carboxaldehyde while compound **2** showed about two times less activity than ferrocenic carboxaldehyde. The free radical scavenging and metal ion chelating activities of all derivatives might be from the ferrocinyl moiety, which has multielectron transfer property and high π-electrons in the molecules, thereby facilitating the electro-oxidations or reduction–oxidation reaction.[21,22] These two anti-oxidative activities may contribute to the above anti-proliferative activity in HeLa cell lines by inhibiting the overproduction of oxidants, which can damage several biomolecules and cause many degenerative disease including cancer.

2.4. Anti-inflammatory Activity. In Table 3, compounds **6** and **7** at 20 mg/kg.bw showed similar in vivo anti-inflammatory activity in rats by hind paw edema to their parent compounds, especially at 1 h after carageenan injection. All derivatives and their parent compounds gave dose response relationship activity. However, the derivatives indicated less prolong anti-inflammatory effect than their parent compounds. At the dose of 20 mg/kg.bw and 1 h after carageenan injection, compound **6** and its parent compound (prednisolone) showed the maximum percentages of edema inhibition at 80.99 ± 13.5 and 82.52 ± 9.2%, while compound **7** and its parent compound (hydrocortisone) gave the maximum percentage of edema inhibition at 68.24 ± 10.4 and 63.59 ± 6.8%, respectively. This has suggested that the ferrocenic modification of prednisolone and hydrocortisone does not affect the anti-inflammatory activity. In fact, a slightly less activity of the derivatives than their parent compounds was observed. As known, the affinity of drugs to their receptors depends on three-dimensional characteristics such as size, stereochemical orientation of its functional groups, types of chemical bond and its physical and electrochemical properties.[23] The more bulky size of the novel compounds **6** and **7** from the ferrocenic attachment may hinder some binding to the target receptors, thereby decreasing the target-receptor interactions and leading to the slight lower anti-inflammatory activity than their parent compounds. In addition, the less prolong anti-inflammatory activity of the derivatives than their parent compounds might be due to the presence of the ferrocenic groups which may facilitate the absorption at duodenal enterocyte and the metabolism of the derivatives in the liver.[24]

Table 3. Comparison of in Vivo Anti-inflammatory Activity in Male Rats by Hind Paw Edema Assay of the Ferrocenic Steroidal Anti-inflammatory Drug Derivatives (Compounds **6** and **7**) and Their Parent Compounds

			edema inhibition (%)		
sample code	compound names	dose (mg/kg·bw)	1 h	2 h	3 h
compd **6**	16-ferrocenic-11β-hydroxy androsta-1,4-diene-3,17-dione	5	50.54 ± 3.1	10.48 ± 0.9	7.72 ± 1.7
		10	70.84 ± 7.7	18.28 ± 3.9	9.16 ± 1.2
		20	80.99 ± 13.5	22.54 ± 3.1	17.83 ± 3.1
(6)	prednisolone	5	46.21 ± 1.7	29.36 ± 2.1	27.33 ± 2.7
		10	74.42 ± 2.5	49.88 ± 8.2	33.57 ± 5.0
		20	82.52 ± 9.2	52.24 ± 9.8	48.41 ± 9.1
compd **7**	16-ferrocenic-11β-hydroxy androst-4-ene-3,17-dione	5	36.04 ± 2.1	6.65 ± 0.53	5.36 ± 0.8
		10	51.64 ± 4.9	12.54 ± 5.3	14.72 ± 7.9
		20	68.24 ± 10.4	15.53 ± 1.7	14.41 ± 4.3
(7)	hydrocortisone	5	32.50 ± 0.6	19.65 ± 3.1	12.83 ± 2.2
		10	52.31 ± 7.4	34.98 ± 3.1	27.70 ± 3.2
		20	63.59 ± 6.8	52.46 ± 7.4	35.58 ± 1.5

$$\%\text{paw edema inhibition} = \left[\frac{\%\text{paw edema of the control group} - \%\text{paw edema of the tested group}}{\%\text{paw edema of the control group}}\right] \times 100$$

