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GAS–LIQUID CHROMATOGRAPHIC STUDIES OF REACTIONS AND STRUCTURAL RELATIONSHIPS OF STEROIDS

PART I. POSITIONS 3, 11 AND 17 IN THE ANDROSTANE SERIES*

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SUMMARY

Qualitative and quantitative effects of classical reactions on steroids observed by gas–liquid chromatography (GLC) under standardized conditions, including the double internal standard technique, are reported. Simple procedures, applicable to nanogram amounts of reactants, that afford excellent yields of the major products are described. Reactions studied include the Wolff–Kishner removal of keto groups (WK), their conversion into hydroxyl groups with sodium borohydride and into dioxolone derivatives with ethylene glycol; the conversion of hydroxyl into keto groups with chromium trioxide and into trimethylsilyl (TMS) ethers with hexamethyldisilazane; and the hydrolysis of dioxolone and TMS derivatives with H^+. Gas–liquid chromatograms of reaction mixtures of single- and multi-step reactions readily provide information on the effects on functional groups at positions 3, 11 and 17 in the androstane series, and the retention times of many steroids unavailable from commercial or other sources. GLC data analysis provides relationships between steroid structure and retention times, from which methods for the calculation of retention times and for steroid identification are designed. The accuracy of the calculation methods is demonstrated and conditions for their efficient application are discussed.

INTRODUCTION

For a number of years, gas–liquid chromatograms of steroid hormones and metabolites extracted from animal tissues and body fluids have been routinely obtained in this laboratory by the application of an improved thin-layer chromatographic–gas–liquid chromatographic (TLC–GLC) method[1–3]. The patterns and retention times of the numerous peaks in chromatograms obtained after extensive TLC fractionation were characteristic of the animal species and sex in a particular physio-

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logical situation, as well as the tissue from which the sample originated. For example, the change in plasma concentration of individual steroids in ewes at different stages of gestation could be readily followed from start to parturition. The method gave access to data on the free and conjugated steroids of neutral and phenolic fractions derived from the same sample: a simple modification of hydrolytic conditions afforded data on either total conjugates or glucuronides only, and on sulphates by difference. As this information could be obtained in a relatively short time at low cost, the method appeared to be potentially valuable to biochemical and physiological studies relevant to animal reproduction as either a fact-finding or a monitoring device. The detailed identification of the GLC peaks was, however, an absolute prerequisite of such applications.

The choice of a peak identification procedure was largely dictated by the following characteristics of the separation method and the resulting GLC data: manipulations were fastest, most convenient and most effective at low sample levels; peaks in gas–liquid chromatograms were clearly distinct and their retention times were highly reproducible. However, partial overlap of peaks corresponding to the 0–5 ng range of steroids was of frequent occurrence; thus the GLC–mass spectrometric method of peak identification could not be used effectively, unless peak separations and sample levels were increased to the point at which important characteristics of the simpler separation method, *viz.*, speed and low cost, were no longer retained.

On the other hand, the remarkable consistency of peak retention times obtained by the double internal standard technique (*cf.*, ref. 4; see also below), *i.e.*, $\pm$ 0.01 min over a period of several years, strongly suggested that a method of identification based on this reliable feature might be successful. Many of the 100–150 peaks in neutral steroid fractions had retention times that matched that of one, and occasionally two or three, commercially available standards. In general, multiple solutions could be reduced to a single solution by making use of the TLC data, *i.e.*, the location of the relevant fraction in the TLC plate[3].

Undoubtedly, some 300 steroid standards available from commercial sources included a large number of compounds relevant to animal tissue. However, the unavailability of many presumably relevant steroids cast considerable doubt on the validity of identifications based on a comparison of retention times with those of available standards.

In view of this problem, we have made an extensive investigation of structural relationships whereby retention times, including those of unavailable steroids, could be calculated. Earlier investigations[1] had suggested that steroid retention times could be derived from the sum of logarithmic increments characteristic of functional groups. Further studies showed, however, that this rule was not of general application, as incremental values for groups such as 11α and 11β varied according to the other groups that were present in the molecule (*cf.*, ref. 4, Table X). However, GLC data on a much larger number of steroids have provided the simple structural relationships described in this paper: the general applicability of these rules and the accuracy of calculations based upon them have been unequivocally demonstrated.

Although retention times of naturally occurring steroids could then be compared with a set of retention times that included those of unavailable steroids, positive identification of any of the structures suggested by this comparison still required independent data supplied by TLC and other discriminatory tests.

Many of the steroids used in this investigation were synthesized in this laboratory from available standards by using simplified procedures of classical reactions standardized for amounts of steroid between 0 and 1 mg, and adapted to GLC analysis of reaction mixtures. The simple procedures afforded excellent yields of major products and gave access to GLC data on key steroids in a matter of hours. As such, these procedures could be used efficiently not only to generate predictable steroids from given structures, but also to identify structures from their reaction products. Thus, effects readily observed by GLC of specific reactions on steroids under investigation led to the unequivocal assignment of the correct structures from those originally suggested by the retention time.

A paper dealing with the systematic use of these effects in steroid identification will follow the publication of the present series.

The present series of papers is concerned mainly with the establishment of structural relationships of steroid retention times and the demonstration of simple methods of calculation based on them. As it is based to a large extent on the retention times of synthesized steroids, the preparation of these compounds is described and their authenticity demonstrated. For convenience in presenting and discussing data relevant to hundreds of compounds, each paper in this series will be confined to groups of steroids characterized by functional groups occupying specific positions.

This introductory paper is concerned with steroids of the androstane series, which feature the following functional groups alone or in all possible combinations: 3α, 3β, 3-keto; 11β, 11-keto; 17β and 17-keto. In addition, it describes the preparative procedures, GLC conditions, methods of data analysis, nomenclature and abbreviations used throughout the present series.

METHODS

Steroid preparation

Common steps. From 0 to 1 mg of steroid or steroid mixture was introduced into a micro-flask either by weighing or by evaporating under nitrogen the required volume of solution. Except in the case of TMS derivatization, the work-up of reaction products involved neutralization followed by extraction with chloroform. The chloroform extract was washed with water and evaporated under a stream of nitrogen in order to remove moisture. The residue, dried *in vacuo* over phosphorus pentoxide, was taken up in chloroform and a portion of this solution was evaporated to dryness and used for GLC analysis.

