Steroids: Physical Methods

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Reviewing the literature mainly between mid 1985 and the end of 1987
(Continuing the coverage of literature in *Natural Product Reports,* 1986, Vol. 3, p. 505)

1 Introduction

The arrangement of this Report is similar to that of the previous one,[1] but with more attention being given to papers concerned with the interactions of steroids with their biological receptors, which is an area of rapidly increasing activity. The section dealing with n.m.r. spectroscopy emphasizes the applications of two-dimensional methods to steroids: these have permitted full assignments of ^{1}H spectra to become almost routine. Probably the other most important development has been the maturing and wide application of h.p.l.c.–(thermospray)–m.s., an analytical technique for the separation and study of highly polar steroids and their conjugates without the need for derivatization.

A new book[2] dedicated to the bile acids reviews the applications of all of the main physical methods of analysis to this important group of compounds, and another,[3] on sterols and bile acids, includes a chapter on those physico-chemical properties of the bile acids (solubility, surface properties, formation of micelles, *etc.*) which are of most importance in the biological context.

2 Structure, Conformation, and Receptor Binding

It is now recognized that neither the biologically active molecular conformation nor the conformation of a molecule in a crystal need correspond exactly with the energy minimum of an isolated steroid molecule. For this reason a systematic comparison of the calculated ('molecular mechanics': MM) and actual crystal conformations of a wide variety of steroids has been undertaken.[4] Disparities may partially reflect the use of inaccurate torsional parameters in the calculations, but still leave room for real deviations of crystal conformations from the calculated energy minima of isolated molecules. The recent examples which follow include several in which crystal-packing forces clearly influence molecular conformations, although none is as clear-cut as the difference between the conformations in solution and in the solid state of 17α-acetoxy-6α-methylpregn-4-ene-3,20-dione, as reported a few years ago.[5]

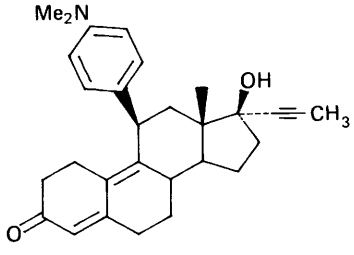
The geometry of progesterone, calculated by the semi-empirical all-valence-electron molecular-orbital (MNDO) method, corresponds very closely to the results that have been obtained by *X*-ray-crystallographic analysis. The only deviation that is considered possibly to be significant is in the preferred conformation of the pregnan-20-one side-chain; the authors[6] point out that they compute a gas-phase structure in which no allowance is made for any solid-state effects which may operate in the crystal. Calculated (by MM) conformations of some progesterones that are substituted at C-16, C-17, and/or C-21 agree with crystallographic data;[7] molecular mechanics has also been used to predict the conformation of the 17β-acetyl side-chain in a 16α,17α-cyclohexano-steroid.[8]

Steric energies and conformations of rings B and C have been calculated for a series of 14-methyloestra-1,3,5(10)-trien-17-ones with the 8β,9α,14α-, 8β,9β,14α-, 8α,9α,14α-, and 8β,9α,14β-configurations, as well as for some 11-oxygenated derivatives. The structures of two such compounds have been confirmed by *X*-ray crystallography.[9]

The butyl acetate solvate of 11β-[4-(dimethylamino)phenyl]-17β-hydroxy-17α-(prop-1-ynyl)oestra-4,9-dien-3-one (1), which is a steroid with powerful antiprogestational and antiglucocorticoid properties, was found to have a rather flat steroid skeleton, with the phenyl group perpendicular to the skeleton, after successful co-crystallization of what had previously been an intractable compound with butyl acetate.[10]

Crystal structures for a series of esters of 11β,17α,21-trihydroxypregn-4-ene-3,20-dione (cortisol; hydrocortisone) and its 9α-fluoro-derivative show two distinct conformations of ring A, corresponding respectively to hexagonal (1α,2β-half-chair) and orthorhombic (inverted conformation) crystal forms.[11] The hexagonal crystals bind solvent strongly, and are readily oxidized in air to give the 11-oxo-derivatives, whereas the orthorhombic forms are stable in air. The implications of these observations for the storage of these pharmaceutical products are obvious. Tetragonal unsolvated crystalline 9α-fluorocortisol acetate has a disordered crystal structure, with ring A in both the normal and the inverted conformation, whereas the propanol solvate has ring A wholly in the normal 1α,2β-half-chair conformation.[12] The difference in free energy between the two conformers must be relatively small.

The iodo-substituent in 16α-iodo-17β-oestradiol has little effect on its molecular geometry, consistent with the high affinity of this steroid for the oestrogen receptor.[13] The 14α-



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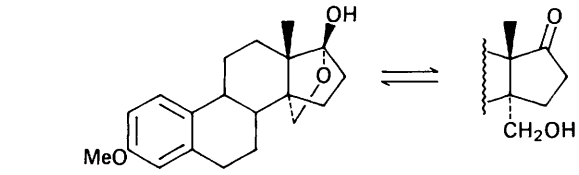

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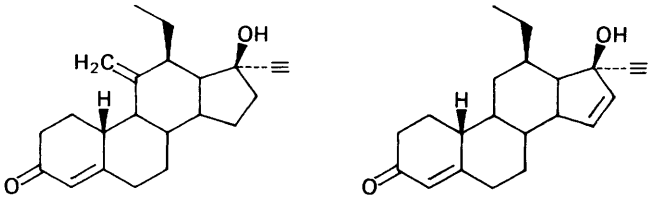

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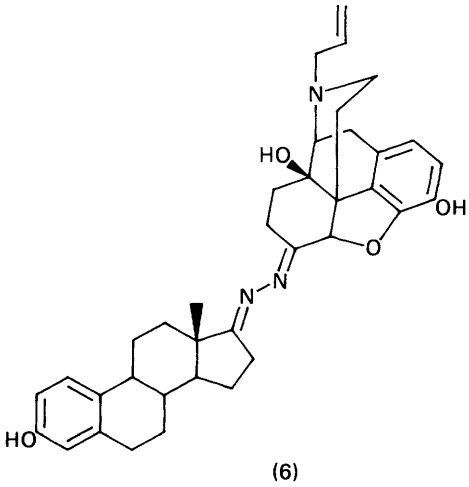


(6)

hydroxymethyloestrone derivative (3), which exists in solution as an equimolar mixture with its hemiacetal (2), crystallizes wholly as the hemiacetal, which is stabilized by intermolecular hydrogen-bonding.[14] 19-Nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17β-diol (17α-ethynyloestradiol), as its hemihydrate, has a crystal structure comprising bilayers of hydrogen-bonded steroid and water molecules, similar to that of oestradiol hemihydrate, but with staggered instead of stacked bilayers.[15] 13-Ethyl-17β-hydroxy-11-methylene-18,19-dinor-17α-pregn-4-en-20-yn-3-one (4) has ring A statistically disordered (1:1), showing a normal 1α,2β-half-chair and an inverted 1β,2α-half-chair.[16] The former is calculated to be marginally the more stable conformer. The related 15-ene (5), lacking the 11-methylene group, has two crystalline forms; in one of these the ethyl group at C-13 has its normal preferred conformation, *anti* to the C/D ring junction, but in the other the ethyl group is twisted to lie above ring D.[17] The stereochemistry of compounds [*e.g.* (6)] in which a steroid hormone is linked to naloxone *via* an azine has been examined by ^{13}C n.m.r. and confirmed by *X*-ray crystallography.[18,19] These complexes are δ-selective opiate antagonists. The configuration around each end of the azine is specifically (*E*) [*anti*].

The photoaddition of acetophenone and *p*-fluoroacetophenone, as guests in deoxycholic acid complexes, at −170 °C comes close to the maximum that is permitted by the crystal packings.[20] Molecular-mechanics calculations have been used to predict the relative steric energies and preferred conformations for a series of stereoisomeric hexahydrobenzo[4,5,6]-cholestanes.[21]

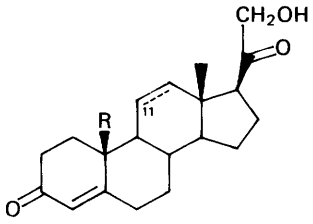
2.1 Receptor Binding of Steroids

Some forty years after conformational analysis was introduced into steroid chemistry, research emphasis has largely shifted from intramolecular manifestations of conformation, affecting physical properties and chemical reactivity, to an intensive study of the interactions between hormonal steroids and their biological receptors and other biological molecules. With the aid of conformational trends determined by *X*-ray crystallography, and even more recently by the computer modelling of molecules and their 'fit' with receptors, considerable progress is being made in the mapping of receptors for steroid hormones, even though much remains to be done before the precise nature of the binding to the receptor, and the three-dimensional structure of the binding site itself, can be defined. Recent work,[22] particularly on the androgen- and progesterone-receptor binding capabilities of a series of hormonally active steroids, has shown that the subtle conformational changes and variations in skeletal flexibility which result from differing patterns of unsaturation and substitution in the steroid can result in a gradual shift from essentially androgenic to essentially progestational receptor binding. It seems probable that the receptors have much in common structurally, differing slightly, but significantly, in the ligand-binding capabilities of their active sites.

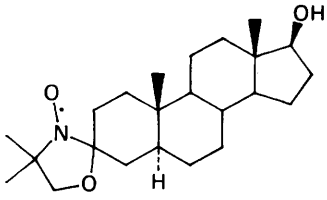
Present views on the structural basis of interaction between steroids and their receptors, based on *X*-ray studies, have been summarized with particular reference to synthetic hormone antagonists.[23] Variations in binding to oestrogen receptors in response to substitution of the steroid at C-17 have been reported for both the 8β- and 8α-oestradiol series.[24] 17α-Methyl, 17α-vinyl, or 17α-ethynyl groups do not interfere significantly with binding, unlike 17α-ethyl (see below). Reversal of the configuration of C-17 (to 17α-OH, 17β-R) reduces the binding affinity of an oestradiol; compounds of the 8α-series are always less effective than their natural 8β-isomers. The extent of steric shielding of the 17β-hydroxyl group of synthetic steroid oestrogens by substituents at the 17α-position correlates with their variety of abilities to elicit the oestrogenic response.[25] Studies on the O–H stretching band in their infrared spectra, and of the O*H* signal in the ^1H n.m.r. spectrum in [$^2H_6$]dimethyl sulphoxide, lead to the conclusion that a 17α-ethyl group, in particular, inhibits hydrogen-bonding interactions between the steroid and its receptor by blocking approach to lone-pairs of electrons on oxygen, whereas 17α-ethynyl groups, at the other extreme, leave hydrogen-bonding unimpaired and allow high biological activity.[25] Molecular-mechanics calculations concerning conformations of the 17-ethyl side-chain have confirmed this conclusion.[26] The abilities of the oestrogen, androgen, and glucocorticoid receptors to bind simpler (non-steroidal) phenols, now assessed for a wide variety of compounds, confirm the strong preference of the oestrogen receptor for *meta*-substituted phenols, which are the closest in structure to the natural oestrogens.[27]

The binding requirements of the aromatase enzyme complex, which is responsible for a key step in the conversion of androst-4-ene-3,17-dione into oestrogens, have been explored by means of a series of potential steroid substrates with differing patterns of substitution. Optical-difference spectra, representing the change in absorbance when the steroid and enzyme interact, were strongest for those steroids that are best able to inhibit the activity of the enzyme complex. Inhibitory steroids include various 6-substituted derivatives, suggesting that the substituent at this position does not interfere appreciably with binding to the enzyme, in contrast to substitution at C-2, C-11, C-16, or C-19.[28]

Molecular-mechanics calculations have been used in a new attempt to assess the energetics and conformational preferences of steroidal 4-en-3-ones that contain and do not contain the methyl group C-19.[29] The 4-en-3-one is essential if a steroid is to bind to the progesterone receptor. Previous studies had indicated that binding forces depend critically on the ability of



(7) R = H
(8) R = Me; 11,12-didehydro



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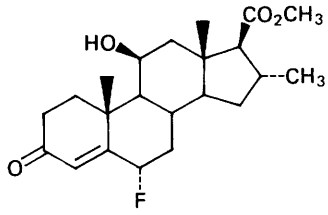


(10)

ring A to adopt the abnormal 1β,2α-half-chair conformation. The new results, however, indicate that a 19-nor-4,9-dien-3-one has somewhat lower affinity for the receptor than has a 19-nor-4-en-3-one, whereas the 4,9-dienone is better able to adopt the inverted conformation. The conformation of the steroid in the steroid–receptor complex is as yet unknown. The topic is clearly still open to study.

