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Skeletal Conformations and Receptor Binding of Some 9,11-Modified Estradiols

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The effect of the modification of the 9-11 positions on the skeletal conformation of estradiol (E_2) has been analyzed by X-ray crystallography and MM2 molecular mechanics. The 11β-hydroxyl and 11-keto analogs of E_2 maintained ring conformations which were similar to the natural hormone (E_2). Introduction of a double bond at position 9-11 induced a flattening of the entire steroid molecule. An 11α-hydroxyl group brought about significant changes in the alicyclic rings of E_2. 9β-Estradiol and 11-keto-9β-estradiol formed ring conformations which were significantly bent from E_2 (below the plane of the A-ring). Examination of the affinity of these C-ring analogs of E_2 for the human estrogen receptor has shown extreme variations. A hydroxyl group placed either α or β at the 11-position yielded ligands with vastly different and reduced affinities for the receptor. The low affinity of 11α-hydroxyestradiol (1/300th of E_2) may be due to the drastic structural change induced in the alicyclic portion of the molecule, as well as, to the steric or electrostatic effects of the α-hydroxyl group upon the receptor protein. An 11β-hydroxyl group diminished the receptor binding to 1/60th that of E_2 without alicyclic ring distortions, whereas a 9-11 unsaturation reduced the binding to 1/5th although this steroid displayed a flattening of rings B, C, and D. The 11-keto function, which had little effect on the conformation of the estrogen nucleus, reduced the affinity of this ligand to 1/1000th that of E_2. The negative bend at the C-ring of 11-keto-9β-estradiol and 9β-estradiol prevented these ligands from binding receptor. Some of the observed receptor interactions were related to structural alterations in the estrogen ring system induced by modifications on the 9-11 region.

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INTRODUCTION

It is now well established that the structure of estrogens and antiestrogens play a critical role in their capacity to bind to the hormone binding domain of the nuclear receptor protein [1–3]. Moreover, the gene regulatory activity induced by the estrogen receptor (ER) complex seems to depend on the structure of the ligand [4, 5]. Although the ER has shown considerable structural tolerance [6–8], there are still fundamental constraints for full activity. For instance, whereas estradiols with the phenolic hydroxy placed on carbons 1, 2, or 3 (E_2) display activity, the 4-hydroxyestratrien-17βol is inactive [5].

Despite the biochemical and pharmacological evaluation of numerous estrogen analogs as well as antiestrogens, a thorough understanding of the structure–activity relationship of estrogenic action has not been achieved. In addition, there appear to be some contradictions in the *in vivo* activity of certain analogs with theoretical models [9]. Clearly, there is a need for further studies to elaborate the structural requirements for the binding of E_2 to the receptor and the consequent transcriptional effects. Some of these requirements have been effectively evaluated through the use of X-ray diffraction studies [10, 11].

Although X-ray diffraction prevails as the analytical tool of choice for structural studies of steroids [9], crystallographic data for a number of steroids are unavailable. This deficiency can be overcome by adopting strategies for those unavailable structures which are based on well established crystalline homologs. However, the use of a particular crystal structure as a model for activity studies is not practical, since conformational analysis by X-ray diffraction is dependent, in

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part, upon the solvent of crystallization. For this reason, several possible conformers may be available as model candidates. For example there are at least three different reported crystal structures for E_2 which differ only in the solvent of crystallization [11]. However, application of a simplified MM2 force field [12, 13] to these structures, provided a convenient procedure for quantitative conformational analysis. These calculations afford a unique structure devoid of lattice energy interactions, that can serve as a model for further studies.

Previously, we have shown that structural changes in rings B, C, and D of the estradiol nucleus can take place by electronic induction from the aromatic ring [10]. Thus, the rotation of the natural 3-hydroxy group around the aromatic A-ring (positions 1, 2 and 4) causes significant changes in the skeletal conformation of the adjacent rings, changes that may affect the overall binding ability of the modified structures. We have now prepared and characterized the structures of four 9,11-estradiol derivatives by X-ray crystallography. In addition, the conformation of two isomers which could not be examined by X-ray diffraction was determined by the molecular modeling geometry optimization method, MM2. The selection of MM2 as the method of minimization of these steroids was based on results of comparative studies using several semi-empirical molecular orbital methods [13]. The influence of the observed structural alterations on the affinity of each estrogen for the human ER is also reported.

EXPERIMENTAL

Synthesis

General methods. Melting points were obtained on a Thomas–Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were performed by M-H-W Laboratories (Phoenix, AZ). ^{1}H-NMR spectra were obtained with a Nicolet QE-300 FT spectrometer. Mass spectra were obtained with a Kratos MS-80 RFA.

9,11-Dehydroestrone (2). To estrone 1 (5 g, 18.5 mmol), dissolved with heating and stirring in 800 ml of ethanol, and then cooled to room temperature was added all at once 2,3-dichloro-5,6-dicyano benzoquinone, DDQ (5 g, 22.0 mmol). After stirring for 1 h, the resulting solution was concentrated in a rotavap (65°C bath temperature) to *ca* 200 ml at which point crystallization occurred. After 2 h at room temperature the solid was collected and washed with ethanol to give 1.5 g of light yellow crystals. A second crystallization afforded an additional 1.0 g for a total yield of 50% of pure 2 (by NMR), m.p. 257–259°C (Ref. [14] 255–258°C).

9,11-Dehydroestradiol (3). A mixture of 2 (3.07 g, 11.4 mmol) dissolved in 100 ml of isopropanol containing $NaBH_4$ (2 g, 50 mmol) was refluxed for 2 h. After neutralization with 6N HCl, the solvent was removed under reduced pressure and the residue was extracted with a mixture of ethyl acetate–hexanes (6:4, v/v), filtered through a plug of silica gel and the filtrate evaporated to produce 2.89 g (10.7 mmol, 94%) of colorless crystals, m.p. 184–186°C (Ref. [14], 183–186°C). H-NMR (DMSO) (not reported previously) δ(ppm) = 9.158 (1H, s, OH), 7.38 (1H, d, arom), 6.52 (1H, d, arom), 6.43 (1H, d, arom), 6.01 (1H, m, H_{11}), 4.51 (1H, m, H_{17}), 0.661 (3H, s, H_{17}) C^{13}-NMR (DMSO) δ(ppm) = 156.572, 137.545, 135.302, 125.998, 125.467, 117.002, 115.338, 114.291, 80.709, 47.547, 41.647, 40.360, 39.507, 30.599, 29.981, 28.453, 24.174, 11.810.

