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Synthesis of New Steroidal Isoxazoles: Inhibitors of Estrogen Synthase

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Abstract—A novel class of steroidal A/B ring isoxazoles have been prepared by two independent reaction schemes using 3β,17β-diacetoxyandrost-5-ene (**1**) and 3β,17β-diacetoxyandrost-4-en-6-one (**4**) as synthetic precursors. The key common intermediate in these syntheses, 3β,17β-diacetoxyandrost-4-eno[6,5,4-*c*,*d*] isoxazole (**3**), was prepared by synthetic methods described in both schemes. Further chemical modification of **3** yielded 3β,17β-dihydroxyandrost-4-eno[6,5,4-*c*,*d*] isoxazole (**6**), androst-3,17-dione-4-eno[6,5,4-*c*,*d*] isoxazole (**7**), and 17β-hydroxyandrost-3-one-4-eno[6,5,4-*c*,*d*] isoxazole (**9**). Human placental estrogen synthase (aromatase) bioassays were conducted to obtain the following IC_{50} values resulting from a 50% reduction of enzymatic activity: **6**, 120.5 μM; **7**, 1.889 μM. **9**, 18.57 μM.

Introduction

The estrogen synthase (aromatase) enzyme system is responsible for the biosynthesis of estrogen hormones in human females. Estrogens are vital for normal growth and development, but will promotes the growth of certain breast cancers. Approximately 30–50% of breast cancers are considered to be hormone-dependent. Consequently, regulation of estrogen biosynthesis has advanced as a potential therapeutic strategy.[1,2] This has led to the development of active-site inhibitors which may have potential for the control of breast cancer.[3]

Several classes of steroidal aromatase inhibitors, both competitive and mechanism-based, have been described in the literature. Among the steroids which have been studied in this context are the 6-hyxroxyiminoandrost-4-en-3-ones, which have shown a high affinity for human placental aromatase, and function as competitive inhibitors of the enzyme.[4]

As an extension of our studies of aromatase inhibition,[5,6] we report here the synthesis of a group of steroidal A/B ring isoxazoles which mimics the 6-oxyimino function found on the 6-hydroxyiminoandrostenes described above.

Results and Discussion

We have prepared three novel steroidal isoxazoles (**6**, **7**, and **9**) using two independent reaction schemes. The first, Scheme 1, utilizes 3β,17β-diacetoxyandrost-5-ene (**1**) as a starting material which is first converted to the C-6 vinyl nitrate[7] and then rearranged photochemically to yield the 3β,17β-diacetoxyisoxazole 3.[8] In the second, Scheme 2, 3β,17β-diacetoxyandrost-4-en-6-one (**4**) is converted to the C-6 oxime derivative (**5**), which was rearranged to **3** by Pb $(OAc)_4$, I_2 (Method 1) or by treatment with KI, I_2 (Method 2[6]), which also gave **3** in good yield. Thus, the common intermediate **3** (3β,17β-diacetoxyandrost-4-eno[6,5,4-*c*,*d*] isoxazole) was prepared by two independent reaction sequences using known methods to prepare isoxazoles (Schemes 1 and 2). However, the rearrangement of oxime **5** to intermediate **3** by Pb $(OAc)_4$, I_2 represents a new synthetic approach to the synthesis of isoxazoles. The intermediate **3** was further modified to produce the final isoxazoles (**6**, **7**, and **9**). In Scheme 3, the diacetate 3 was treated with mild base to produce the 3β,17β-diol **6**. A

