Journal of Steroid Biochemistry, 1977, Vol. 8, pp. 121-126. Pergamon Press. Printed in Great Britain

SPECIFICITY OF ANTISERA TO SEX STEROIDS I— THE EFFECT OF SUBSTITUTION AND STEREOCHEMISTRY

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(*Received* 10 *April* 1976)

SUMMARY

The data so far published on the antisera towards sex steroids is reviewed. There is a good correlation between the stereochemistry of steroid derivatives and the specificity of antisera produced. Steroid derivatives which maintain the coplanarity of the steroid molecule tend to give specific antisera.

INTRODUCTION

In recent years the technique of radioimmunoassay has become one of the most important tools for the measurement of extremely small quantities of chemical substances in biological fluids. For greater simplicity, a highly specific antiserum often permits measurements directly in biological fluids, thus bypassing the step of purification of the sample (usually by chromatography) prior to the assay. It is known that substances having molecular weights of less than 1000 are essentially non-immunogenic [1]. In order to render smaller molecular weight substances immunogenic, they must be made a part of a larger molecule such as albumin or thyroglobulin by covalent bonding, (usually peptide). Using different reagents to make a covalent bond, steroid–protein conjugates have now been prepared against almost all steroid hormones and antisera prepared by immunization of several species of animals. Using such antisera, immunoassays have been developed for these hormones. A very extensive review dealing with different aspects for the development of steroid radioimmunoassays has recently been published [2, 3].

In the early published studies, sex steroids were conjugated in ring-A or ring-D and it was observed that the antisera produced were rather non-specific [4, 5]. However, by chemical modification of different steroids, specific functional groups (in some cases with known stereochemistry) at different positions of the molecule, have been prepared. Using such antigenic steroid–protein conjugates, highly specific antisera have been prepared. This paper reviews the synthesis of sex steroid conjugates and the data thus far published on the specificity of antisera produced using these conjugates. We propose a hypothesis which can, in part, explain the published data on the specificity of antisera toward sex steroids by correlating the conformation of the steroid derivative used for conjugation to the carrier protein and the specificity of antisera produced. Various sex steroid derivatives against which antisera have been produced are shown in Fig. 1.

SYNTHESIS OF ANTIGENIC STEROIDS AND CONJUGATION TO CARRIER PROTEINS

A. Estradiol

When estradiol is treated with one molar equivalent of succinic anhydride in pyridine at 4°C, the major product obtained is the 3-monosuccinate, which can be separated from the mixture using thin layer chromatography. The treatment of estradiol-3-monosuccinate with albumin in the presence of carbodiimide gives the conjugate at 3-position of estradiol. However, treatment of estradiol with excess succinic anhydride in pyridine at room temperature gives estradiol-3,17-disuccinate in crystalline form. When this compound is subjected to mild hydrolysis with sodium carbonate in methanol, estradiol-17-succinate I is formed in about 85% yield. This compound in turn can be purified for conjugation [1]. The estradiol-17-succinate I on treatment with albumin gives the conjugate at C-17.

The synthesis of 6-ketoestradiol is achieved from estradiol following the procedure of Wintersteiner and Moore [6]. Six-ketoestradiol is converted to its carboxymethyl oxime II by treatment with carboxymethyl hydroxyamine hydrochloride in presence of alkali. This derivative has been conjugated to bovine serum albumin, and human serum albumin. In our laboratories, this was conjugated to thyroglobulin [7].

England *et al.*[8] reported the conjugation of estradiol at C-11. Their description is ambiguous as to