9,11-αEpoxyestradiol diacetate (4). The 9,11-dehydroestradiol (3) was dissolved in 20 ml of pyridine, 2 ml of acetic anhydride (18 mmol) was added and the mixture kept protected from moisture for 12 h. The pyridine solution was dissolved in water and extracted with ethyl acetate. The extract was washed with water, dried with sodium sulfate and the solvent removed *in vacuo*. The residue was recrystallized from methanol to furnish 3.59 g (10.1 mmol, 94%) of the acetate as a white solid, m.p. 147–149°C (Ref. [15], 149–150°C).

To a cold (0°C) solution of the acetate (3.4 g, 9.52 mmol) in methylene chloride (20 ml) was added, dropwise and during 30 min, a solution of *m*-chloroperbenzoic acid (3.5 g, 10.08 mmol) in methylene chloride (15 ml). After stirring for 3 h, the mixture was washed with saturated sodium bicarbonate (20 ml × 4), filtered through a plug of silica gel and evaporated to afford 2 g of impure material (2 spots by TLC using hexanes–ethyl acetate, 5:5, v/v). An analytical sample obtained following preparative TLC in the form of white needles, m.p. 127–128°C (Ref. [15], 129–130°C). The remainder of crude 4 was used as such in the next steps.

11α-Hydroxyestradiol (5). A solution of 4 (900 mg, 2.52 mmol) in tetrahydrofuran (THF, 60 ml) was refluxed for 24 h with lithium aluminium hydride (900 mg, 23.6 mmol). After cooling, acetone was added to the mixture which was then evaporated to dryness. The residue was treated with 5% HCl and the mixture was extracted with methylene chloride. The solvent was removed and the residue recrystallized from acetone to produce 550 mg (78%) of colorless crystals, m.p. 247–249°C (Ref. [16], 250–251°C).

11-Keto-9β-estradiol (6). Epoxide 4 (3 g, 8.1 mmol) in methanol (250 ml) was refluxed for 0.5 h with 5% KOH (50 ml) in methanol. After cooling, the mixture was neutralized with 6N HCl, concentrated, and the solid residue extracted with methylene chloride. Crystallization was induced by addition of a mixture of hexanes–ethyl acetate (5:5, v/v) to give 1 g (43%) of colorless crystals, m.p. 245–247°C. (Ref. [8], 238–242°C, Ref. [15], 255–257°C). A second recrystallization from ethanol gave prisms m.p. 250–252°C. Due to discrepancies in melting points, an elemental analy-

sis was performed. *Anal.* Calcd. for $C_{18}H_{22}O_3$: C, 75.52 H, 7.69. Found: C, 75.36; H, 7.68.

11-Ketoestradiol (7). Ketoestradiol **6** (200 mg, 0.7 mmol) in dry THF (10 ml) was treated for 30 min with NaH (2.1 mmol) and then the solution was neutralized with acetic acid. Separation by preparative TLC using hexanes–ethyl acetate (5:5 and then 8:2, 4X, v/v) gave 95 mg (47%) of 7 as a colorless amorphous powder, m.p. 220°C (dec.) (Ref. [15], 219–223°C, dec.). Attempts to crystallize the material from different solvents produced fine needles unsuitable for X-ray studies.

11β-Hydroxyestradiol (8). Ketoestradiol **6** (300 mg, 1.05 mmol) was refluxed in isopropanol (150 ml) with sodium borohydride (1.5 g, 39 mmol) for 24 h. After neutralization with 6N HCl, the mixture was concentrated *in vacuo*, extracted with ethyl acetate–ethanol (8:2, v/v) and filtered through a plug of silica gel. The solid obtained on removal of the solvent was recrystallized from ethanol to produce 250 mg (83%) of 8 as colorless crystals, m.p. 287–289°C (Ref. [17], 289–291°C; Ref. [18], 285–288°C).

9β-Estradiol (9). Dehydroestrone (3) was converted in 95% yield to the 17-ethyleneketal by refluxing the diol (1 g) with an excess of ethyleneglycol and *p*-toluene sulfonic acid (100 mg) in toluene using a Dean-Stark trap. After filtration through a short silica gel plug, the material was hydrogenated for 3 h at 40 lb/in^2 in dry THF with 10% Pd/C. After filtration and concentration the residue was treated for 20 min with 5% HCl in methanol–water (9:1, v/v). The solid obtained after precipitation with water was filtered and recrystallized from ethanol to produce over 90% of estrone. The remaining mixed solid (300 mg) was treated with $NaBH_4$ in ethanol–water, and after workup the **9** was separated by preparative TLC to produce 200 mg of white needles, m.p. 222–224°C (Ref. [19], 217–221°C).

X-ray analysis

General methods. Data were collected on a Nicolet R3 or P2 diffractometer equipped with MoKα radiation ($\lambda = 0.71073$ Å) and a graphite monochromator (**5** and **6**) or CuKα radiation ($\lambda = 1.54178$ Å) and a Ni filter (**8**). The structures were solved by direct methods [16] and refined in a full matrix with the programs of SHELX-76 [20]. All non-hydrogen atoms were refined anisotropically.

9,11-Dehydroestradiol (3). Suitable crystals were grown from acetone. A colorless square plate with dimensions 0.40 × 0.24 × 0.38 mm^3 was used in the study. Half a molecule of water was present in the lattice. A total of 5503 reflections were obtained; of these, 3838 were observed with $I \geqslant 3\ \sigma(I)$. There are two independent molecules in the asymmetric unit. Abbreviated crystal data are as follows: **3**, $C_{18}H_{22}O_2 \cdot 1/2H_2O$, f.w. 279.38 amu, space group C2, $a = 25.135(34)$ Å, $b = 11.835(6)$ Å, $c = 10.373(4)$ Å, $V = 3000(4)$ Å^3, $Z = 8$, $F_{000} = 604\ e^-$, $\mu = 0.75$ cm^{-1}, density(calc) = 1.237 g.cm^{-3}, 2θ range 5–50°. No absorption corrections were applied. Hydrogen atoms were placed in a combination of observed and calculated positions and held fixed with U(H) = 0.08 Å. Lattic constants were derived from 25 high angle ($2\theta > 20°$) reflections. Final conventional and weighted *R*-values were both 0.055. Waters of hydration occupy the space between hydroxy substituents.

