Synthesis of Deuterium-Labeled Androst-5-ene-17β,19-diol and Its 4-Ene Isomer as Internal Standards for the Determination of the 19-Oxygenation of Aromatase Inhibitors Using GC-MS

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Aromatase is a cytochrome P-450 enzyme which catalyzes the conversion of androst-4-ene-3,17-dione (androstenedione) to estrone through three sequential oxygenations of the 19-methyl group.1) Inhibitors of aromatase have recently attracted interest not only in the treatment of advanced estrogen-dependent breast cancer2) but also in the elucidation of the spatial aspects of the active site of the enzyme as well as the still unsolved mechanism of the aromatase reaction.3)

We have previously reported that the androst-4-ene-17-one (2),4) 3-deoxy analog of the natural substrate androstenedione, and its 5-ene isomer 15) are very potent competitive inhibitors of aromatase, although they have no carbonyl group at C-3 which is thought to be essential for tight binding of the substrate to the active site of aromatase. We have recently reported that some competitive inhibitors of aromatase, 6-alkyl6) and 6-oxo7) androstenediones, can serve as substrates for the enzyme to afford estrogens when they are incubated with human placental microsomes. Thus, it was of interest to investigate the relationship between the aromatase inhibitory activity of the 3-deoxy compounds, 1 and 2, and their ability to act as substrates. It was postulated that the aromatase products of compounds 1 and 2, obtained by incubation with placental microsomes, would be a mixture of the 19-hydroxy and 19-oxo derivatives5) as well as their 17β-alcohol analogs produced by the action of 17β-hydroxysteroid dehydrogenase6) in each incubation system. The aromatase-catalyzed 19-oxygenation activity could be determined by GC-MS analysis of the 17β,19-dihydroxy steroids obtained after NaBH₄ reduction of the mixture of 19-oxygenated products.6) Thus, we synthesized the deuterium labeled 17β,19-diols 6 and 15-d₄ as internal standards.

Results and Discussion

We initially focused on the preparation of [3β,7,7,17α-2H₄]androst-5-ene-17β,19-diol (6). [7,7-2H₂]β-Acetoxyandrost-5-en-17-one1) (d₄, 98.6 atom%) was converted [7,7-2H₂]β-p-toluenesulfonyloxym-(tert-butyldimethylsilyl)androst-5-en-17-one (3) according to a previously reported method6) involving the addition of hypobromous acid to a double bond at C-5, followed by the “hypoiodate reaction” (lead tetraacetate, iodine, and hydrochloric acid) and subsequent zinc dust reduction (Fig. 1).10) Reductive elimination of the 3β-hydroxy group, with zinc dust–D₂O in the presence of NaI, produced the 3β-deutero compound 4 (d₄, 89.5 atom%) (Table 1). Deprotection of the 19-tert-butyldimethylsilyl (TBDMS) group of compound 4 with acid followed by reduction of the 17-oxo function with NaBD₄ afforded the deuterated 17β,19-diol 6 (d₄, 79.8 atom%).

We then synthesized another deuterium-labeled steroid, [3β,7,7,17α-2H₄]androst-5-ene-17β,19-diol (6) and [3,3,7,7,17α-2H₅]androst-4-ene-17β,19-diol (15-d₄) were synthesized as internal standards for gas chromatographic-mass spectrometric analysis of the 19-hydroxylation of androst-5-en-17-one (1) and its 4-ene isomer 2, inhibitors of estrogen biosynthesis, using human placental aromatase. Treatment of [7,7-2H₂]β-tosylate 3 with Zn–NaI–D₂O, followed by reduction with NaBD₄ gave compound 6 (d₄, 79.8 atom%). Compound 15-d₄ was synthesized via 3β,17β-dihydroxy-5-en-7-one 10 as a key intermediate. Deoxygenation of the 5-en-7-one 10 and [7,7-2H₂]17β-hydroxy-4-en-3-one 13-d₄, produced from compound 10 in two steps, with AlCl₃, was used for the deuterium labeling reaction, producing compound 15-d₄ (d₄, 93.5 atom%). The 19-oxygenation products of aromatase inhibitors 1 and 2 could be analyzed, after NaBH₄ reduction, as the corresponding 17β,19-diol bis-trimethylsilyl ethers using the internal standards 6 and 15-d₄.