3. Conclusions

Seven novel ferrocenic steroidal drug derivatives from steroidal sex hormones and steroidal anti-inflammatory drugs have been synthesized. Compounds **1** and **2**, which were ferrocenic derivatives produced from testosterone and methyltestosterone, showed dose-dependent anti-proliferative activity on HeLa cell lines and had similar potency to the standard anticancer drug, doxorubicin. All seven derivatives showed anti-oxidative activities evaluated by DPPH scavenging and metal ion chelating activity but lower than the standard vitamin C and EDTA, respectively. However, all seven parent compounds gave no anti-oxidative activity. Compound **1** showed the highest DPPH scavenging activity but lower than ferrocenic carboxaldehyde and vitamin C of 2 and 12 times, respectively. In addition, compound **1** indicated almost the same chelating activity as ferrocenic carboxaldehyde but lower than the standard EDTA by about 5 times. For in vivo anti-inflammatory activity, the two novel ferrocenic derivatives compounds **6** and **7** showed similar strong activity to their parent compounds. However, these two derivatives indicated less prolonged activity than their parent compounds. This study has demonstrated that the modification of chemical structures of steroidal sex hormones by ferrocenic carboxaldehyde can increase anti-proliferative activity on HeLa cell lines by SRB assay and anti-oxidative activities evaluated by DPPH scavenging and metal ion chelating activity. However, ferrocenic modification of the steroidal anti-inflammatory drugs appeared not to affect their activity. The novel compound **1** or 16-ferrocenylidene-3α-hydroxyandrostan-17-one, which was the ferrocenic derivative of testosterone, was the most potential novel compound that can be further developed for the sex hormone related cancer treatment.

4. Experimental Sections

4.1. Synthesis of Ferrocenic Derivatives. Seven ferrocenic derivatives, including 2-ferrocenylidene-17β-hydroxy androst-4-en-3-one (**1**), 2-ferrocenylidene-3‴-ferrocenic benz[4,5,6]-androst-4-en-3-one (**2**), 16-ferrocenylidene-3α-hydroxy androstan-17-one (**3**), 21-ferrocenylidene-3β,17β-dihydroxy pregn-5-en-18-one (**4**), 16-ferrocenylidene-3-hydroxy estra-1,3,5-trien-17-one (**5**), 16-ferrocenylidene-17β-hydroxy androsta-1,4-diene-3,17-dione (**6**), and 16-ferrocenylidene-11β-hydroxy androst-4-ene-3,17-dione (**7**), were synthesized from testosterone (1) (Sigma Chemical Co., St. Louis, MO), methyltestosterone (2) (Sigma Chemical Co., St. Louis, MO), androsterone (3) (Sigma Chemical Co., St. Louis, MO), 17-hydroxypregnenolone (4) (Sigma Chemical Co., St. Louis, MO), estrone (5) (Sigma Chemical Co., St. Louis, MO), prednisolone (6) (Sigma Chemical Co., St. Louis, MO), and hydrocortisone (7) (Sigma Chemical Co., St. Louis, MO), respectively, by an Aldol condensation reaction. Briefly, a mixture of ferrocene carboxaldehyde (0.25 mmol) (Aldrich Chemical Co., St. Louis, MO), potassium hydroxide at 1.0 mmol (Merck KGaA Co., Darmstadt, Germany), and 1, 2, 3, 4, 5, 6, or 7 at 0.25 mmol in 10 mL of 99.8% ethanol were refluxed at 78 °C for 2 h. After cooling, the mixture was hydrolyzed with distilled water and extracted 3 times with 50 mL of ethyl acetate. An amount of 1.0 g of sodium sulfate was added. The organic layer was collected and dried. The resulting residue was purified several times by column chromatography using petroleum ether/ethyl acetate (7:3) as an eluent (mobile phase) and silica gel (Silica gel 60 0.063−0.200 mm Merck, Darmstadt, Germany) as the stationary phase. Briefly, column (50 cm length, 3 cm diameter) was packed with silica gel 60 in petroleum ether. Then 500 mg of the obtained product was loaded into the silica gel column. The fractions were eluted by the mixture of the mobile phase with the flow rate at 10−12 mL/min. Then 10 mL of each fraction was collected and evaporated at 50 °C by a rotary evaporator. The products were identified by thin layer chromatography (TLC) using petroleum ether and ethyl acetate (6:4) as mobile phase. The TLC plate was sprayed with 10% sulfuric acid, heated at 120 °C, and visualized in UV chamber. Further purification of the products was performed by preparative reversed-phase HPLC (Supporting Information). The purity and molecular formula of the compounds were supported by ^{1}H and ^{13}C NMR spectra and HR-ESI-MS, which showed no significant signal other than those of the expected compounds. Compounds **1**−**7** were obtained with the percentage purity more than 95%. All solvents and reagents used were analytical grade.