11α-Hydroxyestradiol (5). Suitable crystals were grown from acetone–ethanol. A pale yellow square tabular crystal with dimensions 0.42 × 0.14 × 0.28 mm^3 was used in the study. No solvent was present in the lattice. A total of 1867 reflections were obtained; of these, 1043 were observed with $I \geqslant 2.5\ \sigma(I)$. Abbreviated crystal data are as follows: **5**, $C_{18}H_{24}O_3$, f.w. 288.39 amu, space group $P2_12_12_1$, $a = 6.154(2)$ Å, $b = 12.516(3)$ Å, $c = 20.000(4)$ Å, $V = 1540.4(8)$ Å^3, $Z = 4$, $F_{000} = 624\ e^-$, $\mu = 0.78$ cm^{-1}, density(calc) = 1.243 g.cm^{-3}, 2θ range 6–52°. No absorption corrections were applied. Hydrogen atoms were placed in a combination of observed and calculated positions and held invariant. Lattice constants were derived from 25 high angle ($2\theta > 20°$) reflections. Final conventional and weighted *R*-values are 0.065 and 0.058, respectively.

11-Keto-9β-estradiol (6). Suitable crystals were grown from acetone. A colorless cylindrical rod crystal with dimensions 0.36 × 0.50 × 0.30 mm^3 was used in the study. No solvent molecules were present in the lattice. A total of 1578 reflections were obtained, 1230 of those as unique data with $I \geqslant 2.5\ \sigma(I)$. Abbreviated crystal data are as follows: **6**, $C_{18}H_{22}O_3$, f.w. 286.37 amu, space group $P2_1$, $a = 6.0322(9)$ Å, $b = 12.153(2)$ Å, $c = 10.236(3)$ Å, $\beta = 91.80\ (2)°$, $V = 750.0(3)$ Å^3, $Z = 2$, $F_{000} = 308\ e^-$, $\mu = 0.79$ cm^{-1}, density(calc) = 1.268 g.cm^{-1}, 2θ range 6–50°. No absorption corrections were applied. Hydrogen atoms were placed in observed positions and held fixed. Non-hydrogen atoms were refined anisotropically. Final conventional and weighted *R*-values are 0.054 and 0.036, respectively.

11β-Hydroxyestradiol (8). Suitable crystals were grown from ethanol. A colorless square rod crystal with dimensions 0.22 × 0.15 × 0.15 mm^3 was used in the study. No solvent molecule was present in the lattice. A total of 1226 reflections were collected; of these 922 were observed with $I \geqslant 2\ \sigma(I)$. Abbreviated crystal data are as follows: **8**, $C_{18}H_{24}O_3$, f.w. 288.39 amu, space group $P2_12_12_1$, $a = 6.4903(4)$ Å, $b = 11.344(1)$ Å, $c = 20.962(4)$ Å, $V = 1543.3(4)$ Å^3, $Z = 4$, $F_{000} = 624\ e^-$, $\mu = 6.24$ cm^{-1}, density(calc) = 1.241 g.cm^{-1}, 2θ range 5–110°. Transmission coefficients applied to the data ranged from 0.882 to 0.858. Hydrogen atoms were placed in observed positions and refined. Final conventional and weighted *R*-values are 0.039 and 0.038, respectively.