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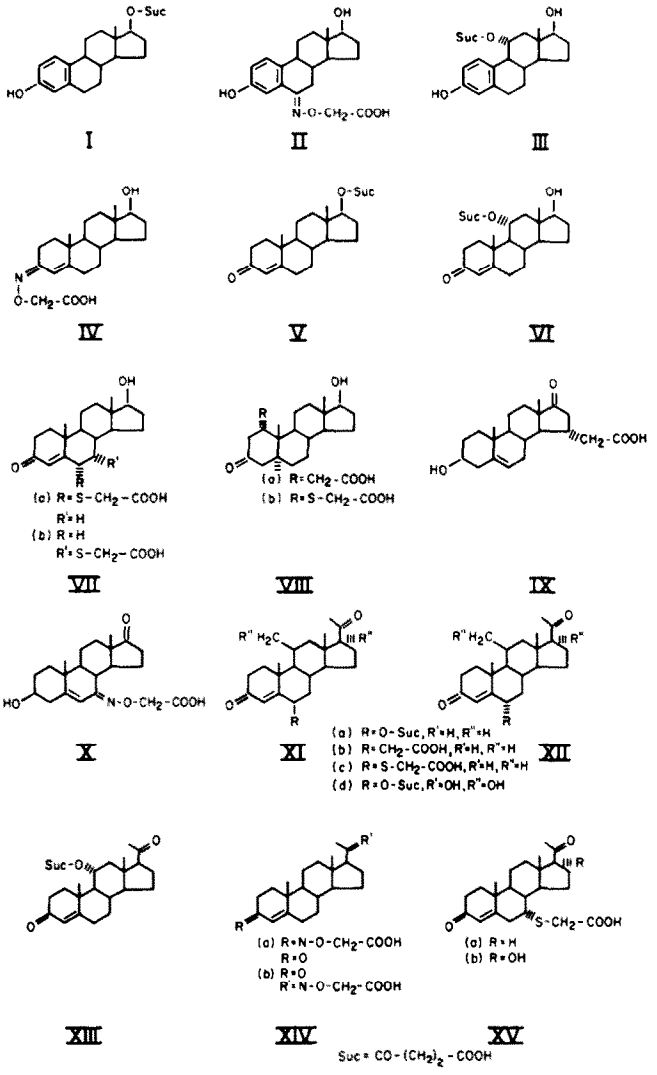

Fig. 1. Chemical structures of sex steroids against which antisera have been produced.

stereochemistry of the conjugate at C-11; the compound is described as estradiol-11α-succinyl in some parts of the paper, 11β-succinyl in others. The authors have not published the method by which the 11-hydroxy-estradiol was prepared. Hollander *et al.* [9] published the preparation of estradiol-11α-succinate III which was conjugated to bovine serum albumin. The 11α-hydroxy-estradiol was prepared by microbial hydroxylation of 19-nor-testosterone and subsequent aromatization. In Table 1 is summarized the relative specificity of the antisera obtained from all the estradiol conjugates.

B. Testosterone

The testosterone molecule has two functional groups; a keto group at C-3 and a hydroxyl group at C-17. As for estradiol, by choosing the suitable derivative or conditions, it is possible to prepare a conjugate at C-3 or C-17. Testosterone-3-carboxymethyl oxime IV was prepared by condensation of testosterone with carboxymethoxyl amine hydrochloride under alkaline conditions; the carboxymethyl oxime produced was conjugated to bovine serum albumin [10]. Testosterone-17β-succinate V was prepared by usual treatment of testosterone with succinic anhydride in pyridine; this was conjugated to bovine serum albumin [11].

Lindner *et al.*[12, 13] have prepared the conjugates of testosterone at C-6 and C-7 position. Bromination of testosterone with N-bromosuccinamide gave the 6-bromo derivative which on treatment with mercapto acetic acid underwent SN_2 displacement giving 6α-carboxymethyl thio-ether of testosterone VIIa. This was conjugated to bovine serum albumin using methods described earlier. Similarly, Michael addition of mercaptoacetic acid to 6-dehydrotestosterone under alkaline conditions gave testosterone-7α-carboxymethyl thio-ether VIIb. The conformation of the derivative at C-7 in VIIb was proved to be axial [13]. These authors[14], employing the same reaction have

Table 1. Cross reaction % of different steroids with three antisera for estradiol

	Cross-Reaction %		
	E_2-6-CMO-Thy.	E_2-17β-Succ. BSA	E_2-11α-Succ. BSA
Estradiol, 17β	100	100	100
Estrone	0.5	35	10
Estriol	0.08	8	0.31
Estradiol, 17α	NT	40	NT
6-Keto-estradiol, 17β	100	NT	NT
Testosterone	<0.01	<0.01	NT
5α-Dihydrotestosterone	<0.01	<0.01	NT
Progesterone	<0.01	<0.01	NT

E_2-6-CMO-Thy = Estradiol-6-carboxymethyl oxime thyroglobulin Succ. BSA = Succinate bovine serum albumin.