Chloroform and aqueous phases were mixed with a vibrator, separated in a clinical centrifuge and transferred or removed with syringes. Solvent evaporations were carried out under a stream of nitrogen in a manifold evaporator described elsewhere (ref. 1, Fig. 2). All solvents and liquid reagents were carefully redistilled.

Reduction of carbonyl groups with sodium borohydride (RD). Ten milligrams of sodium borohydride and 150 μl of 80% aqueous *tert.*-butanol were added to the micro-flask (ref. 5, Fig. 2) containing the steroid. The mixture was stirred until clear and allowed to stand at room temperature for either 2 h (general method) or 0.5 h (partial reduction of 11-keto groups) and then transferred by rinsing with four successive 250-μl volumes of chloroform into a 15-ml centrifuge tube; 1 ml of 0.25 *N* acetic acid was added and the contents were stirred. The chloroform layer was removed after centrif-

ugation, and the aqueous phase extracted twice with 250 μl of chloroform. The total chloroform extract was washed with 250 μl of 1 *N* sodium carbonate solution, evaporated, and the residue placed for 2 h in a vacuum desiccator over phosphorus pentoxide.

Wolff–Kishner removal of carbonyl groups (WK). The reagents used were (A) 5% (v/v) of 99–100% hydrazine hydrate (J. T. Baker, Phillipsburgh, N.J., U.S.A.; N-364) in absolute ethanol and (B) 7.2 g of sodium ethoxide in 35 g of absolute ethanol. These reagents were kept in stoppered bottles at room temperature in the dark.

Reagent A (50 μl) and reagent B (20 μl) were added successively to a micro-tube containing the steroid. For amounts of steroid over 500 μg, these volumes were multiplied by the number of carbonyl groups in the molecule. Up to six micro-tubes could be placed in a reaction vessel (see Appendix, Fig. 5) around a 5-ml flat-bottomed tube containing 4 ml of absolute ethanol. With the cover screwed on about one tenth of a turn only, the vessel was placed in a 160-mm diameter vacuum desiccator which was filled with nitrogen (*cf.*, Appendix). The cover was then tightened quickly by hand and the vessel placed for 5.5 h in an oven kept at 190 and then on an aluminium block to cool to room temperature. The reaction mixture was neutralized with 0.33 *N* hydrochloric acid and extracted twice with 250 μl of chloroform. The extract was processed as described under *Common steps*.

Dioxolone (ketal) derivatives of carbonyl groups (DO). To a 1-ml tube containing the dry steroid were added in succession 10 μl of ethylene glycol, 1 mg of *p*-toluenesulphonic acid and 750 μl of toluene. The tube was placed in the heating block of the evaporator (ref. 3, Fig. 2), which was maintained at about 113 for 6 h. A fine stream of nitrogen directed at the liquid surface was adjusted so as to evaporate about 500 μl of toluene per hour. At the end of each hour, 500 μl of toluene was added to the tube. During the sixth hour, the flow of nitrogen was increased in order to remove as much toluene as possible. After cooling, 250 μl of 0.025 *N* sodium hydroxide solution and 250 μl of chloroform were stirred with the reaction mixture. The chloroform extract was processed as described under *Common steps*.

Chromium trioxide oxidation of hydroxyl groups (OX). The reagent according to Cornforth *et al.*[6] was prepared as follows. Five grams of chromium trioxide were dissolved in 5 ml of water and the solution was poured into 50 ml of pyridine, which was cooled by swirling in ice-water. The reagent was kept in the dark at 2 and was renewed monthly or whenever a dark precipitate started to form.

The oxidation procedure was as follows. Fifty microlitres of reagent were added to a 1- to 2-ml tube containing the steroid; the mixture was stirred and allowed to stand at room temperature. After 5 h, 500 μl of chloroform and 250 μl of water were added, and the mixture was stirred and centrifuged. The chloroform layer was washed three times with 250 μl of water and further processed as described under *Common steps*.

Trimethylsilyl (TMS) ethers[1-7]. To the steroid contained in a micro-flask (ref. 5, Fig. 2) were added, in the following order, 25 μl of redistilled hexamethyldisilazane and 25 μl of freshly prepared 10% (v/v) redistilled trimethylchlorosilane in chloroform. The stoppered flask was allowed to stand overnight at room temperature. The contents were evaporated to dryness (15 min) in a manifold evaporator described elsewhere (ref. 1, Fig. 2) and the required volume of internal standard solution in carbon disulphide was added and the mixture stirred.

Hydrolysis of DO and TMS derivatives (HY). The dry steroid was stirred with 100 μl of absolute ethanol for 2 min, then 100 μl of 0.5 *N* hydrochloric acid was added and the mixture was stirred. The mixture was allowed to stand overnight at room temperature in the stoppered micro-tube. After neutralization with 100 μl of 0.5 *N* sodium hydroxide solution, it was extracted twice with 250 μl of chloroform. The chloroform extract was washed three times with 250 μl of water and further processed as described under *Common steps*. Comparable results were obtained by hydrolysis at 80° for 1 h with occasional stirring.

GLC techniques

A Perkin-Elmer 900 chromatograph equipped with dual flame ionization detectors was used isothermally. The 9 ft. × 1/8 in. O.D. stainless-steel columns were packed with 3% JXR (dimethylpolysiloxane) on 100–120 mesh Gas-Chrom Q and conditioned as described previously[3].

Derivatized or non-derivatized steroids or steroid mixtures were injected in standard carbon disulphide solution containing the TMS derivatives of two internal standards: S1, 3β-hydroxy-5α-androstane (A 2180)*, and S2, 3α-coprostanol (C 5050)* in concentrations of 3.75 and 12.5 ng/μl, respectively, obtained by 40-fold dilution of stock solutions in carbon disulphide. Standard and stock solutions were stored as described previously (ref. 4, p. 444).

Normal conditions under which all measurements were made were established as follows. With the oven temperature and the helium flow-rate set at approximately 230° and 60 ml/min, respectively, 2 μl of standard solution was injected, and the relative retention time, R_t, of S2 *versus* S1 was determined. The oven temperature was then adjusted to bring R_t between 6.700 and 6.648, as close as possible to R_{tN} = 6.674.

