Theoretical Investigations on the Role of Steroid-Skeleton C4=C5 Unsaturation in Competitive Aromatase Inhibition

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Z. Naturforsch. **44c**, 217–225 (1989); received August 29/November 21, 1988

Aromatase Inhibitors, Quantitative Structure-Activity Relationships, MTD Method, Steroid Conformations

A quantitative structure-activity relationship (QSAR) approach by use of the minimal topological difference (MTD) method including 46 derivatives of 4-androstene-3,17-dione and 5α-androstane-3,17-dione is applied to give indications about the role of the C4=C5 double bond in competitive inhibition of human placental aromatase and about sterical requirements in steroid-aromatase interactions. The inhibitory activity is found to correlate with the sterical MTD variable, hydrophobicity, and π-system conjugation in the A,B-ring region.

A comparison of the MTD results reveals a good agreement with interpretations based on free-energy data derived from inhibition constants.

By means of MM2 molecular mechanics and PCILO quantum-chemical calculations, the 4-ene structure is shown to significantly influence conformational features of C19 substituents which are important in enzymatic transformations. While 19-hydroxy-5α-androstane-3,17-dione favour a conformation having the hydroxyl group in the enzyme-directed out-of-ring position, the C4=C5 double bond energetically enables the steroid to adopt a conformation which can be hydroxylated without internal rotations.

According to present theoretical findings, the 4-ene unsaturation thus exerts an indirect conformational influence by hydroxyl positioning appropriate to aromatase interactions and a direct electronic influence by π conjugation.

Introduction

The observation that approximately 35% of breast cancer are estrogen dependent has stimulated research into methods of suppressing the estrogen biosynthesis, especially of reducing the aromatization of 4-en-3-one androgens to phenolic estrogens. The enzyme responsible for this process is a unique cytochrome P-450 monooxygenase complex known as aromatase or estrogen synthetase.

Mechanism-based irreversible inactivation of this enzyme is much more attractive, from a clinical point of view, than competitive inhibition because of much lower intracellular drug concentrations. But it can be assumed that data of competitive reversible inhibition are useful to describe the initial steps of recognition and binding, thus being valid for active-site directed irreversible inhibitors as well. Therefore, activities regarding to competitive inhibition are investigated in the present comparison between steroids possessing and lacking the C4=C5 double bond in order to give a contribution towards an understanding of the underlying molecular binding mechanisms and the role of the 4-ene structural feature found in the substrate and in all potent inhibitors.

In a first part, quantitative structure-activity relationships (QSAR) are derived by means of the minimal topological difference (MTD) method which has already been applied in steroid research [1–6]. The MTD results are compared in part to binding-energy contributions evaluated on the basis of reported inhibition constants from competition experiments. In a second section, a conformational analysis into potential intermediates of both steroid types is presented referring to the first hydroxylation steps in enzyme catalysis.

Materials and Methods

The MTD calculations are performed for a data set including 46 steroids presented in Table I. The classification scheme for competitive inhibition of

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/89/0300–0217 $ 01.30/0

human placental microsomal aromatase by Schwarzel *et al.* [7] is used because this activity determination provides consistent data for both 4-androstene-3,17-dione (4-ene steroid) and 5α-androstane-3,17-dione (5α-H steroid) derivatives. From this source, all but one inhibitory potencies are taken for the MTD analysis and transformed according to $A = \log[x/(100\%-x)]$, with x being the mean %-inhibition values of the classes defined in [7] for a substrate/inhibitor ratio of 1:6 and an incubation time of 15 min. The only compound additionally taken into account is 4-hydroxyandrostenedione (steroid 46 in Table I) which has been included for its importance (*cf.* [8, 9]) and with an activity value corresponding to class 6+ estimated on the basis of reported inhibition [10].

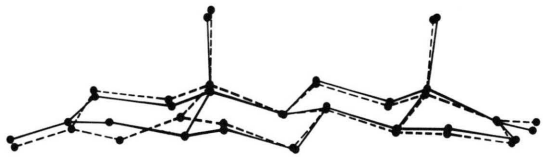

Fig. 1. Crystal structure of 4-androstene-3,17-dione (– – –) [11] superimposed with that of 5α-androstane-3,17-dione (——) [12].

The MTD method and the optimization strategy used is described in detail elsewhere [1, 6]. A structural comparison of 4-ene and 5α-H steroids is given in Fig. 1 which shows a superposition of X-ray crystallographic data of 4-androstene-3,17-dione [11] and 5α-androstane-3,17-dione [12] by a least-squares fit including all non-hydrogen atoms. The largest deviations in the fit are found for the C4 (44 pm) and C5 (38 pm) atomic locations. However, both values are within the limit for unique vertices of 50 pm generally applied in MTD hypermolecule creation [1]. Thus it appears that the direct influence of the C4=C5 double bond on the global steroid-skeleton geometry is small and can be neglected in the rather crude MTD scheme. Since 4α substituents of 5α-H steroids are located equatorial they occupy similar positions as 4-substituents of 4-ene compounds (*cf.* also Fig. 1). Therefore, a common vertex is taken for atoms substituted at both sites. The resulting hypermolecule together with the actual vertex numbering can be taken from Fig. 2.