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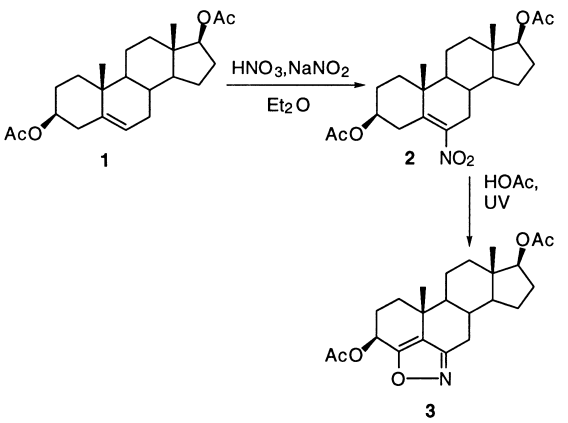
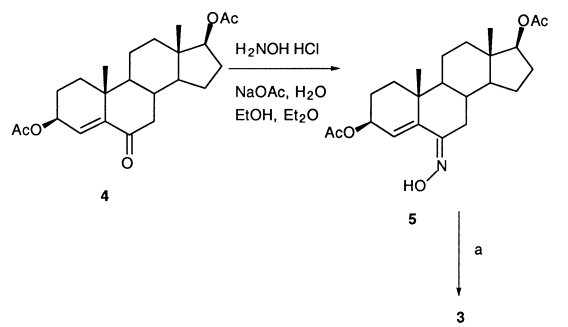


Scheme 1. The synthesis of 3β,17β-diacetoxyandrost-4-eno[6,5,4-*c*,*d*] isoxazole (**3**) from 6-nitro precursor (Method 1).

Swern oxidation[10] of **6** resulted in the 3,17-dione **7**. Also, in Scheme 3 the diacetate **3** was treated with very mild base ($NaHCO_3$), which selectively hydrolyzed the less hindered acetate at C-3 to yield **8**. A Swern oxidation, followed by base catalyzed hydrolysis, gave the final isoxazole **9**.

Microsomes were prepared from term human placentas and stored at −70 °C until used in the aromatase assays. Aromatase assays were performed as described previously.[11,12] The following IC_{50} values resulted from a 50% reduction of enzymatic activity: **6**, 120.5 μM; **7** 1.889, μM; **9** 18.57 μM.

The three steroidal isoxazoles **6**, **7**, and **9** were prepared as mimics of the 6-oxyimino (oxime) function found on the known aromatase inhibitors 17β-hydroxy-6-hydroxyiminoandrost-4-en-3-one and 6-hydroxyiminoandrost-4-ene-3,17-dione.[4] Also, **6**, **7**, and **9** contain a similar oxygen function as found in the well-known aromatase



Scheme 2. The synthesis of 3β,17β-diacetoxyandrost-4-eno[6,5,4-*c*,*d*] isoxazole (**3**) from a 6-oxamine precursor. (a) Method 2. $Pb(OAc)_4$, I_2, $CaCO_3$, cyclohexane, reflux, hv. Method 3. KI, I_2, $NaHCO_3$, THF-H_2O, in dark, reflux.

inhibitor 4-hydroxyandrost-4-ene-3,17-dione.[13] These structural features, which are found in other potent aromatase inhibitors, were the impurus for our attempt to prepare the steroidal A/B ring isoxazoles described herein.

The structural requirements of C_{19} steroids needed for a favorable interaction with the enzymatic site of aromatase have been determined.[14] Those include a ketone functionality at C-3 and a 17-keto or 17β-hydroxyl substituent. Also, steroids with a linear configuration (e.g. 4-en-3-one) in the A and B ring are known to be effective inhibitors. The isoxazoles **7** and **9** exhibited the most potent inhibition and possessed structural features which were most consistent with those described for previous effective inhibitors.

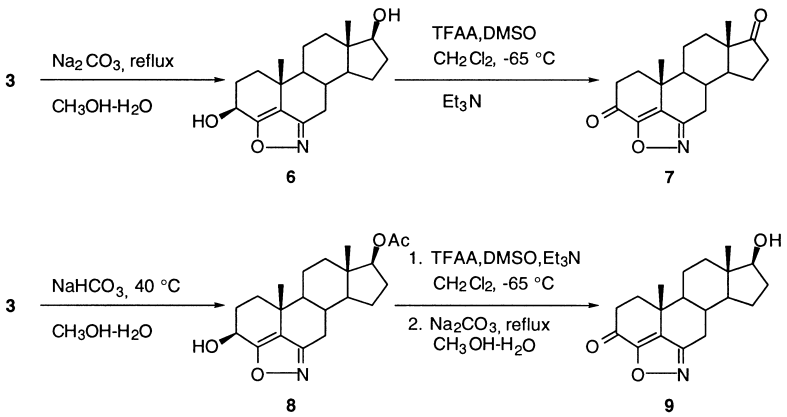
In comparison, the model steroids 17β-hydroxy-6-hydroxyiminoandrost-4-en-3-one, 6-hydroxyimino-androst-4-ene-3,17-dione,[4] and 4-hydroxyandrost-4-ene-3,17-dione[13] have been shown to possess greater inhibitory activity than the described isoxazoles **7** and **9**. However, this is the first report describing the inhibition of aromatase activity by steroidal A/B ring isoxazoles.

