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STRUCTURE-ACTIVITY RELATIONSHIPS FOR GLUCOCORTICOIDS—IV. EFFECTS OF SUBSTITUENTS ON THE OVERALL SHAPE OF STEROIDS WHICH BIND TO THE GLUCOCORTICOID RECEPTOR

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SUMMARY

The lowest molecular energy structure of 24 steroids which bind to the glucocorticoid receptor has been determined by geometry optimisation. In this paper we analyze selected structural parameters which describe the overall shape of the steroid molecule.

The structural influence of 10 different substitutions has been quantitatively assessed by Free and Wilson analysis. The substituents have little if any effect on the distribution of carbon atoms relative to the various mean planes they participate in. Exceptions are the 1-ene unsaturation which flattens the A-ring mean plane and the 6-ene unsaturation which puckers the plane of the C18, C19 methyls. The convexity of the steroid skeleton is increased by the 1-ene and the 11β-hydroxy substitutions while it is decreased by the presence of a 17-hydroxyl group. The twist of the molecule along its long axis is abolished by a 1-ene and reversed by a 7-ene unsaturation. As to the dihedral angle of the side-chain with other planes of reference it is variously perturbed significantly by 7 different substitutions. Some of these effects are illustrated by scaled projected pictures of the steroid molecules. Finally, we have computed molecular surface area and volume parameters and determined to what extent they are modified by the substitutions in our series of steroids.

Together with data presented in the other papers of this series, the studies reported should help investigating the relationships between the structure of steroids and their glucocorticoid properties.

I. INTRODUCTION

To investigate the relationships between the structure and activity of glucocorticoids, we have undertaken a structural and conformational study of a family of steroids which bind to the glucocorticoid receptor [1]. Our approach takes advantage of a geometry optimisation method which provides the lowest energy (fundamental) structure of these molecules [2].

In the preceeding paper [3], we have examined a selection of fifteen steroids among a family of twenty-four and have discussed the influence of some substituents on the different steroid rings. We have also studied, based on the coordinates of the fundamental structures, the conformational potentialities of the side-chain. Consideration of individual coordinates and associated parameters (bond lengths, valence and dihedral angles) shows only very minor differences between the steroids studied. However, the cumulative effect of such differences can introduce significant structural modifications in the whole molecule. Thus, in this paper, we will describe the influence of substituents on the overall molecular shape for the whole family of steroids examined in their lowest energy state. It may seem arbitrary to discuss the structure of molecules in a situation which, unlike in biological fluids, is free of external constraints. However, only in the latter case do the various steroids become comparable to each other. In addition, identification and evaluation of the magnitude of the perturbations introduced in the steroids by the various substituents may help appreciating the type of conformation "preferred" or "induced" by the receptor.

To describe quantitatively the overall shape of the steroid we have studied the distribution of carbon atoms relative to selected mean planes of the molecule. We have also determined both its torsion about the long axis, i.e. the twist (C19, C10, C13, C18 dihedral angle) and its curvature (dihedral angles between selected mean planes). Finally, we have calculated the molecular surface areas and volumes. The 24 steroids examined are listed in Table 1. They are identical to those studied in the preceeding paper [3] (same numbering) with the addition of five 6α-methyl substituted compounds, three steroids with additional double bonds and epicortisol.

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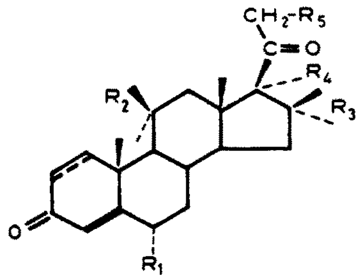
Table 1. The series of steroids selected for geometry optimisation