Hydrophobic properties (f) are expressed as the sum of contributions from all substituents excluding those attached to C3, C4, and C17 since these regions are supposed to be involved in hydrogen bonding or π-system interactions. Rekker's hydrophobic fragmental constants [13] including relevant proximity corrections are used for the calculation of f. As already practised in [6], only half of the proximity effect is applied for electronegative substituents close to hydrogen bonding oxygens because the hydrophobicity interference is restricted to one centre.

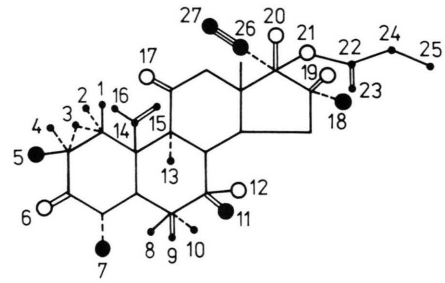

Fig. 2. Schematic representation of the hypermolecule with numerotation of vertices and the results of the optimized MTD map coded as: ——○—— enzyme cavity vertex; ——•—— sterically irrelevant vertex; ——●—— enzyme wall vertex.

Electronic effects in the A- and B-ring region are represented by the π-system resonance energy R (in β units) computed within the Hückel molecular orbital (HMO) theory. Its simplicity is in fair correspondence with the crudeness of the topological treatment. The resonance energy being a measure of π conjugation is evaluated by the π-electronic energy difference between the fully conjugated system and the sum of isolated double-bond subsystems. The following HMO parameters are applied: $\alpha(C)=\alpha$; $\alpha(=O)=\alpha+\beta$; $\alpha(-O-)=\alpha+2\beta$; $\beta(C=O)=\beta(C=C)=\beta$; $\beta(C-C)=\beta(C-O)=0.8\beta$ [14].

Although the inhibition data from [7] have the advantage of compatibility due to uniform experimental conditions and of covering a relatively large data set, they are afflicted with a certain inaccuracy. Therefore, binding-energy contributions of relevant substituents attached to 4-androstene-3,17-dione (ΔG_{obs}) are taken into consideration for comparison. These free energy values are listed in Table II and obtained according to $\Delta G_{obs} = -RT \ln(K_i/K_m)$ for $T = 298$ K and with K_i being the apparent inhibition constant from competition experiments reported in literature. Since K_m data of 4-androstene-3,17-dione differ from 10 to 300 nM in different experiments (larger values omitted from consideration herein), the actually reported K_m values of the substrate are

taken in the ΔG_{obs} calculations instead of a fixed mean value. A positive ΔG_{obs} value indicates that a given substitution increases the total free energy of steroid-enzyme binding whereas negative values are associated with perturbations of the binding-site fit.

In addition to energy data related to experiments, Table II also contains free-energy contributions ΔG_{av} calculated within the model recently proposed by Andrews *et al.* [15]. Energy increments in that model account for average contributions of individual functional groups to non-covalent interactions typical in a large scale of drug-protein binding [15]. Accordingly, positive values of $\Delta G_{diff} = \Delta G_{obs} - \Delta G_{av}$ point to better matching structures than expected in an average binding arrangement. The observed binding is weaker than expected in case ΔG_{diff} is negative which can be found if the side group considered is not involved in binding, the fit is poor, or a high-energy conformation is acting.

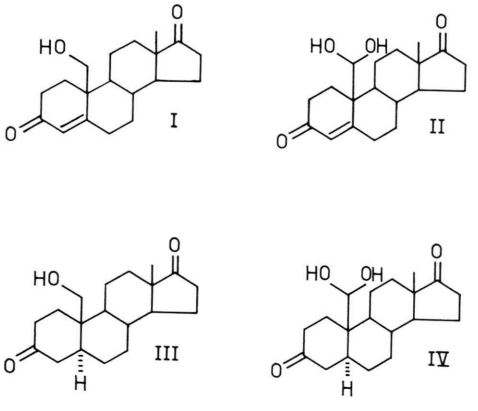

Fig. 3. Hydroxy substituted steroids for conformational analyses.

Investigations on the conformations of 19-hydroxy and 19,19-dihydroxy analogues shown in Fig. 3 are performed by means of Allinger's MM2 molecular mechanics technique [16] and by the quantum-chemical PCILO method [17–19] as well. For all compounds shown in Fig. 3, a completely optimized geometry for each of the three possible 10β side-group conformations is calculated by means of the empirical MM2 scheme using the standard force-field parametrization and including lone pairs at hydroxyl oxygens. Since these MM2 geometries can be regarded as reliable they are also employed in a subsequent step to calculate the PCILO energies on the basis of individually optimized polarities. Only the lone pairs at carbonyl oxygens using standard valence and torsional angles are added to the fixed MM2 coordinates for PCILO computations.

All calculations have been performed on PC 1715 and EC 1056 computers.