In summary, we have described the chemical synthesis of a new series of steroidal A/B ring isoxazoles and have found those possessing a ketone functionality at C-3 to be more potent inhibitors of human placental aromatase.

Experimental

3β,17β-Diacetoxyandrost-5-ene was obtained from the Sigma Chemical Co. (St. Louis, MO, USA). The synthesis of 3β,17β-acetoxyandrost-4-en-6-one (**4**) has been described.[4] Procedures for recording melting points (mp) and of infrared (IR), ^1H NMR, ^{13}C NMR, and mass spectra (MS, electron impact, EI, and chemical ionization, CI) together with details concerning column and thin-layer (TLC) chromatography have been described.[1516] Combustion analyses were performed by Galbraith Laboratories, Inc. (Knoxville, TN, USA).

In a typical assay,[11,12] a 0.9 mL aliquot of microsomal suspension was mixed with 200,000 dpm of [1β^{3}H]-androstenedione (24.56/mmol) (New England Nuclear-DuPont, Boston, MA, USA), 50 nM androstenedione, and 0.1 mL of an NADPH generating system (NADP 5 mg, glucose-6-phosphate 20 mg, glucose-6-phosphate dehydrogenase 25 IU in 0.9 mL phosphate buffer) vortexed vigorously and incubated at 37 °C for 30 min. Microsomes incubated without the cofactor were used as negative controls. Incubations were terminated by placing the tubes in an ice-water bath and adding 2 mL



Scheme 3. The synthesis of androst-3,17-dione-4-ebo[6,5,4-*c*,*d*] isoxazole (**7**) and 17β-hydroxy androst-3-one-4-eno[6,5,4-*c*,*d*] isoxazole (**9**).

chloroform. Estrogen production was measured from the resulting ^3H_2O produced by the release of tritium from the C-1β position during aromatization of radiolabeled substrate. Candidate inhibitors were included in the incubation at concentrations ranging from 10 to 500 nM.

Preparation of 3β,17β-diacetoxy-6-nitroandrost-5-ene (2)

3β,17β-Diacetoxyandriost-5-ene (1.0 g, 2.7 mmol) was dissolved in 20 mL anhydrous ether. It was cooled to 10°C with an ice bath, and 25 mL of 70% HNO_3 was dropped slowly into the ether solution with stirring (keeping the temperature between 35–40 °C). The mixture turned greenish-gray, and then yellow as the temperature decreased. When the internal solution temperature dropped to 5 °C, sodium nitrite (0.21 g, 3.0 mmol) was added in five portions over 25 min. After stirring for another 10 min, the solution was poured into a separatory funnel that contained ice water. The water was drained immediately after pouring without further shaking the funnel. The solution was washed with ice-water and ice-cold 6 N NaOH solution until it was non acidic. Ethyl ether was added to compensate for the ether loss. Finally the solution was dried with anhydrous sodium sulfate. Evaporation of the ether yielded a brownish oil. Product purification was accompanied by column chromatiography on silica gel using 10% Ether-toluene solution as the eluting solvent. Compound **2** (0.91 g, 80% yield) was isolated as a white solid after column chromatography. Mp: 163.5–165.0 °C, MS (CI, NH_3): 437.1 (M + NH_4, 7.3), 420.0 (M + H, 5.3), 402.3 (35.5), 284.3 (100); IR: 1732, 1523, 1437, 1367, 1250, 1036, 604 cm^{-1}; ^1H NMR: 0.814 (s, 3H, H-18), 1.151 (s, 3H, H-19), 4.656 (m, 1H, H-3α), 4.624 (m, 1H, H-17α). ^{13}C NMR: C-1 36.28, C-2 26.85, C-3 71.77, C-4 32.82, C-5 137.56, C-6 146.22, C-7 31.34, C-8 31.35, C-9 48.77, C-10 37.16, C-11 20.41, C-12 36.07, C-13 42.34, C-14 50.44, C-15 23.34 C-16 27.32, C-17 82.13, C-18 11.83, C-19 19.71, two carbonyl carbons on 3,17-diacetyl group 171.01 and 170.00, two methyl carbon on 3,17-diacetyl groups 21.08 and 21.16 (found: C, 65.71; H, 7.83; N, 3.28. $C_{23}H_{33}NO_6$ requires C, 65.85; H, 7.93; N, 3.34%).