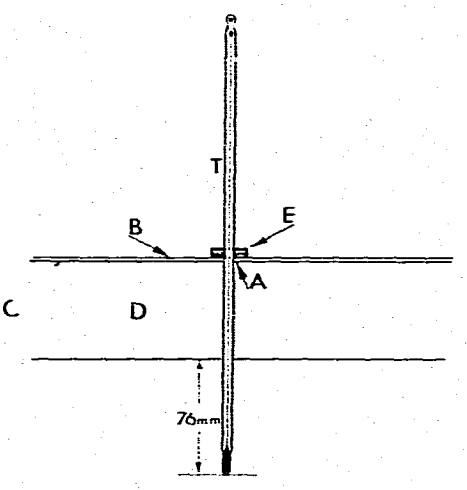

Fig. 1. Precision thermometer (T) (76 mm immersion, 200–250 °, 1/5° division; Cat. No 3548, Brooklyn Thermometer Co. Inc., Farmingdale, N.Y., U.S.A.), inserted through a hole (A) drilled through a 2-mm metal cover (B), 10 cm from the centre of the oven door (C) of a PE 900 Chromatograph. The thermometer crosses loose fibre-glass insulation (D) and can be readily placed or removed. A PTFE ring (E) holds the thermometer at an adjusted level. A telescopic reading lens is used.

* Steraloids Inc., P.O. Box 127, Pawling, N.Y. 12564, U.S.A.

This was achieved by trial and error, making use of the fact that R_t increased by 0.006 for a decrease of 0.1° observed on a precision thermometer (Fig. 1). The carrier gas flow-rate was then adjusted to bring the retention times of internal standards S1 and S2 as close as possible to $A = 190 \times 10^{-2}$ min and $B = 1268 \times 10^{-2}$ min, respectively, A and B being defined ideal normal conditions for which all retention times were normalized by applying the following procedure.

Retention time corrections. Following the injection of a sample, both the timer and recorder chart drive were switched on by actuating a common switch at the precise moment that the pen carriage moved up-scale; retention times being measured from this moment were "corrected retention times", t_R'. The first observable decrease from the maximum peak height was taken to pinpoint the retention time.

Observed t_R' values were further corrected for deviation from normal conditions indicated by differences from nominal A and B values of observed A' and B' values of standard retention times as follows. Let

$$A'' = A'B/B' \tag{1}$$

$$\Delta A = A - A'' \tag{2}$$

$$t_R'' = t_R'B/B' \tag{3}$$

Provided that

$$-0.026 \leq B'/A' - 6.674 \leq 0.026 \tag{4}$$

the corrected retention time, t_{NR}, given by

$$t_{NR} = t_R'' + \Delta A \cdot \frac{B - t_R''}{B - A} \tag{5}$$

is within $\pm 1 \cdot 10^{-2}$ min of the retention time under ideal normal conditions.

The ΔA term in eqn. 5 can be neglected when ΔA is within ± 0.5.

The oven thermometer served primarily to adjust R_t. Its effectiveness was greatest when effects of draughts and changes in room temperature on the emergent stem (Fig. 1) were minimized. Under these conditions, the oven temperature could be accurately determined from thermometer readings after *in situ* calibration described in ref. 4 (p. 440).

Occasional interference of standard peaks S1 or S2 with the peaks of other steroids was encountered. Replacement of S1, S2, or both, by non-interfering steroids required a careful determination of the corrected retention times of the new standards under normal conditions; the new A, B, and R_{tN} values were then used both in adjusting normal conditions and in eqns. 1–5.

All retention times were constant minimum retention times, CMt, defined in ref. 2, p. 3. Sufficient amounts of steroids were injected to obtain CMt, if necessary by increasing the test solution concentration, by injecting larger volumes, or both. Application of this procedure was particularly relevant to unsaturated components (ref. 2, p. 3).

TABLE I

M_R VALUES AND SOURCES OF M-STEROIDS OF THE ANDROSTANE SERIES

Steroid			*Source*
M	*Abbreviation*	M_R	
I	5βA	1887	A 3000
II	5αA	1924	A 700
III	5βA3β	2175	A 3400
IV	5αA3α	2175	A 2150
V	5βA(3)	2184	Prepared; *cf.*, Diagrams 1 and 2
VI	5βA3α	2193	Prepared; *cf.*, Diagram 2
VII	5αA(3)	2228	A 2650
VIII	.14A3β	2256	Calculated; from L_R .14A3β17β − G_R 17β*
IX	.15A3β	2269	A 8290
X	5αA3β	2279	A 2180
XI	.14A(3)	2305	Calculated; from L_R .14A17β(3) − G_R 17β* and L_R .14A(3,17) − G_R (17)**

* *Cf.*, Table X.
** *Cf.*, Table IX.

TLC techniques

All steroids and steroid preparations were routinely submitted to TLC under conditions already described[2]. However, only TLC data relevant to the determination of GLC data are given in this paper (*cf.*, Diagram 14 and Table XI).

GLC data analysis

The data were organized in a manner designed to emphasize both structural similarities and differences, and to provide a GLC parameter for each aspect. This concept led to the grouping of steroids found in Tables I–X. GLC parameters were

TABLE II

M_R VALUES AND SOURCES OF M-STEROIDS OF THE PREGNANE SERIES

Steroid			*Source*	*Calculated values**
M	*Abbreviation*	M_R		
I	5βP	2113	P 5700	M_R 5βA − 226 = 2113
II	5αP	2150	P 1800	M_R 5αA − 226 = 2150
III	5βP3β	2402	Prepared; WK-5βP3β(20)	M_R 5βA3β − 226 = 2402
IV	5αP3α	2401	Calculated	M_R 5αA3α − 226 = 2401
V	5βP(3)	2412	Calculated	M_R 5βA(3) − 226 = 2412
VI	5βP3α	2421	P 7800	M_R 5βA3α − 226 = 2419
VII	5αP(3)	2453	P 4200	M_R 5αA(3) − 226 = 2454
VIII	.14P3β	2483	Calculated	M_R .14A3β − 226 = 2483
IX	.15P3β	2497	Q 5350	M_R .15A3β − 226 = 2495
X	5αP3β	2506	P 3450	M_R 5αA3β − 226 = 2505
XI	.14P(3)	2531	Calculated	M_R .14A(3) − 226 = 2531

* Showing relationship between M_R values of the androstane and pregnane series; see eqn. 17 and text.