Results and Discussion

The following optimized map for steroid-aromatase contacts and the related regression equation are obtained by using the hypermolecule presented in Fig. 2:

$$S^* \begin{cases} j(\varepsilon = -1)\text{: } 6, 12, 17, 19-21 \\ j(\varepsilon = 0)\text{: } 1-4, 8-10, 13-16, 22-25 \\ j(\varepsilon = +1)\text{: } 5, 7, 11, 18, 26, 27 \end{cases}$$

$$A^{theor} = 1.621 - 0.566\ (\pm 0.060)\ \text{MTD} + 0.535\ (\pm 0.052)\ f + 3.12\ (\pm 0.24)\ R \qquad (1)$$

$$n = 46 \quad r = 0.914 \quad s = 0.264 \quad F_{3,42} = 71.3 \quad \alpha < 0.001$$

The vertex characteristics are also illustrated in Fig. 2. The enzyme cavity vertices found for the oxygens at C3 and C17 correspond to hydrogen-bonding facilities generally discussed in the process of recognition and binding by aromatase. In most parts of the regions around C1, C2, and C10 carbon atoms, the attributions of sterically irrelevant vertices ($j = 1-4$) indicate that there is space available for substitutions. Indeed, modifications at C10 or C19 were found to generate powerful inhibitors of aromatase and belong to the most often studied substitution sites of androstenedione [20–29]. Even a bulky trimethylsilyl or trimethylsilylmethyl group attached at C19 is accommodated by aromatase but is obviously too extensive in 1α and 2α positions [29].

According to the wall vertex $j = 5$ (2β position), it should be noted that the assignment of a sterically irrelevant vertex to $j = 5$ within this MTD calculation reveals nearly the same statistical significance. A wall vertex, however, could be explained by direct repulsion from enzyme moieties since one aromatase binding group is strongly supposed to be located on the β-side above the steroidal ring A [25, 30]. An alternative way in which 2β substituents could affect binding properties is to vary preferred conformations of C19 side groups or to influence activation energies to their internal rotations. In the case of 4-ene-3-one structures, 2β substitutions are known to additionally invert the A-ring conformation into an unusual 1β,2α-half chair (crystal: [31], solution: [32]). An inverted boat conformation of ring A in solution was

detected for a 2β substituted 5α-androstan-3-one derivative by NMR [33].

Surprisingly, the $j = 7$ vertex is found to be a wall vertex although the very potent 4-hydroxyandrostenedione has been explicitly included in the data set. The high inhibitory activity of this compound is achieved due to the MTD description presented by the large resonance stabilization. In accordance with this view, 4-substituents of active 4-ene inhibitors reported in literature [9, 10, 34] are linked to the C4 carbon *via* an oxygen or sulfur atom. Considering, however, such extensive 4-substituents as 4-$OCO(CH_2)_5CH_3$ [10] or 4-$S(CH_2)_2CH_3$ [34], the wall vertex $j = 7$ is only understandable if these side chains are oriented into an enzyme cavity on the α side and if a selective repulsive contact of the enzyme occurs from the β side to the atom representing $j = 7$.

Substituents at C6 are classified to not sterically hinder the binding to aromatase. This is in agreement with the recently reported data of 70% and 90% inhibition of human placental microsomal aromatase activity for 6α-Br and 6β-Br derivatives of androstenedione, respectively [35]. But the inhibition of both compounds exhibits different time dependence indicating that both initially formed steroid-enzyme binding complexes have different abilities in subsequent reactions. Also 6α- and 6β-hydroperoxy steroids were found to cause irreversible inactivation of aromatase [36].

It should be noted that some vertices are represented in the data set by one compound only, *e.g.* vertices $j = 11, 12, 17, 18, 19$ each exhibiting a steric contribution in the optimized map. With respect to these interaction sites, the results need further substantiation from independent sources. For substitutions at the 7α position, it was demonstrated that the enzyme can accommodate even side chains which are very much extended in size [37–40]. These results may question the attribution of a wall vertex to $j = 11$. Probably, there are indirect effects of sp^2 hybridization at C7 *via* conformational deformations. In any case, a data point enlargement for some vertices seems to be desirable.

The attribution of irrelevant vertices $j = 22-25$ can be interpreted in two ways: either they describe an enzyme pocket or they suggest a previous transformation of the esters by other enzymes which are contained in the placental microsomes used.

The steric repulsion resulting from occupation of 17α wall vertices $j = 26, 27$ in ethisterone derivatives is in agreement with recent suggestions about different binding orientations with respect to the aromatase surface for steroids such as norethisterone and norethynodrel [41].

Besides the sterical MTD variable, π-conjugation and hydrophobic properties are found to influence the aromatase inhibitory potency. This is in accord with the observed hydrophobic nature of interactions between steroid hormones and their receptors or other steroid-binding proteins [6, 42, 43]. According to equation (1), the introduction of hydrophilic groups, *e.g.* hydroxyl groups at several positions, diminishes the inhibitory activity. The already mentioned loss of inhibition for 19-nor steroids [7, 9, 41] can be rationalized by a binding decrease due to reduced hydrophobicity as well. Since the 10β side group is the target of the enzyme's first hydroxylation attacks, a more mechanism-based contribution of the 10β substituent should be additionally taken into account (*cf.* also [9, 44–46]).