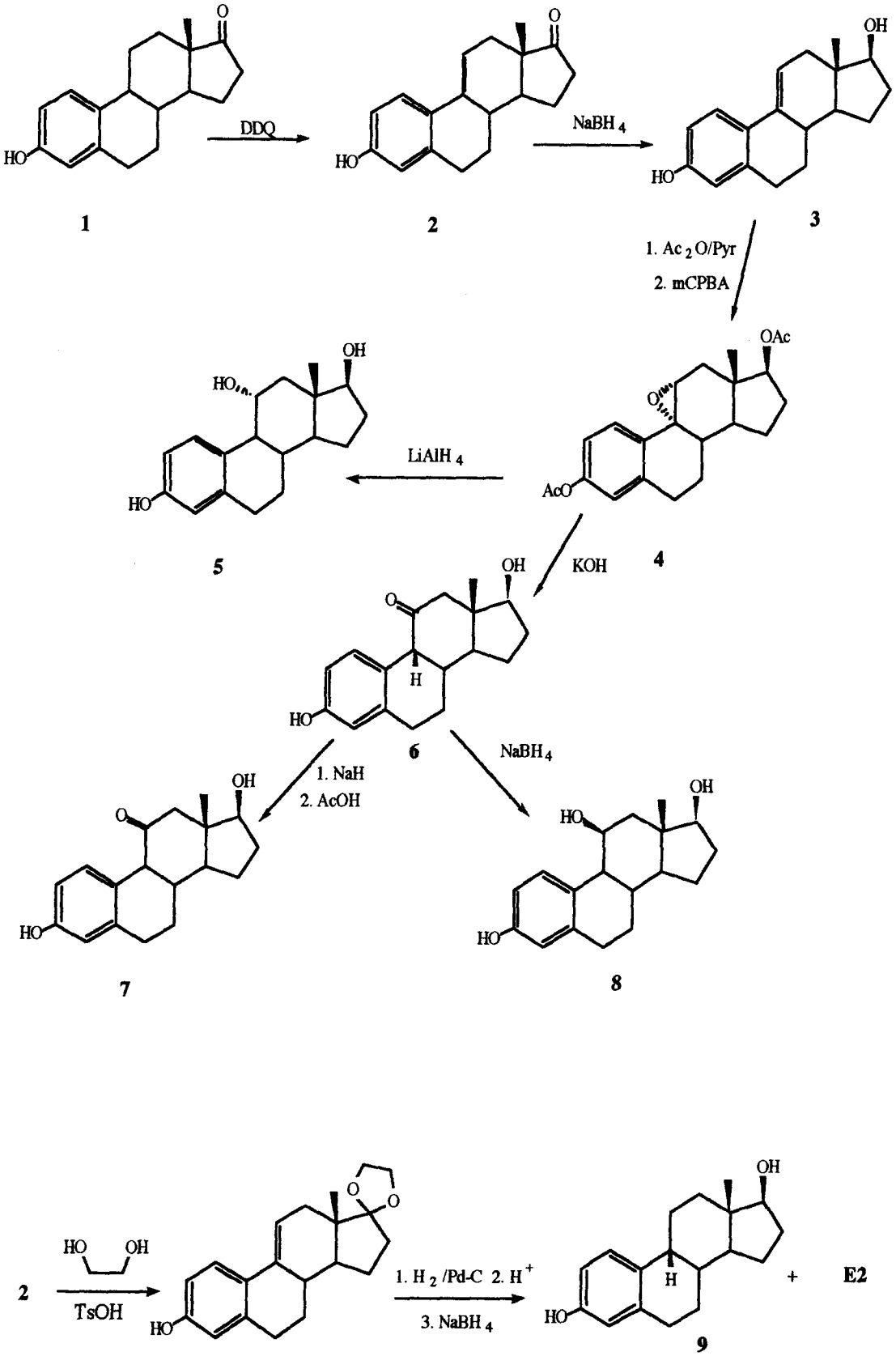

Fig. 1. Schematic pathway for the synthesis of estradiols 3, 5, 6, 7, 8, and 9.

Receptor binding studies

MCF-7 cells were grown to confluence in minimal essential medium with Hank's basic salt solution, 10% calf serum, antibiotics and insulin as described previously [21]. Following homogenization, the 100,000*g* supernatant was collected as a source of ER [22]. Cytosolic ER assays were carried out according to classical Scatchard [23] and competitive binding methods [24] utilizing dextran-coated charcoal (DCC). The incubations (in triplicate) carried out to generate a Scatchard plot contained 0.4 ml cytosol and [2,4,6-^{3}H]E_2 (100 Ci/mmol, New England Nuclear, Boston, MA) over a concentration range of 0.1–1.5 nM with and without 200-fold unlabeled E_2. Competitive binding assays (triplicate) contained the same volume of cytosol with 1.5 nM tritiated E_2 and five different concentrations of the E_2 analog being tested. Concentrations selected were 1.5, 3.0, 30, 300 and 3000 nM. The tubes were incubated overnight at 4°C before treatment with DCC and measurement of the radioactivity in the DCC treated supernatant [22].

RESULTS

Synthesis

The synthetic methodology used in the preparation of the 9,11-modified E_2 analogs (Fig. 1) follows the literature with several significant modifications and improvements. Thus, the synthesis of the key starting material 11-dehydroestrone 2 was simplified from that

Table 1. *Atomic positional parameters for 11α-hydroxy-(5), 11β-hydroxy-(8), 9-11 ene-(3), and 11-keto-9α-(6) estradiols*

Atom	x	y	z	x	y	z
		(5)			(8)	
C1	1.200 (1)	−0.0249 (5)	0.6892 (3)	0.1405 (8)	0.1135 (5)	0.5091 (2)
C2	1.315 (1)	−0.0829 (6)	0.7375 (3)	0.1747 (9)	0.1926 (4)	0.4598 (3)
C3	1.249 (1)	−0.1877 (6)	0.7527 (3)	0.0333 (8)	0.1992 (4)	0.4110 (2)
C4	1.067 (1)	−0.2315 (5)	0.7219 (3)	−0.1366 (9)	0.1262 (5)	0.4108 (2)
C5	0.951 (1)	−0.1727 (5)	0.6748 (3)	−0.1712 (7)	0.0470 (4)	0.4602 (2)
C6	0.752 (1)	−0.2202 (5)	0.6444 (3)	−0.3645 (9)	−0.0275 (5)	0.4580 (3)
C7	0.702 (1)	−0.1836 (5)	0.5721 (3)	−0.3578 (9)	−0.1331 (4)	0.5025 (2)
C8	0.847 (1)	−0.0906 (5)	0.5464 (3)	−0.2788 (8)	−0.0992 (4)	0.5683 (2)
C9	0.886 (1)	−0.0117 (5)	0.6047 (3)	−0.0592 (8)	−0.0500 (4)	0.5645 (2)
C10	1.018 (1)	−0.0685 (5)	0.6581 (3)	−0.0311 (7)	0.0402 (4)	0.5107 (2)
C11	0.992 (1)	0.0943 (5)	0.5788 (3)	0.0183 (8)	−0.0090 (4)	0.6301 (2)
C12	0.872 (1)	0.1415 (5)	0.5194 (3)	−0.0005 (9)	−0.1051(5)	0.6809 (3)
C13	0.859 (1)	0.0607 (5)	0.4618 (3)	−0.2128 (8)	−0.1667 (4)	0.6832 (2)
C14	0.738 (1)	−0.0363 (5)	0.4882 (3)	−0.2714 (8)	−0.2020 (4)	0.6155 (2)
C15	0.692 (2)	−0.1033 (5)	0.4228 (3)	−0.455 (1)	−0.2853 (5)	0.6241 (3)
C16	0.660 (1)	−0.0145 (5)	0.3690 (3)	−0.411 (1)	−0.3474 (6)	0.6881 (3)
C17	0.706 (1)	0.0923 (5)	0.4046 (3)	−0.2143 (9)	−0.2894 (4)	0.7142 (2)
C18	1.085 (1)	0.0373 (6)	0.4349 (4)	−0.373 (1)	−0.0878 (5)	0.7149 (3)
O3	1.3566 (8)	−0.2475 (4)	0.8000 (2)	0.0545 (7)	0.2773 (4)	0.3610 (2)
O11	0.9970 (9)	0.1708 (3)	0.6331 (2)	−0.0882 (6)	0.0972 (3)	0.6477 (2)
O17	0.7950 (7)	0.1730 (3)	0.3605 (2)	−0.2024 (7)	−0.2898 (4)	0.7825 (2)
		(3)			(6)	
C1	0.2069 (2)	0.7162 (9)	0.4349 (4)	−0.3547 (9)	−0.2121 (5)	−0.5305 (5)
C2	0.1512 (2)	0.7402 (5)	0.3893 (4)	−0.4818 (9)	−0.2218 (5)	−0.4420 (5)
C3	0.1274 (2)	0.8208 (4)	0.4551 (4)	−0.451 (1)	−0.3103 (6)	−0.3396 (5)
C4	0.1590 (2)	0.8760 (4)	0.5647 (4)	−0.293 (1)	−0.3892 (5)	−0.3678 (5)
C5	0.2138 (2)	0.8520 (4)	0.6112 (4)	−0.1623 (9)	−0.3820 (5)	−0.4762 (5)
C6	0.2456 (2)	0.9157 (4)	0.7323 (4)	0.006 (1)	−0.4706 (5)	−0.4990 (5)
C7	0.3005 (2)	0.8600 (4)	0.7948 (4)	0.1699 (8)	−0.4402 (5)	−0.6035 (5)
C8	0.3314 (2)	0.8297 (4)	0.6900 (4)	0.0508 (7)	−0.3886 (5)	−0.7233 (4)
C9	0.2986 (1)	0.7450 (4)	0.5931 (4)	−0.0591 (8)	−0.2798 (5)	−0.6820 (5)
C10	0.2392 (2)	0.7702 (4)	0.5453 (4)	−0.1946 (9)	−0.2922 (5)	−0.5607 (5)
C11	0.3219 (2)	0.6527 (4)	0.5572 (4)	−0.2062 (8)	−0.2305 (5)	−0.7929 (4)
C12	0.3822 (2)	0.6223 (4)	0.5961 (4)	−0.3691 (8)	−0.3088 (6)	−0.8603 (4)
C13	0.4158 (2)	0.7251 (4)	0.6529 (4)	−0.2405 (9)	−0.4107 (5)	−0.9077 (4)
C14	0.3875 (2)	0.7820 (5)	0.7520 (4)	−0.1184 (8)	−0.4645 (5)	−0.7876 (4)
C15	0.4311 (2)	0.8648 (6)	0.8286 (6)	−0.0465 (9)	−0.5772 (5)	−0.8406 (5)
C16	0.4866 (2)	0.8108 (7)	0.8202 (8)	−0.231 (1)	−0.6085 (5)	−0.9426 (5)
C17	0.4710 (2)	0.6987 (5)	0.7473 (4)	−0.3883 (8)	−0.5090 (5)	−0.9481 (4)
C18	0.4230 (2)	0.8045 (5)	0.5422 (5)	−0.0824 (8)	−0.3781 (5)	−1.0155 (5)
O3	0.0731 (1)	0.8481 (4)	0.4161 (3)	−0.5810 (7)	−0.3204 (0)	−0.2334 (3)
O11	—	—	—	−0.1945 (6)	−0.1346 (4)	−0.8216 (3)
O17	0.5156 (1)	0.6631 (4)	0.6938 (3)	−0.4898 (6)	−0.4928 (4)	−1.0791 (3)