A curious correlation has been noted between ^{13}C chemical shifts (for C-17) and the abilities of various derivatives and analogues of 17α-ethynyl-17β-hydroxyoestr-4-en-3-one ('norethisterone') to bind to the human endometrial progesterone receptor.[30] The receptor-binding and hormonal activities of 9β,10α-steroids have been considered in the context of steric requirements of the receptors.[31] Some des-A-steroids are bound surprisingly well by the androgen receptor.[32] Compounds of the aldosterone and corticosterone type, which bind to the mineralocorticoid receptor, do so with increasing affinity the flatter the molecule.[33] New *X*-ray analyses have added 21-hydroxy-19-norpregn-4-ene-3,20-dione (19-nor-11-deoxycorticosterone) (7) and 21-hydroxypregna-4,11-diene-3,20-dione (8) to the list of those whose structures are known in detail. The 19-nor-compound (7) has the flattest structure of those studied, and bonds most strongly to the receptor, exceeding the affinity (though not the biological activity) of the main natural mineralocorticoid aldosterone. Electronic effects in this series, although detectable by small variations in the ^{13}C chemical shift (especially of C-5), appear to be of secondary importance in affecting affinity for the receptor.[33] The long-held view that mineralocorticoid and glucocorticoid hormones are differentiated by oxygenation at C-18 and C-17, respectively, has been challenged.[34] New clinical evidence indicates that the main natural glucocorticoid cortisol (see above) is strongly bound and active at the *mineralocorticoid* receptor but is normally prevented from reaching it by 11β-hydroxysteroid dehydrogenase, which converts cortisol into inactive cortisone. The mineralocorticoid receptor is clearly much less specific in its requirements than was previously believed.

The spin-labelled spiro-doxyl androstane derivative (9) binds at the substrate-binding site of crystalline Δ^5-3-keto-steroid isomerase [steroid Δ^5-isomerase; E.C. 5.3.3.1].[35] The location of the steroid has been found (by *X*-ray crystallography and n.m.r. spectroscopy) to be in a hydrophobic cavity which is accessible from the external environment, and probably corresponds closely to the docking position of androst-5-ene-3,17-dione, which is the natural substrate for the enzyme.

Computer modelling of the receptor site for cardiac steroids of Na^+/K^+-transporting ATPase, aided by experimental data from *X*-ray crystallography, site-specific labelling, and biological studies, suggests that cardiac glycosides are bound mainly by interactions involving the carbonyl group in the steroid side-chain, the sugar molecule that is attached directly to the steroid, and possibly also any 16β-ester group.[36]

As with receptors, the ability of an antibody to recognize and bind a steroid is thought to be enhanced if the steroid molecule has sufficient conformational mobility to allow it to adapt easily to the requirements of the antibody. Unsaturation in steroid rings, by generally lowering the difference in free energy between conformers, appears to increase the likelihood of cross-reactivity with antibodies.[37]

Other *X*-ray analyses of steroids which have come to the Reporter's attention are collected in Table 1. The list is not necessarily exhaustive, because a few *X*-ray analyses that are reported incidentally in papers concerned mainly with synthesis may have been overlooked.

3 N.M.R. Spectroscopy

3.1 ^1H Spectra and ^1H–^{13}C Correlated Spectra

Considerable progress has been made since the last Report[1] on complete assignments of the high-field ^1H n.m.r. spectra of steroids. Two-dimensional (2D) techniques (known by their acronyms!) are now almost routinely applied to assist the interpretation of high-field ^1H spectra. The 2D methods that have so far been used for steroids are usefully reviewed,[94] with emphasis on problem solving and on those methods which can be informative with samples at or below the milligram level. If the size of a sample is not a problem, a full and reliable ^{13}C analysis can be carried out by the ^{13}C–^{13}C connectivity (INADEQUATE) method. The assignment of ^{13}C spectra forms the basis for a ^1H–^{13}C 2D heteronuclear correlated (HETCOR) spectrum which provides chemical shifts for the protons that are attached to each carbon atom. When sample size is insufficient for these procedures, 2D ^1H homonuclear shift-correlated (COSY) spectra often provide enough information to enable a partial, if not complete, analysis of the ^1H spectrum. Details, including configurational assignments, can be filled in by either 1D [nuclear Overhauser effect (n.O.e) or decoupling difference] or 2D (NOESY) methods.

Full ^1H n.m.r. (400 MHz) analyses for sixteen 5α-androstanes, including the parent hydrocarbon and a series of derivatives that are substituted mainly at C-3 and C-17, as well as for methyl 6α-fluoro-11β-hydroxy-16α-methyl-3-oxoandrost-4-ene-17β-carboxylate (10) made use of the 2D COSY and HETCOR methods, complemented by n.O.e. difference spectra where necessary.[95] Spectral simulation by computer was valuable for refinement of shift and spin-coupling data when signals overlapped. Spin couplings, supported by molecular-mechanics calculations, revealed that there are conformational variations, especially in ring D. Substituent increments in ^1H chemical shifts due to carbonyl, hydroxyl, and halogeno-groups were evaluated, and have been interpreted in terms of a combination of anisotropy and electric-field effects.[95]

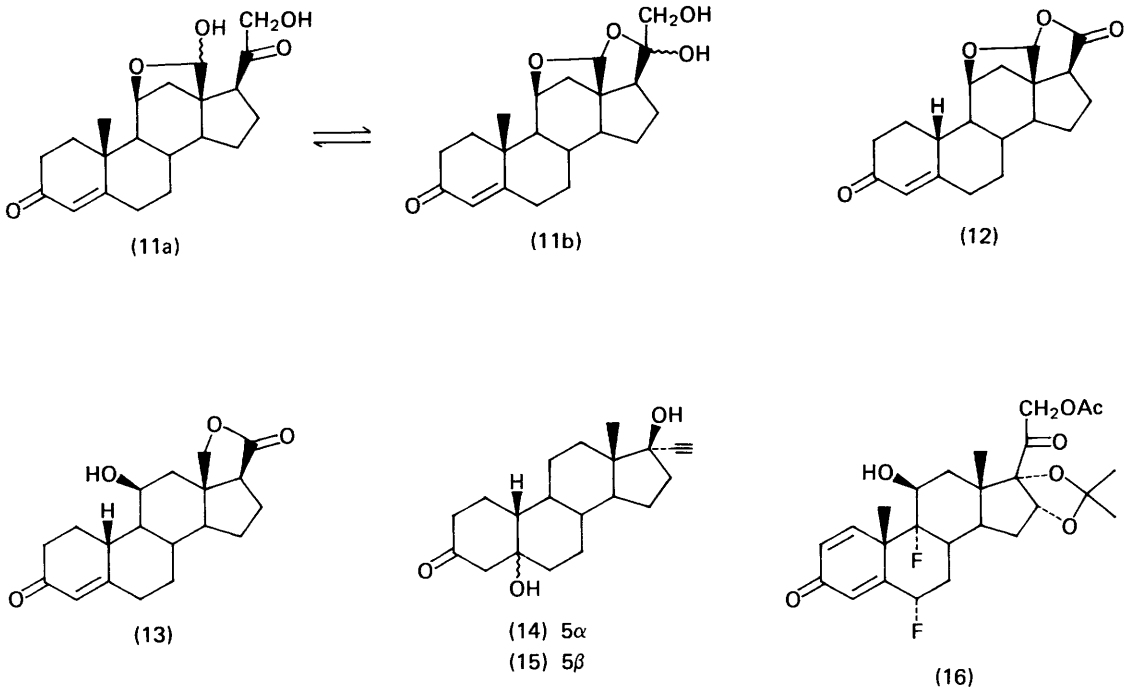
Accurate experimentally determined ^1H–^1H coupling constants for the protons at C-1 and C-2 in a series of steroidal 4-en-3-ones[96] and some 2-methyl-4-en-3-ones[97] have been used to establish the preferred conformations of ring A in solution.

Table 1 Steroids that have been studied by *X*-ray crystallography. (Compounds with references 9–37 are mentioned also in the text).