No.	Molecule	Formula	R_1	R_2	R_3	R_4	R_5
I	4-pregnen-11β,17,21-triol-3,20-dione cortisol, hydrocortisone	C21 H30 O5	H	βOH	H	OH	OH
II	1,4-pregnadien-11β,17,21-triol-3,20-dione prednisolone, 1-dehydrocortisol	C21 H28 O5	H	βOH	H	OH	OH
III	4-pregnen-17,21-diol-3,20-dione cortexolone, Reichstein "S"	C21 H30 O4	H	H	H	OH	OH
IV	4-pregnen-17-ol-3,20-dione 17-hydroxyprogesterone	C21 H30 O3	H	H	H	OH	—
V	4-pregnen-11β,21-diol-3,20-dione corticosterone	C21 H30 O4	H	βOH	H	H	OH
VI	4-pregnen-21-ol-3,20-dione deoxycorticosterone, DOC	C21 H30 O3	H	H	H	H	OH
VII	4-pregnen-16α-methyl-3,20-dione 16α-methylprogesterone	C22 H32 O2	H	H	αCH_3	H	—
VIII	4-pregnen-16α-methyl-17,21-diol-3,20-dione 16α-methylcortexolone	C22 H32 O4	H	H	αCH_3	OH	OH
IX	4-pregnen-3,20-dione progesterone	C21 H30 O2	H	H	H	H	—
X	4-pregnen-16β-methyl-3,20-dione 16β-methylprogesterone	C22 H32 O2	H	H	βCH_3	H	—
XI	4-pregnen-16α-methyl-17-ol-3,20-dione 16α-methyl-17-hydroxyprogesterone	C22 H32 O3	H	H	αCH_3	OH	—
XII	4-pregnen-11β-ol-3,20-dione 11β-hydroxyprogesterone	C21 H30 O3	H	βOH	H	H	—
XIII	4-pregnen-11α-ol-3,20-dione 11α-hydroxyprogesterone	C21 H30 O3	H	αOH	H	H	—
XIV	4-pregnen-16β-methyl-17,21-diol-3,20-dione 16β-methylcortexolone	C22 H32 O4	H	H	βCH_3	OH	OH
XV	4-pregnen-16β-methyl-17-ol-3,20-dione 16β-methyl-17-hydroxyprogesterone	C22 H32 O3	H	H	βCH_3	OH	—
XVI	1,4-pregnadien-6α-methyl-11β,17,21-triol-3,20-dione 6α-methylprednisolone	C22 H30 O5	CH_3	βOH	H	OH	OH
XVII	4-pregnen-11α,17,21-triol-3,20-dione epicortisol	C21 H30 O5	H	αOH	H	OH	OH
XVIII	4-pregnen-6α-methyl-11β,17,21-triol-3,20-dione 6α-methylcortisol	C22 H32 O5	CH_3	βOH	H	OH	OH
XIX	4-pregnen-6α-methyl-3,20-dione 6α-methylprogesterone	C22 H32 O2	CH_3	H	H	H	—
XX	4-pregnen-6α,16α-dimethyl-3,20-dione 6α,16α-dimethylprogesterone	C23 H34 O2	CH_3	H	αCH_3	H	—
XXI	1,4-pregnadien-3,20-dione 1-dehydroprogesterone	C21 H28 O2	H	H	H	H	—
XXII	4-pregnen-6α-methyl-21-ol-3,20-dione 6α-methyldeoxycorticosterone	C22 H32 O3	CH_3	H	H	H	OH
XXIII	1,6-pregnadien-11β,17,21-triol-3,20-dione 6-dehydrocortisol	C21 H28 O5	H	βOH	H	OH	OH
XXIV	1,4,7-pregnatrien-11β,17,21-triol-3,20-dione 7-dehydroprednisolone	C21 H26 O5	H	βOH	H	OH	OH

II. METHOD

The steroids were optimized in terms of their molecular energy computed by the GEMO [4] program, based on a Westheimer type equation as published [2, 3]. The optimized structures were described with the help of the programs Mean Plane [5], APLGRAF (Carleton University Computing Services) for the pictures, and a modified version of MOLAREA [6] extended up to 70 atoms for the surfaces. The computations were carried out on a Xerox CP/V computer.

Statistical analyses were performed with the SPSS programs [7]. All coordinates of the optimized structures are available upon request and will be published elsewhere.