TABLE III

L_K AND G_K VALUES AND SOURCES OF STEROIDS OF GROUP (11)

Steroid				*Source*
M	*Abbreviation*	L_K	G_K*	
I	5βA(11)	2033	146	Prepared; *cf.*, Diagram 13
II	5αA(11)	2076	152	Prepared; *cf.*, Diagram 7
III	5βA3β(11)	2325	150	Calculated; M_R 5βA3β → G_K(11)*
IV	5αA3α(11)	2327	151	Prepared; *cf.*, Diagrams 9 and 11
V	5βA(3,11)	2308	124**	Prepared; *cf.*, Diagram 14
VI	5βA3α(11)	2326	133**	Prepared; *cf.*, Diagram 13
VII	5αA(3,11)	2360	132**	Prepared; *cf.*, Diagram 10
VIII	14A3β(11)	2390	133**	Calculated; L_K 14A3β17β(11) → $1G_K$***
IX	15A3β(11)	2420	151	Calculated; M_R 15A3β → G_K(11)*
X	5αA3β(11)	2431	152	Prepared; *cf.*, Diagram 10
XI	14A(3,11)	2421	116**	Calculated; L_K 14A(11,17) → $1G_K$***

* Average G_K-normal = G_R(11) = 150.5.
** G_K-odd steroid.
*** For L_K value, see appropriate Table; for appropriate $1G_K$ value, see Table XII.

then tested for fit in functions expressing simple relationships that provided the basis for retention time calculations.

The basic concept considers two distinct sets of structural features in the steroid molecule: M-features, which affect the structure of ring A, and G-features, which affect the remainder of the molecule. Steroids with identical G-features form distinct groups, the members of which differ in respect of M-features only (*cf.*, Tables I–X). Steroids devoid of G-features, the M-steroids of the androstane and the pregnane series, are listed in Tables I and II, respectively.

TABLE IV

L_K AND G_K VALUES AND SOURCES OF STEROIDS OF GROUP 11β

Steroid				*Source*
M	*Abbreviation*	L_K	G_K*	
I	5βA11β	2097	210**	Prepared; *cf.*, Diagram 13
II	5αA11β	2145	221	Prepared; *cf.*, Diagram 7
III	5βA3β11β	2390	215**	Calculated; L_K 5βP3β11β → $1G_K$***
IV	5αA3α11β	2385	210**	Prepared; *cf.*, Diagrams 8 and 11
V	5βA11β(3)	2390	206**	Calculated; L_K 5βA11β(3,17) → $1G_K$§
VI	5βA3α11β	2392	199**	Prepared; *cf.*, Diagram 14
VII	5αA11β(3)	2450	222	Calculated; L_K 5αA11β(3,17) → $1G_K$§
VIII	14A3β11β	2465	208**	Calculated; L_K 14A3β11β17β → $1G_K$§
IX	15A3β11β	2491	222	Calculated; L_K 15A3β11β17β → $1G_K$§
X	5αA3β11β	2504	225	Prepared; *cf.*, Diagrams 9 and 10
XI	14A11β(3)	2525	220	Calculated; L_K 14A11β(3,17) → $1G_K$§

* Average G_K-normal = G_R 11β = 222.
** G_K-odd steroid.
*** L_K 5βP3β11β = 2617; $1G_K$ P11βA11β = 227 (see text).
§ For L_K values, see appropriate Table; for appropriate $1G_K$ value, see Table XII.

TABLE V

L_R AND G_R VALUES AND SOURCES OF STEROIDS OF GROUP (11,17)

Steroid				*Source*
M	*Abbreviation*	L_R	G_R*	
I	5βA(11,17)	2248	361	Prepared: *cf.*, Diagram 16
II	5αA(11,17)	2287	363	Prepared: *cf.*, Diagrams 7 and 12
III	5βA3β(11,17)	2535	361	Calculated: M_R 5βA3β → G_R(11,17)*
IV	5αA3α(11,17)	2534	359	A 2280
V	5βA(3,11,17)	2526	342**	A 4010
VI	5βA3α(11,17)	2541	348**	A 3460
VII	5αA(3,11,17)	2578	350**	Prepared: *cf.*, Diagram 12
VIII	.14A3β(11,17)	2599	342**	Calculated: L_R .14A3β17β(11) → .1G_R***
IX	.15A3β(11,17)	2627	358	SRC
X	5αA3β(11,17)	2643	364	Prepared: OX (1 h) 5αA3β11β(17)§
XI	.14A(3,11,17)	2629	324**	Calculated: L_R .14A17β(3,11) → .1G_R***

* Average G_R-normal value = G_R(11,17) = 361.
** G_R-odd steroid.
*** For L_R value, see appropriate Table; for appropriate .1G_R value, see Table XII.
§ 95% 5αA3β(11,17) + 5% 5αA(3,11,17).

As shown in Tables I–X, M-features considered in the present study were limited to the following series, where each feature corresponds to a figure in roman numerals, and where (3) stands for 3-one:

I. 5β	V. 5β(3)	IX. .15 3β
II. 5α	VI. 5β3α	X. 5α3β
III. 5β3β	VII. 5α(3)	XI. .14(3)
IV. 5α3α	VIII. .14 3β	

TABLE VI

L_R AND G_R VALUES AND SOURCES OF STEROIDS OF GROUP 11β(17)

Steroid				*Source*
M	*Abbreviation*	L_R	G_R*	
I	5βA11β(17)	2350	463**	Prepared: *cf.*, Diagram 16
II	5αA11β(17)	2399	475	Calculated: L_R 5αA11β → .1G_R***
III	5βA3β11β(17)	2643	468**	Calculated: L_R 5βP3β11β(20) − 214 (see text)
IV	5αA3α11β(17)	2634	459**	A 1330
V	5βA11β(3,17)	2644	460**	Prepared: *cf.*, Diagram 15
VI	5βA3α11β(17)	2645	452**	A 3120
VII	5αA11β(3,17)	2704	476	A 2360
VIII	.14A3β11β(17)	2720	463**	Calculated: L_R .14A3β11β17β → .1G_R§
IX	.15A3β11β(17)	2746	477	Calculated: L_R .15A3β11β17β → .1G_R§
X	5αA3β11β(17)	2757	478	A 1500
XI	.14A11β(3,17)	2779	474	A 6630

* Average G_R-normal value = G_R 11β(17) = 477.
** G_R-odd steroid.
*** L_R 5αA11β: *cf.*, Table IV; .1G_R: *cf.*, Table XII.
§ For L_R value, see appropriate Table; for appropriate .1G_R value, see Table XII.