Table 2. Cross reaction of different steroids with four antisera for testosterone

	Cross-Reaction %			
	T-3-CMO·BSA	T-17β-Succ·BSA	T-11α-Succ·BSA	T-7α-CMTE·BSA
Testosterone	100	100	100	100
5α-Dihydrotestosterone	65.0	61	15	55
Androst-r-ene-3,17-dione	1.6	0.061	2	<1
Epitestosterone	0.3	NT	0.9	1
Dehydroepiandrosterone	<0.06	NT	<0.1	<0.5
Androst-5-ene-3β 17β-diol	0.9	0.25	0.16	NT
5α-Androstane-3α 17β-diol	3.0	0.8	—	17
11-Oxotestosterone	1.9	NT	17	NT
11β-Hydroxytesterone	2.5	NT	25	NT
Cortisol	<0.006	0.008	<0.01	NT

T = Testosterone CMTE = Carboxymethyl thio-ether. Other abbreviations as in Table 1.

Table 3. Cross reaction % of different steroids with seven antisera for progesterone

	Cross-Reaction %						
	P-6β-CMTE BSA	P-11α-Succ. BSA	P-7α CMTE BSA	P-6α Succ. BSA	P-6β Succ. BSA	P-6α CM BSA	P-6β CM BSA
Progesterone	100	100	100	100	100	100	100
17α-OH Progesterone	4	1.4	15	0.026	0.32	1.0	0.016
11α-OH Progesterone	0.4	500	NT	0.75	0.92	7.3	4.1
Pregnenolone	14	2.5	4	0.16	0.34	11.4	13.6
Deoxycorticosterone (DOC)	2	9.7	0.5	NT	NT	0.79	0.62
5β Pregnane 3,30-dione	100	18	NT	NT	NT	19.0	8.4
5α Pregnane 3,20-dione	100	NT	NT	27	30	100	67
Dihydrotestosterone	NT	2.3	NT	<0.001	<0.001	<0.03	<0.03
Testosterone	0.3	NT	<0.1	<0.003	<0.003	<0.03	<0.03

P = Progesterone CM = Carboxymethyl. Other abbreviations as in Tables 1 and 2.

prepared 17β-hydroxy-5α-androstane-3-one-1α-carboxymethyl thio-ether VIIIb.*

Hillier *et al.*[11] have prepared antisera against testosterone-11α-succinate conjugated to bovine serum albumin. The antigen testosterone-11α-succinate VI was not synthesized by these authors but was obtained as a gift from Organon Ltd., Scotland. The specificity of antisera from different testosterone derivatives is described in Table 2.

C. *Progesterone*

Utilizing the same reactions they described for testosterone, Lindner *et al.*[13, 14] have also prepared conjugates of progesterone at the C-6 position XIIc and C-7α position XVa, and conjugates of 17α-hydroxy progesterone at the C-7α position XVb.

* Using an analogous reaction sequence, the synthesis of 1α-carboxymethyl-17β-hydroxy-5α-androstane-3-one VIIIa has been reported but the data on the specificity of antisera have not been published.