To evaluate the dependence of observed perturbations on the nature and position of the various substituents, we have analyzed the data by the method of Free and Wilson [8].

$$Y = \Sigma a_i X_i + \mu$$

where Y is the dependent variable, μ is the contribution of the steroid skeleton in the absence of substituents (e.g. progesterone), a_i is the mathematical contribution of the i^{th} substituent, and X_i equals 1 when the substituent is present and zero when it is absent.

III. RESULTS AND DISCUSSION

A. *Distribution of carbon atoms relative to selected mean planes*

The general shape of the steroids can be described by dihedral angles between selected planes of the molecule. Before presenting these data, it is appropriate to determine to what extent the actual distribution of individual carbon atoms fits to these mean planes and how this distribution may be affected by the various substituents. This parameter may be quantitatively estimated by the R.M.S., namely the square root of the mean square of the distances of carbon atoms to the particular plane

$$\text{R.M.S.} = \left[\frac{\sum_{i=1}^{n} \delta_i^2}{n}\right]^{1/2} \cdot \text{Å}$$

where n is the number of carbon atoms in the plane and δ the distance between these atoms and the computed mean plane [5].

The results are presented in Table 2. It can be seen that, first, the plane of the methyls (defined by atoms C9, C10, C13, C17, C18, C19) is very flat. Second, apart from introduction of a Δ1 or Δ6 double bond, the substituents have little influence on the R.M.S. of the rings. Although statistically significant, the effect of the 16-methyl substitutions on the R.M.S. of the D-ring is very minor. A 6α-methyl substituent also significantly modifies the R.M.S. of the plane of the methyls and of the A-ring, but here again, the changes appear negligible. Third, as expected, a Δ1 double bond drastically flattens the A-ring and increases somewhat the R.M.S. of the methyls mean plane, while a Δ6 double bond produces a major increase in the latter parameter. Finally, the absence of significant perturbation of the R.M.S. of the B-C rings makes this plane a good reference for describing variations in the steroid curvature (see below).

B. *Curvature*

1. *Dihedral angles.* These angles give a quantitative estimation of the tridimensional features of the steroid, which may be of particular importance for the interaction with the receptor site. Among the numerous possibilities we have chosen the angles between the rings A and B-C, B-C and D, and A and D, as a measure of the convexity of the steroid; the angle between the mean plane of the side-chain and either that of the methyls or that of the D-ring to describe the relief of the β-face of the molecule;

Table 2. Distribution of carbon atoms relative to mean planes (R.M.S.)†

Planes	Mean value	Range	μ	Effect of substituents		
A-ring	0.146 ± 0.012	0.005 (XVI) 0.184 (XXIII)	0.177	−0.160 ± 0.004	−0.009 ± 0.003	n.s.
substituent (*n*)				1-ene (3)***	6α-methyl (5)*	n.s.
B-C rings	0.227 ± 0.001	0.218 (V) 0.246 (XXIII)	0.225	n.s.	n.s.	n.s.
D-ring	0.208 ± 0.002	0.160 (XVI) 0.215 (XX)	0.217	−0.008 ± 0.001	+0.002 ± 0.001	n.s.
substituent (*n*)				16β-methyl (3)*	16α-methyl (4)*	n.s.
Methyls	0.069 ± 0.002	0.060 (IX) 0.114 (XXIII)	0.060	+0.043	+0.014 ± 0.0003	+0.005 ± 0.0002
substituent (*n*)				6-ene (1)	1-ene (3)***	6α-methyl (5)***

† Data in Å ± S.E. The R.M.S. is defined in the text. The methyls mean plane is defined by C9, C10, C13, C17, C18, C19 carbon atoms. n.s.: not significant. The substituents indicated are those which, by Free and Wilson analysis, have a statistically significant effect on the R.M.S. (Thus, the R.M.S. of B-C rings was not significantly influenced by any substituent). For each of these substituents we have examined the pairs of molecules (n is the number of these pairs) which, within a pair, differ from each other by this substituent only. The data listed under "effect of substituents" are the mean of difference in R.M.S. calculated within each pair. A Student's t-test performed on these differences gave the following levels of significance for the influence of the substituents listed: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The mean value, range and μ of the R.M.S. have been calculated on the 24 molecules of Table 1, and Roman numerals refer to this table. μ is the contribution of the steroid skeleton obtained from the Free and Wilson statistical analysis, namely the value of the R.M.S. for a steroid without any substituent (e.g. progesterone).