TABLE VII

L_R AND G_R VALUES AND SOURCES OF STEROIDS OF GROUP 17β(11)

Steroid				*Source*
M	*Abbreviation*	L_R	G_R*	
I	5βA17β(11)	2395	508	Calculated: L_R 5βA(11,17) + .1G_R***
II	5αA17β(11)	2432	508	Prepared; *cf.*, Diagram 12
III	5βA3β17β(11)	2682	507	Calculated: M_R 5βA3β + G_R 17β(11)*
IV	5αA3α17β(11)	2681	506	Prepared; *cf.*, Diagram 3
V	5βA17β(3,11)	2668	484**	Calculated: L_R 5βA(3,11,17) + .1G_R***
VI	5βA3α17β(11)	2671	478**	Prepared; *cf.*, Diagram 4
VII	5αA17β(3,11)	2721	493**	Calculated: L_R 5αA(3,11,17) + .1G_R***
VIII	.14A3β17β(11)	2746	489**	Prepared; *cf.*, Diagram 6
IX	.15A3β17β(11)	2775	506	Prepared: 0.5 h RD of .15A3β(11,17)
X	5αA3β17β(11)	2787	508	Prepared: 0.5 h RD of 5αA3β(11,17)
XI	.14A17β(3,11)	2776	471**	SRC

* Average G_R-normal value = G_R 17β(11) = 507.
** G_R-odd steroid.
*** For L_R value, see appropriate Table; for appropriate .1G_R value, see Table XII.

The logarithmic expression, L_R, of retention time, $t'_{NR}\cdot 10^{-2}$ min, is defined by[4]

$$L_R = 10^3 \cdot t'_{NR} \tag{6}$$

For the retention time, t'_{MR}, of M-steroids, this expression is

$$M_R = 10^3 \cdot t'_{MR} \tag{7}$$

L_R may be considered to result from a modification of M_R by G-features. Let G_R be the G-feature contribution; then,

$$L_R = M_R + G_R \tag{8}$$

TABLE VIII

L_R AND G_R VALUES AND SOURCES OF STEROIDS OF GROUP 11β17β

Steroid				*Source*
M	*Abbreviation*	L_R	G_R*	
I	5βA11β17β	2432	545**	Prepared; *cf.*, Diagram 16
II	5αA11β17β	2487	563	Prepared; *cf.*, Diagram 7
III	5βA3β11β17β	2728	543**	Calculated: L_R 5βP3β11β20β − 214 (see text)
IV	5αA3α11β17β	2729	554**	A 2780
V	5βA11β17β(3)	2735	551**	Calculated: L_R 5βA11β(3,17) + .1G_R***
VI	5βA3α11β17β	2714	521**	A 3940
VII	5αA11β17β(3)	2795	567	Calculated: L_R 5αA11β(3,17) + .1G_R***
VIII	.14A3β11β17β	2811	554**	Prepared; *cf.*, Diagram 6
IX	.15A3β11β17β	2837	560	Prepared; 0.5 h RD of .15A3β(11,17)
X	5αA3β11β17β	2850	571	A 2800; also SRC
XI	.14A11β17β(3)	2870	565	Calculated; L_R .14A11β(3,11) + .1G_R***

* Average G_R-normal value = G_R 11β17β = 568.
** G_R-odd steroid.
*** For L_R value, see appropriate Table; for appropriate .1G_R value, see Table XII.

TABLE IX

L_R AND G_R VALUES AND SOURCES OF STEROIDS OF GROUP (17)

Steroid				*Source*
M	*Abbreviation*	L_R	G_R*	
I	5βA(17)	2148	261	Prepared: OX 5βA17β
II	5αA(17)	2187	263	SRC
III	5βA3β(17)	2441	266	A 3670
IV	5αA3α(17)	2439	264	A 2420
V	5βA(3,17)	2445	261	A 3270
VI	5βA3α(17)	2460	267	A 3610
VII	5αA(3,17)	2489	261	A 1630
VIII	.14A3β(17)	2520	263	Calculated: M_R .14A3β − G_R(17)*
IX	.15A3β(17)	2528	259	A 8500
X	5αA3β(17)	2542	263	A 2490
XI	.14A(3,17)	2566	261	A 8020

* Average G_R-normal value = $G_R(17)$ = 263.

This expression implies that M_R is the same for all steroids with the same M-features. Thus M_R and G_R are GLC parameters for similarity and dissimilarity, respectively.

As eqn. 8 can be written as

$$G_R = L_R - M_R \tag{9}$$

the G_R value of a steroid can be calculated from its L_R value and the M_R value corresponding to its M-features taken from Tables I or II.

From eqns. 6 and 7, eqn. 9 can be written as

$$G_R = 10^3 \cdot \log \left(\frac{t'_{XR}}{t'_{MR}} \right) \tag{10}$$

TABLE X

L_R AND G_R VALUES AND SOURCES OF STEROIDS OF GROUP 17β

Steroid				*Source*
M	*Abbreviation*	L_R	G_R*	
I	5βA17β	2230	343	A 3430
II	5αA17β	2270	346	A 2250
III	5βA3β17β	2521	346	A 3060
IV	5αA3α17β	2520	345	A 1170
V	5βA17β(3)	2523	339**	A 3730
VI	5βA3α17β	2528	335**	A 3030
VII	5αA17β(3)	2573	345	A 2570
VIII	.14A3β17β	2603	347	A 5600
IX	.15A3β17β	2618	349	A 7830
X	5αA3β17β	2629	350	A 1220
XI	.14A17β(3)	2652	347	A 6950

* Average G_R-normal value = G_R 17β = 346.5.
** G_R-odd steroid.

Hence, the G_R value of a steroid can also be obtained as 10^3 times the logarithm of its relative retention time *versus* the corresponding M-steroid.

Eqn. 8 suggests a simple test for G_R values of steroids of the same group. A plot of L_R *versus* M_R values, the LM plot, yields a straight line of 45° slope for all steroids of a group with the same G_R value: the intercept with the L_R axis ($M_R = 0$) gives the G_R value of the group. Some groups yield LM lines fitted by all members. In general, however, a group includes members that fit, the G_R-normal steroids, and the G_R-odd steroids which have a variable G_R value considerably below the G_R value of the group.

It follows from the above that the G_R value of a group can also be obtained through eqn. 9 or 10 as the G_R value of any G_R-normal steroid of the group. Conversely, the L_R value of any G_R-normal steroid can be calculated from the G_R value of the group.