Compound	Ref.
Oestranes	
17α-Azidomethyl-17β-hydroxyoestr-4-en-3-one	38
17α-Chloromethyl-17β-hydroxyoestr-4-en-3-one	39
17α-Cyanomethyl-17β-hydroxyoestr-4-en-3-one	40
10β-Hydroxyoestra-1,4-diene-3,17-dione	41
2,4-Dibromo-10β,17β-dihydroxyoestra-1,4-dien-3-one	42
17α-Azidomethyl-17β-hydroxyoestra-4,9-dien-3-one	43
11β-[4-(dimethylamino)phenyl]-17β-hydroxy-17α-(prop-1-ynyl)oestra-4,9-dien-3-one	10
11β-[4-(dimethylamino)phenyl]-17β-hydroxy-17α-(prop-2-enyl)oestra-4,9-dien-3-one	44
Oestra-1,3,5(10)-triene	45
16α-Iodo-oestra-1,3,5(10)-triene-3,17β-diol	13
(+)- and (−)-3-Methoxy-18-methyl-8α-oestra-1,3,5(10)-trien-17-one	45
14-Hydroxy-3-methoxy-18-methyl-14β-oestra-1,3,5(10)-trien-17-one	46
(±)-14-Hydroxy-3-methoxy-18-methyl-8α,9β,14β-oestra-1,3,5(10)-trien-17-one	45
3-Methoxy-14-hydroxymethyloestra-1,3,5(10)-trien-17-one hemiacetal	14
(±)-3-Methoxy-14-methyl-9β-oestra-1,3,5(10)-trien-17β-ol	9
(±)-3-Methoxy-14-methyloestra-1,3,5(10)-triene-11α,17β-diol	9
16α-(1-Acetoxyethyl)-3,15α-dimethoxy-17β-phenyl-14,17α-etheno-oestra-1,3,5(10)-triene	47
(±)-15α-Methyl-8-aza-16-oxa-13α-estra-1,3,5(10)-trien-17-one	48
Oestrone naloxone azine	19
17α-Ethynyl-17β-hydroxyoestrane derivatives: *see* 19-nor-17α-pregn-20-ynes (below)	
Androstanes	
4-Chloromercurioandrosta-4,6-diene-3,17-dione (acetone solvate)	49
5,17β-Dihydroxy-A-nor-5β-androstan-3-one 17-acetate 5-(*R*)-methanesulphinate	50
(±)-14β-Hydroxy-1β,4β-methano-5β,8α,9β-androstane-7,17-dione	51
3β-Acetoxy-17,20-epoxy-17-picolylandrost-5-en-7-one (17,20-isomers)	52
4α-Carbomethoxy-15α-cyano-4β-methyl-5α,13α-androstane	53
17β-Hydroxy-4-aza-5β-androst-1-en-3-one	54
17β-Acetoxy-3-aza-A-homoandrost-4a-en-4-one	54
Methyl (4′*S*,16*S*)-3β-acetoxy-17-oxospiro[androst-5-ene-16,3′-(4,5-dihydropyrazole)]-4′-carboxylate	55
17α-Ethynyl-17β-hydroxyandrostane derivatives: *see* 17α-pregn-20-ynes (below)	
Pregnanes	
11β-Hydroxymethyl-5α-pregnane-3β,20β-diol 3,20-diacetate	56
(20*R*)-19-Nor-5β,14β-pregnane-3β,14,20-triol 3,20-diacetate	57
21-Hydroxy-19-norpregn-4-ene-3,20-dione [19-nordeoxycorticosterone]	33
21-Hydroxypregna-4,11-diene-3,20-dione	33
17α,21-Dihydroxypregn-4-ene-3,11,20-trione 21-acetate [cortisone acetate (modification IVac)]	58
Cortisone acetate (modification Vaq)	59
Esters of 11β,17α,21-trihydroxypregn-4-ene-3,20-dione [cortisol; hydrocortisone]:	
21-butyrate (hexagonal and orthorhombic forms)	11
21-propionate (orthorhombic)	11
21-pentanoate (hexagonal)	11
21-decanoate (hexagonal)	11
21-(3-cyclopentylpropionate) (hexagonal)	11
9α-Fluorocortisol 21-butyrate (hexagonal)	11
9α-Fluorocortisol 21-pentanoate (hexagonal)	11
9α-Fluorocortisol 21-acetate	12
6α,9α-Difluoro-11β,16α,17α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 21-acetate 16,17-acetonide ['fluocinonide']	60
20β-Hydroxy-16α,17α-cyclohexanopregn-4-en-3-one	61
11-Methylene-19-nor-17α-pregn-4-en-20-yn-17β-ol	62
13-Ethyl-17β-hydroxy-11-methylene-18,19-dinor-17α-pregn-4-en-20-yn-3-one ['3-ketodesogestrel']	16
13-Ethyl-17β-hydroxy-18,19-dinor-17α-pregna-4,15-dien-20-yn-3-one ['gestogene']	17
19-Nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17β-diol ['ethynyloestradiol']	15
13-Ethyl-18,19-dinor-17α-pregn-4-en-20-yn-17β-ol	63
11β-Chloro-13-ethyl-18,19-dinor-17α-pregn-4-en-20-yn-17β-ol (methanol solvate)	64
11β-(4-Fluorophenyl)-17β-hydroxy-19-nor-17α-pregna-4,9-dien-20-yn-3-one	65
11β-(4-Fluorophenyl)-17α-hydroxy-19-nor-13α-pregna-4,9-dien-20-yn-3-one	65
1,11β,17,21-Tetrahydroxy-4-methyl-19-norpregna-1,3,5(10),8(14)-tetraen-20-one 1,21-diacetate	66
Sterols and miscellaneous	
Cholesteryl formate	67
Cholesteryl *cis*-dec-9-enoate	68
Cholesteryl *trans*-dec-9-enoate	68
Cholesteryl *cis*-octadec-9-enoate [oleate]	69
Cholesterol–cholesteryl oleate binary mixtures	70
Cholesteryl perfluoropropionate	71
Cholesteryl *p*-hexyloxybenzoate	72
(25*S*)-Cholest-5-ene-3β-26-diol	73
5α-Cholestane-3β,6β-diol 3-acetate 6-[3-(4-iodophenyl)phenyl]acetate	74
(23*R*,24*R*)-4α,23,24-Trimethyl-5α-cholestan-3β-ol	75
3β-Acetoxy-7α-aza-B-homocholest-5-eno[7a,7-*d*]tetrazole	76
(1*S*,10*S*)- and (1*R*,10*R*)-1,5;5,10-diepoxy-5,10-secocholestan-3β-yl acetates	77
17,25-Epoxy-20-oxo-21,22,23,27-tetranor-16,25-cyclocholest-5-en-3β-yl acetate	78
Calciol (vitamin D_3) 5,6;7,8-diepoxide	79
Calciol (vitamin D_3) 5,6;7,8;10,19-triepoxide	80
3α,7β-Dihydroxy-5β-cholan-24-oic acid [ursodeoxycholic acid]	81
Sodium taurodeoxycholate (monohydrate)	82
(25*R*)-Spirost-5-ene-3β,17α-diol [pennogenin; hemihydrate]	83
Spirosta-5,25(27)-diene-1β,3β,11α-triol (monohydrate)	84
(25*R*)-Spirost-5-ene-3β,12β,15α-triol [bahamagenin]	85
22,23-Dibromo-10-methyl-19-noranthraergosta-5,7,9,14-tetraene	86
2β,3α,22,23-Tetrabromo-18-nor-17-iso-5α-ergosta-8,11,13-triene	86
19-Nordigitoxigenin	87
14-Hydroxy-3β-(α-L-rhamnosyloxy)-14β-bufa-4,20,22-trienolide [proscillaridin]	88
3β,11α,14-Trihydroxy-5β,14β-bufa-20,22-dienolide [gamabufotalin]	89
3β,11α,14-Trihydroxy-12-oxo-5β,14β-bufa-20,22-dienolide [arenobufagin]	89
Deoxycholic acid–ethyl acetate (2:1 complex)	90
Deoxycholic acid–ferrocene (2:1 complex)	91
Deoxycholic acid–phenylacetylene (2:1 complex)	92
Deoxycholic acid–thiocamphenilone	93

Only one of the compounds that were investigated, 2β,17β-diacetoxyandrost-4-en-3-one, has the inverted 1β,2α-half-chair conformation, as reported previously. The authors caution against equating signal splittings, measured directly from the spectrum, with coupling constants for an ABX or a more complicated spin system. Such a simple first-order analysis is likely to be unreliable, even at high fields. We are advised,[96] when reporting such data, to state clearly that they are only estimates of 'apparent' *J* values. In the work under review, computer simulation of spectra was used to refine approximate chemical shifts of the four coupled protons at C-1 and C-2 and to refine the initial estimates of *J* values, obtained from 2D COSY spectra. Iterative analyses gave chemical shifts which are quoted to four decimal places, and values of ^2J and ^3J to two decimal places.

A two-dimensional ^{13}C–^{13}C connectivity experiment (INADEQUATE) at natural abundance has provided all ^{13}C n.m.r. assignments for each of the two principal tautomers (11a) and (11b) of aldosterone in the equilibrating mixture in solution. The results were used, *via* two-dimensional ^1H–^{13}C heteronuclear spectroscopy, together with phase-sensitive double-quantum-filtered COSY, 2D *J*-resolved ^1H spectra, and



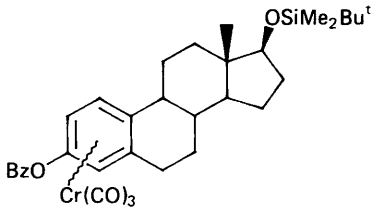
a few selective n.O.e. measurements, to determine all ^1H chemical shifts and most of the ^1H–^1H coupling constants for each tautomer.[98] Two-dimensional ^1H n.m.r. has been used to confirm the structures of the γ-lactones (12) and (13), which were obtained by oxidation of 19-noraldosterone and 11β,18,21-trihydroxy-19-norpregn-4-ene-3,20-dione, respectively with periodate.[99]

The known ^{13}C chemical shifts for 5β-cholan-24-oic acid and its common hydroxylated derivatives [*i.e.* 3α-OH, lithocholic acid; 3α,7α-$(OH)_2$, chenodeoxycholic acid; 3α,7β-$(OH)_2$, ursodeoxycholic acid; 3α,12α-$(OH)_2$, deoxycholic acid; and 3α,7α,12α-$(OH)_3$, cholic acid] have been used, *via* two-dimensional ^1H–^{13}C heteronuclear correlated spectroscopy, to assign all of the ^1H signals for these compounds.[100] This is the first major series of 5β-steroids to be subjected to full ^1H analyses, providing valuable reference data for others of the 5β class. The increments in ^1H chemical shift for individual hydroxyl substituents show good additivity within this series. Fully assigned ^1H and ^{13}C spectra of sodium cholate and sodium deoxycholate have been used to probe the effects of concentration on the association of these bile salts in solution. Two details of an earlier ^1H assignment (for 6α-H and 8β-H) have been reversed.[101] The rotations of the methyl groups in micelles of sodium deoxycholate have been studied by observing the intensities of forbidden peaks in double-quantum-filtered one-dimensional spectra as a function of excitation time, in combination with T_1 and T_2 datasets.[102]

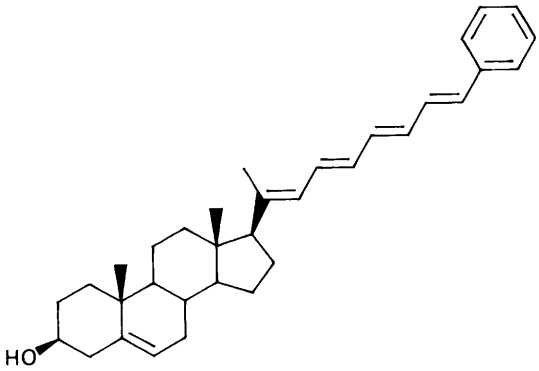
The binding of Ca^{2+} and of Na^+ by glycocholate and taurocholate ions has been studied by measuring the ^1H shifts that are induced by the lanthanide ion Dy^{3+}, as a paramagnetic isomorphous replacement for Ca^{2+}, alone and in competition with added Ca^{2+} or Na^+. Derived dissociation constants for the metal–bile salt complexes show that Ca^{2+} is more strongly bound by the carboxylate group of glycocholate than by the sulphonate group of taurocholate. In all cases the ionic terminus of the side-chain provides the main binding site for the metal ion.[103] The Dy^{3+} ion has also been used in estimating the metal-ion-induced relaxation rates of protons in glycocholate ions, and thence to construct a model of the glycocholate–Dy^{3+} complex in submicellar concentrations in aqueous solution. The metal ion appears to associate strongly with one carboxylate oxygen atom of a glycine residue, and at longer range with the peptide carbonyl oxygen and the other carboxylate oxygen, but not with skeletal hydroxyl groups.[104] Proton chemical shifts (only for protons at C-18, C-19, and C-21, and geminal to hydroxyl groups at C-3, C-7, and/or C-12) have been reported for all of the isomeric methyl 5α-cholanoates that contain from one to three hydroxyl groups, or from one to three oxo-groups, at positions 3, 7, and 12. Additivity of substituent effects is again observed.[105]

A complete assignment of the ^1H and ^{13}C n.m.r. spectra of 17α-ethynyl-3-methoxyoestra-1,3,5(10)-trien-17β-ol ('mestranol') made use of both homonuclear and heteronuclear two-dimensional methods, including spin-echo *J*-correlated spectroscopy (SECSY). Spectral differences were then used to aid the structural elucidation of photo-oxidation products of mestranol, modified in rings B and C.[106] Proton assignments, confirmed by computer simulation, have also been reported for the 3-methyl ethers of oestrone, oestradiol, and their 14-methyl derivatives.[107] Full assignments (^1H), obtained by the COSY method, have been reported for the isomeric phototransformation products (14) and (15) which were obtained from 17β-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one (norethisterone).[108] High-field n.m.r. spectroscopy, leading to full ^1H and ^{13}C assignments of the n.m.r. spectra of 17β-hydroxy-19-nor-5α,17α-pregn-20-yn-3-one and its 5β-isomer, has provided vicinal coupling constants for conformational analysis of these compounds. The conformations of the molecules in solution that were determined in this way agree well with the conformations that have been derived by *X*-ray-crystallographic and molecular-mechanics methods.[109] COSY and n.O.e. difference spectra, aided by computer simulation, were employed in a full analysis (^1H, ^{13}C, and ^{19}F) of the spectra of 6α,9-difluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione 21-acetate 16,17-acetonide ('fluocinonide') (16) which included the measurement of long-range ^1H–^{19}F coupling constants (*e.g.* 7α-H,6-F = 13 Hz; 7β-H,6-F = 2.5 Hz, and 12α-H,9-F = 3.1 Hz).[110]

COSY spectra, under conditions that were chosen to emphasize long-range ^1H–^1H couplings, simplify the assignment of signals in oestrogens. The ^4J and ^6J couplings from protons at C-6 and C-9 to protons of the aromatic ring provide entry points for analysis of the COSY cross-peaks relating to protons in rings B and C, with the advantage that the method is



(17) α-Cr(CO)$_3$
(18) β-Cr(CO)$_3$



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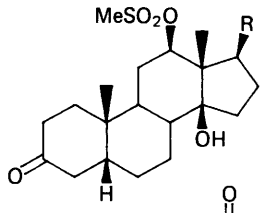
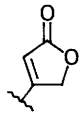
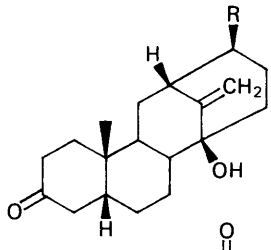