finally, the dihedral angle C19, C10, C13, C18 to estimate the twist of the steroid about its long axis. By Free and Wilson analysis, we have computed the value of these angles in the absence of substituents, namely the contribution of the steroid skeleton (μ) and then have evaluated the perturbations of this value introduced by the various substituents in the 24 molecules of our series (Table 3).

As said above, the B-C rings mean plane is rigid. Thus, the curvature of the steroid can be modified following perturbations of the A- and D-rings. The bending of the A-ring is increased upon introduction of a 1-ene (+52%), 6α-methyl (+9%) or 11β-hydroxy (+9%) substituent. In contrast, 6-ene unsaturation decreases the A/B-C angle by 26%. The other extremity of the molecule is sensitive to the presence of 6-ene (+36%), 7-ene (+25%), and 11β-hydroxy (+28%) substitutions which increase the B-C/D angle, while 17-hydroxy and 21-hydroxy substituents decrease it by 40% and 14%, respectively. All these changes result (A/D angle) in either an increased curvature of 30% for the 1-ene and of 14% for the 11β-hydroxy substitution, or in a decrease of 14% for the 17-hydroxy substitution. Although it is premature to draw any conclusions, it is worth mentioning that 1-ene and 11β-hydroxy substitutions both increase the affinity for the receptor, while addition of a 17-hydroxyl group decrease it [1].

We found that the dihedral angle between B-C rings mean plane and that of the methyls is constant ($\mu = 81.72°$) whatever the substituents, except for a small effect of the 7-ene (+5.0°) and the 1-ene (+1.2°) unsaturations. Thus, in addition to the D-ring, the latter plane has been used as a reference for defining the position of the side-chain mean plane. Table 3 shows that the substituents most perturbing for the S.C/D dihedral angle are the 16β-methyl (an increase of 33%) and the 17-hydroxyl (a decrease of 37% in this angle). These opposite effects on the S.C/D dihedral angle can be related to the observation that a 16β-methyl substitution reduces the inhibitory influence of a 17-hydroxyl group [1] on glucocorticoid-receptor binding. For instance, betamethasone (9α-fluoro, 16β-methyl-prednisolone) has a higher affinity than its 16α-epimer dexamethasone (unpublished). Moreover, we found [3] that upon rotation of the side-chain of compound XIV (16β-methylcortexolone) about its C17-C20 double bond, the rotational energy "barrier" usually associated with the 17-hydroxyl substitution was almost abolished. In these studies, the height of this barrier appeared directly correlated with the decrease in affinity for the receptor.

As to the S.C/methyl plane it appears to be sensitive to more substituents. It is decreased by the 7-ene (−28%), the 17-hydroxy (−22%) and the 6α-methyl (−7%) substituents. It is increased by 6% following either a 11β-hydroxy, a 16α-methyl or a 21-hydroxy substitution and by 13% upon addition of a 16β-methyl group. Finally, the twist is abolished by introduction of a 1-ene double bond, while the sign of its angle is changed by the 7-ene substitution.

2. *Projected pictures.* To illustrate some of the changes reported above, we have compared the scaled projected pictures of the molecules. One way to appreciate the curvature is to superimpose, on the screen of a computer graphic terminal, the C14 and C12 carbon atoms of the steroid. Thus, the reference mean plane B-C is perpendicular to the projection plane and allows one to emphasize the effect of substituents. Molecules can then be compared to each other by making, on the same picture, their respective C10 carbon atoms and C13 carbon atoms to coincide.