Given steroids a and b in different groups,

$$\Delta G_R(a,b) = G_R(a) - G_R(b) \tag{11}$$

For M-corresponding members of two groups:

$$M_R(a) \equiv M_R(b) \tag{12}$$

Hence, from eqns. 8, 11 and 12

$$\Delta G_R(a,b) = L_R(a) - L_R(b) \tag{13}$$

or, from eqn. 6

$$\Delta G_R(a,b) = 10^3 \cdot \log\left(\frac{t'_{xR}(a)}{t'_{xR}(b)}\right) \tag{14}$$

Also, from eqn. 13

$$L_R(a) = L_R(b) + \Delta G_R(a,b) \tag{15}$$

When $\Delta G_R(a,b)$ defined by eqn. 13 is the same for all pairs of M-corresponding members of two groups, the groups are related and the unique $\Delta G_R(a,b)$ value is the ΔG_R value of the groups. In this instance a plot of $L_R(a)$ *versus* $L_R(b)$, the LL plot, yields a straight line of 45° slope fitted by both G_R-normal and G_R-odd steroids. The intercept of the LL plot is the ΔG_R value of the groups. Three other ways of obtaining ΔG_R are suggested: from eqn. 11, as the difference between the G_R values of the groups; from eqn. 13, as the difference between the L_R values of two M-corresponding members of the groups; and from eqn. 14, as 10^3 times the logarithm of the relative retention time of a member of one group *versus* the M-corresponding member in the other group. Conversely, the ΔG_R value of two related groups affords several ways in which the L_R value of members of such groups can be calculated.

Abbreviations for steroids

The abbreviations used in this series of papers begin with 5α, 5β, 14 or 15 followed by A (androstane) or P (pregnane). Next come the hydroxyl groups followed by the carbonyl groups in parentheses. For example:

5αA3β	designates	3β-hydroxy-5α-androstane
5βA3β11β	designates	3β,11β-dihydroxy-5β-androstane
14A11β(3,17)	designates	11β-hydroxyandrost-4-ene-3,17-dione
15P3β17α(20)	designates	3β,17α-dihydroxypregn-5-ene-20-one
5αP17α21(3,11,20)	designates	17α,21-dihydroxy-5α-pregnane-3,11,20-trione.

In the figures, M-features of steroids are indicated by the corresponding roman numerals.

THE DATA

Diagrams 1–16 describe preparations of steroids starting from available standards. The reaction used on the whole mixture of products from the preceding step is indicated on the left by two capital letters, such as RD or OX (*cf.*, *Steroid preparation*). Percentages of all compounds in the reaction mixture are indicated in bold type; component derivation from products of the preceding step, and retention times in 10^{-2} min, are also indicated. Retention times correspond to derivatized mixtures, D. In a few instances, *e.g.*, in Diagrams 1, 2, 7, 15 and 16, retention times for the non-derivatized mixtures, N, are given also in order to illustrate a point in the Discussion. For similar reasons, retention times are occasionally followed by the corresponding L_R value in parentheses, *e.g.*, in Diagram 16.

L_R values in Tables I–X were obtained from compounds which, as such, or as components of a reaction mixture, had been submitted to TMS derivatization. Except for 11β (*cf.* Discussion), all hydroxyl groups were derivatized in the process.

G_R values indicated for each steroid in Tables III–X were obtained from the L_R values by subtracting the corresponding M_R value (eqn. 9) taken from Table I. The origin of each compound is indicated under source(s): a capital letter followed by four digits indicates the catalogue number of Steraloids Inc., from whom the steroid was purchased; SRC indicates a gift from the Steroid Reference Collection; prepared indicates a compound prepared in this laboratory, and in this case reference to the method of preparation is given; calculated indicates an L_R value computed by one of the methods described under *GLC data analysis* (the method is identified).

Table XI gives a list of standard compounds from which the L_R values of 88 steroids listed in Tables III–X can be derived. Table XI gives the retention times, L_R values, source(s) and TLC R_h values of the standard compounds.

In Table XII, experimental G_R and ΔG_R values are shown in parentheses above the corresponding G_R and ΔG_R values from the standards listed in Table XI. The derived G_R and ΔG_R values were used in order to calculate L_R values for the steroids listed in Tables III–X. A comparison of the calculated and experimental L_R values indicated errors; Table XIII shows these errors expressed in seconds of retention time.

Reaction		5βA3β(17) D 276	
WK			
	90 5βA3β D 149.5		**10** 5βA3β17β D 331
OX			
	90 5βA(3) D,N 153		**10** 5βA(3,17) D,N 280

Diagram 1. Synthesis of 5βA3β and 5βA(3).

Reaction		5βA3α(17) D 290	
WK			
	91 5βA3α D 156		**9** 5βA3α17β D 337
OX			
	88 5βA(3) D,N 153		**12** 5βA(3,17) D,N 280

Diagram 2. Synthesis of 5βA3α and 5βA(3).

Reaction		5αA3α(11,17) D 342	
RD **(0.5 h)**	**35** 5αA3α17β(11) D 480		**65** 5αA3α11β17β D 534

Diagram 3. Synthesis of 5αA3α17β(11).

Reaction		5βA(3,11,17) N.D 332	
RD **(0.5 h)**	**1** 5βA3α(11,17) D 342	**5.4** 5βA3α17β(11) D 468	**93** 5βA3α11β17β D 518

Diagram 4. Synthesis of 5βA3α17β(11).

		5βA3α11β(17) D 442
Reaction		
RD (2 h)		**99** 5βA3α11β17β D 517

Diagram 5. Synthesis of 5βA3α11β17β.

				.14A(3,11,17) N,D 431		
Reaction						
RD						
(0.5 h)	**1.7** .14A3β(11,17) D 404	**5.1** 5βA3α17β(11) D 467	**45** .14A3β17β(11) D 558	**6.7** 5αA3β17β(11) D 612	**36** .14A3β11β17β D 647	**5.6** 5αA3β11β17β D 709

Diagram 6. Synthesis of .14A3β17β(11) and .14A3β11β17β.

		5αA11β(3,17) D 506	
Reaction			
WK			
	94 5αA11β D 139.5 N 139.5	**5** 5αA11β17β D 307 N 268	**1** 5αA3β11β17β D 708 N 612
OX			
	94 5αA(11) D 119 N 119	**5** 5αA(11,17) D 194 N 194	**0.5** 5αA(3,11,17) D 379 N 379

Diagram 7. Synthesis of 5αA(11), 5αA11β, 5αA(11,17) and 5αA11β17β.