Key words  aromatase inhibitor; 19-oxygenation; deuterium-labeled steroid; synthesis; internal standard; GC-MS

1
2

Chart 1. Structures of 3-Deoxy Steroids

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Fig. 1. Synthesis of Deuterated 17β,19-dihydroxy-5-ene 6
a) Zn dust, NaI, D₂O, diglyme.  b) NaBH₄, MeOD.  c) NaBD₄, MeOD.

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[3,3,7,7-2H4]androst-4-ene-17β,19-diol (15-d4). Non-labeled compound 15 has been synthesized previously using desulfurization of the 3,3-ethylenedithio derivative of 19-(tert-butyl-dimethylsiloxy)androst-4-ene-3,17-dione with a sodium-liquid ammonia–MeOH system. 4) This synthesis was unsuitable for introduction of deuterium atoms at C-3 because deuterated liquid ammonia is not readily available. We therefore employed another route to synthesize the deuterated compound 15. 3β-Acetoxy-19-(tert-butyldimethylsiloxy)androst-5-en-17β-ol (7), obtained by NaBH4 reduction of the known 17-oxo derivative,12) was converted to 3β,17β-diacetate, 8. Treatment of this with tert-butylhydroperoxide and pyridinium dichromate in the presence of Celite 54513) gave the 7-oxo compound, 9, in 63% yield from compound 7 (Fig. 2). After hydrolysis of acetate 9 with K2CO3, the reductive deoxygenation with AlCl3H2 or AlCl3D2 (generated from LiAlH4 and AlCl3 or LiAlD4 and AlCl3 in ether, respectively) yielded the 5-ene compound, 11, or its 7,7-deuterio analog (11-d2, d2->99 atom%) in good yield. This 3β,17β-diol, 11 or 11-d2, was oxidized with Jones reagent to the 3,17-dione, 12 or 12-d2, followed by treatment with 0.48 mol eq of NaBH4 to give the 17β-ol, 13, or its 7,7-deuterio analog, 13-d2, in 15% yield from compound 11 or 11-d2. Treatment of 4-en-3-one steroid, 13 or 13-d2, with AlCl3H2 or AlCl3D2 as described above, and subsequent hydrolysis of the 19-siloxy group gave the 3-deoxy compound, 15, or its 3,3,7,7-deuterio analog, 15-d4, (d4, 93.3 atom%) respectively in good yield.

Table 1. Deuterium Content of Labeled Compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular or Relative intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fragment ion (m/z)</td>
</tr>
<tr>
<td>[7,7-2H2]3β-Acetoxy-androst-5-en-17-one</td>
<td>M−=−60(270)</td>
</tr>
<tr>
<td>4</td>
<td>M−=−57(345)</td>
</tr>
<tr>
<td>5</td>
<td>M−(288)</td>
</tr>
<tr>
<td>6</td>
<td>M−(290)</td>
</tr>
<tr>
<td>6-bis TMS</td>
<td>M−=−90(344)</td>
</tr>
<tr>
<td>11-bis TMS</td>
<td>M−=−57(570)</td>
</tr>
<tr>
<td>15-d1</td>
<td>M−(290)</td>
</tr>
<tr>
<td>15-d1-bis TMS</td>
<td>M−=−90(344)</td>
</tr>
</tbody>
</table>

a) Analysis by GC-MS. b) Analysis by direct MS.

Fig. 3. Mass Spectra of the Trimethylsilyl Ether Derivatives of Compounds 6 (A) and 15-d1 (B)

Fig. 2. Synthesis of 17β,19-Dihydroxy-4-ene 15 and Its [3,3,7,7-2H4] Analog

a) (CH3CO)2O, pyridine. b) tert-ButOOH, pyridinium dichromate, Celite, benzene. c) K2CO3, MeOH, H2O. d) LiAlH4 or LiAlD4, AlCl3, Et2O. e) Jones reagent, acetone. f) NaBH4, MeOH. g) dil. HCl, THF, 2-propanol.
The deuterium content of compounds 6 and 15-\_d4 was very high making them suitable internal standards for GC-MS analysis.