Preparation of 3β,17β-diacetoxyandrost-4-eno [6, 5, 4-*c*,*d*] isoxazole (3)

Three methods were used to prepare this compound. Method 1. Compound **2** (1.0 g, 2.4 mmol) was dissolved in 50 mL acetic acid. The mixture was warmed to 75 °C, then placed into a photochemical reactor and exposed to a long wavelength (> 300 nm) UV light, while the reaction was monitored by TLC. When the reaction was complete (about 3.5 h) the solution was extracted with ethyl ether and washed with water, sodium bicarbonate, saturated sodium chloride, and the solution dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was subjected to silica gel column chromatography using ether-toluene (500 mL of 5% ether and 500 mL of 10% ether in sequence) as the eluting solvent. 3β,17β-Diacetoxyandrost-4-eno[6,5,4-*c*,*d*] isoxazole (**3**, 0.20 g, 21% yield) was isolated after column chromatography as a white solid. Mp: 164.5–166.0 °C; IR: 2967, 1746, 1352, 1235, 1030 cm^{-1}; MS (CI, NH_3): 402.1 (M + H, 5.7), 357.2 (13.2), 331.1 (6.3), 43.1 (100); ^1H NMR: 0.867 (s, 3H, H- 18), 1.228 (s, 3H, H-19), 3 and 17 acetate methyl 2.056 and 1.970, 5.785 (dd, 1H, H-3α), 4.559 (dd, 1H, H-17α). ^{13}C-NMR: C-1 36.82, C-2 26260, C-3 65.24, C-4 161.11, C-5 125.76, C-6 158.53, C-7 25.52, C-8 32.10, C-9 49.31, C-10 32.62, C-11 20.31, C-12 36.08, C-13 42.81, C-14 51.33, C-15 23.63, C-16 27.21, C-17 82.23, C-18 12.01, C-19 20.09, two carbonyl carbons on 3,17-diacetyl group 170.97 and 170.31, two methyl carbons on 3,17-diacetyl group, 21.08 and 21.16. (Found: C, 68.71; H, 7.62; N, 3.38. $C_{23}H_{31}NO_5$ requires C, 68.80; H, 7.78; N, 3.49%.)

Method 2. 3β,17β-Diacetoxyandrost-4-en-6-one (**4**, 1.0 g, 2.6 mmol) was dissolved in 150 mL ethanol–ether–water (2:2:1) mixed solvent. Hydroxylamine hydrochloride (1.1 g, 16 mmol) and sodium acetate (1.1 g, 17 mmol) were added. The resulting reaction mixture was stirred at room temperature for 24 h, then extracted with ether, and washed with water and brine. Evaporation of the solvent give 3β,17β-diacetoxy androst-4-ene-6-oxime (**5**) (1.0 g) as a brownish oil. The crude oxime (1.0 g) dissolved in 100 mL of cyclohexane, and added to a reaction mixture of lead tetracetate (5.0 g, 13 mmol), calcium carbonate (2.0 g) and iodine (1.0 g, 3.9 mmol) in 150 mL of cyclohexane. The reaction mixture was heated at reflux under a 300 watt tungsten lamp until the iodine color faded (about 1.5 h). The reaction mixture was filtered, and evaporation of the filtrate gave a brownish oil. The oil was purified by silica gel column chromatography, using ether–toluene (500 mL of 5% ether and then 500 ml of 10% ether in sequence) as the eluting solvent. 3β,17β-Diacetoxy-androst-4-eno[6,5,4-*c*,*d*] isoxazole (**3** 0.51 g, 49% yield) was isolated as a white crystalline solid after column chromatography. The spectral and physical data were identifical to the product made by method 1.