			5αA3α11β(17)				
Reaction			D 431				
WK							
	95		**0.2**	**4.5**			
	5αA3α11β		5αA3β11β	5αA3α11β17β			
	D 242		D 318	D 535			

Diagram 8. Synthesis of 5αA3α11β.

				5αA3α(11,17)			
Reaction				D 342			
WK							
	2	**1**	**4**		**90**	**1**	**2**
	5αA11β	5αA3α	5αA3α(11)		5αA3α11β	5αA3α17β	5αA3α11β17β
	D 140	D 150	D 212		D 243	D 340	D 537
OX							
	2	**1**		**93**		**1**	**2**
	5αA(11)	5αA(3)		5αA(3,11)		5αA(3,17)	5αA(3,11,17)
	D 119	D 171		D 230		D 308	D 379
RD							
(2 h)	**2**	**1**	**1**	**2**	**91**	**1**	**1**
	5αA11β	5αA3β	5αA(3,11)	5αA3β(11)	5αA3β11β	5αA3β17β	5αA3β11β17β
	D 139.5	D 190	D 230	D 271	D 319	D 426	D 708

Diagram 9. Synthesis of 5αA3α(11), 5αA3α11β, 5αA(3,11) and 5αA3β11β.

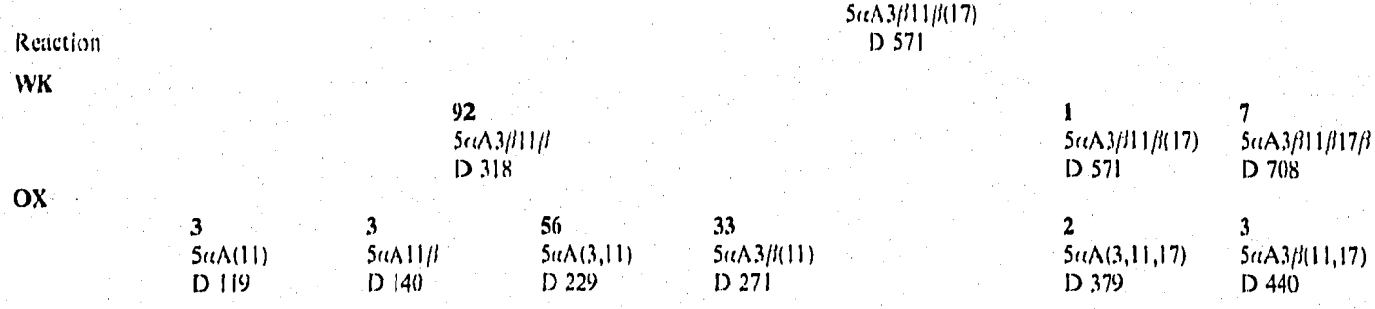

Diagram 10. Synthesis of 5αA(3,11), 5αA3β(11) and 5αA3β11β.

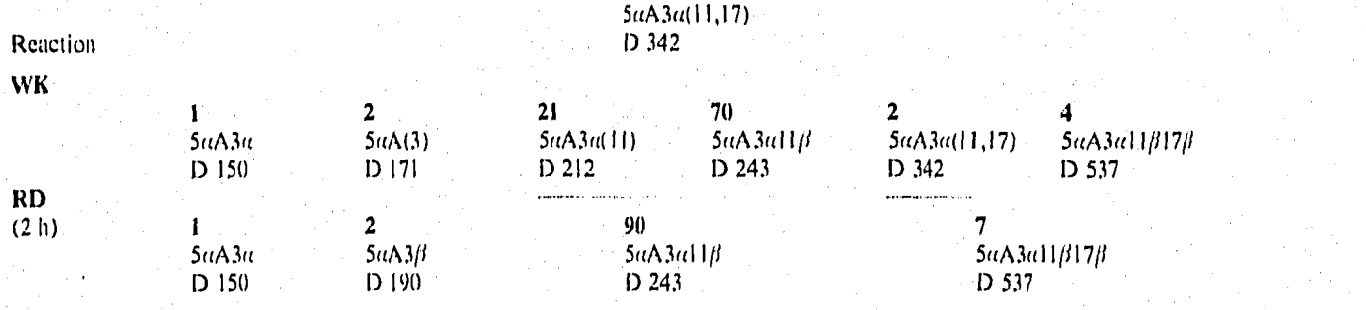

Diagram 11. Synthesis of 5αA3α(11) and 5αA3α11β.