A change from the 6-oxo compound 1 (*cf.* Table I) to the corresponding 6-methylene steroid would increase both hydrophobicity and slightly the HMO resonance energy, thus leading to an enhanced inhibition. In accord with this theoretical suggestion, the 6-methylene analogue of androsta-1,4-diene-3,17-dione was recently proved to be a powerful inhibitor [47, 48].

An additional C1=C2 unsaturation should further pronounce the resonance energy and, thus, give rise to an inhibition increase. This effect was found in the case of androsta-1,4,6-triene-3,17-dione [7], but Marsh *et al.* [10] reported a slight inhibition reduction for 4-OH and 4-OAc androsta-1,4-diene-3,17-dione upon introduction of the 1-ene feature.

In the following, the MTD results described above are compared to interpretations by use of free-energy data collected in Table II.

4-Androstene-3,17-dione is predicted in the model by Andrews *et al.* [15] to bind with an average free energy of 26.0 kJ mol^{-1} if the whole molecule is bound. Applying a mean K_m constant of about 40 nM, the corresponding experimental value amounts to 42.2 kJ mol^{-1} which shows that this compound matches the enzyme well.

This close fit of the bound steroid is in agreement with the overall hydrophobicity influence found in the MTD correlation.

For the triene compound 47 in Table II, a theoretically unexpected increase in the free energy of bind-

Table I. Data of aromatase inhibitors for MTD calculations.

No.	Steroid designation	A^{exp}	A^{theor}	MTD	f	R	Vertex occupation
1.	6-Keto-AD	0.75	0.25	4	−2.162	0.656	6, 9, 14, 20
2.	17β-OAc-A	0.48	0.42	4	0.0	0.341	6, 14, 21, 22, 23, 24
3.	17β-OCHO-A	0.48	0.42	4	0.0	0.341	6, 14, 21, 22, 23
4.	17β-OCOEt-A	0.21	0.42	4	0.0	0.341	6, 14, 21, 22, 23, 24, 25
5.	19-Cl-AD	0.21	0.36	4	−0.125	0.341	6, 14, 16, 20
6.	1β-OH-T	−0.05	−0.46	4	−1.652	0.341	1, 6, 14, 21
7.	16-Keto-A	−0.69	−0.73	4	−2.162	0.341	6, 14, 19
8.	Ethisterone	−0.69	−0.71	6	0.0	0.341	6, 14, 21, 26, 27
9.	1α-OH-AD	−0.69	−0.46	4	−1.652	0.341	2, 6, 14, 20
10.	1β-OH-AD	−0.69	−0.46	4	−1.652	0.341	1, 6, 14, 20
11.	9α-OH-AD	−0.33	−0.46	4	−1.652	0.341	6, 13, 14, 20
12.	16α-OH-AD	−1.28	−0.87	5	−1.363	0.341	6, 14, 18, 20
13.	17α-OH-A	−0.69	−0.71	6	0.0	0.341	6, 14, 26
14.	6-Keto-17β-OAc-A	−0.05	0.25	4	−2.162	0.656	6, 9, 14, 21, 22, 23, 24
15.	7-Keto-17β-OAc-A	−1.28	−1.30	5	−2.162	0.341	6, 11, 14, 21, 22, 23, 24
16.	5α-Androstane	−1.28	−1.68	6	0.182	0.0	14
17.	2α-Br-5α-H-AD	−0.69	−0.36	4	0.538	0.0	4, 6, 14, 20
18.	2α,4α-diBr-5α-H-AD	−1.28	−0.92	5	0.538	0.0	4, 6, 7, 14, 20
19.	5α-H-AD	−0.05	−0.55	4	0.182	0.0	6, 14, 20
20.	1α,2α-Epoxy-5α-H-AD	−1.28	−1.44	4	−1.488	0.0	3, 6, 14, 20
21.	2α-F-17β-OAc-5α-H-A	−0.69	−0.74	4	−0.187	0.0	4, 6, 14, 21, 22, 23, 24
22.	2β-F-17β-OAc-5α-H-A	−1.28	−1.31	5	−0.187	0.0	5, 6, 14, 21, 22, 23, 24
23.	4α-F-17β-OAc-5α-H-A	−1.28	−1.11	5	0.182	0.0	6, 7, 14, 21, 22, 23, 24
24.	10β-Ac-19-nor-5α-H-AD	−1.28	−1.42	4	−1.461	0.0	6, 14, 15, 16, 20
25.	19-OH-5α-H-AD	−1.28	−1.43	4	−1.470	0.0	6, 14, 16, 20
26.	5α-H-T	−0.05	−0.55	4	0.182	0.0	6, 14, 21
27.	17β-OAc-5α-H-A	−0.33	−0.55	4	0.182	0.0	6, 14, 21, 22, 23, 24
28.	5α-Androstan-3-one	−1.28	−1.11	5	0.182	0.0	6, 14
29.	5α-Androstan-17-one	−1.28	−1.11	5	0.182	0.0	14, 20
30.	11-Keto-5α-H-AD	−1.28	−1.14	3	−1.980	0.0	6, 14, 17, 20
31.	19-Nor-5α-H-AD	−1.28	−0.82	4	−0.337	0.0	6, 20
32.	6α,6β-diF-19-nor-AD	−0.05	−0.10	4	−0.968	0.341	6, 8, 10, 20
33.	6α,6β-diF-19-nor-T	−0.05	−0.10	4	−0.968	0.341	6, 8, 10, 21
34.	6α,6β-diF-NE	−1.28	−1.23	6	−0.968	0.341	6, 8, 10, 21, 26, 27
35.	1β-OH-19-nor-T	−0.69	−0.74	4	−2.171	0.341	1, 6, 21
36.	10β-OH-19-nor-T	−0.69	−0.74	4	−2.171	0.341	6, 14, 21
37.	19-Norethisterone	−1.28	−0.99	6	−0.519	0.341	6, 21, 26, 27
38.	1β-OH-19-nor-AD	−1.28	−0.74	4	−2.171	0.341	1, 6, 20
39.	2α-OH-19-nor-AD	−0.69	−0.59	4	−1.882	0.341	4, 6, 20
40.	2β-OH-19-nor-AD	−0.69	−1.15	5	−1.882	0.341	5, 6, 20
41.	6β-OH-19-nor-AD	−0.69	−0.74	4	−2.171	0.341	6, 8, 20
42.	7β-OH-19-nor-AD	−0.33	−0.17	3	−2.171	0.341	6, 12, 20
43.	10β-OH-19-nor-AD	−0.69	−0.74	4	−2.171	0.341	6, 14, 20
44.	6-Keto-19-nor-AD	−0.05	−0.03	4	−2.681	0.656	6, 9, 20
45.	19-Nor-AD	0.21	0.14	4	−0.519	0.341	6, 20
46.	4-OH-AD	0.75	0.47	5	0.0	0.538	6, 7, 14, 20