Method 3. Method 2 was used to obtain the crude 6-oxime (**5**). The crude 6-oxime (0.46 g, 1.2 mmol) was dissolved in 20 mL of THF. Sodium bicarbonate (0.32 g, 3.8 mmol), iodine (0.29 g, 1.2 mmol), potassium iodide (0.50 g, 0.6 mmol) and 7.0 mL water was added. The reaction mixture was heated at reflux for 4 h in the dark, and then cooled to room temperature and extracted with ethyl acetate. The organic layer was washed with sodium thiosulfate and brine. The solvent was removed under reduced pressure to give a brownish oil, which was purified by silica gel column chromatography using ether-toluene (500 mL of 5% ether and then 500 mL of 10% ether in sequence) as the eluting solvent. 3β,17β,-Diacetoxyandrost-4-eno[6,5,4-*c*,*d*] isoxazole (**3** 0.38 g, 82% yield) was isolated after column chromatography. The spectral and physical data was identical to the produce made by method 1.

Preparation of 3β,17β-dihydroxyandrost-4-eno[6,5,4-*c*,*d*] isoxazole (6). 3β,17β-Diazetoxyandrost-4-eno[6,5,4-*c*,*d*] isoxazole (**3**, 0.30 g, 0.75 mmol) was dissolved in 20 mL methanol, and sodium carbonate (0.12 g) was added. The solution was heated at reflux for 2 h, and the solvent evaporated. The residue was extracted with ethyl acetate (3×20 mL), and the combined extracts were washed with aqueous sodium bicarbonate, water, saturated NaCl solution, and dried with anhydrous sodium sulfate. Evaporation of the solvent yielded 0.23 g of pure 3β,17β-dihydroxy-androst-4-eno[6,5,4-*c*,*d*] 6-isoxazole as a white solid in 95 yield. Mp: 150 °C (decomposed); IR: 3289, 2945, 1653, 1406, 1250.3, 1061, 708 cm^{-1}; MS(CI, NH_3): 318.2 (M + H, 2.3), 282.3 (M + H-2H_2O, 9.2); 1H-NMR: 0.812 (s, 3H, H-18), 1.240 (s, 3H, H-19), 4.716 (t, 1H, H-3α), 3.612 (t, 1H, H-17α). ^{13}C-NMR: C-1 38.12, C-2 30.43, C-3 64.68, C-4 160.17, C-5 125.21, C-6 152.76, C-7 26.32, C-8 33.67, C-9 51.21, C-10 33.67, C-11 21.63, C-12 37.27, C-13 44.41, C-14 52.87, C-15 24.42, C-16 31.17, C-17 82.21, C-18 11.59, C-19 20.59. (Found: C, 71.81; H, 8.46; N 4.38. $C_{19}H_{27}NO_3$ requires C, 71.84; H, 8.57; N, 4.41%.)