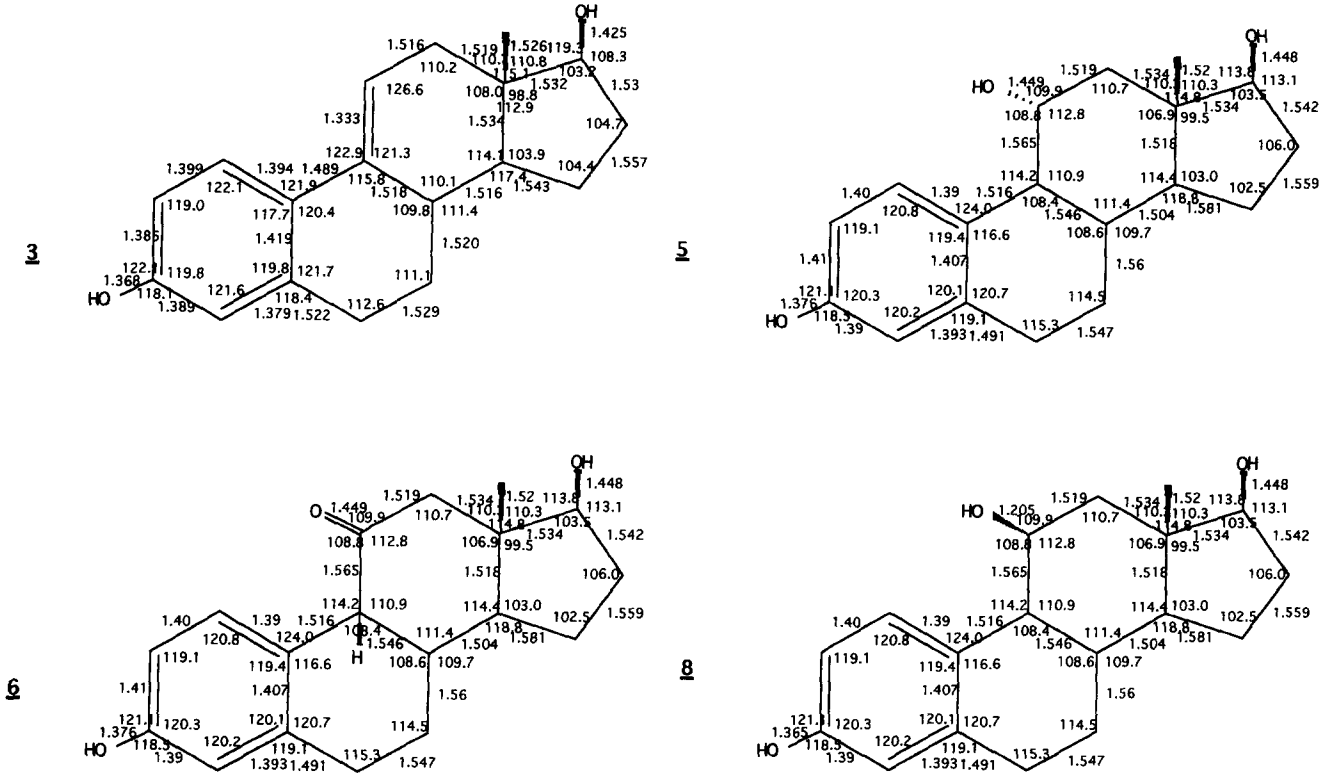

Fig. 2. Bond distances(Å) and bond angles(°) of 9-11 ene-(3), 11-keto-9β-(6), 11α-hydroxy- (5) and 11β-hydroxy-(8) estradiols.

of Gabbard *et al.* [14] by solvent change and allowing crystallization of pure material to occur directly in solution. Preparation of the triol 5 was executed from the protected epoxy diacetate 4 rather than from the 17-keto isomer as reported by Hasegawa *et al.* [16], since it is known that lithium aluminium hydride reduction of 17-keto steroids tends to produce a somewhat large proportion of the α isomer.