Figure 1 shows how dramatic the difference can be between two molecules in our series (**XVI** and **XXIV**). Since these two steroids bind to the receptor,

Table 3. Influence of substituents on dihedral angles between selected mean planes†

Angle	A/B-C	B-C/D	A/D	S.C/D	S.C/Me	Twist
μ	24.42	9.80	34.74	61.21	115.78	5.12
1-ene	12.64***	−1.05*	10.26***	−1.82	1.77	−5.12***
6α-Me	2.24**	−0.62	0.54	−9.11**	−8.06*	−0.29
6-ene	−6.41***	3.52***	−2.47	4.62	8.81	1.49
7-ene	1.15	2.46**	0.17	7.84	−32.86***	−11.09***
11α-OH	−0.72	−1.50	−2.74	6.19	7.52	0.78
11β-OH	2.15***	2.70***	4.86**	6.14	6.94*	−0.62
16α-Me	−0.71	−0.26	−1.26	6.39	7.05*	0.64
16β-Me	−0.72	−0.10	−1.44	20.29***	15.02**	0.88
17-OH	−0.22	−3.92***	−4.73***	−22.82***	−25.12***	−0.46
21-OH	−0.55	−1.40**	−2.30	5.06	6.69*	0.27
R^2	0.955	0.954	0.932	0.928	0.948	0.934

† The mean planes are defined as follows: A includes all carbon atoms involved in A-ring; B-C includes all carbon atoms involved in B- and C-rings; D includes all carbon atoms involved in D-ring; S.C(side-chain): atoms C17, C20, O20, C21; Me (methyls): C9, C10, C13, C17, C18, C19. The twist is the dihedral angle between carbon atoms C19, C10, C13, C18. μ is the contribution of the steroid skeleton (e.g. progesterone). All data (in degrees) are a_i values from a Free and Wilson analysis on the 24 structures defined in Table 1. Statistical significance (F-distribution): * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$. R^2 is the proportion of variation explained by the variables included in the regression equation.

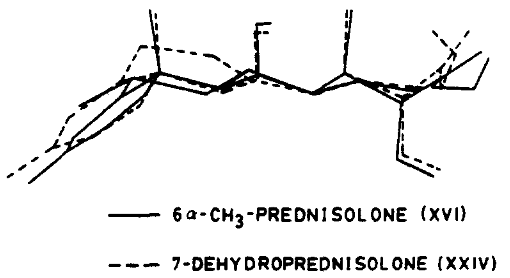


Fig. 1. Greatest differences in the curvature of the steroid molecule seen in the series of optimized steroids studied. Each molecule is projected to the scale by superimposition of the C14 on the C12 carbon atom. The two molecules are compared by coincidence of the corresponding C10 and C13 carbon atoms.

this may have important implications which will be discussed elsewhere (in preparation). Figure 2a underscores the characteristic influence of the 11β-hydroxy substitution on the curvature of progesterone. The increased convexity of the β-face of the steroid results from the interaction of the 11β-hydroxyl group with the C18- and C19-methyls. In contrast, the perturbations in the progesterone molecule associated with a 11α-hydroxy substitution are very subtle. Because this substituent does not interact significantly with the methyl groups, it has only a very minor influence on the molecule shape (Fig. 2b). Thus, since both progesterone and 11α-hydroxyprogesterone have quite a similar skeleton, steric hindrance might be one of the main reasons why 11α-hydroxyprogesterone has a negligible affinity for the receptor as compared to progesterone [1]. Figure 3 shows how the shape of the cortisol molecule can be modified by a 6α-methyl group (Fig. 3a) or by a Δ1 double bond (Fig. 3b). While the latter affects the curvature only by increasing the tilt of the A-ring, the 6α-methyl substituent influences both the A-ring and the side-chain dihedral angles (see also Table 3). Although many other factors may govern the interaction between steroids and the glucocorticoid receptor, it is obvious that such effects of substituents on the overall shape of the steroids can be a major determinant of this interaction.