AD: 4-androstene-3,17-dione; A: 4-androsten-3-one; 5α-H-AD: 5α-androstane-3,17-dione; 5α-H-A: 5α-androstan-3-one; 5α-H-T: 17β-hydroxy-5α-androstan-3-one; T: testosterone; NE: 19-norethisterone.

ing due to the enlarged unsaturation is experimentally found. This is consistent with an additional stabilization which is explained in the MTD scheme by the conjugation effect. The same tendency is found in the case of steroid 48. However, the data reported for the 4,6-diene 49 are in conflict with a pure conjugation effect. Either the resonance stabilization is not the most appropriate measure for general use or an additional, *e.g.* sterical, effect brings about an opposite contribution to the interaction in this case.

The binding affinity of the 4-hydroxy derivative 50 in Table II is smaller than theoretically estimated. This corresponds with the wall vertex attributed to $j = 7$ in the MTD map. A 6α-Br substitution en-

Table II. Differences in free energy of binding to aromatase (in kJ mol^{-1}) relative to 4-androstene-3,17-dione. (ΔG_{obs} based on experimental K_i (inhibitor)/K_m (androstenedione) ratios; ΔG_{av} based on theoretical functional group contributions according to [15]; $\Delta G_{diff} = \Delta G_{obs} - \Delta G_{av}$.)

No.	Inhibitor	Ref.	Structure coding [15]								Free-energy contributions		
			DOF	Csp^2	Csp^3	N	OH	C=O	O, S	X	ΔG_{obs}	ΔG_{av}	ΔG_{diff}
47	1,4,6-ATD	[24]	0	6	11	0	0	2	0	0	2.7	−1.7	4.4
48	1-Me-1,4-ADD	[24]	0	4	14	0	0	2	0	0	3.2	2.5	0.7
49	4,6-ADD	[44]	0	4	13	0	0	2	0	0	−1.7	−0.8	−0.9
50	4-OH-AD	[24]	1	2	15	0	1	2	0	0	5.2	7.5	−2.4
51	6α-Br-AD	[35]	0	2	15	0	0	2	0	1	2.7	5.4	−2.8
52	7α-S$(CH_2)_3CH_3$-AD	[39]	4	2	19	0	0	2	1	0	0.3	6.3	−6.0
53	7α-SCH_2ph-AD	[39]	3	8	16	0	0	2	1	0	−0.2	16.8	−17.0
54	7α-SCH_2CH_2ph-AD	[39]	4	8	17	0	0	2	1	0	0.0	17.2	−17.1
55	7α-SCH_2phOMe-AD	[39]	4	8	17	0	0	2	2	0	1.8	21.8	−20.0
56	7α-SphNH_3-AD	[39]	3	8	15	1	0	2	1	0	3.1	18.4	−15.3
57	19-Nor-AD	[49]	0	2	14	0	0	2	0	0	−1.3	−3.4	2.1
58	10β-OH-AD	[50]	1	2	14	0	1	2	0	0	−9.9	4.2	−14.0
59	10β-OOH-AD	[50]	2	2	14	0	1	2	1	0	−6.8	5.9	−12.7
60	19-SMe-AD	[24]	2	2	16	0	0	2	1	0	5.2	2.1	3.1
61	(19*R*)-Oxirane	[30]	1	2	16	0	0	2	1	0	6.4	5.0	1.3
62	(19*R*)-Thiirane	[30]	1	2	16	0	0	2	1	0	10.3	5.0	5.3
63	19-OH-AD	[27]	2	2	15	0	1	2	0	0	−3.8	4.6	−8.4
64	19-Oxo-AD	[27]	1	2	14	0	0	3	0	0	−4.3	8.0	−12.3
65	19-CCH-AD	[27]	1	4	15	0	0	2	0	0	1.6	2.9	−1.4
66	19-CCH-19-OH-AD	[27]	2	4	15	0	1	2	0	0	−9.2	10.5	−19.7
67	19-CCH-19-oxo-AD	[27]	1	4	14	0	0	3	0	0	−7.5	13.8	−21.3
68	19-Me-AD	[28]	1	2	16	0	0	2	0	0	0.3	0.4	−0.1
69	(19*R*)-19-OH-19-Me-AD	[28]	2	2	16	0	1	2	0	0	−11.6	8.0	−19.6
70	19-Me-19-oxo-AD	[28]	1	2	15	0	0	3	0	0	−1.2	11.3	−12.5
71	Testosterone	[49]	1	2	16	0	1	1	0	0	−1.1	−3.4	2.3
72	Norethisterone	[41]	1	4	15	0	1	1	0	0	−15.6	−0.8	−14.7
73	Norethynodrel	[41]	1	4	15	0	1	1	0	0	−15.0	−0.8	−14.1
74	Testolactone	[24]	0	4	13	0	0	2	1	0	−10.4	3.8	−14.2