Triol 8 was produced in a simple, one step operation, from 11-keto-9β-estradiol 6 using the known base-directed, kinetically controlled, formation of the 9α isomer previous to the reduction step. The conversion of the 11-keto-9β-estradiol 6 to 11-ketoestradiol 7 was performed as a two-step, one-pot operation, avoiding the protection–deprotection scheme reported previously [15].

The synthesis of 9 followed that of Gabbard and Segaloff [19]. However, in our hands the yield of this isomer of E_2 was far below the reported value and, in addition, required purification by preparative TLC.

Crystallography and modeling

In contrast to many E_2 analogs, the 11-oxo derivatives do not cocrystallize with solvent molecules, a fact that is related to their close intermolecular packing. Olefin 3 crystallized as the hemihydrate. Table 1 lists the observed atomic positional parameters for the crystal structure of steroids 3, 5, 6, and 8. Bond distances, bond angles, and torsion angles of the same crystals are shown in Figs 2 and 3.

Although 11-ketoestradiol 7 and 9 crystallized as fine needles, every attempt to generate crystals suitable for X-ray analysis failed. This failure prompted the three-dimensional generation of the structures using a computational approach based on the conformation of three crystal structures: E_2, 11α-(5), and 11β-(8) hydroxyestradiol for 7, and E_2 and 6 for 9. When the three models were modified to produce 7 and then the generated structures were subjected to geometry optimization using a simplified MM2 method [12], a single model of 7 was obtained. A similar computational approach was used to obtain an optimized structure of 9 from E_2 and 6 and to generate E_2 from three published X-ray crystal structures [11]. The crystal structures of 3, 5, 6, and 8 were optimized using the same MM2 method, and these, along with the computer generated structures of 9, 7 and E_2, were used to study the relationship of electronic induction to conformational changes. Figure 4 depicts the MM2 optimized structures of 3, 5, 6, 7, 8, 9 and E_2 viewed parallel to the plane of the aromatic ring (ring A).

Despite a pronounced flattening of rings B and C, compound 3 shares some important structural values with E_2 and analogs 7 and 8. The last three compounds exhibit similar half-chair conformations for ring B and an envelope for ring D. The angle formed between the

aromatic C3 and C5 with the angular methyl group, C18, is 14.0° for E_2, 14.4° for 7 and 12.8° for 8. This angle compares quite well with a value of 15.0° for 3. The distances between O3 and O17 are 10.993, 10.973, and 11.068 Å for E_2, 7 and 8, respectively, in close similarity with a value of 11.039 for 3. These results contrast with the α-hydroxy analog 5 in which a larger C3-C5-C18 angle of 17.7° corresponds to a shorter O3-O17 distance of 10.793 Å (Table 2). In 5, ring B is modified significantly to a slightly twisted chair as is ring D. In general compound 5 has been modified structurally to a chair conformation. Compound 6 on the other hand, presents a C3-C5-C18 angle of −14.3°, significantly different than the value of −13.0 found for 9. This result is in clear agreement with the expected distortion created by the electronic repulsion of the oxygen in C11 with adjacent carbon atoms, mainly C1. This repulsion rotates O = C11 modifying the position of rings C and D, and making the distance between O3 and O17 shorter (9.038 Å) than in the relaxed structure 9 (O3 ... O17 = 9.319 Å, Table 2).

Receptor affinity

Table 2 also includes the results of experiments to examine the binding of these E_2 analogs to cytosolic ER. Relative to E_2, 9,11-dehydroestradiol, 3 was the best binder with 1/5th the affinity of E_2. The 11β-hydroxylated derivative 8 displayed 1/60th the affinity for the receptor whereas its isomer, the 11α-hydroxy, 5, was 1/5th of that value. The 11-ketoestradiol (7) bound to the receptor 1/1000th as tightly as the natural E_2, whereas 11-keto-9β-estradiol, 6, displayed an affinity which was too low to measure. 9β-Estradiol, 9, bound receptor with very low, but measurable, affinity.

DISCUSSION

It has been postulated that the binding domain of ER contains a transactivation function (TAF-2) which seems to play a crucial role in transcription activation [25]. More recently, the hypothesis of protonated amino acid residues inside or near the active site of the E_2 receptor [26] has gained support in the evaluation of certain positively charged organometallic-labeled E_2 derivatives [27]. Additionally, the substitution of a hydroxyl group on the E_2 nucleus may alter the solvation of the ligand. Therefore the aqueous environment surrounding the receptor may in itself alter the binding characteristics of different estrogens. These fundamentals underline the difficulties involved in mapping the estrogen binding domain, and more importantly, evaluating its sterochemical requirements. In these investigations, a more traditional approach has been employed to examine the binding of analogs which are stereochemically related to the natural E_2. The variable in this study has been the skeletal