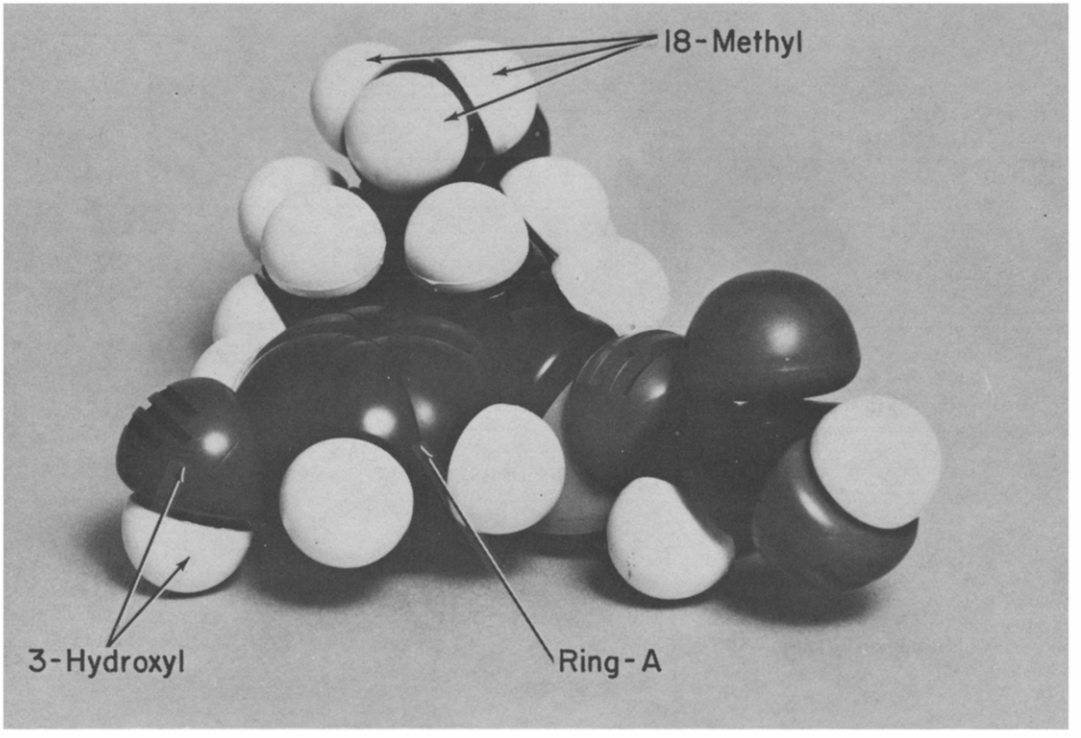

Fig. 2. CPK model of estradiol-6-carboxymethyl oxime.

Niswender[15] has published the preparation of the conjugates, progesterone-6α-succinate XIIa and progesterone-6β-succinate XIa to bovine serum albumin. The synthesis of 6α-hydroxy and 6β-hydroxy progesterone was achieved by the previously described procedure [16, 17]. Jones and Mason[18] have synthesized 6α-carboxymethyl progesterone XIIb and 6β-carboxymethyl progesterone XIb start-

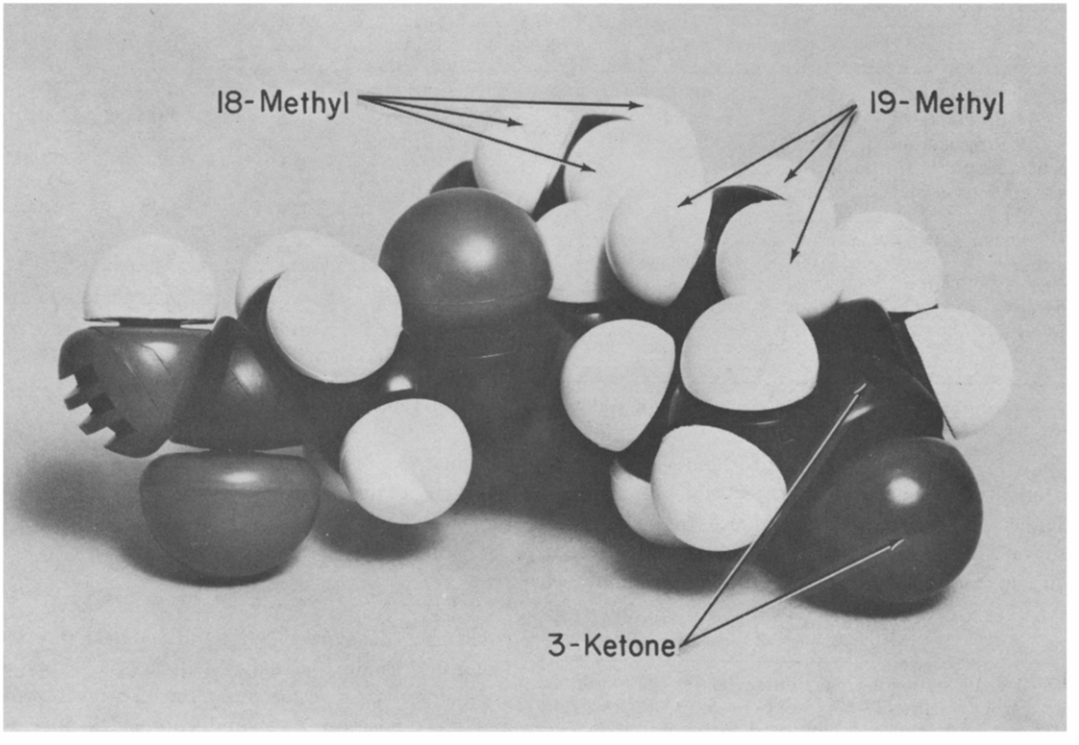

Fig. 3. CPK model of testosterone-11α-succinate.

