1,4-Addition reaction with thiols and conformational analysis with PM3 molecular orbital calculations of 19-oxygenated androst-4-ene-3,6,17-triones

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Reactions of androst-4-ene-3,6,17-trione ***(1)*** *and its 19-hydroxy or 19-oxo derivative (**2** or **3**), suicide substrates of aromatase, with thiols were initially studied. Treatment of 4-ene-3,6-diones **1–3** with benzyl-mercaptan in MeOH at room temperature gave the corresponding 4α-benzylthio-5α-androstane-3,6-diones **(4–6)** as the major products in 24–80% yields. The C_{18} steroid, estr-5(10)-ene-3,6,17-trione **(7)**, was also isolated on the treatment of 19-oxo steroid **3**. Oxidation with $NaIO_4$ and reduction with Raney Ni of the adducts gave the corresponding 4-ene-3,6-dione and desulfurized products, respectively. The results show that 19-oxygenated steroids **2** and **3** react with a thiol in a 1,4-addition manner. By means of PM3 molecular orbital calculations, the conformational features of the 19-oxygen functions of 4-ene-3,6-diones **2** and **3**, 5α-3,6-diones **10** and **11**, and their 4α-methylthio derivatives **14** and **15**, model compounds of 1,4-adducts **5** and **6**, were determined. In the compounds examined, the 19-hydroxy steroids favor a conformation having the hydroxyl group above the A-ring, whereas the 19-oxo substituent is oriented in the out-of-ring position (not above either A- or B-ring). These calculations suggest that compound **1** would inactivate aromatase by the same steric course of the oxygenation at C-19 as that of the natural substrate, androstenedione. (Steroids* **58**:423–428, 1993)

Keywords: 19-Oxygenated androst-4-ene-3,6,17-trione; 19-oxygenated 4α-alkylthio-5α-androstane-3,6,17-trione; 1,4-addition reaction; aromatase inhibitor; PM3 molecular orbital calculation; stereomechanism; steroids

Introduction

Androst-4-ene-3,6,17-trione (**1**) is a suicide inactivator of aromatase.[1,2] It has been reported that the inactivation proceeds through the 19-hydroxy and 19-oxo derivatives (**2** and **3**),[3,4] and we recently have shown that the 19-oxygenated steroids disappear rapidly in a pseudo-first order manner with half-lives 25 minutes and 20 seconds, respectively, upon treatment with a nucleophile, *N*-acetyl-L-cysteine to give steroid-amino acid adducts.[4] The adduct formation seems to be involved in the aromatase inactivation process by the 19-oxygenated steroids. However, the structures of the adducts have not been unambiguously determined, although it has previously been reported that 4-ene-3,6-dione **1** reacts with a thiol to give a 1,4-Michael adduct, 4α-alkylthio-5α-3,6-dione along with other minor products.[5]

On the other hand, the androstenedione aromatization is thought to proceed through 19-hydroxyandrostenedione and 19,19-dihydroxyandrostenedione which is converted into 19-oxoandrostenedione after stereospecific dehydration.[6–10] The molecular conformations of the 19-hydroxyl and oxo groups of the intermediates have been discussed with respect to understanding the stereochemistry of the aromatization reaction or the binding aspects of the substrate and the intermediates. Osawa and co-workers[11–13] have proposed that aromatase delivers oxygen to the 19-position of the substrate androstenedione from the out-of-ring position, *trans* to the C(5)—C(10) bond. The relevance of conformational positions in enzyme interactions has further been supported by a comparison of X-ray crystallographic structures and inhibition experiments of (19R)- and (19S)-10β-oxiranyl steroids[14] and also by recent MM2 molecular mechanics calculations.[15,16] Thus, the conformational analysis of the 19-hydroxy-

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and 19-oxo-4-ene-3,6-diones **2** and **3** would be important for understanding not only the molecular change of compound **1** in the aromatase inactivation process but also the substrate specificity of the aromatase enzyme.