AD: 4-androstene-3,17-dione; ADD: androstadiene-3,17-dione; ATD: androstatriene-3,17-dione; Me: methyl; ph: phenyl; CCH: ethynyl.

hances the enzyme binding but less than expected (steroid 51). The MTD correlation provides the explanation of some hydrophobic contribution and a missing sterical contact. The data of steroids 52 to 56 indicate different enzyme interactions of the 7α substituents under study. The negative ΔG_{diff} values of these conformationally flexible side chains suggest that only a small part of the substituent is in binding contact with the aromatase. The removal of the 10-methyl group (compound 57) causes a diminished reduction in free energy according to the hydrophobicity change and the sterically irrelevant vertex $j = 14$ in the MTD results. In further agreement with the hydrophobic influence described, hydrophilic substituents at the C10 carbon atom (steroids 58, 59) and at the C19 one (steroids 63, 64) induce a loss in the binding affinity. The (19*R*) substituents in compounds 61 and 62 are found to be well matched to the enzyme, *i.e.* positive ΔG_{diff} values which could also lead to the conclusion that the crystallographically observed conformation [25] is retained in the bound state. Conformational hindrances might be supposed in the cases of 19-disubstitutions (steroids 66, 67, 69) since the free-energy decrease is much stronger than the sum of those for corresponding monosubstitutions. Whereas the data of testosterone in Table II indicate a good fit even for 17β-hydroxy steroids, the strong negative ΔG_{diff} values of compounds 72 and 73 support the MTD finding that the 17α region is occupied by aromatase moieties (wall vertices $j = 26$, 27). Furthermore, the enzyme does also not accommodate substantial modifications in the D-ring structure as provided by the compound 74. In summary, the MTD results can be used to interpret energetic data based on more accurate binding measurements than applied in the MTD calculations.

Apart from influencing hydrophobicity and conjugation, modifications in the A,B-ring region can also affect the C10 side-chain conformational characteristics. The conformational preference of the out-of-ring hydrogens in the first two enzymatic hydroxylations of the substrate was demonstrated by Osawa *et al.* [51–53] on the basis of crystallographical results and isotope labeling experiments. The relevance of conformational properties in enzyme interactions was further supported by a comparison of X-ray structures and inhibition data of (19*R*)- and (19*S*)-configurated 10β-oxiranyl steroids [25]. In a recent conformational analysis on 19-hydroxyandrost-4-ene-3,17-dione and 19-hydroxyandrosta-4,6-diene-3,17-dione by Beusen *et al.* [54], nearly equivalent molecular ratios of the two conformations relevant to enzymatic out-of-ring hydroxylations were determined for both compounds on the basis of MM2 molecular mechanics calculations. A similar stereospecificity of 19-proR hydrogen removal which was observed for both steroids is in full agreement with the lack of differences in MM2 results. However, the energetic stabilization of the preferred conformation computed to be only 2.9 kJ mol^{-1} (*cf.* Table III) is too low to explain the extreme stereospecificity of 99%. Therefore, we provide relative PCILO energies in Table III for comparison. The PCILO data, which are generally expected to be most reliable, confirm both the order of magnitude given by the MM2 calculations and the major tendencies in conformational preference. Possible explanations of further stabilizing the energetically most preferred conformation may be an entropic contribution, intramolecular or additional intermolecular interactions. While an intramolecular hemiketal formation was discussed by Covey *et al.* [44], intermolecular stabilization is consistent with recent interpretations of an enzyme binding-site positioning above steroidal ring A concluded from spectral data [30], crystal structure-activity findings with 10β-oxiranes and 10β-thiiranes [25, 26] as well as model reactions [46].