The mass spectra and deuterium content of the trimethylsilyl derivatives of the deuterated steroids 6 and 15-\_d4 are shown in Fig. 3 and Table 1. In every case, no molecular ion (M^+ , m/z 434) was observed and the base ion peak (m/z 344) was a fragment ion corresponding to M^+ - 90, with a deuterium content different from that of the M^+ ion obtained by the direct MS method. During fragmentation of the trimethylsilyl (TMS)-derivative, a deuterium incorporated at C-3 of the 4-ene steroid, 15-\_d4, would be lost. However, the deuterium content (d4, 61.0 and 64.8 atom\% for 6 and 15-\_d4, respectively) is still sufficient for use as an internal standard for GC-MS analysis.

Using a selected-ion monitoring method with the fragment ion M^+ - 90 (m/z 344 and 348 for non-labeled steroid and internal standard, respectively), compounds 6 and 15, potential metabolites of inhibitors 1 and 2 of aromatase, could be detected in amounts as low as 50—80 pg (s/n = 10).

Experimental

Melting points were measured on a Yanagimoto melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer Fourier transform (FT)-IR 1725 spectrophotometer and UV spectra in 95% EtOH solution. 1H-NMR spectra were obtained in CDCl3 solution with a JEOL EX 270 (270 MHz) spectrometer using tetramethylsilane as an internal standard, and direct MS with a JEOL JMS-DX 303 spectrometer. GC-MS was conducted with silica-gel (E. Merk, 70—230 mesh). Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was obtained from Tokyo Kasei Kogyo Co. The reaction mixture was diluted with AcOEt (30 ml), washed with 5% HCl, 5% NaHCO3, and brine, washed with water (1 ml), and dried (Na2SO4). After evaporation of the solvent, the residue was recrystallized from AcOEt-EtO to give compound 6 as colorless prisms (15 mg, 73\%), mp 133—134 °C (lit.3 137—138 °C for the non-labeled form). 1H-NMR: δ 0.82 (3H, s, 18-Me), 3.59 and 3.86 (1H each, d, J = 11.2 Hz, 19-CH2), 5.67 (1H, s, 6-H). FT-IR (KBr): 3433 (OH) cm\^-1.

3β-Acetoxy-19-(tert-butyldimethylsiloxy)androst-5-en-17\_d1 (7) To a solution of 3β-acetoxy-19-(tert-butyldimethylsiloxy)androst-5-en-17-one (244, 0.53 mmol) in EtOH (12 ml), NaBH4 (10 mg, 0.26 mmol) was added under ice-cooling. The reaction mixture was stirred at 0 °C for 4 h. After extraction with AcOEt (60 ml×2), the organic layer was washed with 5% HCl, 5% NaHCO3, and brine, sequentially, and dried (Na2SO4). After evaporation of the solvent, the oily product obtained was purified by column chromatography. Elution with hexane—AcOEt (3:1) gave compound 7 as colorless prisms (191 mg, 88\%), mp 123—124 °C (acetone). 1H-NMR: δ 0.04 and 0.05 (3H each, s, 19-OSiMe2), 0.78 (3H, s, 18-Me), 0.88 (9H, s, 19-OSiMe2), 2.06 (3H, s, 3\_d_Ac), 3.63 (1H, m, 17e-H), 3.59 and 3.74 (1H each, d, J = 10.6 Hz, 19-CH2), 4.62 (1H, m, 3z-H), 5.56 (1H, m, 6-H). FT-IR (KBr): 3449 (OH), 1734 (C=O) cm\^-1. Anal. Calc'd for C29H46O6Si: C, 70.8; H, 10.02. Found: C, 70.37; H, 10.35.