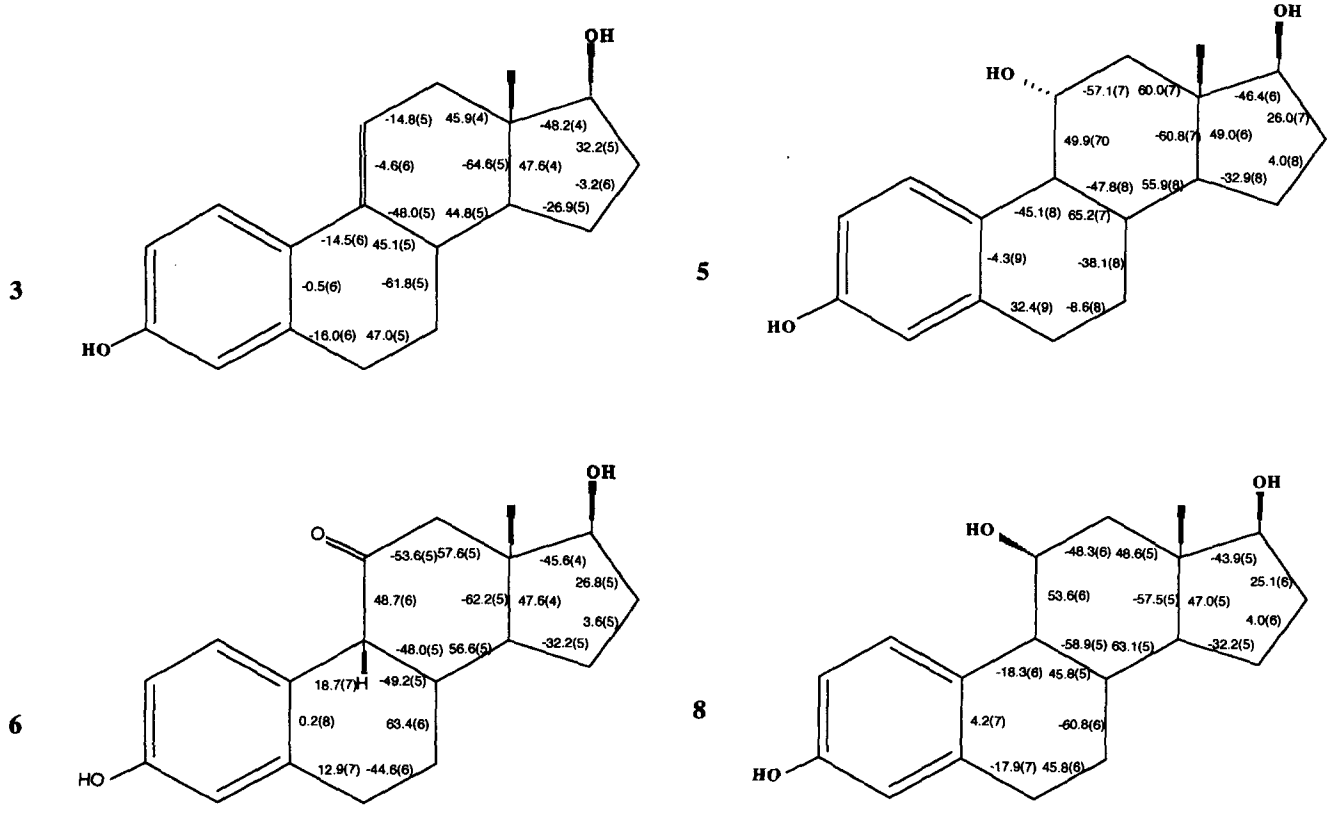

Fig. 3. Torsion angles (°) of 9-11 ene- (3), 11-keto-9β- (6), 11α-hydroxy- (5) and 11β-hydroxy- (8) estradiols.

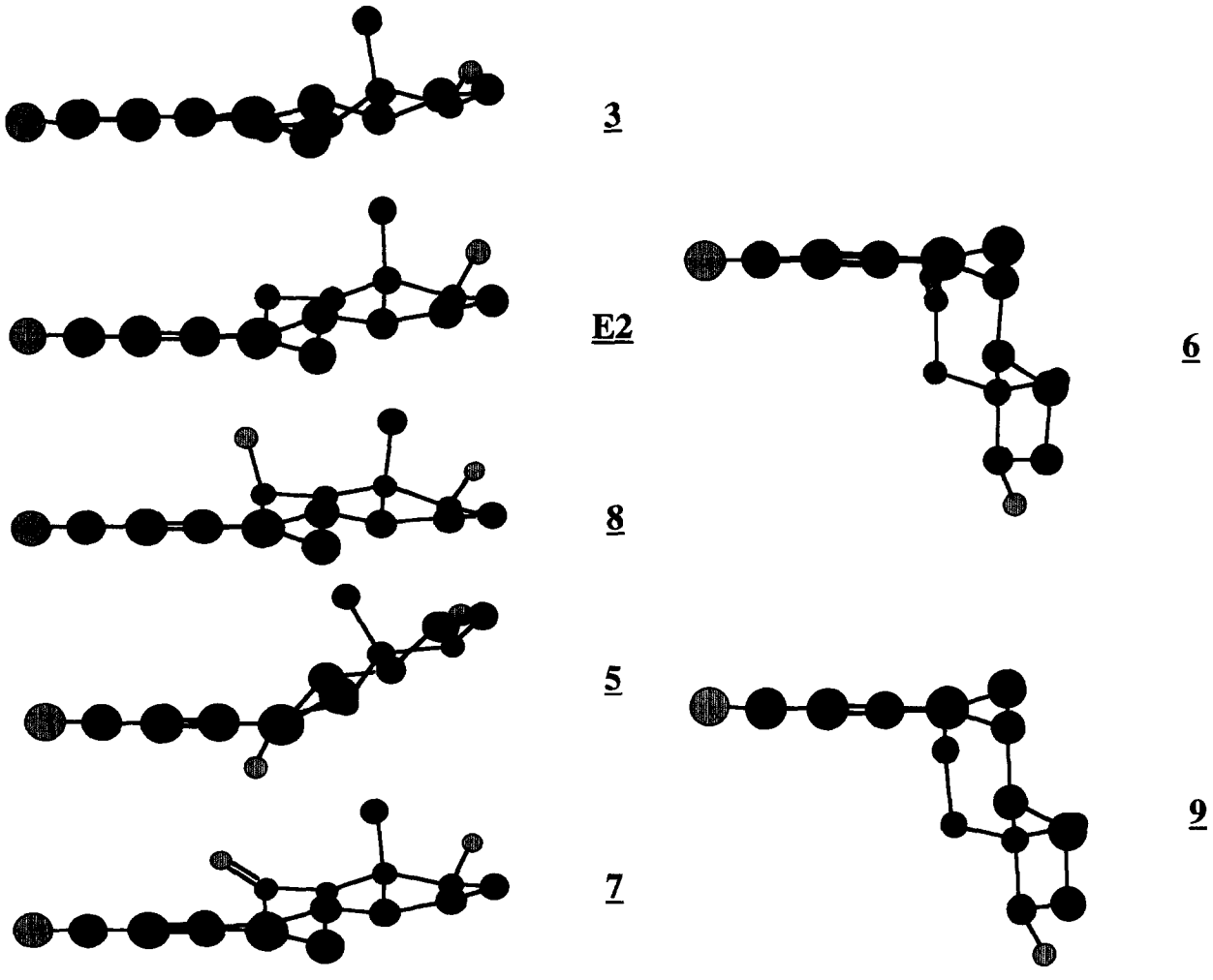

Fig. 4. Crystallographic conformations of estradiols 3, 8, 5, and 7 (MM2) viewed parallel to the aromatic ring and compared to E_2 and of estradiols 6 and 9 (MM2) viewed parallel to the aromatic ring.

conformation of the C-ring analogs which was accurately resolved with X-ray crystallography or computer modeling.