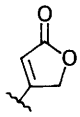
(20) a; R =



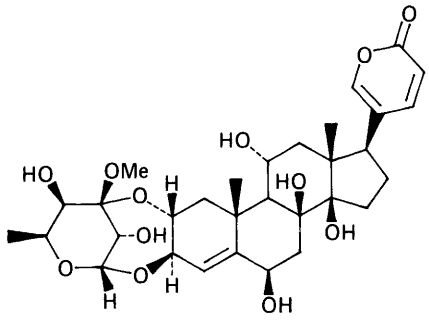
b; R = C(O)Me



(21) a; R =



b; R = C(O)Me



(22)

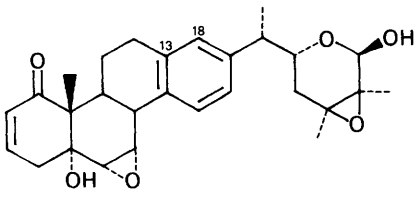
applicable to quite small samples.[111] The diastereoisomers (17) and (18), in which a tricarbonylchromium moiety is attached to the α- or the β-face of the aromatic ring of an oestradiol derivative, are distinguished by their high-field n.m.r. spectra (500 MHz for ^{1}H; 125 MHz for ^{13}C). Full assignments, achieved by the two-dimensional COSY and SECSY techniques as well as ^{1}H–^{13}C heteronuclear-shift-correlated spectra, show that there is strong deshielding of neighbouring protons on the same face as the tricarbonylchromium. The ^{13}C spectra have been tabulated for oestradiol and fifteen of its derivatives in this series.[112] The 500 MHz ^{1}H n.m.r. spectra for the cluster complexes formed from 17α-propynyloestra-1,3,5(10)-triene-3,17β-diol and $Co_2(CO)_8$ or $(\eta^5\text{-}C_5H_5)_2Mo_2(CO)_4$ have been fully assigned by the 2D COSY method. Anisotropic effects of the propynyl group cause very large down-field shifts of signals from neighbouring protons, especially those at the 12α- and 14α-positions.[113]

Fourier-transform ^{1}H n.m.r. at 300 MHz has been proved to be capable of giving a useful spectrum with as little as 5 μg of compounds of the vitamin D_2 series, by using standard n.m.r. tubes of 5 mm internal diameter, fitted with glass inserts, and isotopically enriched (99.96%) $CDCl_3$. Some re-assignments of signals have been reported.[114] The equilibrium between the two conformations of ring A in solutions of compounds of the vitamin D series is sensitive to the polarity of the solvent. Polar solvents (methanol and DMSO) favour the conformer with an equatorial hydroxyl group. Inversion is too fast for the individual conformers to be observed by n.m.r., so the conformational preferences in a range of solvents were obtained from the variations in vicinal ^{1}H–^{1}H coupling constants.[115]

A ^{1}H and ^{13}C n.m.r. study of the products of dienone–phenol rearrangement of 1,4-dien-3-ones in deuteriated acidic media showed that deuterium was incorporated only into the phenolic ring and at C-6, in proportions depending upon the reagents that were used. No deuterium was found at C-8, at C-9, or elsewhere, showing that these sites are not subject to exchange during the reaction. Even C-6 is probably only involved *via* a reversible pre-enolization of the dienone.[116] Proton n.m.r. spectra of a series of D-homo-5α-androstan-17a-one derivatives, reporting chemical shifts for up to half of the ring protons in each case, have been analysed to provide values of the increments in the shifts that are induced by hydroxyl groups at various positions.[117] Proton n.m.r. studies of solutions in sulphuric acid at various concentrations have shown that the chromogenic reaction of 17α-hydroxyprogesterone involves a rapid D-homo-annulation followed by dehydration and migration of C-18, leading to coloured cationic species.[118,119] Proton n.m.r. spectra at 500 MHz permit easy distinction between α- and β-anomers of some steroid glucuronides; the synthetic method is critical in deciding which anomer is the predominant product.[120] Fully assigned ^{1}H and ^{13}C spectra have been reported for 3β-acetoxypregn-5-en-20-one (pregnenolone acetate) and a series of derivatives with modified side-chains (including 20β-OH), leading to the pregn-20-ene derivative,[121] and 2D n.m.r. has been used to determine the structures of two oxidation products of cholesterol, which include a dimeric steroid.[122]

Proton and carbon-13 spectroscopy, including 2D experiments, has defined the geometries of the unsaturated side-chains in a series of fluorescent analogues of cholesterol, *e.g.* (19), that had been designed as membrane probes.[123] Proton and ^{13}C spectra have been related to the configurations of the alkyl groups at C-24 in sterols from members of the Cucurbitaceae.[124] The 13,17-seco-12,17-cyclo [17(13 → 12) *abeo*] structures (21a) and (21b) of the solvolysis products of the 12β-mesylates (20a) and (20b) have been determined by 2D n.m.r. spectroscopy.[125] The ^{1}H (nearly complete) and ^{13}C assignments have been reported for three novel bufadienolide glycosides ('orbicusides A–C') from *Cotyledon orbiculata*[126] and for rubellin (22), which is a novel bufadienolide glycoside.[127]

A combination of n.m.r. relaxation studies and MM calculations for a series of unsaturated steroids indicates that the rotational barriers of the angular methyl groups are highly sensitive to their interactions with axial hydrogens; some of these interactions are relieved by the presence of unsaturation nearby.[128] N.m.r. relaxation rates for protons of water have been used to probe the interaction between cholesterol and its side-chain-cleavage enzyme cytochrome *P*450. A molecule of water that was initially at a distance of only *ca.* 2.5 Å from the



(23)

haem iron in the cytochrome, compatible with direct binding, is displaced to *ca.* 4 Å if cholesterol is present.[129]

3.2 ^{2}H-Labelled Compounds

Cholesteryl acetate and 3β-acetoxypregn-5-en-20-one are among compounds that can be used to illustrate the detection of sites of deuterium labelling by means of two-dimensional ^{1}H–^{13}C heteronuclear shift correlation, with ^{2}H decoupling. Only signals from incompletely deuteriated sites (CDH, CDH_2 or CD_2H) can be observed. This method has established that catalytic reduction of cholesteryl acetate with deuterium gas exchanges the 7α-proton selectively to give the 5α,6α,7α-$^2H_3$-labelled species.[130] The ^{2}H n.m.r. spectra of specifically deuteriated cholesterol (3α-^{2}H-, 7,7-$^2H_2$-, and 2,2,4,4,6-$^2H_5$-labelled) and its palmitate have been used to study its organization in multilamellar dispersions of dipalmitoylglycerophosphocholine.[131] Deuteron n.m.r. has provided evidence for the orientation of [3α-^{2}H]cholesterol and [3β-^{2}H]epicholesterol in dipalmitoylglycerophosphocholine liposomes[132] and measurements of the spin–lattice relaxation of ^{2}H were used to study the complex motions of [2,2,3,4,4,6-$^2H_6$] cholesterol in model membranes of dimyristoylglycerophosphocholine.[133] Cholesteryl oleate, selectively deuteriated in the acyl chain, was the subject of ^{2}H n.m.r. experiments to determine its orientation, ordering, and mobility in association with low-density lipoprotein.[134] Isotopic incorporation experiments, with ^{2}H n.m.r. spectroscopy, showed that the aromatic ring of the antifeedant steroid NiC-1 (23) is formed with incorporation of the angular methyl group (C-18) into ring D at the 17α-position.[135]

3.3 ^{13}C Spectra

Three pseudopolymorphic forms of testosterone (one anhydrous and the others polymorphic monohydrates) give distinctive infrared spectra. Their ^{13}C solid-state CP/MAS spectra also have interesting features. The α-form (anhydrous) showed two peaks each for most of the carbon atoms, corresponding to the two independent molecules which make up the asymmetric unit in the crystal. C-17 showed the largest splitting, with resonance signals separated by 2.3 p.p.m. The two hydrated polymorphs each showed its own characteristic pattern of ^{13}C chemical shifts.[136]

Examples of the use of 2D-INADEQUATE experiments have already been mentioned (Section 3.1) and 2D-INADEQUATE experiments have also been used to determine carbon connectivities and all of the $^1J(^{13}C–^{13}C)$ coupling constants for stigmasterol and androsta-1,4-diene-3,11,17-trione. One conclusion that has been drawn from the results is that $J_{10,19}$, *i.e.* the coupling constant to the methyl group C-19, is reduced significantly by its proximity to more unsaturation in the latter compound.[137] It remains to be seen whether this effect is general. A down-field shift of the ^{13}C signals that is seen for all carbon atoms *except* those that are α to carbonyl when the solvent is changed from $CDCl_3$ to $CDCl_3$–dioxane (1:4) offers a simpler and more versatile alternative to α-deuterium exchange as an aid to assignment of ^{13}C spectra.[138]

Rather few steroids of 5β configuration have been subjected to a full analysis of their ^{13}C spectra (the same is true at present of high-field ^{1}H spectra!), so the publication of full ^{13}C assignments for a series of 23 compounds of the 5β type is welcome. They comprise methyl 5β-cholanoates, 5β-pregnan-20-ones, and 5β-androstane-17β-carboxylates; conformational and substituent effects associated with the various side-chains are analysed.[139] The available collections of ^{13}C n.m.r. data are also usefully supplemented by the publication of fully analysed spectra for 247 compounds of the cardenolide and bufadienolide series. These compounds include many in which the configurations (5β, 14β, and 17α), unsaturation ($\Delta^{8(14)}$, Δ^{14}, and Δ^{16}), and substitution (*e.g.* 5β-OH, 8β-OH, 12β-OH, 14β-OH, 15α-OH, 16β-OH, 19-OH, 19-oxo, and various epoxides) were not previously well represented among compounds for which fully assigned ^{13}C spectra were available.[140] The corresponding increments in chemical shifts will have wider application. Another group[141] of workers has reported ^{13}C spectra of thirty substituted 5β,14β-dihydroxy-steroids, with particular emphasis on the effects of 12α- and 12β-substituents. Carbon-13 n.m.r. data have also been listed for 130 steroidal sapogenins and saponin derivatives for which spectra were published up to 1983: most of the sapogenins are of the spirostan type, but a few others are included. The sugar components of the saponins, and their anomeric configurations, can readily be distinguished by comparing ^{13}C data.[142] Lanthanide-induced shifts {*e.g.* by [Yb(fod)$_3$]} have aided the full assignment of ^{13}C n.m.r. spectra for a number of spirostan derivatives;[143] data for sixteen (25*R*)-5α-spirostanes have been tabulated.[144] Other compounds for which ^{13}C spectra have been assigned include all 26 isomeric methyl 5α-cholan-24-oates that possess one, two, or three hydroxyl groups at positions 3,7 and 12,[145] epimeric 22,23-epoxy derivatives of sterols,[146] over a hundred sterols and triterpene alcohols,[147] a series of 3-methyl-substituted 5α-cholestanes with 3α- and 3β-OH, -Cl, -F, and -OAc (as well as discussion of carbon–halogen stretching frequencies in the infrared spectra),[148] some bromo-derivatives of 5α-cholestan-3-one and cholest-4-en-3-one,[149] 6-methylated steroids,[150] and 3β-acetoxypregn-5-en-20-one derivatives in which there is an additional ring fused at C-16 and C-17.[151]

The exact fate of hydrogen atoms originating in [2-^{13}C, $^2H_3$]acetate in the biosynthesis of sitosterol has been studied by ^{13}C n.m.r.[152] Earlier assignments of ^{13}C n.m.r. spectra for ecdysterone, polypodiene B, and pterosterone have been revised on the basis of 2D spectroscopy.[153] Carbon-13 n.m.r. has been used to determine the temperature dependence of molecular motions in liquid-crystalline and isotropic liquid phases of some cholesteryl esters.[154]

4 Other Spectroscopic Methods

Methoxymethyl-protected hydroxyl groups are characterized by three strong absorption bands in the infrared, due to coupled vibrations of the $H_3C–O–CH_2–O–C$ group, in the region 1200–1000 cm^{-1}.[155] Differences have been observed between the infrared spectra of some B-nor-steroids and the corresponding normal steroids.[156]