19-(tert-Butyldimethylsiloxy)androst-5-en-3β-\_d1 Diacetate (8) A solution of compound 7 (200 mg, 0.30 mmol) in pyridine (2 ml) and Ac2O (1 ml) was allowed to stand at room temperature for 12 h. After this time, the reaction mixture was poured into ice-water (20 ml), and then extracted with AcOEt (50 ml). The organic layer was washed with 5% HCl, 5% NaHCO3, and brine, sequentially, and dried (Na2SO4). After evaporation of the solvent, the crystalline solid obtained was recrystallized from hexane to give compound 8 as colorless needles (191 mg, 88\%), mp 121—123 °C. 1H-NMR: δ 0.03 and 0.04 (3H each, s, 19-OSiMe2), 0.83 (3H, s, 18-Me), 0.86 (9H, s, 19-OSiMe2), 2.03 (6H, s, 3\_d- and 17β-OAc), 3.59 and 3.74 (1H each, d, J = 10.6 Hz, 19-CH2), 4.58 (1H, m, 3z-H), 4.64 (1H, m, 3z-H), 5.16 (1H, m, 6-H). FT-IR (KBr): 1736 (C=O) cm\^-1. Anal. Calc'd for C31H48O8Si: C, 69.00; H, 9.58. Found: C, 68.80; H, 9.94.

3β,17\_d2-Diacetoxy-19-(tert-butyldimethylsiloxy)androst-5-en-7\_d1 (9) tert-BuOOH (0.26 ml) was added dropwise to a mixture of compound 8 (252 mg, 0.50 mmol), benzene (7.7 ml), Celite 574 (775 mg), and pyridinium dichromate (952 mmol, 0.40 mmol) under ice-cooling and the resulting mixture was stirred at room temperature for 3 d. The oil obtained was filtered through a pad of Celite 545 which was washed with AcOEt (50 ml). The filtrate was concentrated with 5% HCl and 5% NaHCO3, and brine, sequentially, and dried (Na2SO4). After evaporation of the solvent, the crystalline solid obtained was recrystallized from hexane to give compound 9 as colorless prisms (15 mg, 73\%), mp 133—134 °C. FT-IR: 1739 (C=O) cm\^-1. Anal. Calc'd for C29H46O6Si: C, 69.00; H, 9.58. Found: C, 68.80; H, 9.94.

3β,17-Dihydroxy-19-(tert-butyldimethylsiloxy)androst-5-en-7\_d1 (10) To a solution of compound 9 (170 mg, 0.33 mmol) in MeOH (6 ml) and water (1 ml), K2CO3 (90 mg, 0.65 mmol) was added and the mixture was heated at 60 °C for 3 h. After this time, the reaction mixture was concentrated to about 2 ml and extracted with AcOEt (50 ml). The organic layer was washed with brine, and dried (Na2SO4). After evaporation of the solvent, the crystalline solid obtained was recrystallized from acetone to give compound 10 (140 mg, 98\%) as colorless prisms, mp 185—186.5 °C. 1H-NMR: δ 0.03 and 0.06 (3H each, s, 19-OSiMe2), 0.79 (3H, s, 18-Me), 0.85 (9H, s, 19-OSiMe2), 3.65 (1H, m, 17e-H), 3.72 (1H, m, 3z-H), 3.83 and 3.93 (1H each, d, J = 10.6 Hz, 19-CH2), 5.87 (1H, dd, J = 7.2, 17-H), 4.73 (1H, m, 3z-H), 5.88 (1H, dd, J = 7.2, 17-H). FT-IR (KBr): 1736, 1672 (C=O) cm\^-1. UV λmax nm (ε): 237 (1200). Anal. Calc'd for C29H46O7Si: C, 67.14; H, 8.94. Found: C, 67.24; H, 8.96.

3β,17-Dihydroxy-19-(tert-butyldimethylsiloxy)androst-5-en-3\_d1 (11) and Its [7,7\_d2-H] Analog Aluminum chloride (277.5 mg, 2.08 mmol) was carefully added to dry Et2O (4 ml) at 0 °C, then a solution of LiAlH4 (22.8 mg,
0.60 mmol) in dry EtO (4 ml) was added dropwise at 0 °C to this suspension. The mixture was heated under reflux for 30 min under N₂, and then cooled to room temperature. A solution of compound 10 (100 mg, 0.23 mmol) in dry THF (2 ml) was added to the mixture, and then heated under reflux for 2.5 h and cooled. Water was carefully added to the reaction mixture, followed by extraction with AcOEt (100 ml). The organic layer was washed with 5% HCl, 5% NaHCO₃ and brine, sequentially, and dried (Na₂SO₄). Evaporation of the solvent afforded a solid which was recrystallized from acetone to give compound 11 (77 mg, 79%) as colorless prisms, mp 205—206 °C. ¹H-NMR δ: 0.30 and 0.04 (3H each, s, 19-OSiMe₃), 0.78 (3H, s, 18-Me), 0.87 (9H, s, 19-OSiMe₃), 3.56 (1H, m, 3-H), 3.67 and 3.79 (1H each, d, J = 10.6 Hz, 19-CH₃), 5.54 (1H, m, 6-H). FT-IR (KBr): 3402 (OH), 1637 (C=O) cm⁻¹. Anal. Calcd for C₂₅H₄₀O₃Si: C, 72.08; H, 9.66. Found: C, 72.27; H, 9.66.