We now report reactions of steroids **2** and **3** with ethanethiol (EtSH) and benzylmercaptan ($PhCH_2SH$) as model reactions of the amino acid adduct formation. Additionally, the conformational analysis of the 19-substituents of 4-ene-3,6-diones **2** and **3,** 5α-3,6-diones **10** and **11,** and 4α-methylthio steroids **14** and **15** was carried out by means of PM3 molecular orbital calculations. The reactions with thiols gave the 1,4-adduct, 4α-alkylthio derivatives in each case. The 19-hydroxyl function of the steroids was assigned to the conformation in which it is above the A-ring while the 19-carbonyl was in the out-of-ring position (not above either A- or B-ring).

Experimental

Materials and general methods

Androst-4-ene-3,6,17-trione **(1)**[5] and its 19-oxygenated compounds **2** and **3**[4] are synthesized to the methods previously reported. Raney Ni was purchased from Nikko's Chemicals (Tokyo, Japan).

Melting points were measured on a Yanagimoto melting point apparatus and were uncorrected. Infrared (IR) spectra were recorded in KBr pellet on a Perkin Elmer FT-IR 1725X spectrophotometer. Proton nuclear magnetic resonance (^1H NMR) spectra were obtained with a JEOL GX 400 (400 MHz) spectrometer using tetramethylsilane as an internal standard, and high-resolution mass spectra (HRMS) with a JEOL JMS-DX 303 spectrometer. Thin-layer chromatography (TLC) was performed with E. Merck pre-coated TLC plates, silica gel 60-254, layer thickness 0.25 mm. Silica gel column chromatography was conducted with E. Merck Kieselgel 60 (70-230 mesh).

General procedure for reactions of androst-4-ene-3,6-diones with thiols

To a solution of each steroid (0.48 mmol) in MeOH (23 ml) was added EtSH or $PhCH_2SH$ (7.7 mmol). The mixture was stirred at room temperature for an appropriate time under N_2 atmosphere, and then the solvent was removed under reduced pressure below 40 C. The residue was chromatographed on silica gel eluted with hexane/AcOEt.

4α-Benzylthio-5α-androstane-3,6,17-trione (4)

Compound **4** (163 mg, 80%) was prepared using 28-hour reaction time: mp 162–167 C (from MeOH); ^1H NMR ($CDCl_3$) δ 0.76 (3H, s, 19-Me), 0.86 (3H, s, 18-Me), 3.68 (1H, d, J = 10.6 Hz, 4β-H), 3.86 and 3.91 (1H each, d, J = 12.8 Hz, 4-SCH_2). 7.25–7.36 (5H, m, benzyl aromatic protons); FT-IR 1737, 1717, and 1688 (C=O) cm^{-1}. Analysis Calculated for $C_{26}H_{32}O_3S$: C, 73.55; H, 7.60. Found: C, 73.56; H, 7.43.

4α-Benzylthio-19-hydroxy-5α-androstane-3,6,17-trione (5)

Compound **5** (120 mg, 57%) was obtained using 8-hour reaction time: mp 166–168 C (from acetone); ^1H NMR ($CDCl_3$) δ 0.82 (3H, s, 18-Me), 2.46 (1H, m, 5α-H), 3.67 (1H, dd, J = 3.3 and 8.9 Hz, 4β-H), 3.79 (1H, dd, J = 3.0 and 5.9 Hz, 19-Ha), 3.90 (1H, m, 19-Hb), 3.94 and 3.97 (1H each, d, J = 13.2 Hz, 4-SCH_2), 7.23–7.38 (5H, m, benzyl aromatic protons); FT-IR 3415 (OH), 1737 and 1713 (C=O) cm^{-1}. Analysis Calculated for $C_{26}H_{32}O_4S$: C, 70.87; H, 7.32. Found: C, 70.57; H, 7.37.