A transient triplet excited species (lifetime *ca.* 10 μs) has been detected from the laser flash excitation of 17β-hydroxyandrosta-4,6-dien-3-one at 355 nm. It has been suggested that this may be the species that is involved in photocycloaddition of olefins to the dienone.[157] The fluorescence characteristics of ergosterol in hydrocarbon matrices at low temperatures[158] and the fluorescence characteristics of ergosta-5,7,9(11),22-tetraen-3β-ol and cholesta-5,7,9(11)-trien-3β-ol have been reported.[159]

Cotton effects ($n \rightarrow \pi^*$) for over 30 saturated and α,β-unsaturated steroidal ketones in the strongly associating solvent 1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP) show differences from the spectra in heptane which roughly parallel those previously observed for 2,2,2-trifluoroethanol. Some Cotton effects are strongly enhanced, some others are only weakly effected, while those in a third group are reversed in sign. All show blue-shifts of the circular dichroism maximum in HFIP. The results may be partially and qualitatively rationalized in terms of differing contributions of structural features to the

Cotton effect, according to the strength of association with the solvent, and the weaker solvation of those ketones which are subject to most steric hindrance.[160]

The '1,2-glycol' component of the side-chain of 5β-cholestane-3α,7α,12α,25,26-pentaol, which is a product of impaired biosynthesis of bile acids, has the (25*S*)-configuration. This was established from the negative sign of the Cotton effect that was induced in the circular dichroism spectrum in the presence of the lanthanide reagent [Eu(fod)$_3$], and was confirmed by comparison of the ^{13}C chemical shifts of carbon atoms in the side-chain with those for related compounds whose structure had previously been established by *X*-ray crystallography.[161] Measurements of rotational strength at 303, 273, and 208 nm have been used to study the binding of testosterone to human serum albumin and the effects of varying pH and concentrations of urea and salt which alter the conformations of the protein.[162] Circular dichroism studies have also been reported for the pentadienolide chromophore in bufadienolides,[163] to quantify some corticosteroids,[164] and for steroidal 4-en-3-ones in drug preparations.[165]

Free-radical species that are generated in single crystals of cholest-4-en-3-one by irradiation with *X*-rays have been characterized by e.s.r. and ENDOR methods. Three distinct free radicals were recognized, one being formed by hydrogen abstraction (from C-6), another by addition of hydrogen (to oxygen), and the third probably by abstraction of the methyl group C-19.[166] Electron spin resonance spectroscopy of the radicals that were generated by γ-irradiation of a single crystal of androst-4-ene-3,17-dione indicated that hydrogen atoms are abstracted, leaving the unpaired electron in an orbital that is delocalized over C-6, C-4 and O-3; the spectrum showed appropriate hyperfine splitting.[167] γ-Irradiation of crystals of testosterone monohydrate[168] and of 17β-hydroxy-5α-androstan-3-one[169] also gave radicals by loss of hydrogen atoms. An e.s.r. study of γ-irradiated cholesterol, and some of its derivatives, confirms that the ring B allylic radical and C-25 radical are the main species formed. In the presence of oxygen the corresponding peroxy-radicals are produced; these are the species that are believed to be implicated in the formation of stable auto-oxidation products in solid cholesterol.[170] The nitroxide spin-probe 3-doxylcholestane has been incorporated into liquid crystals for e.s.r. and ENDOR study of their ordering and dynamic behaviour.[171]

5 Mass Spectrometry and Gas Chromatography–Mass Spectrometry

An authoritative and wide-ranging review of mass spectrometry applied to steroid and peptide research discusses its use for analysis of urinary and plasma steroids, including the g.c.–m.s. of derivatized steroids, their characteristic fragmentations, and the use of selected ion monitoring with deuteriated internal standards for quantification.[172] Gas chromatography–mass spectrometry with selected ion monitoring has been applied to the determination of androgens, with 19-$^2H_3$-labelled internal standards.[173] A special issue of *Steroids*, dedicated to vitamin D, includes a review of methods for assay of hydroxylated metabolites in serum or plasma. Most methods so far used have depended upon saturation analysis, using a binding protein which may not, however, be specific in its affinity. A reliable procedure based on gas chromatography–mass fragmentography, with deuteriated internal standards, is described. Although too costly in equipment to be suitable for routine assays, the g.c.–m.s. method allows accurate evaluation and calibration of the other assay procedures which are available.[174] Recent developments in this field are discussed in another paper from the same group.[175] Desorption-chemical-ionization (DCI) mass spectrometry has been used to quantify vitamin D sulphate in human milk.[176]

The daughter-ion spectra of $[M+H]^+$ ions derived from tandem mass spectrometry of steroid glucuronides, generated by fast atom bombardment (FAB), are sensitive to experimental parameters, especially analyte concentrations.[177] FAB mass spectra of some steroidal oligoglycosides that contain two to four sugar units gave intense $[M+H]^+$ ions. Collision-activated mass spectra of these mass-selected ions yield information on the sequence of the oligoglycoside while avoiding interference from impurities.[178] Negative ions that are produced by FAB from salts of bile acids show little fragmentation, and hence give limited structural information. Collision-activated decomposition spectra, in contrast, give products of fragmentation remote from the charged site, which can be useful in assigning detailed structures and for quantifying individual components of a mixture of bile salts.[179]

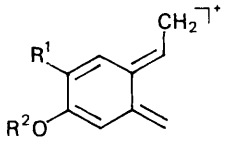
Gas chromatography–mass spectrometry with chemical ionization (by NH_3) and negative-ion scanning gives good mass spectra of steryl esters, allowing identification of both the sterol and the acyl components.[180] The mass-spectrometric identification of saturated and unsaturated sterols has been reviewed.[181] The negative-ion mass spectra of some brassinosteroids show intense molecular ions: a curious $[M-4]^-$ ion appears for such compounds if there is a vicinal diol system in the side-chain.[182] The high-temperature (up to 350 °C) g.c.–m.s. of derivatized ecdysteroids and steryl esters has been reviewed.[183] Other reports provide g.c.–m.s. data for trimethylsilylated ecdysteroids[184] and g.c. retention data for 56 bile acids of the 5α- and the 5β-series, derivatized as their methyl ester or their trimethylsilyl or ethyldimethylsilyl ethers.[185] Positive- and negative-ion mass spectra have been obtained by laser-desorption–Fourier-transform mass spectrometry from α-solanine, α-tomatine, and three cardenolide glycosides.[186]

Pentafluorobenzyl (PFB) esters of bile acids give good negative-ion mass spectra (g.c.–m.s.) in which the $[M-181]^-$ ion, resulting from loss of the PFB group, is most abundant. Further derivatization of hydroxyl groups in the PFB esters (as ethyldimethylsilyl ethers) allowed the common bile acids to be separated by gas chromatography.[187] 3,5-Bis(trifluoromethyl)-benzoyl derivatives of steroid alcohols are detectable in electron-capture negative-ion chemical-ionization mass spectra to as little as 1 pg, and have been proposed for the quantitative analysis of mixtures of steroids by g.c.–m.s. These esters formed at skeletal hydroxyl groups normally give the molecular ion as the base peak.[188] (2-Cyanoethyl)dimethylsilyl (CEDMS) derivatives of hydroxyl groups are suitable for g.c. analysis with the very sensitive nitrogen–phosphorus detector. The mass-spectral fragmentation of the CEDMS derivative of cholesterol has been illustrated.[189] Mass spectrometry has been used to characterize 16-amino-oestrogen derivatives[190] and metabolites of the aromatase inhibitor 4-hydroxyandrost-4-ene-3,17-dione.[191] Fragment ions (24), formed *via* a retro-Diels–Alder reaction, were observed in the mass spectra of a series of oestra-1,3,5(10)-trienes.[192] Protium–deuterium exchanges were observed in the E.I. mass spectra of the steroidal amides (25).[193]

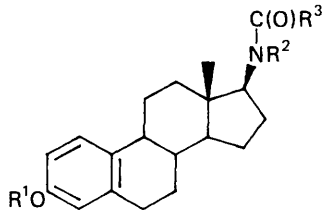
The translational energy that is released when a methyl group (C-18 or C-19) is lost from unsaturated steroids has been investigated.[194] The translational energy that is released during the loss of the methyl group C-19 of cholesterol and related sterols is greater if dehydration occurs first. The effect is less pronounced for C-18.[195]

6 High-Performance Liquid Chromatography (H.P.L.C.) and Liquid Chromatography–Mass Spectrometry (L.C.–M.S.)

A new and detailed review of analytical procedures for steroids concentrates on methods that are aimed at determining, in a single assay, the full range of steroids present in plasma or urine samples taken from patients suffering from a variety of clinical disorders and congenital hormone deficiencies. Steroid 'profiles' that have been obtained by h.p.l.c., preferably in conjunction with m.s., are illustrated.[196] The hormonal steroids and their metabolites are the subjects of another review of h.p.l.c. methods for their separation and quantitation in the



(24) R^1 = Ac, HO, CHO, or MeO
R^2 = H or Me



(25) R^1 = Me, D, $(CD_3)_2CH$, or $CD_3C(O)$
R^2 = D, H, or Me
R^3 = H or Me

clinical context, with practical examples given to illustrate the techniques employed.[197]

The separation of isotopically labelled from unlabelled steriods by h.p.l.c. has been used as the basis for isotope-dilution analysis of various hormonal steroids and their metabolites in serum. The labelled internal standards contained six or more deuterium atoms, which had been introduced by chemical exchange, and were easily separated from the natural substrates; peaks were quantified by conventional immuno-assay methods.[198]

Ferrocenoyl azide reacts readily with steroidal alcohols and other alcohols to give urethanes which are suitable for separation by h.p.l.c., with electrochemical detection, showing maximum sensitivity at +0.4 V *vs* a silver–silver chloride reference electrode, and a detection limit of 0.5 pmol.[199] In another h.p.l.c. application of the electrochemical detection of ferrocene, 2-ferrocenylethylamine has been condensed with the carboxyl group of steroid glucuronides. The resulting ferrocenyl amides are also detectable to a limit of *ca.* 0.5 pmol.[200]

Separations of ercalcidiol and calcidiol (25-hydroxy-vitamins D_2 and D_3) by automated h.p.l.c. use an ultraviolet-absorbing internal standard.[201] Increased resolution of steroid mixtures has been achieved by h.p.l.c. at low temperatures; with MeCN–MeOH as the mobile phase, a reverse-phase column could be used at temperatures as low as −50 °C.[202] Optical resolution of racemic norgestrel, achieved by h.p.l.c. with γ-cyclodextrin as a chiral additive in the mobile phase,[203] provides a method that is likely to find wider application. A β-cyclodextrin column has been used for separation of epimeric steroids by h.p.l.c.[204] Equations have been developed for predicting reversed-phase h.p.l.c. retention data for steroids.[205]

Thermospray l.c.–m.s. of steroids, and of other classes of compounds of biomedical interest, with mixed aqueous solvent systems that contain 0.1 mol dm^{-3} of ammonium acetate generally gives a positive-ion spectrum in which there is a strong $(M+H)^+$ peak.[206] In the case of highly hydroxylated steroids, a series of peaks, with masses $(M+H-18n)^+$, correspond to sequential losses of up to n molecules of water. Corticosteroids that contain the dihydroxyacetone side-chain are characterized by the $(M+H-60)^+$ ion, resulting from loss of the whole side-chain, as the base peak. Steroid glucuronides give negative-ion spectra comprising the $(M-H)^-$ ion, with only very weak peaks for fragment ions, whereas their positive-ion spectra include $(M+NH_4)^+$ peaks and show the usual losses of water molecules. Another report on thermospray negative-ion mass spectrometry of steroid glucuronides, in which a salt-free aqueous solvent system was used, shows that they give molecular anions only: with gradient-elution h.p.l.c. and selected ion monitoring, mixtures of glucuronides can be analysed with a detection limit of 100 pg.[207]

The thermospray method is excellent for selected ion monitoring.[206] Thermospray l.c.–m.s., with dilution with a stable isotope, provides a fast quantitative assay for cortisol in serum, although the reliability of the results has not yet reached the high level that can be achieved by the more tedious g.c.–m.s. method.[208] The use of thermospray l.c.–m.s., in combination with isotope dilution, for the quantitative analysis of various compounds, including cortisol, testosterone, and 1,25-dihydroxyvitamin D_3 is described in a short review.[209] Thermospray l.c.–m.s. has also been applied effectively to the identification of aldosterone, 18-hydroxycorticosterone, and related metabolites from rat adrenals. Most of the mass spectra are dominated by either $[M+H]^+$ or $[M-H_2O+H]^+$ ions, with little other fragmentation.[210] Liquid chromatography–mass spectrometry, with gradient elution, has been applied to the study of bile acids.[211] Statistical methods have been applied to the selection of optimized solvent systems for two-dimensional t.l.c. of steroids.[212]