4.2. Structural Determination. All derivatives (compounds **1**−**7**) were structurally determined by NMR spectroscopy using a JEOL JNM-GX400 spectrometer (^{1}H, 400 MHz, ^{13}C, 100 MHz, JEOL, Tokyo, Japan) or a JEOL JMS-ECP-500 spectrometer (^{1}H, 500 MHz, ^{13}C, 125 MHz) in $CDCl_3$ with tetramethylsilane as an internal standard. ESI-MS and high-resolution (HR)-ESI-MS in a positive-ion mode were analyzed by a Shimadzu LCMS-2000A system and an Agilent 1100 LC/MSD TOF system. Structural determination of compounds **1**−**7** and the NMR signal assignments cited below were supported from the analysis of ^{13}C DEPT, ^{1}H−^{1}H COSY, HMQC, and HMBC spectra.

2-Ferrocenylidene-17β-hydroxy androst-4-en-3-one, compound **1**, was a red−purple solid, ESI-MS m/z 500 ($[M + H]^+$, 100%). HR-ESI-MS m/z 499.1928, calculated m/z 499.1935 $[C_{30}H_{33}O_2Fe + H_2O]^+$. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.54 (1H, d, J = 2.3 Hz, H-1′), 6.54 (1H, s, H-4), 4.55 (1H, s, H-4′), 4.51 (1H, s, H-3′), 4.49 (1H, s, H-5′), 4.46 (1H, s, H-5′), 4.18 (5H, s, H-6′), 3.73 (1H, m, H-17), 3.34 (1H, d, *J* = 14.6 Hz, H-1), 2.73 (1H, dd, *J* = 3.7, 16.2 Hz, H-6), 2.41 (1H, dd, *J* = 2.3, 14.6 Hz, H-1), 2.12 (1H, m, H-16), 2.07 (1H, dd, *J* = 12.3, 16.2 Hz, H-6), 1.98 (2H, m, H-7), 1.93 (1H, m, H-8), 1.84 (1H, m, H-11), 1.68 (1H, m, H-15), 1.58 (1H, m, H-11), 1.54 (1H, m, H-16), 1.50 (1H, m, H-9), 1.34 (1H, dd, *J* = 6.0, 12.4 Hz, H-15), 1.26 (2H, m, H-12), 1.16 (1H, m, H-14), 1.11 (3H, s, H-18), 0.82 (3H, s, H-19). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 200.4 (C, C-3), 157.3 (C, C-5), 138.5 (CH, C-1′), 128.6 (CH, C-4), 127.7 (C, C-2), 81.4 (CH, C-17), 78.6 (C, C-2′), 71.8 (CH, C-4′), 71.0 (2CH, C-5′), 70.2 (CH, C-3′), 69.5 (5CH, C-6′), 51.2 (CH, C-14), 49.8 (CH, C-9), 46.0 (CH_2, C-6), 42.9 (C, C-13), 40.3 (C, C-10), 39.7 (CH_2, C-1), 36.1 ($2CH_2$, C-7, 12), 33.7 (CH, C-8), 30.4 (CH_2, C-16), 23.2 (CH_2, C-15), 21.0 (CH_2, C-11), 20.6 (CH_3, C-18), 11.0 (CH_3, C-19).