Preparation of androst-3,17-dione-4-eno[6,5,4-*c*,*d*] isoxazole (7). Trifluoracetic anhydride (0.52 g 2.5 mmol) in 5 mL dry methylene chloride (fresh distilled over CaH_2) was added dropwise into a solution of dimethyl sulfoxide (0.25 g 3.2 mmol) in 5 mL of dry methylene chloride, which was cooled in an acetone–dry ice bath, under dry nitrogen for 0.5 h. The resulting solution was stirred for another 15 min. A solution of 3β,17β-dihydroxyandrost-4-eno[6,5,4-*c*,*d*] isoxazole (**6**, 0.20 g, 0.63 mmol) in 5 mL of fresh distilled THF was added into the solution dropwise for 0.5 h. The temperature was maintained at −65 °C in the above process. Then the solution was allowed to warm to room temperature slowly and stirred another 0.5 h. Triethylamine (1 mL) and methylene chloride (20 mL) was added. The solution was washed with water and brine, and dried with anhydrous magnesium sulfate. Evaporation of the solvent gave a brownish oil residue, which was purified by silica gel column chromatography using ether–toluene (200 mL of 5% ether and then 600 mL of 10% ether in toluene in sequence) as the eluting solvent. Androst-3,17-dione-4-eno[6,5,4-*c*,*d*] isoxazole (**7**, 0.16 g, 81% yield) was isolated as white crystals after column chromatography. Mp: 190 °C decomposed; IR: 2973, 2948, 1736, 1701, 1458, 1318, 1078.35, 1015 cm^{-1}; MS(EI): 313.00 (M, 2.8), 298.2 (M-CH_3, 3.1), 224.3 (100). ^{1}H NMR: 0.975 (s, 3H, H-18), 1.348 (s, 3H, H-19). ^{13}C NMR: C-1 38.20, C-2 36.75, C-3 185.21, C-4 159.08, C-5 138.10, C-6 156.60, C-7 25.20, C-8 32.15, C-9 48.93, C-10 33.51, C-11 20.18, C-12 30.83, C-13 48.00, C-14 51.79, C-15 22.12, C-16 35.66, C-17 219.50, C-18 13.81, C-19 19.27. (Found: C, 72.71; H, 7.35; N, 4.41. $C_{19}H_{23}NO_3$ requires C, 72.82; H, 7.40; N, 4.47%.)

Preparation of 17β-hydroxyandrost-3-one-4-eno[6,5,4-*c*,*d*] isoxazole (9). 3β,17β-Diacetoxyandrost-4-eno[6,5,4-*c*,*d*] isoxazole (3, 0.40 g, 1.0 mmol) was dissolved in 40 mL methanol, and sodium biocarbonate (0.10 g) was added. The solution was stirred at 35 °C overnight and the solvent evaporated. The residue was extracted with ethyl acetate (3×20 mL). The extracts were combined and washed with sodium bicarbonate solution, water, saturated NaCl solution, and dried over anhydrous sodium sulfate. After evaporating the solvent 3β-hydroxy-17β-acetoxyandrost-4-eno[6,5,4-*c*,*d*] isoxazole (**8**) was obtained as a brownish oil (0.35 g). The crude

compound (**8**) was oxidized to 17β-acetoxyandrost-3-one-4-eno[6,5,4-*c*,*d*]isoxazole by Swern oxidation without further purification. The same procedure for the oxidation of **6** to **7** was used to conduct the oxidation. The 17β acetoxy group on the crude oxidized product was hydrolyzed by heating the methanol solution with sodium carbonate at reflux. 17β-Hydroxyandrost-3-one-4-eno[6,5,4-*c*,*d*] isoxazole (**9**, 0.20 g, 62% yield) was isolated as light yellow crystals after column chromatography. Mp: 158.0–159.5 °C; IR: 3451, 2973, 2948, 2874, 1701, 1318, 1078, 1053, 1030 cm^{-1}; MS (CI,NH_3): 333.2 ($M+NH_4$, 2.4), 316.2 ($M+H$, 3.5), 298.1 (7.5); ^1H NMR: 0.844 (s, 3H, H-18), 1.320 (s, 3H, H-19), 3.72 (t, 1H, H-17a). ^{13}C NMR: C-1 38.38, C-2 36.83, C-3 H-19), 3.72 (t, 1H, H-17a). ^{13}C NMR: C-1 38.38, C-2 36.83, C-3 185.54, C-4 159.59, C-5 138.51, C-6 156.53, C-7 25.81, C-8 32.64, C-9 48.93, C-10 33.53, C-11 20.56, C-12 35.95, C-13 43.35, C-14 51.54, C-15 23.73, C-16 30.29, C-17 81.52, C-18 11.27, C-19 19.32. (Found: C, 72.78; H, 8.12; N, 4.35. Requires C, 72.35; H, 7.99; N, 4.44%.)

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