7 Immunoassay, and Miscellaneous Physical Methods

Applications of aminophthalhydrazides, aminonaphthohydrazides, and acridinium esters as labels for luminescence immunoassays of steroids[213] and chemiluminescence immunoassays of steroids and peptide hormones in body fluids[214] have been reviewed. *N*-(Fluoranthen-3-yl) maleimide and its derivatives can serve as fluorescent tracers in immunoassays for progesterone.[215]

With chelated europium(III) as the label for either the antibody or the antigen, time-resolved fluorescence immunoassays have been developed for progesterone and oestradiol; solid-phase supports were used, to simplify the experimental procedure.[216] Sensitivity is high. A time-resolved fluoro-immunoassay for testosterone uses antibodies that are conjugated to isothiocyanatophenyldiethylenetriaminepenta-acetic acid–europium chelate as a marker.[217] The separated (*Z*)- and (*E*)-isomers of the 3-carboxymethyl-oxime of 11-deoxycortisol gave antisera with enhanced specificity and low cross-reactivity; the (*Z*)-isomer was particularly effective.[218]

Electron diffraction has been used to study the mesomorphic behaviour of cholesteryl myristate.[219] A theoretical and experimental study has been made of the optical properties of blue phases of cholesteryl nonanoate.[220] The uses of polarizing microscopy, differential scanning calorimetry, ^{13}C n.m.r., *X*-ray diffraction, and other techniques to study phase characteristics of cholesteryl esters have been reviewed.[221]

Some cardiac glycosides which adsorb strongly on Hg electrodes may be determined quantitatively, at nanomole sensitivity, by adsorptive stripping voltammetry.[222] A novel microdetermination of mercury (as Hg^{2+}) depends upon its ability to inhibit the oxidation of deoxycholic acid by 3α-hydroxysteroid dehydrogenase.[223] The natural level of ^{13}C in cholesterol from human food, tissues, and serum showed little difference; no isotope fractionation was observed during its isolation and analysis. However, there were unexplained variations in the ^{13}C content of cholesterol in prawns from different parts of the world![224]

8 References

1 D. N. Kirk, *Nat. Prod. Rep.*, 1986, **3**, 505.
2 'The Bile Acids: Methods and Applications' (Volume 4 in the series 'The Bile Acids: Chemistry, Physiology, and Metabolism'), ed. K. D. R. Setchell, D. Kritchevsky, and P. P. Nair, Plenum, New York, 1988.
3 'Sterols and Bile Acids', ed. H. Danielsson and J. Sjövall, Elsevier Amsterdam, 1985.
4 W. L. Duax, J. F. Griffin, and D. C. Rohrer, *Stud. Org. Chem. (Amsterdam)*, 1985, **20**, 385.
5 M. W. Barrett, R. D. Farrant, D. N. Kirk, J. D. Mersh, J. K. M. Sanders, and W. L. Duax, *J. Chem. Soc., Perkin Trans. 2*, 1982, 105.
6 J. M. Adelantado and W. G. Richards, *J. Chem. Soc., Perkin Trans. 2*, 1986, 1253.
7 L. Carballeira and M. A. Rios, *An. Quim., Ser. A.*, 1986, **82**, 46.
8 M. Bohl, H. Kasch, and I. S. Levina, *Z. Chem.*, 1986, **26**, 378.
9 K. Bischofberger, J. R. Bull, J. L. M. Dillen, and P. H. van Rooyen, *S. Afr. J. Chem.*, 1987, **40**, 123.
10 V. J. van Geerestein, J. A. Kanters, P. van der Sluis, and J. Kroon, *Acta Crystallogr., Sect. C*, 1986, **42**, 1521.
11 S. R. Byrn and D. W. Kessler, *Tetrahedron*, 1987, **43**, 1335.
12 P. A. Sutton and S. R. Byrn, *J. Pharm. Sci.*, 1987, **76**, 253.
13 R. B. Hochberg, J. E. Zielinski, W. L. Duax, and P. Strong, *J. Steroid Biochem.*, 1986, **25**, 615.
14 J. L. M. Dillen, *Acta Crystallogr., Sect. C*, 1986, **42**, 1204.
15 V. J. van Geerestein, *Acta Crystallogr., Sect. C*, 1987, **43**, 1206.
16 V. J. van Geerestein, J. A. Kanters, and J. Kroon, *Acta Crystallogr., Sect. C*, 1987, **43**, 2398.
17 V. J. van Geerestein, A. J. M. Duisenberg, M. H. G. Duits, J. A. Kanters, and J. Kroon, *Acta Crystallogr., Sect. C*, 1987, **43**, 2402.
18 V. M. Kolb, D. H. Hua, and W. L. Duax, *NIDA Res. Monogr.*, 1986, **75**, 45.
19 V. M. Kolb, D. H. Hua, and W. L. Duax, *J. Org. Chem.*, 1987, **52**, 3003.
20 H. C. Chang, R. Popovitz-Biro, M. Lahav, and L. Leiserowitz, *J. Am. Chem. Soc.*, 1987, **109**, 3883.
21 J. R. Bull, J. L. M. Dillen, and L. M. Steer, *S. Afr. J. Chem.*, 1987, **40**, 155.
22 T. Ojasoo, J. Delettré, J. P. Mornon, C. Turpin-Van Dycke, and J. P. Raymond, *J. Steroid Biochem.* 1987, **27**, 255.
23 W. L. Duax and J. F. Griffin, in 'Receptor Mediated Antisteroid Action', ed. M. K. Agarwal, Walter de Gruyter, Berlin, 1987.
24 P. Kaspar and H. Witzel, *J. Steroid Biochem.* 1985, **23**, 259.
25 P. Kaspar and H. Witzel, *J. Steroid Biochem.*, 1985, **23**, 611.
26 M. Bohl, *Z. Naturforsch., Sect. C*, 1987, **42**, 221.
27 C. Chang and S. Liao, *J. Steroid Biochem.*, 1987, **27**, 123.
28 L. Tan and P. Rousseau, *Biochem. Biophys. Res. Commun.*, 1987, **147**, 1259.
29 M. Bohl, G. Kaufmann, M. Hübner, G. Reck, and R. G. Kretschmer, *J. Steroid Biochem.*, 1985, **23**, 895.
30 H. O. Hoppen and P. Hammann, *Acta Endocrinol. (Copenhagen)*, 1987, **115**, 406.
31 J. R. Bull, *S. Afr. J. Chem.*, 1985, **38**, 155.
32 H. Morales-Alanis, M. J. Brienne, J. Jacques, M. M. Bouton, L. Nedelec, V. Torelli and C. Tournemine, *J. Med. Chem.*, 1985, **28**, 1796.
33 M. Yamakawa, K. Ezumi, M. Shiro, H. Nakai, S. Kamata, T. Matsui, and N. Haga, *Mol. Pharmacol.*, 1986, **30**, 585.
34 P. M. Stewart, A. M. Wallace, R. Valentino, D. Burt, C. H. L. Shackleton, and C. R. W. Edwards, *Lancet*, 1987, 821.
35 A. Kuliopulos, E. M. Westbrook, P. Talalay, and A. S. Mildvan, *Biochemistry*, 1987, **26**, 3927.
36 D. S. Fullerton, K. Ahmed, A. H. L. From, R. H. McParland, D. C. Rohrer, and J. F. Griffin, *Top. Mol. Pharmacol.*, 1986, **3**, 257.
37 M. Fránek, *J. Steroid Biochem.*, 1987, **28**, 95.
38 G. Reck, M. Hübner, M. Bohl, and P. Leibnitz, *Cryst. Res. Technol.*, 1986, **21**, 1041.
39 G. Reck, M. Hübner, and D. Pfeiffer, *Cryst. Res. Technol.*, 1986, **21**, 1185.
40 G. Reck, M. Hübner, M. Bohl, G. Bannier and R. G. Kretschmer, *Cryst. Res. Technol.*, 1986, **21**, 1035.
41 J. H. Bierte, R. Prewo, J. L. Brianso, and J. F. Piniella, *Acta Crystallogr., Sect. C*, 1985, **41**, 1530.
42 Z. Galdecki, P. Grochulski, Z. Wawrzak, W. L. Duax, M. Numazawa, and Y. Osawa, *Acta Crystallogr., Sect. C*, 1987, **43**, 967.
43 G. Reck, M. Hübner, G. Bannier, and P. Scharfenberg, *Cryst. Res. Technol.*, 1986, **21**, 1191.
44 V. J. van Geerestein, J. A. Kanters, and J. Kroon, *Acta Crystallogr., Sect. C*, 1987, **43**, 319.
45 M. H. Dzafarov, M. V. Dodonov, S. N. Ananchenko, A. S. Platonova, and S. P. Ionov, *Bioorg. Khim.*, 1986, **12**, 970.
46 M. H. Dzafarov, M. V. Dodonov, S. N. Ananchenko, A. V. Platonova, and S. P. Ionov, *Bioorg. Khim.*, 1986, **12**, 981.
47 D. Schomburg, M. Thielmann, and E. Winterfeldt, *Tetrahedron Lett.*, 1986, **27**, 5833.
48 N. M. Galitskii, G. A. Yasnitskii, V. A. Gritsuk, L. G. Lis, and F. A. Lakhvich, *Zh. Strukt. Khim.*, 1987, **28**, 98.
49 M. G. B. Drew, J. Mann, and B. Pietrzak, *J. Chem. Soc., Perkin Trans. 1*, 1985, 1049.
50 M. Przbylska and E. R. Ahmed, *Acta Crystallogr., Sect. C*, 1985, **41**, 1467.
51 S. P. Douglas, J. F. Sawyer, and P. Yates, *Acta Crystallogr., Sect. C*, 1987, **43**, 1372; *Tetrahedron Lett.*, 1985, **26**, 5955.
52 D. Miljkovic, K. Gasi, M. Kindjer, S. Stankovic, and G. Argay, *Tetrahedron*, 1987, **43**, 631.
53 G. Precigoux, F. Leroy, B. Arreguy-San Miguel, and B. Delmond, *Acta Crystallogr., Sect. C*, 1987, **43**, 730.
54 X. Solanz, J. F. Piniella, J. L. Brianso, and C. Miravitlles, *Acta Crystallogr., Sect. C*, 1987, **43**, 2372.
55 P. Gluzinski, J. W. Krajewski, Z. Urbanczyk-Lipkowska, G. Argay, and L. S. Parkanyi, *Acta Crystallogr., Sect. C*, 1985, **41**, 1630.
56 F. R. Ahmed and C. P. Huber, *Acta Crystallogr., Sect. C*, 1986, **42**, 1596.
57 D. Pfeiffer, J. Weiland, and P. Leibnitz, *Cryst. Res. Technol.*, 1987, **22**, 389.
58 V. J. van Geerestein and J. A. Kanters, *Acta Crystallogr., Sect. C*, 1987, **43**, 136.
59 V. J. van Geerestein and J. A. Kanters, *Acta Crystallogr., Sect. C*, 1987, **43**, 936.
60 S. I. Cho and B. M. Craven, *Acta Crystallogr., Sect. C*, 1987, **43**, 917.
61 A. E. Kapul'skii, V. I. Simonov, I. S. Levina, L. E. Kulikova, and A. V. Kamernitskii, *Kristallografiya*, 1985, **30**, 1096.
62 V. J. van Geerestein, *Acta Crystallogr., Sect. C*, 1987, **43**, 2232.
63 V. J. van Geerestein, J. A. Kanters, A. J. M. Duisenberg, and J. Kroon, *Acta Crystallogr., Sect. C.* 1986, **42**, 469.
64 V. J. van Geerestein, *Acta Crystallogr., Sect. C*, 1987, **43**, 2233.
65 C. Hermann, G. A. Hoyer, G. Neef, D. Schomburg, and R. Weichert, *Liebigs Ann. Chem.*, 1986, 1317.
66 G. Reck and G. Langbein, *Cryst. Res. Technol.*, 1985, **20**, 1643.
67 B. K. Kang, M. J. Chung, and Y. J. Park, *Bull. Korean Chem. Soc.*, 1985, **6**, 333.
68 S. I. Cho and B. M. Craven, *J. Lipid Res.*, 1987, **28**, 80.
69 Q. Gao and B. M. Craven, *J. Lipid Res.*, 1986, **27**, 1214.
70 B. Dubini, P. Mariani, M. G. Ponzi-Bossi, F. Rustichelli and S. Melone, *Nuovo Cimento Soc. Ital. Fis. D*, 1987, **9**, 86.
71 H. Tamura, T. Sato, K. Ogawa, S. Yano, K. Sakaguchi, and Y. Matsuura, *Chem. Lett.*, 1987, 503.
72 A. P. Polishchuk, M. Yu. Antipin, T. V. Timofeeva, V. I. Kulishov, and Yu. T. Struchkov, *Kristallografiya*, 1986, **31**, 671.
73 A. Uomori, S. Seo, T. Sato, Y. Yoshimura, and K. Takeda, *J. Chem. Soc., Perkin Trans. 1*, 1987, 1713.
74 U. Maitra and R. Breslow, *Tetrahedron Lett.*, 1986, **27**, 3087.
75 G. R. Pettit, D. L. Herald, C. L. Herald, W. C. M. C. Kokke, and C. Djerassi, *Steroids*, 1986, **47**, 321.
76 T. K. Chattopadhyay, J. N. Lisgarten, R. A. Palmer, H. Singh, and R. K. Malhotra, *J. Crystallogr. Spectrosc. Res.*, 1987, **17**, 51.
77 B. Tinant, J. P. Declercq, M. van Meerssche, L. Lorenc, and M. L. Mihailovic, *Bull. Soc. Chim. Belg.*, 1987, **96**, 35.
78 F. Brisse, M. J. Olivier, G. Cavallo, and D. Gravel, *Acta Crystallogr., Sect. C*, 1986, **42**, 1833.
79 W. Reischl, H. Bernhard, C. Kratky, and E. Zbiral, *Monatsh. Chem.*, 1985, **116**, 831.
80 H. Bernhard, C. Kratky, W. Reischl, and E. Zbiral, *Monatsh. Chem.*, 1985, **116**, 1221.
81 P. F. Lindley and M. C. Carey, *J. Crystallogr. Spectrosc. Res.*, 1987, **17**, 231.
82 A. R. Campanelli, S. Candeloro de Sanctis, E. Giglio, and L. Scaramuzza, *J. Lipid Res.*, 1987, **28**, 483.
83 M. Soriano-Garcia, I. Lopez y Celis, R. A. Toscano, J. M. Barba Chavez, P. Enriquez, R. A. Hernandez, and A. Rodriguez, *Acta Crystallogr., Sect. C.*, 1987, **43**, 1163.
84 A. Kalman, G. Argay, B. Ribar, D. Zivanov-Stakic, and S. Vladimirov, *Acta Crystallogr., Sect. C*, 1985, **41**, 1645.
85 D. Pfeiffer, L. Kutschabsky, R. G. Kretschmer, F. Coll, and G. Adam, *Z. Chem.*, 1985, **25**, 183.