Crystallographic and modeling studies reveal that minimal skeletal variations have occurred in the overall conformation of E_2 when an 11-keto (7), or an 11β-hydroxy (8) group has been introduced in the C-ring. The 11α-hydroxy isomer (5), on the other hand, has undergone a structural modification with respect to E_2 that converted ring B into a twisted envelope with additional twist variations in rings C and D. Similar observations have been reported for the 11α and β methoxy ethynyl estradiols [28]. Molecule 5 represents a relaxed chair in which the angle C3-C5-C18 has been increased over three degrees from E_2. By comparison, the distance between the phenolic O3 and alcohol O17,

Table 2. Selected angles, interatomic distances and relative binding (RBA) of crystal and minimized E_2 analogs

	<C3-C5-C18°		O3-O17Å		O11-H1Å		
	MM2	Crystal	MM2	Crystal	MM2	Crystal	RBA
Estradiol (E_2)	14.0	15.7	10.993	10.925	—	—	1000
9β-Estradiol (9)	−13.0	NA	9.319	NA	—	—	0.7
9-11 Ene-E_2 (3)	15.0	14.9	10.829	11.039	—	—	196
11β-Hydroxy-E_2 (8)	12.8	12.8	11.068	11.056	3.120	3.019	16.8
11α-Hydroxy-E_2 (5)	17.7	18.2	10.793	10.812	2.550	2.117	3.1
11-Keto-E_2 (7)	14.4	NA	10.973	NA	2.476	NA	0.9
11-Keto-9β-E_2 (6)	−14.3	−13.8	9.038	8.940	2.732	2.571	<0.5

NA, not available.

displayed minimal modification. This result indicates that the relative decrease in binding affinity of 5 compared to 8 may be due either to the presence of the polar hydroxy group in the alpha face, or to changes in the conformation of the hydrophobic region of the molecule. The importance of the alpha and beta faces of estrogens seems to be of relevance in the presence of some cationic substituents since the inclusion of hydrophobic moieties on the aromatic or 17α regions of the molecule causes little change in the binding affinities of the molecules [27].

It is of interest to speculate as to the reasons for the extensive structural effect an 11α-hydroxyl group (5) exerts on the triol as compared to the minor structural modifications brought about by an 11β-hydroxyl (8). The small crystallographic distance between O11 and H1 in 5 (2.117 Å) favors their interaction. Conceivably the O11 acquires a positive charge from H1 creating a negative polarization on C1, which in turn will change the relative polarization of the aromatic ring with a concomitant effect on the alicyclic rings. This is not possible in the case of the 11β-hydroxyl (8) in which O11 is separated from C1 by 3.120 Å. Employing X-ray crystallography we have shown previously [10] that pronounced changes in the skeletal B–C–D alicyclic rings occur as a consequence of electronic influences of substituents on the aromatic A-ring. For example, the relocation of the 3-hydroxyl group of E_2 to the 2 position causes a partial flattening of the B ring with conformational consequences to rings C and D.

The importance of the aromatic A-ring and the position of the phenolic substituent in the binding of the ligand to ER has been amply described for the human receptor [2, 4, 6, 10]. In addition, several other structure–activity relationships have been established. For example, the 17β-hydroxyl group appears to be involved in providing the proper orientation for the binding of E_2, possibly by hydrogen bonding to a group on the receptor which lies above ring D [4]. Furthermore, substituents on the α and β face of the steroid seem to have varying effects with both rodent and human ER, as in the case of the 11β-alkyl substituted estradiols which have been reported to be either strongly estrogenic [29] or antiestrogenic [30] whereas the 7α-amidoalkyl derivatives have pure antiestrogenic activity [31]. Nevertheless, the incorporation of an alcohol function at either side of the 11-position lead to a significant decrease in affinity for the ER (Table 2). The relative binding affinity (RBA) of the 11β-hydroxy derivative (8) was 1/60th of that of E_2, whereas the α isomer (5) bound ER much less tightly (K_a was 1/300th that of E_2, Table 2). This variation between the two isomers may be related to the drastic structural change in the alicyclic hydrophobic portion of the molecule as discussed above, or to a particular steric or electrostatic requirement of the α side binding region of the receptor. The introduction of a keto group at the 11-position of E_2 (7) caused an even larger decrease (1/1000th) in the relative binding (Table 2) without affecting the overall conformation of the molecule (Fig. 4). This clearly is a substituent effect that relates to the perturbation in electrostatic energy around the 11-position, since the 11-methylene homolog has been shown to possess a strong binding affinity [32]. The latter fact explains in part the relative affinity of the olefin **3**, which displayed the best binding activity of the modified analogs assayed, despite a severe flattening of rings B, C, and D. It is apparent that the electrostatic influence of an endocyclic C–C double bond cannot be compared with that of an exocyclic C=O.

A more predictable result occurred with the binding of the 9β isomers of 7 and E_2 (**6** and **9**, respectively, Table 2). Although these steroids displayed atom distances and angles similar to those of E_2, a bent conformation for both apparently precludes a proper alignment with the receptor. It is worth mentioning that the 17-keto homolog of **6** reportedly has similar *in vivo* estrogenic activity as estrone [9]. However, the reported values for restoration of uterine weight for **9** (relative potency 0.78 vs 100 for E_2) are more in line with the RBA of the present study [33]. Interestingly, **6** has been shown to yield four conformers with a potential energy difference within 5 kcal [13]. Since two of these conformers are not bent, it is conceivable that the forces involved in binding to the receptor *in vivo* may overcome this energy barrier.

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