2-Ferrocenylidene-3‴-ferrocenic benz[4,5,6]-androst-4-en-3-one, compound **2**, was a red−purple solid, ESI-MS *m*/*z* 719 ($[M + H]^+$, 100%). HR-ESI-MS *m*/*z* 718.2201, calculated *m*/*z* 718.2196 $[C_{44}H_{46}O_2Fe_2]^+$. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.94 (1H, d, *J* = 7.8 Hz, H-1′), 7.79 (1H, brs, H-1″), 7.74 (1H, d, *J* = 7.8 Hz, H-2′), 4.62 (1H, br s, H-4″), 4.57 (1H, br s, H-5″), 4.51 (1H, br s, H-3‴), 4.50 (1H, br s, H-4‴), 4.48 (1H, br s, H-3″), 4.45 (1H, br s, H-5″), 4.36 (1H, br s, H-5‴), 4.35 (1H, br s, H-5‴), 4.18 (5H, s, H-6″), 4.15 (5H, s, H-6‴), 3.40 (1H, d, *J* = 14.6 Hz, H-1), 3.15 (1H, dd, *J* = 5.3, 17.2 Hz, H-7), 2.39 (1H, d, *J* = 14.6 Hz, H-1), 2.28 (1H, dd, *J* = 11.0, 17.2 Hz, H-7), 1.92 (1H, m, H-11), 1.85 (1H, m, H-16), 1.81 (1H, m, H-8), 1.77 (1H, m, H-16), 1.74 (1H, m, H-15), 1.69 (1H, m, H-12), 1.62 (1H, m, H-11), 1.51 (1H, m, H-12), 1.45 (1H, m, H-9), 1.39 (1H, m, H-14), 1.38 (1H, m, H-15), 1.30 (3H, s, H-20), 1.17 (3H, s, H-19), 0.92 (3H, s, H-18). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 186.0 (C, C-3), 146.9 (C, C-5), 143.0 (C, C-3′), 138.2 (CH, C-1″), 132.0 (C, C-6), 131.0 (C, C-4), 129.3 (C, C-2), 129.3 (CH, C-2′), 125.8 (CH, C-1′), 86.7 (C, C-2‴), 81.6 (C, C-17), 79.1 (C, C-2″), 72.1 (CH, C-4″), 71.7 (CH, C-4‴), 70.9 (CH, C-5″), 70.8 (CH, C-3″), 70.0 (CH, C-5″), 69.7 (5C, C-6‴), 69.4 (5CH, C-6″), 69.3 (CH, C-3‴), 68.4 (CH, C-5‴), 68.2 (CH, C-5‴), 51.6 (CH, C-14), 47.2 (CH, C-9), 45.3 (C, C-13), 39.1 (CH_2, C-1), 39.0 (CH_2, C-16), 36.6 (C, C-10), 34.8 (CH_2, C-7), 32.7 (CH, C-8), 31.5 (CH_2, C-12), 25.9 (CH_3, C-20), 25.0 (CH_3, C-19), 23.6 (CH_2, C-15), 21.1 (CH_2, C-11), 13.9 (CH_3, C-18).