Our MM2 results for steroid I recorded in Table III are in full agreement with the potential curve given in [54] and the crystallographically observed conformation of 4-en-3-one steroids locating the hydroxyl group over ring A [52, 55]. In contrast, steroid III lacking the C4=C5 double bond favours the conformation with the OH group in the out-of-ring position. This conformer which was also detected in a crystal structure analysis [56] cannot be enzymatically hydroxylated according to the above-mentioned mechanism unless an internal rotation takes place. Energy requirements for that process are in the same order as calculated for rotations of 4-ene steroids.

In the case of dihydroxylated compounds, differences between 4-ene and 5α-H steroids can be found with respect to the conformation which places the hydroxyls above rings A and B. As can be taken from Table III, an introduction of the C4=C5 double bond increases the relative stability of this conformation (compound II *vs.* IV). Accordingly, the

Table III. Results of conformational analyses.

Steroid No. (*cf.* Fig. 3)	10β side chain conformation (#)	Torsional angle C5–C10–C19–O19 (MM2 optimized)	Relative energy [kJ mol^{-1}] ΔE(MM2)	ΔE(PCILO)
I	A	−47°	0.0	0.0
I	B	+73°	2.9	3.9
I	O	−160°	4.1	4.8
II	A/O	−38°/−160°	0.0	0.0
II	A/B	−41°/ +80°	0.3	2.6
II	B/O	+77°/−159°	2.2	2.0
III	A	−52°	2.3	2.4
III	B	+72°	3.2	1.4
III	O	−163°	0.0	0.0
IV	A/O	−36°/−158°	0.0	4.1
IV	A/B	−47°/ +76°	6.0	8.3
IV	B/O	+75°/−161°	1.9	0.0

(#) Conformation designation: A: −OH group above ring A; B: −OH group above ring B; O: −OH group in out-of-ring position.

MM2 results contradict the argumentation given in [52] that a low stability of this conformation would prevent the 4-ene steroid from forming a trihydroxyl intermediate.

In summary, C4=C5 unsaturation is shown to energetically favour conformations in which an oxygen function at C19 can occupy a position above the steroidal ring A. This is most useful for the interaction with the aromatase binding sites (oxygen over ring A, hydrogen in out-of-ring position) due to recent conceptions about the molecular mode of action. Furthermore, there are indications from MTD correlations that the 4-ene double bond increases the steroidal inhibitory activity by a conjugation effect, while direct steric effects seem to play only a minor role.

Acknowledgements

We wish to thank Dr. B. Schönecker (Central Institute of Microbiology and Experimental Therapy, Jena) for valuable discussions.