4α-Benzylthio-5α-androstane-3,6,17,19-tetraone (6)

Column chromatography of the products obtained using 5-hour reaction time gave compound **6** (51 mg, 24%) as the less polar product: mp 185–188 C (from acetone); ^1H NMR ($CDCl_3$) δ 0.80 (3H, s, 18-Me), 3.75 (1H, dd, J = 1.7 and 10.1 Hz, 4β-H), 3.86 and 3.90 (1H each, J = 13.1 Hz, 4-SCH_2), 9.68 (1H, s, 19-H); FT-IR 1752 and 1708 (C=O) cm^{-1}. Analysis Calculated for $C_{26}H_{30}O_4S \cdot 1/2H_2O$: C, 69.79; H, 6.93. Found: C, 69.42; H, 6.82.

Estr-5(10)-ene-3,6,17-trione (7)

Compound **7** (12 mg, 12%) was also separated as the more polar product by the chromatography; mp 167–169 C (from acetone); ^1H NMR ($CDCl_3$) δ 0.93 (3H, s, 18-Me), 2.98 and 3.25 (1H each, d, J = 22.0 Hz, 4-H_2); FT-IR 1740, 1721, and 1662 (C=O) cm^{-1}; UVλ_{max} (EtOH) 248.4 nm (ε = 1.37×10^4). HRMS (EI) calculated for $C_{18}H_{22}O_3$ 286.1569, found 286.1559.

4α-Ethylthio-19-hydroxy-5α-androstane-3,6,17-trione (8)

Compound **8** (82 mg, 45%) was prepared using 18-hour reaction time: mp 195–198 C (from acetone); ^1H NMR ($CDCl_3$) δ 0.82 (3H, s, 18-Me), 1.29 (3H, t, J = 7.3 Hz, 4-$SCH_2\underline{Me}$), 3.71 (1H, dd, J = 3.3 and 9.0 Hz, 4β-H), 3.80 (1H, m, 19-Ha), 3.92 (1H, dd, J = 1.8 and 9.2 Hz, 19-Hb); FT-IR 3499 (OH), 1730 and 1709 (C=O) cm^{-1}. Analysis Calculated for $C_{21}H_{30}O_4S$: C, 66.63; H, 8.01. Found: C, 66.87; H, 8.13.

Raney Ni Reduction of 4α-Alkylthio Steroids

To a solution of 4α-alkylthio steroids **5** and **6** (50 mg, ca. 0.11 mmol) in EtOH (5 ml) was separately added Raney Ni and the suspension was stirred for 30 minutes or 2 hours at room temperature. After removal of the nickel by filtration, evaporation of the solvent left a residue of solid or oil which was purified by preparative TLC (hexane/AcOEt) and recrystallized to give the corresponding desulfurization products, 5α-3,6-diones **10** and **11** and their 3β-hydroxy derivatives **12** and **13,** respectively, in the experiments with 30-minute reaction time, while only the latter products were isolated by the experiments with 2-hour reaction time.

19-Hydroxy-5α-androstane-3,6,17-trione (10)

Yield: 46% from **5** (30-minute reaction time). mp 169–171 C (from MeOH); ^1H NMR ($CDCl_3$) δ 0.96 (3H, s, 18-Me), 3.84 and 4.22 (1H each, d, J = 8.8 Hz, 19-H_2); FT-IR 1737, 1698 (C=O) cm^{-1}; HRMS (EI) calculated for $C_{19}H_{26}O_4$ 318.1831, found 318.1825.

5α-Androstane-3,6,17,19-tetraone (11)

Yield: 42% from **6.** mp 198–200 C (from MeOH); ^1H NMR ($CDCl_3$) δ 0.84 (3H, s, 18-Me), 9.89 (1H, s, 19-H); FT-IR 1737 and 1703 (C=O) cm^{-1}. HRMS (EI) cacld for $C_{19}H_{24}O_4$ 316.1675, found 316.1674.