16-Ferrocenylidene-3α-hydroxy androstan-17-one, compound **3**, was a red−purple solid, ESI-MS *m*/*z* 487 ($[M + H]^+$, 100%). HR-ESI-MS *m*/*z* 486.2209, calculated *m*/*z* 486.2209 $[C_{30}H_{38}O_2Fe]^+$. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.26 (1H, s, H-1′), 4.55 (1H, s, H-4′), 4.54 (1H, s, H-3′), 4.43 (1H, s, H-5′), 4.40 (1H, s, H-5′), 4.16 (5H, s, H-6′), 4.07 (1H, brs, H-3), 2.69 (1H, dd, *J* = 6.3, 15.3 Hz, H-15), 2.09 (1H, ddd, *J* = 2.7, 12.7, 15.3 Hz, H-15), 1.91 (1H, m, H-7), 1.89 (1H, m, H-11), 1.71 (1H, m, H-6), 1.68 (1H, m, H-8), 1.57 (1H, m, H-5), 1.55 (2H, m, H-2, H-4) 1.49 (1H, m, H-1), 1.41 (1H, m, H-2), 1.38 (2H, m, H-12), 1.34 (1H, m, H-11), 1.33 (1H, m, H-14), 1.32 (1H, m, H-6), 1.30 (1H, m, H-1), 1.26 (1H, m, H-4), 1.16 (1H, m, H-7), 0.92 (3H, s, H-18), 0.88 (1H, m H-9), 0.85 (3H, s, H-19). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 209.4 (C, C-17), 133.9 (CH, C-1′), 132.7 (C, C-16), 79.2 (C, C-2′), 71.7 (CH, C-4′), 71.0 (2CH, C-5′), 69.8 (CH, C-3′), 69.7 (5CH, C-6′), 66.4 (CH, C-3), 54.5 (CH, C-9), 49.3 (CH, C-14), 47.6 (C, C-13), 39.1 (CH, C-5), 36.3 (C, C-10), 35.8 (CH_2, C-2), 34.6 (CH, C-8), 32.0 (CH_2, C-1), 31.6 (CH_2, C-11), 31.1 (CH_2, C-7), 29.0 (CH_2, C-4), 28.7 (CH_2, C-15), 28.3 (CH_2, C-12) 20.1 (CH_2, C-6), 14.7 (CH_3, C-18), 11.2 (CH_3, C-19).

21-Ferrocenylidene-3*β*,17*β*-dihydroxy pregn-5-en-18-one, compound **4**, was a red−purple solid, ESI-MS *m*/*z* 529 ($[M + H]^+$, 100%). HR-ESI-MS *m*/*z* 528.2322, calculated *m*/*z* 528.2326 $[C_{32}H_{40}O_3Fe]^+$. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 6.59 (d, *J* = 15.1 Hz, H-1′), 6.15 (1H, d, *J* = 15.1 Hz, H-21), 5.37 (1H, brs, H-6), 4.40 (1H, br s, H-3′), 4.34 (1H, br s, H-4′), 4.29 (2H, br s, H-5′), 4.10 (5H, br s, H-6′), 3.54 (1H, m, H-3), 2.63 (1H, m, H-16), 2.51 (1H, dd, *J* = 4.6, 14.7 Hz, H-16), 2.33 (1H, dd, *J* = 3.2, 12.4 Hz, H-4), 2.24 (1H, m, H-7), 2.17 (1H, m, H-4), 2.15 (1H, m, H-15), 1.88 (1H, m, H-1), 1.84 (1H, m, H-2), 1.66 (1H, m, H-14), 1.59 (1H, m, H-12), 1.50 (1H, m, H-15), 1.54 (1H, m, H-7), 1.47 (1H, m, H-2), 1.45 (1H, m, H-8), 1.36 (1H, dd, *J* = 3.5, 12.6 Hz, H-12), 1.33 (2H, m, H-11), 1.08 (1H, m, H-1), 1.03 (1H, m, H-9), 0.99 (3H, s, H-19), 0.81 (3H, s, H-18). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 211.9 (C, C-20), 140.7 (C, C-5), 130.4 (CH, C-1′), 122.8 (CH, C-21), 120.9 (CH, C-6), 84.6 (C, C-17), 77.0 (C, C-2′), 71.6 (CH, C-3), 69.1 (2CH, C-5), 69.1 (5CH, C-6′), 67.2 (CH, C-4′), 66.7 (CH, C-3′), 49.3 (CH, C-9), 45.8 (C, C-13), 45.7 (CH, C-14), 42.0 (CH_2, C-4), 37.0 (C, C-10), 36.7 (CH_2, C-1), 36.7 (CH_2, C-16), 32.8 (CH, C-8), 32.1 (CH_2, C-7), 31.8 (CH_2, C-12), 31.5 (CH_2, C-2), 26.1 (CH_2, C-15), 19.9 (CH_2, C-11), 19.3 (CH_3, C-19), 14.2 (CH_3, C-18).