[3,3,7,7-²H₄]Compound 11-d₄, mp 135—138 °C, was obtained from [3,3,7,7-²H₄]compound 11-d₄ essentially according to the above method used for the synthesis of non-labeled 13-δ₄.

17β-Hydroxy-19-(tert-butyldimethylsilyloxy)androst-4-ene-3,17-dione (12) and Its [7,7-²H₄]Analog

Jones reagent (0.17 ml) was added to a solution of compound 11 (100 mg, 0.24 mmol) in acetonitrile under ice-cooling. The resulting mixture was stirred for 25 min. After this time, the reaction mixture was poured into ice-water (50 ml), extracted with AcOEt (100 ml), washed with 5% NaHCO₃ and brine, and dried (Na₂SO₄). Evaporation of the solvent afforded an oily product (105 mg) which was dissolved in acetone (2 ml). Toluenesulfonic acid monohydrate (3.7 mg, 0.02 mmol) was added to this solution and then allowed to stand at room temperature for 3 h, diluted with AcOEt (50 ml), and washed with 5% NaHCO₃ and brine, and dried (Na₂SO₄). Evaporation of the solvent gave an oily product which was purified by preparative silica-gel TLC (two developments with hexane–AcOEt (1:1)) to give compound 17-δ₁ as colorless prisms (27 mg, 75%), mp 135—138 °C, was obtained from [7,7-²H₄]compound 11-d₄ essentially according to the above method used for the synthesis of non-labeled 13-δ₁.

Androst-4-ene-17β,19-diol (15) and Its [3,3,7,7-²H₄]Analog

To a solution of compound 14 (50 mg, 0.123 mmol) in THF (0.9 ml) and 2-propanol (1.4 ml), 3 mol/l HCl (0.9 ml) was added, and the resulting mixture was allowed to stand at room temperature. After 3 d, the reaction mixture was diluted with AcOEt (50 ml), washed with 5% NaHCO₃ and brine, and dried (Na₂SO₄). Evaporation of the solvent yielded an oily product which was purified by preparative silica-gel TLC (three developments with hexane: AcOEt=2:1) to give compound 15 as colorless prisms (27 mg, 75%), mp 176—179 °C (acetone) (lit.® mp 97—101 °C). ¹H-NMR δ: 0.75 (3H, s, 18-Me), 3.53 and 3.95 (1H each, d, J = 10.4 Hz, 19-CH₃), 3.62 (1H, t, J = 8.4 Hz, 17-α-H), 5.72 (1H, m, 4-H).

[3,3,7,7-²H₄]Compound 15-d₄, mp 138—140 °C, was obtained as described above from [3,3,7,7-²H₄]compound 14-d₄.

GC-MS Gas chromatographic conditions: column, 30 m×0.25 mm i.d. fused-silica DB5 MS (J & W Scientific, CA, U.S.A.); column temperature, from 50 °C at 25°C/min to 250 °C and then at 10°C/min to 280 °C; carrier gas, He at a flow rate of 1 ml/min. Mass spectrometric conditions: ionization energy, 70 eV; ion source temperature, 150 °C.

Derivatization of Compounds 15 and 16 with BSTFA

BSTFA (20 μl) was added separately to a solution of the steroid 15 and 16-d₄ in pyridine (50 μl). The mixture was heated at 60 °C for 30 min and then the solvent was removed under a stream of N₂. The residue was dissolved in anhydrous hexane containing 0.5% pyridine (50 μl), and 1 μl of the solution was subjected to analysis.

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References