3β,19-Dihydroxy-5α-androstane-6,17-dione (12)

Yield: 43% (30-minute reaction time) and 85% (2-hour reaction time) from **5.** mp 232–234 C (from acetone); ^1H NMR (acetone-

d_6) δ 0.94 (3H, s, 18-Me), 3.52 (1H, broad m, 3α-H), 3.63 and 3.90 (1H each, d, J = 8.4 Hz, 19-CH_2). FT-IR 3368 (OH), 1736 and 1703 (C=O) cm^{-1}; HRMS (EI) calcd for $C_{19}H_{28}O_4$ 320.1988, found 320.1983.

3β-Hydroxy-5α-androstane-6,17,19-trione (13)

Yield: 38% (30-minute reaction time) and 82% (2-hour reaction time) from **6**. mp 156–159 C (from acetone); ^1H NMR ($CDCl_3$) δ 0.90 (3H, s, 18-Me), 3.56 (1H, broad m, 3α-H), 9.69 (1H, s, 19-H); FT-IR 3434 (OH), 1737 and 1720 (C=O) cm^{-1}. HRMS (EI) calcd for $C_{19}H_{26}O_4$ 318.1831, found 318.1831.

Raney Ni reduction of 5α-3,6-dione derivatives

Raney Ni was added to a solution of compound **10** or **11** (20 mg, 0.06 mmol) in EtOH (2 ml) and the suspension was stirred for 3 hours at room temperature. After the same work-up as above, the 3β-reduced product **12** (91%) or **13** (86%) was isolated by preparative TLC. Compound **12** or **13** obtained by this experiment was completely identical with that by the above desulfurization reaction, respectively.

Treatment of 4α-alkylthio steroids with $NaIO_4$

To a solution of compounds **5** or **6** (20 mg, ca. 0.05 mmol) in MeOH (4 ml) was added $NaIO_4$ (15 mg, 0.07 mmol)[17] and the reaction mixture was stirred at room temperature for 4 days. After filtration, the filtrate was concentrated, diluted with AcOEt (30 ml), washed with saturated NaCl solution, and dried (Na_2SO_4). After evaporation of the solvent, the residue was dissolved in MeOH (3 ml) and heated under reflux for 1 hour. Evaporation of the solvent yielded a solid product, which was purified by preparative TLC to afford compound **2** (12 mg, 85% from **5**) or **3** (14 mg, 96% from **6**). The products were completely identical with the authentic samples, respectively.

Conformational Analysis

Semiempirical molecular orbital calculations by the PM3 method[18,19] were performed with MOPAC program (Quantum Chemistry Program Exchange No. 455), which was revised as Version 6.01 to adapt it for HITAC and UNIX machines. The Version 6.01 was obtained through the Japan Chemistry Program Exchange (Tokyo).[20] For all compounds determined, final geometries and energetics for each of the three possible 10β side-chain conformations were determined by optimizing the total molecular energy with respect to all structural variables. No attempt was made to account for solvation.

Results and discussion

Chemistry

Reaction of 4-ene-3,6-diones **1–3** with a large excess of $PhCH_2SH$ was initially carried out in MeOH at room