16-Ferrocenylidene-3-hydroxy estra-1,3,5-trien-17-one, compound **5**, was a red−purple solid, ESI-MS *m*/*z* 468 ($[M + H]^+$, 100%). HR-ESI-MS *m*/*z* 467.1643, calculated *m*/*z* 467.1673 $[C_{29}H_{31}O_3Fe]^+$. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.29 (1H, brs, H-1′), 7.17 (1H, d, *J* = 8.2 Hz, H-1), 6.65 (1H, dd, *J* = 1.8, 8.2 Hz, H-2), 6.60 (1H, d, *J* = 1.8 Hz, H-4), 4.60 (2H, brs, H-5′), 4.49 (1H, brs, H-4′), 4.46 (1H, brs, H-3′), 4.18 (5H, s, H-6′), 2.91 (2H, m, H-6), 2.80 (1H, dd, *J* = 5.5, 14.7 Hz, H-15), 2.41 (1H, m, H-11), 2.30 (1H, m, H-9), 2.22 (1H, m, H-15), 2.10 (1H, m, H-12), 2.08 (1H, m, H-7), 1.70 (1H, brt, *J* = 10.7 Hz, H-8), 1.59 (1H, m, H-11), 1.57 (1H, m, H-12), 1.56 (1H, m, H-14), 1.49 (1H, m, H-7), 0.95 (3H, s, H-18). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 209.2 (C, C-17), 153.5 (C, C-3), 138.0 (C, C-5), 134.5 (CH, C-1′), 132.3 (C, C-16), 132.2 (C, C-10), 126.5 (CH, C-1), 115.3 (CH, C-4), 112.8 (CH, C-2), 79.4 (C, C-2′), 71.9 (2CH, C-5′), 71.3 (2CH, C-3′, 4′), 70.0 (5C, C-6′), 48.3 (CH, C-14), 47.8 (C, C-13), 44.0 (CH, C-9), 37.9 (CH, C-8), 31.6 (CH_2, C-12), 29.5 (CH_2, C-6), 28.6 (CH_2, C-15), 26.8 (CH_2, C-7), 26.0 (CH_2, C-11), 14.8 (CH_3, C-18).