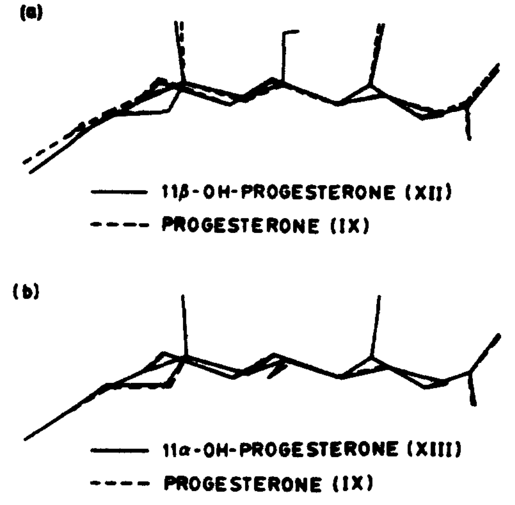


Fig. 2. Effect of 11-hydroxy substituents on the curvature of progesterone. Details in legend of Fig. 1.

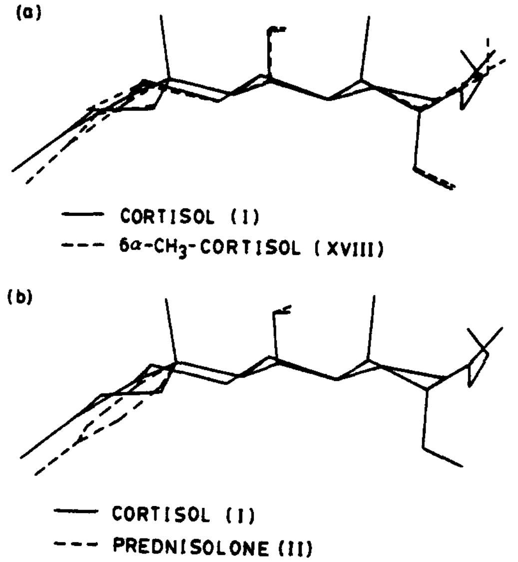


Fig. 3. Effect of 6α-methyl and Δ1-substituents on the curvature of cortisol. Details in legend of Fig. 1.

C. *Surface areas and volumes*

Hydrophobic forces may contribute significantly to the energy of the receptor-steroid interaction. Therefore, it may be of interest to calculate the surface area of the steroid molecule including the percent contribution of the various atomic species, and to determine to what extent these parameters are influenced by different substituents. Likewise, since the energy of the Van der Waals interactions decreases as a function of the sixth power of the distance, more or less adequate fitting of the steroid in the receptor binding site may be crucial for determining the affinity of receptor-steroid binding. For this reason we also have computed the molecular volumes of the various steroids in our series. Computation of the surfaces and volumes include the curved glossing, namely the surroundings of the steroid molecule inaccessible to the solvent assumed to be water. Water radius was taken as 1.45 Å, the half average distribution of distances between water molecules [see p. 261 of ref. 9].

Of course, it would be premature at this stage to expect a correlation between receptor binding and these parameters since only part of the steroid molecule might be involved in this interaction. However, recent studies by Wolff and coworkers [10] suggest that most of the steroid molecule is "buried" in the receptor site. In addition, the present study helps estimating the sensitivity of our method as well as the magnitude of perturbations introduced by specific substituents.

Table 4. Molecular surface area and volume of cortisol*

Contribution	Surface area ($Å^2$)	%	Contribution	Volume ($Å^3$)	%	
Carbons	47	8.3 (8–14)	Sum of spheres	746 (712–785)	100	
Oxygens	104	18.3 (11–20)	Spheres overlap	591 (560–633)	79	
Hydrogens	417	73.4 (68–78)	Actual volume	155	21	53
Total	568 (541–589)	100.0	Curved glossing	138 (115–148)		47
			Final volume	293 (254–310)		100

* All determinations were based on optimized structures and include the solvent radius (1.45 Å) assumed to be water. Spheres: Van der Waals spheres. Curved glossing: molecular surroundings inaccessible to the solvent. For comparison, the range of extreme variations among the 24 steroids in our series is shown in parentheses.