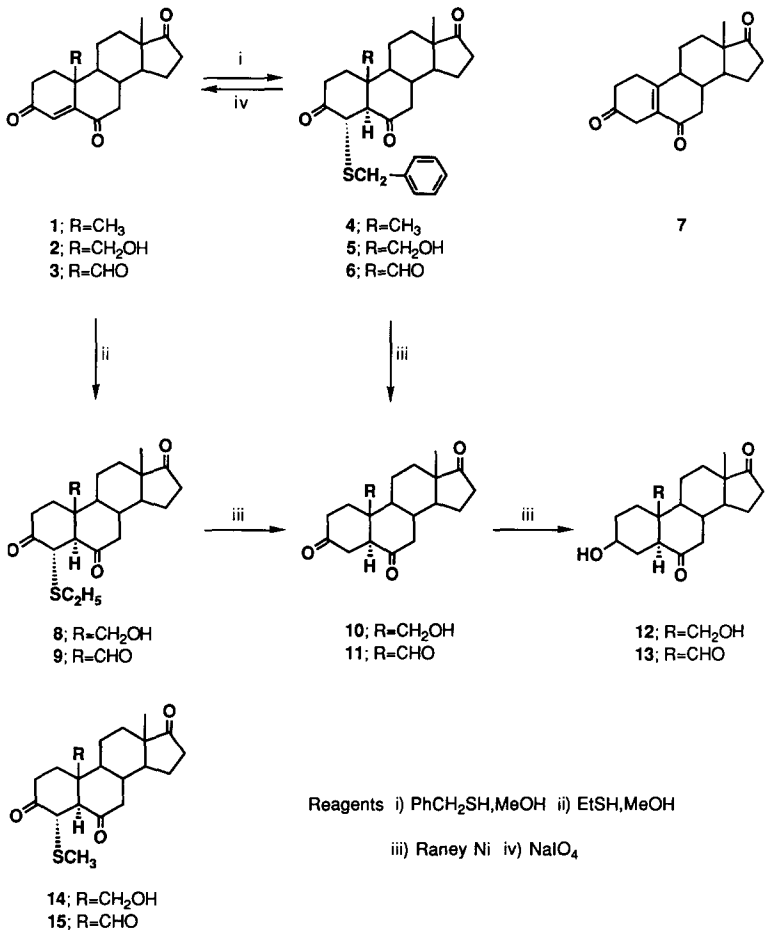

Figure 1 Reaction pathways and structures of steroids.

temperature, giving the corresponding 4α-benzylthio-5α-3,6-dione derivatives **4** (80%), **5** (57%), and **6** (24%) (Figure 1). The low yield of compound **6** was principally due to production of 5(10)-ene-3,6-dione **7** (12%). The spectral data and elemental analysis of the product are fully consistent with the assigned structures. The stereochemistry at C-4 of the products was determined by ^{1}H NMR spectroscopy. The C-4 proton signal resonance appeared at δ 3.68 as a doublet (J = 10.6 Hz) for steroid **4,** δ 3.67 as a doublet of doublets (J = 3.3 and 8.9 Hz) for steroid **5,** and δ 3.75 as a doublet of doublets (J = 1.7 and 10.1 Hz) for steroid **6.** The J values 8.9–10.6 Hz correspond to axial-axial interactions of the H—C(4)—C(5)—H dihedral angle in the chair conformation of the A-ring of 5α-androstane. Moreover, the 4-proton at δ 3.68 of steroid **2** showed NOE (12%) upon irradiation at the 19-methyl proton resonance frequencies (δ 0.76). Treatment of 19-alcohol **2** with EtSH under the above condition also afforded 4α-adduct **8** (45%) whereas on the similar treatment of 19-oxo steroid **3,** the 4α-adduct **9** could not be isolated in a pure form because it easily decomposed to the starting material **3** during the isolation procedure (column and thin-layer chromatographies with silica gel).

To further confirm the structures of 4α-adducts **5, 6,** and **8,** they were separately subjected to desulfurization reaction with Raney Ni at room temperature. Each experiment using 30-minute reaction time yielded a mixture of desulfurized products, 5α-3,6-dione **10** or **11** and its 3β-reduced compound **12** or **13** but the 3β-alcohol was exclusively produced using a longer reaction time (2 hours) in high yield. Moreover, when compounds **10** and **11** were similarly treated with the nickel, 3β-ols **12** and **13** were obtained in high yields. These show that the 3β-ols are produced essentially through the corresponding 3-ones during the desulfurization reaction. The 3β-configuration was deduced by ^{1}H NMR spectroscopy (the 3α-proton at δ 3.52 and 3.56: broad multiplet with ~30 Hz of half-widths)[21] and by the stereospecificity of Raney Ni reduction of a 3-carbonyl function of 5α-steroid.[22] Previous studies demonstrate that a carbonyl group at C-3 is reduced selectively in the presence of the C-6 or C-17 carbonyl on treatment with the nickel,[23] supporting the present results. On the other hand, oxygenation of steroids **5** and **6** with $NaIO_4$[17] predictably afforded the corresponding parent steroids **2** and **3** in high yields. These chemical aspects of the thio steroids also support their assigned structures.