16-Ferrocenylidene-17*β*-hydroxy androsta-1,4-diene-3,17-dione, compound **6**, was a red−purple solid, ESI-MS *m*/*z* 497 ($[M + H]^+$, 100%). HR-ESI-MS *m*/*z* 496.1681, calculated *m*/*z* 496.1700 $[C_{30}H_{32}O_3Fe]^+$. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.31 (1H, brs, H-1′), 7.30 (1H, d, *J* = 9.3 Hz, H-1), 6.29 (1H, d, *J* = 9.3 Hz, H-2), 6.05 (1H, s, H-4), 4.54 (3H, brs, H-11, 3′, 4′), 4.44 (2H, brs, H-5′), 4.13 (5H, s, H-6′), 2.74 (1H, dd, *J* = 5.8, 15.4 Hz, H-15), 2.65 (1H, dd, *J* = 5.0, 13.3 Hz, H-6), 2.42 (1H, m, H-6), 2.37 (1H, m, H-8), 2.28 (1H, m, H-7), 2.25 (1H, m, H-15), 2.05 (1H, m, H-12), 1.62 (1H, dd, *J* = 2.4, 14.4 Hz, H-12), 1.53 (3H, s, H-19), 1.30 (1H, m, H-14), 1.26 (3H, s, H-18), 1.22 (1H, m, H-7), 1.15 (1H, dd, *J* = 2.8, 10.1 Hz, H-9). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 207.3 (C, C-17), 186.4 (C, C-3), 169.4 (C, C-5), 155.0 (CH, C-1), 135.1 (CH, C-1′), 131.6 (C, C-16), 128.0 (CH, C-2), 122.7 (CH, C-4), 78.6 (C, C-2′), 71.7 (CH, C-11), 71.0 (2CH, C-5′), 69.9 (CH, C-3′), 69.7 (CH, C-4′), 69.5 (5CH, C-6′), 56.0 (CH, C-9), 49.8 (CH, C-14), 44.1 (C, C-10), 46.6 (C, C-13), 41.1 (CH_2, C-12), 32.9 (CH_2, C-7), 31.8 (CH_2, C-6), 30.6 (CH, C-8), 28.7 (CH_2, C-15), 21.2 (CH_3, C-19), 16.7 (CH_3, C-18).

16-Ferrocenylidene-11*β*-hydroxy androst-4-ene-3,17-dione, compound **7**, was a red−purple solid, ESI-MS *m*/*z* 499 ($[M + H]^+$, 100%). HR-ESI-MS *m*/*z* 498.1864, calculated *m*/*z* 498.1857 $[C_{30}H_{34}O_3Fe]^+$. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.31 (1H, brs, H-20), 5.71 (1H, brs, H-4), 4.53 (2H, brs, H-5′), 4.50 (1H, brs, H-11), 4.46 (1H, brs, H-3′), 4.42 (1H, brs, H-4′), 4.13 (5H, s, H-6′), 2.75 (1H, dd, *J* = 6.3, 14.9 Hz, H-15), 2.47 (1H, m, H-2), 2.40 (1H, m, H-2), 2.35 (2H, m, H-6), 2.30 (1H, m, H-8), 2.25 (2H, m, H-7), 2.20 (2H, m, H-1, 15), 2.05 (1H, brd, *J* = 14.6 Hz, H-12), 1.88 (1H, dt, *J* = 4.2, 13.4 Hz, H-1), 1.62 (1H, m, H-12), 1.50 (3H, s, H-19), 1.33 (1H, m, H-14), 1.24 (3H, s, H-18), 1.05 (1H, dd, *J* = 2.8, 10.9 Hz, H-9). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 207.4 (C, C-17), 199.3 (C, C-3), 171.3 (C, C-5), 135.1 (CH, C-1′), 131.6 (C, C-16), 122.6 (CH, C-4), 78.7 (C, C-2′), 71.7 (CH, C-11), 71.0 (CH, C-3′), 69.7 (2CH, C-5′), 69.6 (5CH, C-6′), 68.1 (CH, C-4′), 56.8 (CH, C-9), 50.4 (CH, C-14), 46.4 (C, C-13), 41.3 (CH_2, C-12), 39.4 (C, C-10), 34.9 (CH_2, C-1), 33.8 (CH_2, C-2), 31.8 (CH_2, C-6), 31.7 (CH_2, C-7), 30.8 (CH, C-8), 28.6 (CH_2, C-15), 21.1 (CH_3, C-19), 16.8 (CH_3, C-18).