ing from bis ethylene ketal of progesterone and epoxidation of 5,6 double bond. The 5α,6α-epoxide formed was opened with allyl magnesium bromide after which the terminal carbon-carbon double bond was oxidized to give 6α-carboxymethyl progesterone XIIb. Similarly from 5β,6β-epoxide the 6β-carboxymethyl progesterone XIb was synthesized. These were conjugated to bovine serum albumin by conventional methods. The specificity of antisera from different progesterone derivatives is described in Table 3.

The synthesis of 15α-carboxymethyl-dehydroepiandrosterone IX and 7-carboxymethyl oxime of dehydroepiandrosterone X has been reported by Condom *et al.*[19].

DISCUSSION

From the data so far published, it appears that the antibodies directed toward estradiol-6-carboxymethoxyl oxime II are more specific than estradiol-11α-succinate III and these two antisera are more specific than estradiol-17β-succinate I. Similarly, testosterone-11α-succinate VI resulted in relatively more specific antibodies than testosterone-3-carboxymethyl oxime IV, testosterone-17β-succinate V, and testosterone-7α-carboxymethyl thio-ether VIIb. In the case of progesterone, the antibodies directed at progesterone-11α-succinate XIII are more specific than progesterone-3-carboxymethyl oxime XIVa, progesterone-20-carboxymethyl oxime XIVb, progesterone-6α-carboxymethyl thio-ether XIIc and progesterone-7α-carboxymethyl thio-ether XVa. Surprisingly, progesterone-6α and 6β succinate, XIIa and XIa respectively, gave equally specific antibodies as did 6α and 6β carboxymethyl progesterone XIIb and XIb. Cortisol-6α and 6β-succinate XIId and XId behaved in a similar manner [20, 21]. Dehydroepiandrosterone, when conjugated at 15 position via 15α-carboxymethyl dehydroepiandrosterone IX, or at C-7 position via dehydroepiandrosterone-7-carboxymethyl oxime X gave very specific antibodies as compared to those directed toward dehydroepiandrosterone-3-succinate or dehydroepiandrosterone-17 carboxymethyl oxime.

It has been suggested [22] that antibodies directed toward rings B and C of steroids are specific because the conjugation to the protein is achieved away from the active site of the molecule. However, antibodies prepared against testosterone-7α-carboxymethyl thio-ether VIIb have been shown to be nonspecific. It has also been proposed [4] that the antibodies for progesterone-11α-succinate XIII are more specific because the conjugate is made on the α-side of the molecule so exposing β-side only to immune response. Accepting this reasoning as correct, then the antibodies made from testosterone-7α-carboxymethyl thio-ether VIIb and progesterone-7α-carboxymethyl thio-ether XVa where the 7α-bond is axially oriented should yield specific antibodies which does not seem to be the case.

If one makes the Drieding and CPK color coded models of 6-ketoestradiol, and its carboxymethyl oxime (Fig. 2), it may be observed that the 6-keto group is coplanar with the aromatic ring-A and so also is the 6-carboxymethyl oxime of estradiol (Fig. 2). Though this derivative can take many conformations, it favors a sterically coplanar conformation, especially when conjugated to the protein. Similarly, the Dreiding models of testosterone-11α-succinate (Fig. 3) show two important features: (a) 11α-bond is an equatorial bond, and not an axial bond; and (b) in the succinate derivative, each bond is free to rotate along its own axis and thus can take several conformations and not only the one below the molecule, as was suggested by Niswender and Midgely[4]. However, the succinyl group, once bound to the protein, will take a fixed conformation as directed by the non-bonded interactions, van der Waals' forces, and hydrogen bonding. Even in the CPK model of testosterone-11α-succinate (Fig. 3), the succinyl group also takes a sterically favored conformation which is in the plane of the steroid molecule; this is not the case if the derivative is axially oriented. In other words, in these two cases, the steroid takes a conformation which is in a plane perpendicular to the protein, although it is free to rotate.