[1] Z. Simon, A. Chiriac, S. Holban, D. Ciubotaru, and G. I. Mihalas, Minimum Steric Difference, Res. Studies Press, Letchworth 1984.
[2] Z. Simon, N. Dragomir, M. G. Plauchithiu, S. Holban, H. Glatt, and F. Kerek, Eur. J. Med. Chem. **15,** 521 (1980).
[3] I. Motoc, in: Topics in Current Chemistry, **Vol. 114,** p. 93, Springer Verlag, Berlin 1983.
[4] M. Bohl and Z. Simon, Z. Naturforsch. **40c,** 858 (1985).
[5] V. Popoviciu, S. Holban, I. I. Bădilescu, and Z. Simon, Studia Biophys. **69,** 75 (1978).
[6] M. Bohl, Z. Simon, A. Vlad, G. Kaufmann, and K. Ponsold, Z. Naturforsch. **42c,** 935 (1987).
[7] W. C. Schwarzel, W. G. Kruggel, and H. J. Brodie, Endocrinol. **92,** 866 (1973).
[8] A. M. H. Brodie, W. M. Garrett, J. R. Hendrickson, C. H. Tsai-Morris, and J. G. Williams, J. Steroid Biochem. **19,** 53 (1983).
[9] D. F. Covey and W. F. Hood, Mol. Pharmacol. **21,** 173 (1982).
[10] D. A. Marsh, H. J. Brodie, W. Garrett, C.-H. Tsai-Morris, and A. M. H. Brodie, J. Med. Chem. **28,** 788 (1985).
[11] B. Busetta, G. Comberton, C. Courseille, and M. Hospital, Cryst. Struct. Comm. **1,** 129 (1972).
[12] V. M. Coiro, E. Giglio, A. Lucano, and R. Puliti, Acta Cryst. **B 29,** 1404 (1973).
[13] R. F. Rekker and H. M. de Kort, Eur. J. Med. Chem. **14,** 479 (1979).
[14] W. P. Purcell and J. A. Singer, J. Chem. Eng. Data **12,** 235 (1967).
[15] P. R. Andrews, D. J. Craik, and J. L. Martin, J. Med. Chem. **27,** 1648 (1984).
[16] N. L. Allinger and Y. H. Yuh, Computer program MM2, University of Georgia, Athens (U.S.A.) 1980.
[17] S. Diner, J. P. Malrieu, and P. Claverie, Theoret. Chim. Acta **13,** 1 (1969).
[18] S. Diner, J. P. Malrieu, F. Jordan, and M. Gilbert, Theoret. Chim. Acta **15,** 100 (1969).
[19] J. R. Lochmann, Computer program PCILO, Karl-Marx University, Leipzig (G.D.R.) 1985.
[20] P. A. Marcotte and C. H. Robinson, Cancer Res. **42,** 3322s (1982).
[21] P. A. Marcotte and C. H. Robinson, Steroids **39,** 325 (1982).
[22] J. A. Lovett, M. V. Darby, and R. E. Counsell, J. Med. Chem. **27,** 734 (1984).
[23] P. J. Bednarski, D. J. Porubek, and S. D. Nelson, J. Med. Chem. **28,** 775 (1985).
[24] D. Henderson, U.-F. Habenicht, Y. Nishino, U. Kerb, and M. F. El Etreby, J. Steroid Biochem. **25,** 867 (1986).
[25] M.-J. Shih, M. H. Carrell, H. L. Carrell, C. L. Wright, J. O'N. Johnston, and C. H. Robinson, J. Chem. Soc., Chem. Commun. **1987,** 213.
[26] W. E. Childers and C. H. Robinson, J. Chem. Soc., Chem. Commun. **1987,** 320.
[27] D. F. Covey, W. F. Hood, and V. D. Parikh, J. Biol. Chem. **256,** 1076 (1981).
[28] D. D. Beusen, H. L. Carrell, and D. F. Covey, Biochem. **26,** 7833 (1987).
[29] J. P. Burkhart, P. M. Weintraub, C. L. Wright, and J. O'N. Johnston, Steroids **45,** 357 (1985).
[30] J. T. Kellis jr., W. E. Childers, C. H. Robinson, and L. E. Vickery, J. Biol. Chem. **262,** 4421 (1987).
[31] W. L. Duax, C. Eger, S. Pokrywiecki, and Y. Osawa, J. Med. Chem. **14,** 295 (1971).
[32] K. Marat, J. F. Templeton, and V. P. S. Kumar, Magn. Reson. Chem. **25,** 25 (1987).
[33] K. Marat, J. F. Templeton, R. K. Gupta, and V. P. S. Kumar, Magn. Reson. Chem. **25,** 730 (1987).
[34] Y. J. Abul-Hajj, J. Med. Chem. **29,** 582 (1986).
[35] Y. Osawa, Y. Osawa, and M. J. Coon, Endocrinol. **121,** 1010 (1987).
[36] L. Tan and A. Petit, Biochem. Biophys. Res. Commun. **128,** 613 (1985).
[37] A. J. Solo, C. Caroli, M. V. Darby, T. McKay, W. D. Slaunwhite, and P. Hebborn, Steroids **40,** 603 (1982).
[38] R. W. Brueggemeier, E. E. Floyd, and R. E. Counsell, J. Med. Chem. **21,** 1007 (1978).
[39] R. W. Brueggemeier, C. E. Snider, and R. E. Counsell, Cancer Res. **42,** 3334s (1982).
[40] M. V. Darby, J. A. Lovett, R. W. Brueggemeier, M. P. Groziak, and R. E. Counsell, J. Med. Chem. **28,** 803 (1985).
[41] D. F. Covey, W. F. Hood, and P. C. McMullan, Biochem. Pharmacol. **35,** 1671 (1986).
[42] M. E. Wolff, J. D. Baxter, P. A. Kollman, D. L. Lee, I. D. Kuntz, E. Bloom, D. T. Matulich, and J. Morris, Biochem. **17,** 3201 (1978).
[43] M. Bohl, G. Schubert, M. Koch, G. Reck, J. Strecke, M. Wunderwald, R. Prousa, and K. Ponsold, J. Steroid Biochem. **26,** 589 (1987).
[44] D. F. Covey and W. F. Hood, Cancer Res. **42,** 3327s (1982).

[45] Y. J. Abul-Hajj, Steroids **41,** 783 (1983).
[46] P. A. Cole and C. H. Robinson, J. Am. Chem. Soc. **110,** 1284 (1988).
[47] D. Giudici, G. Ornati, G. Briatico, F. Buzzetti, P. Lombardi, and E. DiSalle, J. Steroid Biochem. **30,** 391 (1988).
[48] H. Wagner, K. Ponsold, and G. Schumann, D. D. WP in proof (1987).
[49] W. G. Kelly, D. Judd, and A. Stolee, Biochem. **16,** 140 (1977).
[50] D. F. Covey, W. F. Hood, D. D. Beusen, and H. L. Carrell, Biochem. **23,** 5398 (1984).
[51] Y. Osawa, K. Shibata, D. Rohrer, C. Weeks, and W. L. Duax, J. Am. Chem. Soc. **97,** 4400 (1975).
[52] W. L. Duax and Y. Osawa, J. Steroid Biochem. **13,** 383 (1980).
[53] W. L. Duax, C. M. Weeks, and D. C. Rohrer, in: Recent Progress in Hormone Research, **Vol. 32,** p. 81, Academic Press, New York 1976.
[54] D. D. Beusen, B. L. Kalman, and D. F. Covey, Biochem. **25,** 662 (1986).
[55] C. M. Weeks, P. Strong, and Y. Osawa, Cryst. Struct. Commun. **5,** 915 (1976).
[56] G. I. Birnbaum, Acta Cryst. **B 29,** 1426 (1973).