4.3. Anti-proliferation Activity. The anti-proliferation activity of ferrocenic sex steroidal hormone derivatives, Compounds

1−5 were investigated on human cervical adenocarcinoma cell lines (HeLa) by sulforhodamine B (SRB) assay as previous described.[15] Briefly, HeLa cell lines (National Cancer Institute, Bangkok, Thailand) were grown in DMEM medium (Sigma-Aldrich Biotechnology, St. Louis, MO), supplemented with 10% fetal calf serum (Sigma-Aldrich Corporation, St. Louis, MO), 50 IU/mL of penicillin, and 50 mg/mL of streptomycin (Gibco BRL, Gaithersburg, MD). The cells were incubated at 37 °C in a 5% CO_2 incubator. The cells were treated with various concentrations (1×10^{-8} to 10 mg/mL) of the derivatives and further incubated for 24 h. The absorbance was measured at 570 nm by a microplate reader (model 680 BIO-RAD, USA). The GI_{50} values which were the concentrations of the samples that gave 50% growth inhibition were determined. Doxorubicin (Dabur Pharma, Thailand) at 1×10^{-7} to 1 mg/mL was used as a standard control.

4.4. Anti-oxidative Assay. 4.4.1. Free Radical Scavenging Activity. Compounds **1−7** were assayed for free radical scavenging activity by the modified 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method.[16] Briefly, the derivatives at various concentrations (1×10^{-3} to 5 mg/mL) were dissolved in absolute methanol. An amount of 100 μL of the sample was mixed with 0.2 mM of DPPH (Sigma Chemical Co., St. Louis, MO). The mixture was incubated at 28 °C for 30 min and measured for the absorbance at 515 nm by a spectrophotometer (model 680 BIO-RAD, USA). The percentages of DPPH radical scavenging activity were calculated. The SC_{50} values which were the concentrations of the samples, providing 50% scavenging activity were determined.

4.4.2. Metal Ion Chelating Activity. The chelating activity of compounds **1−7** were assayed by the method previously described.[17] Briefly, 0.1 mL volume of various concentrations of the derivatives (1×10^{-4} to 4.0 mg/mL) was added to 0.05 mL of 2 mM $FeCl_2$ (Merck KGaA Co., Darmstadt, Germany). The reaction was initiated by the addition of 0.2 mL of 5 mM ferrozine (Merck KGaA Co., Darmstadt, Germany), adjusted to 0.25 mL by 99.8% v/v ethanol and incubated at 25 °C for 10 min. The absorbance at 570 nm was measured by a spectrophotometer. The MC_{50} values,which were the concentrations of the samples providing 50% inhibition of metal ion chelating, were determined.

4.5. In Vivo Anti-inflammatory Activity Test. The anti-inflammatory activity in rats of compounds **6** and **7** were performed by hind paw edema method.[18] Sprague−Dawley male rats from National Laboratory Animal Centre, Mahidol University, Bangkok, Thailand (200−250 g body weight, age 6−7 weeks) were used. The animals were injected subcutaneously with 0.1 mL of 1% carrageenan suspension (Type IV, Sigma Co., St. Louis, MO) into the plantar side of the hind paw. All samples were dispersed in distilled water and fed orally by a gastric tube 1 h prior to carrageenan injection. The positive control groups were fed with prednisolone or hydrocortisone. Distilled water was used as a negative control. Paw edema was determined by volume displacement method using a plethysmometer (Ugo Basile 7150, USA) at initial, 1, 2, and 3 h after carrageenan injection. The percentage edema inhibition was calculated according to the following equation:

$$\begin{aligned}\%\text{paw edema inhibition} &= [(\%\text{paw edema of the control} \\ &\quad 12ptgroup - \%\text{paw edema of the tested group})/ \\ &\quad (\%\text{paw edema of the control group})] \times 100\end{aligned}$$

4.6. Statistical Analysis. All results were performed from three independent and separate experiments. Mean and standard deviations were evaluated. Statistical analysis of the results was performed by the analysis of variance (ANOVA).

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Supporting Information Available: Purification of the synthesized ferrocenic derivatives by reversed-phase HPLC, ^{13}C, ^{1}H, and HMBC NMR data, and ^{1}H NMR spectra of compounds **1−7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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