Table 4 gives the surface areas, including the relative contribution of C, O and H atoms, the volumes and the related parameters of cortisol, based on standard Van der Waals radii. It can be seen that about half of the volume is contributed by the curved glossing. The other half contributed by the compounded Van der Waals spheres is only one fifth of the total volume of these spheres, emphasizing the importance of their overlap within the steroid molecule. Concerning the surfaces, about three-quarters are contributed by hydrogen atoms, about 20% by oxygen and less than 10% by carbon atoms. Although volume changes do not prejudge changes in the shape, one can compute from the ratio of the volume to' the surface the extent to which the perturbation diverts the molecular shape from an idealized sphere. For cortisol, for instance, this ratio is 0.52 Å, while that of a sphere with the same volume would be 1.37 °A.

Table 5 shows that the magnitude of the perturbations introduced by a given substituent in the surface areas and volumes not only depends on the nature of the substituent, as expected, but also on its position on the steroid molecule. This is essentially due to the variable contribution of this substituent to the curved glossing since the change in overlap is virtually independent from the position of the substituent (data not shown). Thus, although the Van der Waals volume (adjusted to the solvent) of a hydroxy substituent is only one third of that of a methyl, its introduction at the C17 position has the same effect on the volume of the steroid as the addition of a 16β-methyl group.

CONCLUSION

In conclusion, we have shown in this paper how the various substituents can influence the general shape of the C-21 steroids molecular skeleton. The results emphasize differences which were barely detectable when individual geometric coordinates or even isolated rings are examined [3]. We have discussed elsewhere [2, 3] how data obtained by the geometry optimisation method used here fit with the

Table 5. Influence of substituents on volume and surface parameters†

Substituent	n	Position	Δ area ($Å^2$)	Δ volume ($Å^3$)	Δ C.G ($Å^3$)
Hydroxy	2	11α	6.28	7.01	2.15
(102 $Å^2$)	3	11β	6.81	8.01	3.07
(11.5 $Å^3$)	5	17	1.20*	12.56*	7.06*
	6	21	13.71*	8.40	2.26
Methyl	5	6α	22.66	17.76	10.37
(305 $Å^2$)	4	16α	21.84	14.68	7.46
(36.9 $Å^3$)	3	16β	19.28*	12.01*	4.15*
Ene	3	1	−4.42	−8.90	−7.90
(−176.5 $Å^2$)	1	6	−3.44	−7.97	−6.24
(−14.4 $Å^3$)	1	7	−2.02*	−17.07*	−16.34*
μ			544.72	264.99	126.85
R^2			0.998	0.991	0.979

† Data in parentheses refer to the surface and volume contributions of isolated substituents. n indicates the number of pairs of molecules which differ within each pair by the particular substituent only. Except for μ and R^2 all other data are the a_i parameters from a Free and Wilson analysis. * Data differing significantly ($P < 0.05$ or less) from the others in the same group. C.G is the curved glossing as defined in the legend of Table 4 and in the text. μ is the contribution of the steroid skeleton and R^2 the proportion of variation explained by the variables included in the regression equation.

crystallographic ones. In addition, the computer analysis permits a quantitative determination of the differences between the various steroids for complex parameters sensitive enough to adequately describe the overall conformation of these steroids. For instance, in the Free and Wilson analysis, the mathematical contribution of each substituent is additive. Therefore, the curvature of individual molecules possessing any combination of the substituents listed in Table 3 can be calculated with a reasonable approximation. As an example, the A/D dihedral angle of cortisol (11β,17,21-trihydroxyprogesterone) is 32.57° from such a computation, as compared to an actual value of 32.60°. Obviously, such an approach could be applied to describe the effect of substituents on any other structural feature of the steroids.

From the work presented in this series of papers [see refs 1–3] it should be possible to characterize some relations between the structure and the glucocorticoid properties of steroids. Indeed, we have seen how various combinations of substituents influence the affinity for the receptor and determine the class (i.e. agonist or antagonist) of activity [1]. We know now how these combinations could affect the molecular structures and conformations. Studies are in progress to relate these two sets of observations and, hopefully, provide new insights into the mechanism of action of glucocorticoid hormones.

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