86 G. Ferguson, B. Kaitner, A. F. Somogyvari, V. I. Bendall, W. B. Whalley, C. L. Yeats, R. Edmunds, and J. M. Midgley, *J. Chem. Soc., Perkin Trans. 1*, 1985, 1337.
87 D. Scharfenberg-Pfeiffer, E. Hoehne, and M. Wunderwald, *Cryst. Res. Technol.*, 1987, **22**, 1403.
88 H. Wiedenfeld, F. Knoch, I. Naidis, and B. Kopp, *Sci. Pharm.*, 1987, **55**, 167.
89 G. Argay, A. Kalman, B. Ribar, S. Vladimirov, and D. Zivanov-Stakic, *Acta Crystallogr., Sect. C*, 1987, **43**, 922.
90 L. R. Nassimbeni, M. L. Niven, D. A. Stuart, and K. J. Zemke, *J. Crystallogr. Spectrosc. Res.*, 1986, **16**, 557.
91 K. Miki, N. Kasai, H. Tsutsumi, M. Miyata, and K. Takemoto, *J. Chem. Soc., Perkin Trans. 1*, 1987, 545.
92 E. Giglio, F. Mazza, and L. Scaramuzza, *J. Inclusion Phenom.*, 1985, **3**, 437.
93 K. Padmanabhan, V. Ramamurthy, and K. Venkatesan, *J. Inclusion Phenom.*, 1987, **5**, 315.
94 W. R. Croasmun and R. M. K. Carlson, *Methods Stereochem. Anal.*, 1987, **9** (Two-dimensional NMR Spectroscopy), 387.
95 H. J. Schneider, U. Buchheit, N. Becker, G. Schmidt, and U. Siehl, *J. Am. Chem. Soc.*, 1985, **107**, 7027.
96 K. Marat, J. F. Templeton, and V. P. S. Kumar, *Magn. Reson. Chem.*, 1987, **25**, 25.
97 K. Marat, J. F. Templeton, R. K. Gupta, and V. P. S. Kumar, *Magn. Reson. Chem.*, 1987, **25**, 730.
98 B. G. Carter, D. N. Kirk, and P. J. Burke, *J. Chem. Soc., Perkin Trans. 2*, 1987, 1247.
99 M. Harnik, Y. Kashman, S. Carmely, M. Cojocaru, S. L. Dale, M. M. Holbrook, and J. C. Melby, *Steroids*, 1986, **47**, 67.
100 D. V. Waterhous, S. Barnes, and D. D. Muccio, *J. Lipid Res.*, 1985, **26**, 1068.
101 M. Campredon, V. Quiroa, A. Thevand, A. Allouche, and G. Pouzard, *Magn. Reson. Chem.*, 1986, **24**, 624.
102 L. E. Kay and J. H. Prestegard, *J. Am. Chem. Soc.*, 1987, **109**, 3829.
103 E. Mukidjam, S. Barnes, and G. A. Elgavish, *J. Am. Chem. Soc.*, 1986, **108**, 7082.
104 E. Mukidjam, G. A. Elgavish, and S. Barnes, *Biochemistry*, 1987, **26**, 6785.
105 T. Iida and T. Shinohara, *Nihon Daigaku Kogakubu Kiyo, Bunrui A*, 1987, **28**, 175.
106 A. G. J. Sedee, G. M. J. Beijersbergen van Henegouwen, M. E. de Vries, and C. Erkelens, *Steroids*, 1985, **45**, 101.
107 K. Bischofberger, J. R. Bull, and A. A. Chalmers, *Magn. Reson. Chem.*, 1987, **25**, 780.
108 A. G. J. Sedee, G. M. J. Beijersbergen van Henegouwen, W. Guijt, and C. A. G. Haasnoot, *Pharm. Weekbl., Sci. Ed.*, 1985, **7**, 202.
109 A. G. J. Sedee, G. M. J. Beijersbergen van Henegouwen, W. Guijt, and C. A. G. Haasnoot, *J. Org. Chem.*, 1985, **50**, 4182.
110 V. J. Robinson, A. D. Bain, and C. A. Rodger, *Steroids*, 1986, **48**, 267.
111 E. T. K. Haupt, *Spectrosc. Lett.*, 1986, **19**, 1091.
112 S. Top, G. Jaouen, A. Vessières, J.-P. Abjean, D. Davoust, C. A. Rodger, B. G. Sayer, and M. J. McGlinchey, *Organometallics*, 1985, **4**, 2143.
113 M. Savignac, G. Jaouen, C. A. Rodger, R. E. Perrier, B. G. Sayer, and M. J. McGlinchey, *J. Org. Chem.*, 1986, **51**, 2328.
114 N. J. Koszewski, T. A. Reinhardt, D. C. Beitz, J. L. Napoli, E. G. Baggiolini, M. R. Uskokovic, and R. L. Horst, *Anal. Biochem.*, 1987, **162**, 446.
115 B. Helmer, H. K. Schnoes, and H. F. Deluca, *Arch. Biochem. Biophys.*, 1985, **241**, 608.
116 A. G. Avent, J. R. Hanson, Y. Z. Ling, and I. H. Sadler, *J. Chem. Soc., Perkin Trans. 1*, 1985, 2129.
117 S. A. Campbell, T. A. Crabb, and R. O. Williams, *Magn. Reson. Chem.*, 1986, **24**, 803.
118 H. Takagi, T. Hayakawa, T. Miura, and M. Kimura, *Chem. Pharm. Bull.* 1985, **33**, 3224.
119 H. Takagi, T. Hayakawa, T. Miura, and M. Kimura, *Chem. Pharm. Bull.*, 1985, **33**, 3317.
120 P. N. Rao, A. M. Rodriguez, and D. W. Miller, *J. Steroid Biochem.*, 1986, **25**, 417.
121 R. D. Dawe and J. L. C. Wright, *Can. J. Chem.*, 1987, **65**, 666.
122 S. M. Koll, M. Vuilhorgne, B. Dang Vu, D. Ba, S. Bourdon and R. Bourdon, *Tetrahedron Lett.*, 1986, **27**, 2613.
123 J. Drew, J. R. Brisson, P. Morand, and A. G. Szabo, *Can. J. Chem.*, 1987, **65**, 1784.
124 T. Akihisa, S. Thakur, F. U. Rosenstein, and T. Matsumoto, *Lipids*, 1986, **21**, 39.
125 G. G. Habermehl and P. E. Hammann, *Z. Naturforsch., Teil B*, 1985, **40**, 656.
126 P. S. Steyn, F. R. van Heerden, R. Vleggaar, and L. A. P. Anderson, *J. Chem. Soc., Perkin Trans. 1*, 1986, 1633.
127 P. S. Steyn, R. F. van Heerden, and R. Vleggaar, *S. Afr. J. Chem.*, 1986, **39**, 143.
128 J. M. Bernassau, M. Fetizon, L. Hanna, and J. A. Pinheiro, *J. Phys. Chem.*, 1986, **90**, 3941.
129 R. E. Jacobs, J. Singh, and L. E. Vickery, *Biochemistry*, 1987, **26**, 4541.
130 P. B. Reese, L. A. Trimble, and J. C. Vederas, *Can. J. Chem.*, 1986, **64**, 1427.
131 R. S. Chana, R. J. Cushley, S. R. Wassall, I. C. P. Smith, and E. J. Dufourc, *Chem. Phys. Lipids*, 1985, **37**, 345.
132 R. Murari, M. P. Murari, and W. J. Baumann, *Biochemistry*, 1986, **25**, 1062.
133 E. J. Dufourc and I. C. P. Smith, *Chem. Phys. Lipids*, 1986, **41**, 123.
134 W. D. Treleaven, Y. I. Parmar, H. Gorrissen, and R. J. Cushley, *Biochim. Biophys. Acta*, 1986, **877**, 198.
135 H. K. Gill, R. W. Smith, and D. A. Whiting, *J. Chem. Soc., Chem. Commun.*, 1986, 1457.
136 R. A. Fletton, R. K. Harris, A. M. Kenwright, R. W. Lancaster, K. J. Packer, and N. Sheppard, *Spectrochim. Acta, Part A*, 1987, **43**, 1111.
137 A. Neszmelyi, W. E. Hull, G. Lukacs, and W. Voelter, *Z. Naturforsch., Teil B*, 1986, **41**, 1178.
138 J.-C. Gramain and J. C. Quirion, *Magn. Reson. Chem.*, 1986, **24**, 938.
139 A. M. Seldes, M. E. DeLuca, and E. G. Gros, *Magn. Reson. Chem.*, 1986, **24**, 185.
140 W. Robien, B. Kopp, D. Schabl, and H. Schwarz, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1987, **19**, 131.
141 G. G. Habermehl, P. E. Hammann, and V. Wray, *Magn. Reson. Chem.*, 1985, **23**, 959.
142 P. K. Agrawal, D. C. Jain, R. K. Gupta, and R. S. Thakur, *Phytochemistry*, 1985, **24**, 2479.
143 P. K. Agrawal and R. S. Thakur, *Indian J. Chem., Sect. B*, 1986, **25**, 469.
144 J. A. R. Garzía and H. T. V. Castro, *Magn. Reson. Chem.*, 1987, **25**, 831.
145 T. Iida, F. C. Chang, J. Goto, and T. Nambara, *Chem Phys. Lipids*, 1987, **45**, 1.
146 M. G. Sierra, D. A. Bustos, M. E. Zudenigo, and E. A. Rúveda, *Tetrahedron*, 1986, **42**, 755.
147 T. Akihisa and T. Matsumoto, *Yukagaku*, 1987, **36**, 301.
148 M. van Robays, R. Busson, and H. Vanderhaeghe, *J. Chem. Soc., Perkin Trans. 1*, 1986, 251.
149 J. Römer, D. Scheller, and G. Grossmann, *Magn. Reson. Chem.*, 1987, **25**, 135.
150 T. Iida, T. Tamura, and T. Matsumoto, *Magn. Reson. Chem.*, 1987, **25**, 558.
151 V. S. Bogdanov, E. G. Cherepanova, I. S. Levina, L. E. Kulikova and V. N. Ignatov, *Izv. Akad. Nauk SSSR, Ser. Khim.*, 1986, 2315.
152 S. Seo, U. Sankawa, H. Seto, A. Uomori, Y. Yoshimura, Y. Ebizuka, H. Noguchi, and K. Takeda, *J. Chem. Soc., Chem. Commun.*, 1986, 1139.
153 N. Nishimoto, Y. Shiobara, M. Fujino, S. Inoue, T. Takemoto, F. de Oliveira, G. Akisue, M. K. Akisue, G. Hashimoto, O. Tanaka, R. Kasai, and H. Matsuura, *Phytochemistry*, 1987, **26**, 2505.
154 D. H. Croll, D. M. Small, and J. A. Hamilton, *J. Chem. Phys.*, 1986, **85**, 7380.
155 S. Vašíčková, V. Pouzar, I. Cerný, P. Drašar, and M. Havel, *Collect. Czech. Chem. Commun.*, 1986, **51**, 90.
156 R. Schwartz, A. Juhasz, and P. Coltea, *Rev. Roum. Chim.*, 1986, **31**, 481.
157 D. I. Schuster, D. A. Dunn, and R. Bonneau, *J. Photochem.*, 1985, **28**, 413.
158 N. Nakashima, S. R. Meech, A. R. Auty, A. C. Jones, and D. Phillips, *J. Photochem.*, 1985, **30**, 207.
159 G. Smutzer, B. F. Crawford, and P. L. Yeagle, *Biochim. Biophys. Acta*, 1986, **862**, 361.
160 J. K. Gawroński, K. Gawrońska, and J. Koput, *Pol. J. Chem.*, 1986, **60**, 45.
161 B. Dayal, G. Salen, V. Toome, and G. S. Tint, *J. Lipid Res.*, 1986, **27**, 1328.
162 S. Watanabe, *J. Steroid Biochem.*, 1985, **23**, 177.
163 B. Green, F. Snatzke, G. Snatzke, G. P. Pettit, Y. Kamano, and M. L. Niven, *Croat. Chem. Acta*, 1985, **58**, 371.