In the case of testosterone-7α-carboxymethyl thio-ether, (Fig. 4), the 7α-bond is axial and though here also the carboxymethyl thio-ether group can take many conformations, it can in no case be coplanar with the steroid molecule but instead takes a favored crooked conformation. Thus, when bound to the protein, the steroid will not be in a plane perpendicular to the protein, but will be bent at a considerable angle.

From the data on the specificity of antisera for sex steroids, it appears that derivatives which are coplanar with the steroid molecule tend to give specific antibodies; examples being, estradiol-6-carboxymethyl oxime II, estradiol-11α-succinate III, testosterone 11α-succinate VI, 15α-carboxymethyl dehydroepiandrosterone IX and dehydroepiandrosterone-7-carboxymethyl oxime X. For steroid derivatives taking a crooked conformation, the antibodies tend to be non-specific; e.g., progesterone-6α-carboxymethyl thio-ether XIIc and testosterone-7α-carboxymethyl thio-ether VIIb. It should be mentioned that in the case of progesterone, the derivatives like 6β and 6α succinate, XIa, XIIa, and 6β and 6α-carboxymethyl, XIb, XIIb, and 5α-dihydrotestosterone-1α-carboxymethyl thio-ether VIIIb give fairly specific antibodies.

This hypothesis can in part rationalize the published data on the specificity of antisera for sex steroids and provides a working hypothesis for future work which can be done in this area. The stereochemistry of the steroid derivative and its coplanarity adds one more parameter among many such as dose of the conjugate, site of injection and animal variation which have been considered to impart specificity to the antisera.

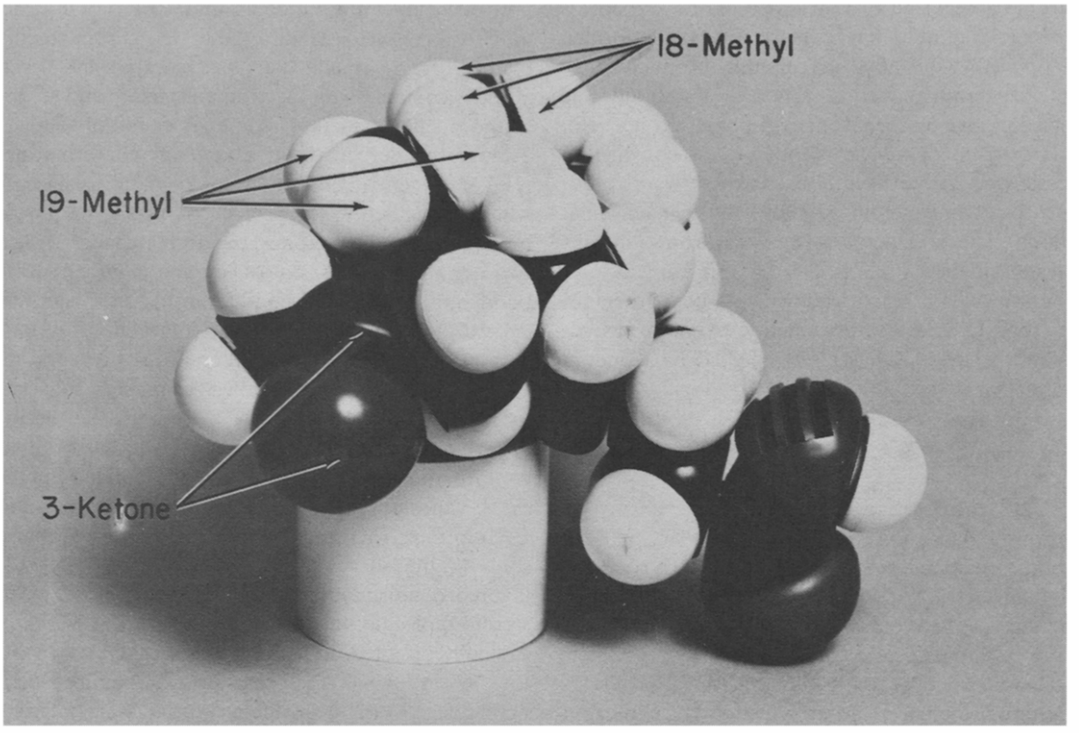

Fig. 4. CPK model of testosterone-7α-carboxymethyl thio-ether.

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