Thus, condensation of the 19-oxygenated 4-ene-3,6-diones with a thiol should proceed in a 1,4-Michael addition manner through the less sterically hindered α-face, giving the 4α-alkylthio derivatives similarly as the reaction with compound **1** reported previously.[5]

Molecular orbital calculations

We performed PM3 molecular orbital calculations on 19-oxygenated 4-ene-3,6-diones **2** and **3** to gain access to conformational features of the 6-oxo steroid in the stereospecificity of the 19-oxygenation. Moreover, the PM3 calculations of 4α-methylthio compounds **14** and **15,** model steroids of the 1,4-adducts, along with their 5α-reduced steroids **10** and **11,** were also carried out in order to learn the effect of the 4α-substituent on the conformations of the 19-substituents. The results of the PM3 calculations are shown in Table 1. The 19-hydroxyl group of the lowest energy conformer of the 6-oxo steroids determined oriented above the A-ring while the 19-oxo function favors the out-of-ring conformation, irrespective of the A-ring structure (Figure 2). It has been reported that the 19-hydroxyl is oriented above the A-ring and the 19-oxo in the out-of-ring position, respectively, in a series of androstenedi-

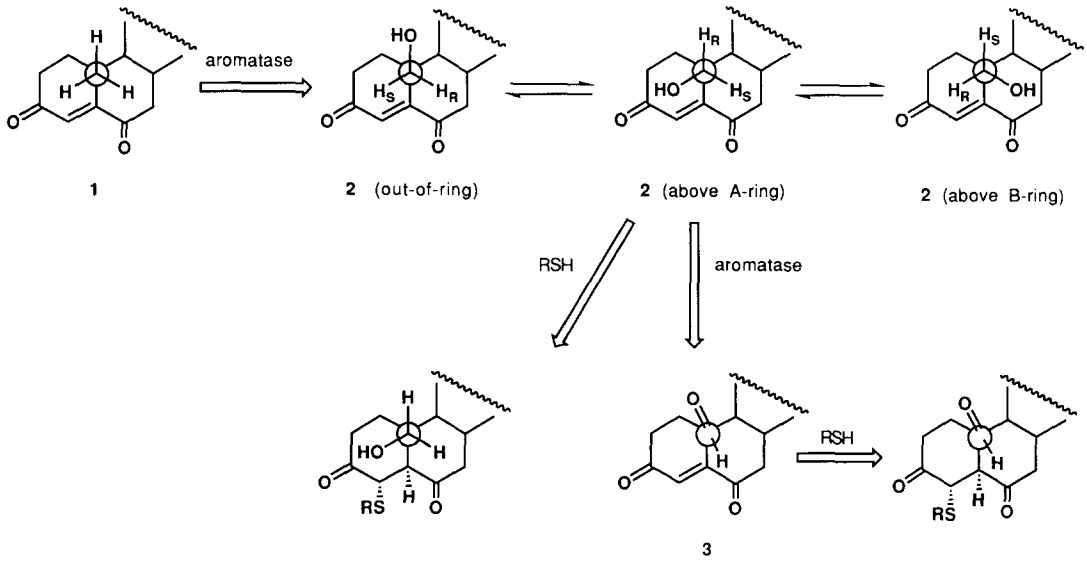

Figure 2 Proposed stereomechanism of the aromatase reaction of compound **1.**

Table 1 Results of conformational analysis with PM3 calculations

Steroid	10β-Side-chain conformation[a] (relative population, %)	Torsional angle[b] C(5)—C(10)—C(19)—O(19) PM3 optimized	Relative energy (kJ mol^{-1}) ΔE
2	A (45)	−58°	0
	B (42)	+42°	0.14
	O (13)	−163°	3.3
3	A (42)	−36°	0.12
	B (14)	+91°	2.7
	O (44)	−130°	0
10	A (47)	−62°	0
	B (11)	+41°	3.7
	O (42)	−163°	0.30
11	A (22)	−33°	2.9
	B (8)	+89°	5.3
	O (70)	−148°	0
14	A (51)	−63°	0
	B (7)	+45°	4.8
	O (42)	−164°	0.47
15	A (25)	−58°	0.61
	B (6)	+68°	6.1
	O (69)	−148°	0