164 A. Gergely, G. Szasz, and J. Soos, *Fresenius' Z. Anal. Chem.*, 1986, **323**, 157.
165 A. Gergely and G. Szasz, *Anal. Chem. Symp. Ser.*, 1985, **23**, 581.
166 M. F. Andersen, E. Sagstuen, T. Henriksen, and M. Lindgren, *J. Magn. Reson.*, 1987, **71**, 461.
167 R. Krzyminiewski, A. M. Hafez, A. Szyczewski, and J. Pietrzak, *J. Mol. Struct.*, 1987, **160**, 127.
168 A. Szyczewski, R. Krzyminiewski, and J. Pietrzak, *Ser. Fiz. (Uniw. im. Adama Mickiewicza Poznaniu)*, 1985, **53**, 201.
169 A. M. Hafez, R. Krzyminiewski, and J. Pietrzak, *Ser. Fiz. (Uniw. im. Adama Mickiewicza Poznaniu)*, 1985, **53**, 205.
170 C. L. Sevilla, D. Becker, and M. D. Sevilla, *J. Phys. Chem.*, 1986, **90**, 2963.
171 B. Kirste, *Z. Naturforsch., Teil A*, 1987, **42**, 1296.
172 C. H. L. Shackleton and W. Chai, *Endocrine Rev.*, 1985, **6**, 441.
173 T. Yamaguchi, K. Konno, K. Kawabe, K. Yasuda, H. Mori, T. Suzuki, T. Yanaihara, and T. Nakayama, *Endocrinol. Jpn.*, 1985, **32**, 279.
174 R. D. Coldwell, C. E. Porteous, D. J. H. Trafford, and H. L. J. Makin, *Steroids*, 1987, **49**, 155.
175 C. E. Porteous, R. D. Coldwell, D. J. H. Trafford, and H. L. J. Makin, *J. Steroid Biochem.*, 1987, **28**, 785.
176 N. Le Boulch, L. Cancela, L. Miravet, and C. Lange, *Biomed. Environ. Mass Spectrom.*, 1986, **13**, 53.
177 R. B. Cole, C. R. Guenat, and S. J. Gaskell, *Anal. Chem.*, 1987, **59**, 1139.
178 Y.-Z. Chen. N.-Y. Chen., H.-Q. Li, F.-Z. Zhao, and N. Chen, *Biomed. Environ. Mass Spectrom.*, 1987, **14**, 9.
179 K. B. Tomer, N. J. Jensen, M. L. Gross, and J. Whitney, *Biomed. Environ. Mass Spectrom.*, 1986, **13**, 265.
180 R. P. Evershed and L. J. Goad, *Biomed. Environ. Mass Spectrom.*, 1987, **14**, 131.
181 M. Dumazer, M. Farines, and J. Soulier, *Rev. Fr. Corps Gras*, 1986, **33**, 151.
182 J. Schmidt, H. M. Vorbrodt, and G. Adam, *ZfI-Mitt.*, 1986, **115**, 165 (*Chem. Abstr.*, 1986, **105**, 191 470).
183 R. P. Evershed, M. C. Prescott, L. J. Goad, and H. H. Rees, *Biochem. Soc. Trans.*, 1987, **15**, 175.
184 T. Iida, T. Momose, T. Mamura, T. Matsumoto, J. Goto, T. Nambara, and F. C. Chang, *J. Chromatogr.*, 1987, **389**, 155.
185 R. P. Evershed, J. G. Mercer, and H. H. Rees, *J. Chromatogr.*, 1987, **390**, 357.
186 M. L. Coates and C. L. Wilkins, *Biomed. Environ. Mass Spectrom.*, 1986, **13**, 199.
187 J. Goto, K. Watanabe, H. Miura, T. Nambara, and T. Iida, *J. Chromatogr.*, 1987, **388**, 379.
188 A. Murray and D. Watson, *J. Steroid Biochem.*, 1986, **25**, 255.
189 M. J. Bertrand, S. Stefanidis, and B. Sarrasin, *J., Chromatogr.*, 1986, **351**, 47.
190 W. Schade, B. Schoenecker, and J. Vokoun, *J. Prakt. Chem.*, 1985, **327**, 589.
191 A. B. Foster, M. Jarman, J. Mann, and I. B. Parr, *J. Steroid Biochem.*, 1986, **24**, 607.
192 X.-Z. Yuan, *Org. Mass Spectrom.*, 1985, **20**, 792.
193 P. Longevialle, *Org. Mass Spectrom.*, 1985, **20**, 644.
194 Z. V. I. Zaretskii, Z. Kustanovich, E. E. Kingston, J. H. Beynon, R. Lauber, U. P. Schlunegger and C. Djerassi, *Org. Mass Spectrom.*, 1985, **20**, 471.
195 Z. V. I. Zaretskii, Z. Kustanovich, E. E. Kinston, J. H. Beynon, C. Djerassi, and L. Toekes, *Org. Mass Spectrom.*, 1986, **21**, 125.
196 C. H. L. Shackleton, *J. Chromatogr.*, 1986, **379**, 91.
197 J. W. Honour, in 'H.p.l.c. of Small Molecules', ed. C. K. Lim, IRL Press, Oxford, 1986, p. 117.
198 J. J. Pratt, *Ann. Clin. Biochem.*, 1986, **23**, 251.
199 K. Shimada, S. Orii, M. Tanaka, and T. Nambara, *J. Chromatogr.*, 1986, **352**, 329.
200 K. Shimada, E. Nagashima, S. Orii, and T. Nambara, *J. Pharm. Biomed. Anal.*, 1987, **5**, 361.
201 E. B. Mawer and J. T. Hann, *J. Chromatogr.*, 1987, **415**, 305.
202 S. U. Shikh and J. C. Touchstone, *J. Liq. Chromatogr.*, 1987, **10**, 2489.
203 M. Gazdag, G. Szepesi, and L. Huszar, *J. Chromatogr.*, 1986, **351**, 128.
204 J. Kirschbaum and L. Kerr, *LC Mag.*, 1986, **4**, 30.
205 S. D. West, *J. Chromatogr. Sci.*, 1987, **25**, 122.
206 D. J. Liberato, A. L. Yergey, N. Esteban, C. E. Gomez-Sanchez, and C. H. L. Shackleton, *J. Steroid Biochem.*, 1987, **27**, 61.
207 D. Watson, G. W. Taylor, and S. Murray, *Biomed. Environ. Mass Spectrom.*, 1986, **13**, 65.
208 S. J. Gaskell, K. Rollins, R. W. Smith, and C. E. Parker, *Biomed. Environ. Mass Spectrom.*, 1987, **14**, 717.
209 A. L. Yergey, N. V. Esteban, and D. J. Liberato, *Biomed. Environ. Mass Spectrom.* 1987, **14**, 623.
210 D. Watson, G. W. Taylor, S. Laird, and G. P. Vinson, *Biochem. J.*, 1987, **242**, 109.
211 Y. Ito, T. Takeuchi, D. Ishii, M. Goto, and T. Mizuno, *J. Chromatogr.*, 1986, **358**, 201.
212 D. Nurok, S. Habibi-Goudarzi, and R. Kleyle, *Anal. Chem.*, 1987, **59**, 2424.
213 W. Klingler, G. Wiegand, and R. Knuppen, *J. Steroid Biochem.*, 1987, **27**, 41.
214 G. J. R. Barnard, J. B. Kim, J. L. Williams, and W. P. Collins, *Biolumin. Chemilumin.: Instrum. Appl.*, 1985, **1**, 151.
215 C. Parini, M. A. Bacigalupo, S. Colombi, L. Ferrara, F. Franceschetti, and R. Saita, *Steroids*, 1985, **46**, 903.
216 T. N. E. Lövgren, *J. Steroid Biochem.*, 1987, **27**, 47.
217 E. J. Bertoft, O. K. Maentausta, and T. N. E. Lövgren, *Anal. Chem. Symp. Ser.*, 1985, **23**, 279.
218 H. Galons, J. Fiet, B. Gueux, R. Bonete, J. M. Villette, R. Julien, M. Miocque, and C. Dreux, *C.R. Acad. Sci., Ser.* 2, 1987, **304**, 75.
219 D. L. Dorset, *J. Lipid Res.*, 1985, **26**, 1142.
220 V. A. Belyakov, E. I. Demikhov, V. E. Dmitrienko, and V. K. Dolganov, *Zh. Eksp. Teor. Fiz.*, 1985, **89**, 2035.
221 G. S. Ginsburg, D. Atkinson, and D. M. Small, *Prog. Lipid Res.*, 1985, **23**, 135.
222 J. Wang, J. S. Mahmoud, and P. A. M. Farias, *Analyst (London)*, 1985, **110**, 855.
223 A. Papanastasiou-Dimandi and P. A. Siskos, *Microchem. J.*, 1985, **32**, 237.
224 D. Mendelsohn, A. R. Immelman, J. C. Vogel, and G. von La Chevallerie, *Biomed. Environ. Mass Spectrom.*, 1986, **13**, 21.