[a] Conformation designation: oxygen function (OH or C=O) above A-ring (A), above B-ring (B), and in out-of-ring position (O).
[b] A negative angle corresponds to a counterclockwise torsion as viewed along the C(10)—C(19) bond.

ones,[11–13,15] while the 19-hydroxyl of the 5α-reduced derivative favors the out-of-ring conformation.[16] The lowest energy conformers for compounds **2** and **3** are comparable for the corresponding androst-4-ene-3,17-dione derivatives. However, the conformation (above A-ring) of the 19-hydroxyl of 5α-3,6-dione **10** is different from that (out-of-ring position) of its 6-deoxy compound, 5α-androstane-3,17-dione.

The relative population of each conformer of 19-alcohol **2** (45% above A-ring, 42% above B-ring, 13% out-of-ring position) is almost the same as that obtained previously for 19-hydroxyandrosta-4,6-diene-3,17-dione by the MM2 calculations (46% above A-ring, 40% above B-ring, 14% out-of-ring).[15] This demonstrates that the 6-oxo function shifts the conformer population, making the conformer having the 19-hydroxyl above the B-ring more favorable, similar to the effect of a 6-double bond.

The stereospecificity of aromatization for 19-hydroxyandrostenedione and its 6-dehydro derivative has been found to be the same.[15] On the other hand, in our preliminary experiments, it has been shown that compound **1** is indeed converted into both the 19-oxygenated steroids **2** and **3** and the aromatized product, 6-oxoestrone, by incubation with human placental microsomes in the presence of NADPH.[24] Considering these previous results along with the effect of a 6-double bond on the conformer population, it is suggested that the 19-position of 19-alcohol **2** would be oxygenated by aromatase through the same stereomechanism to that operating in the androstenedione aromatization; the 19-pro-R hydrogen of 19-alcohol **2** is released to water, presumably by oxygen insertion into the 19-pro-R carbon-hydrogen bond during the oxygenation (Figure 2).

Furthermore, introduction of the carbonyl group at C-6 in the 5α-steroid changes the lowest energy conformer from out-of-ring (5α-androstane-3,17-dione)[16] to the above the A-ring (compound **10**). Presumably, this is due to the lack of a 6β-axial hydrogen, and the consequent decrease in 1,3-diaxial interactions as compared to those of 19-hydroxy-5α-androstane-3,17-dione.

4α-Methylthio adducts **14** and **15** have nearly identical relative populations of each conformer to that of 5α-steroids **10** and **11**, respectively. Moreover, the lowest energy conformations of the adducts are almost identical with those of the corresponding 4-ene-3,6-diones. These results suggest that the addition reaction may proceed without the rotation of the 19-oxygen function around the C(10)—C(19) bond (Figure 2).

Conclusions

19-Oxygenated androst-4-ene-3,6-diones **2** and **3** were converted to the corresponding 4α-alkylthio compounds on treatment with thiols. Similar reactions have been observed with the 19-methyl steroid **1**.[5] This establishes that a 1,4-addition reaction is involved in the extremely rapid reaction of compounds **2** and **3** with N-acetyl-L-cysteine, observed previously,[4] although the exact reason why the 19-oxygenated functions accelerate the addition reaction is not clear. Since aromatase has an SH group which is essential for the expression of the enzyme activity, it is speculated that the 1,4-addition reaction may be in part involved in the irreversible inactivation of aromatase caused by the 19-oxygenated steroids.

The PM3 calculations indicate for the first time the conformation of the 19-hydroxyl and 19-oxo groups of compounds **2** and **3**, showing that the stereospecific 19-oxygenation of 19-alcohol **2** may occur during the inactivation process, similarly to the aromatization process of androgens with a 4-en-3-one system.

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