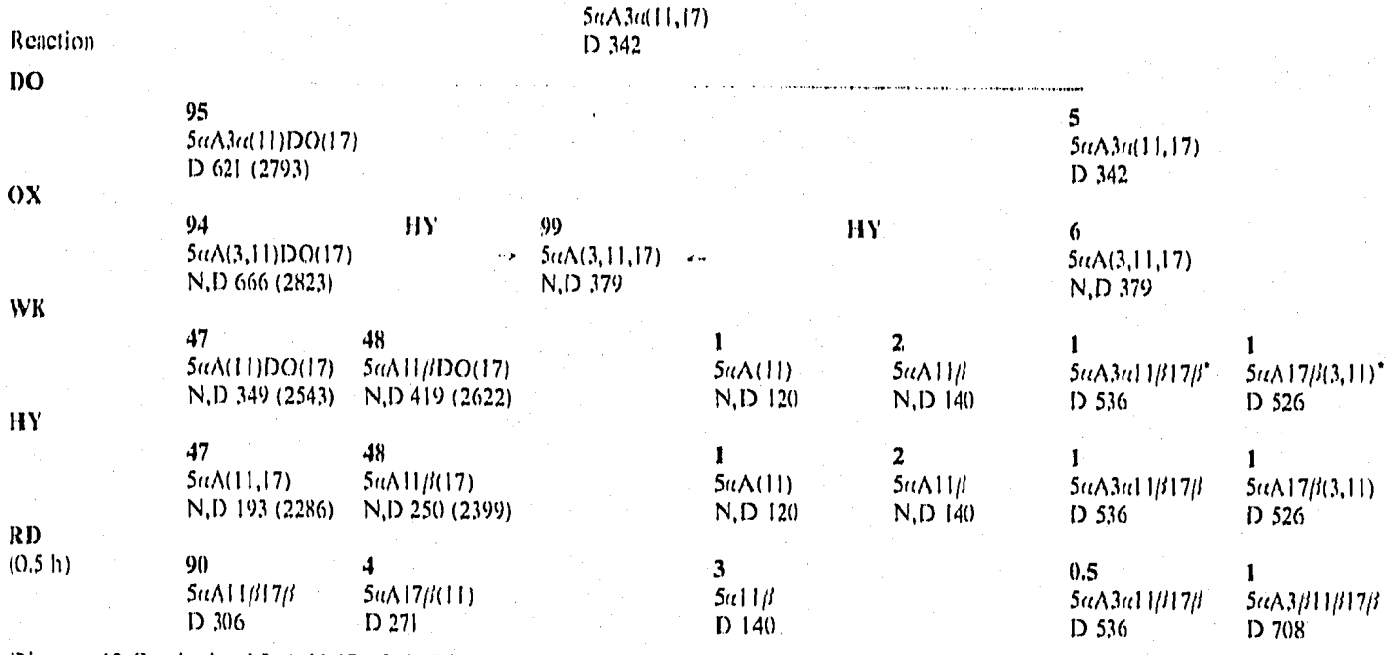

Diagram 12. Synthesis of 5αA(11,17), 5αA17β(11), 5αA11β(17) and 5αA11β17β. * Separable by TLC.

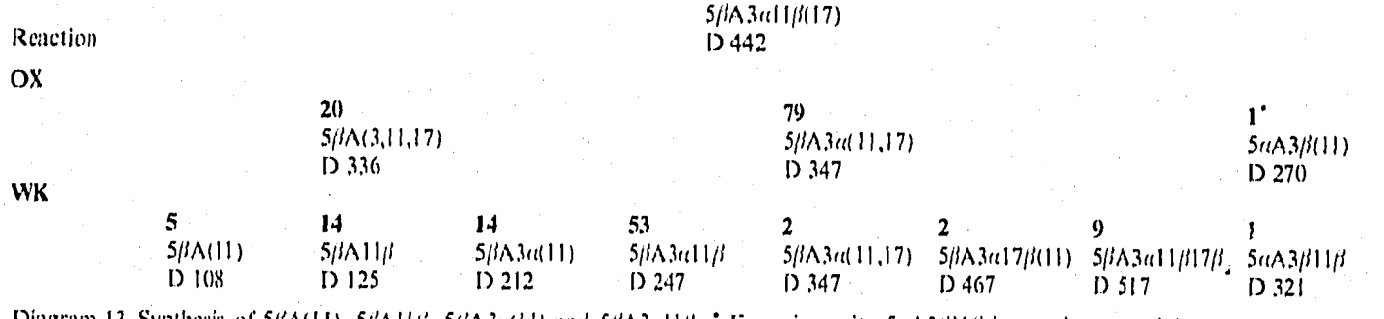

Diagram 13. Synthesis of 5βA(11), 5βA11β, 5βA3α(11) and 5βA3α11β. * From impurity 5αA3β11β in starting material.

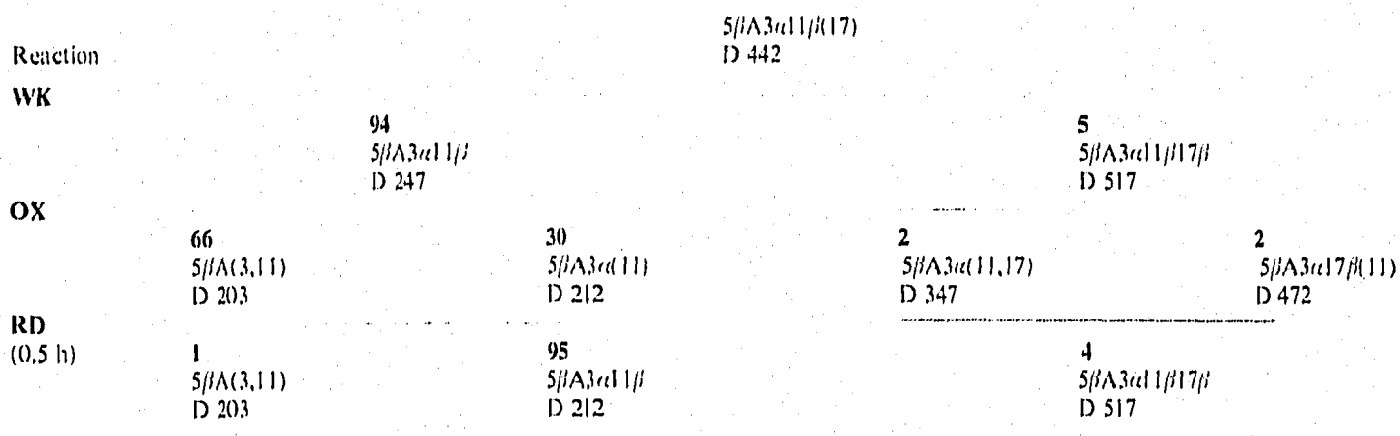

Diagram 14. Synthesis of 5βA(3,11), 5βA3α(11), 5βA3α11β and 5βA3α11β17β.

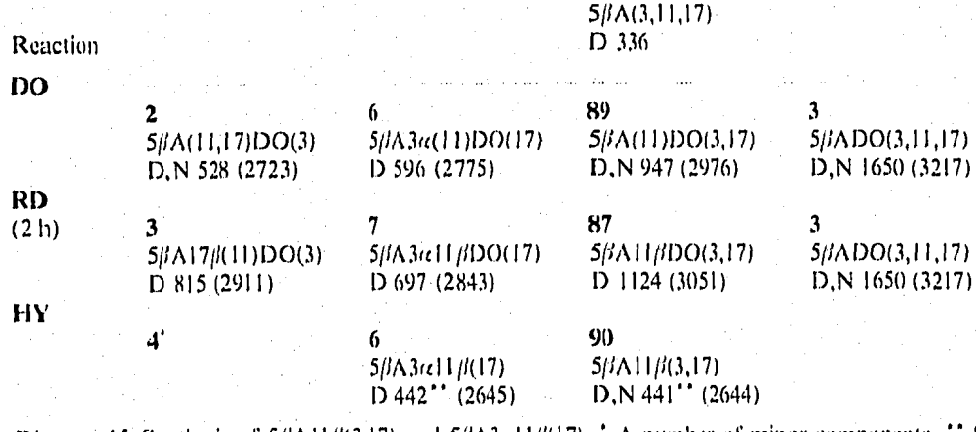

Diagram 15. Synthesis of 5βA11β(3,17) and 5βA3α11β(17). * A number of minor components. ** Separated by TLC.

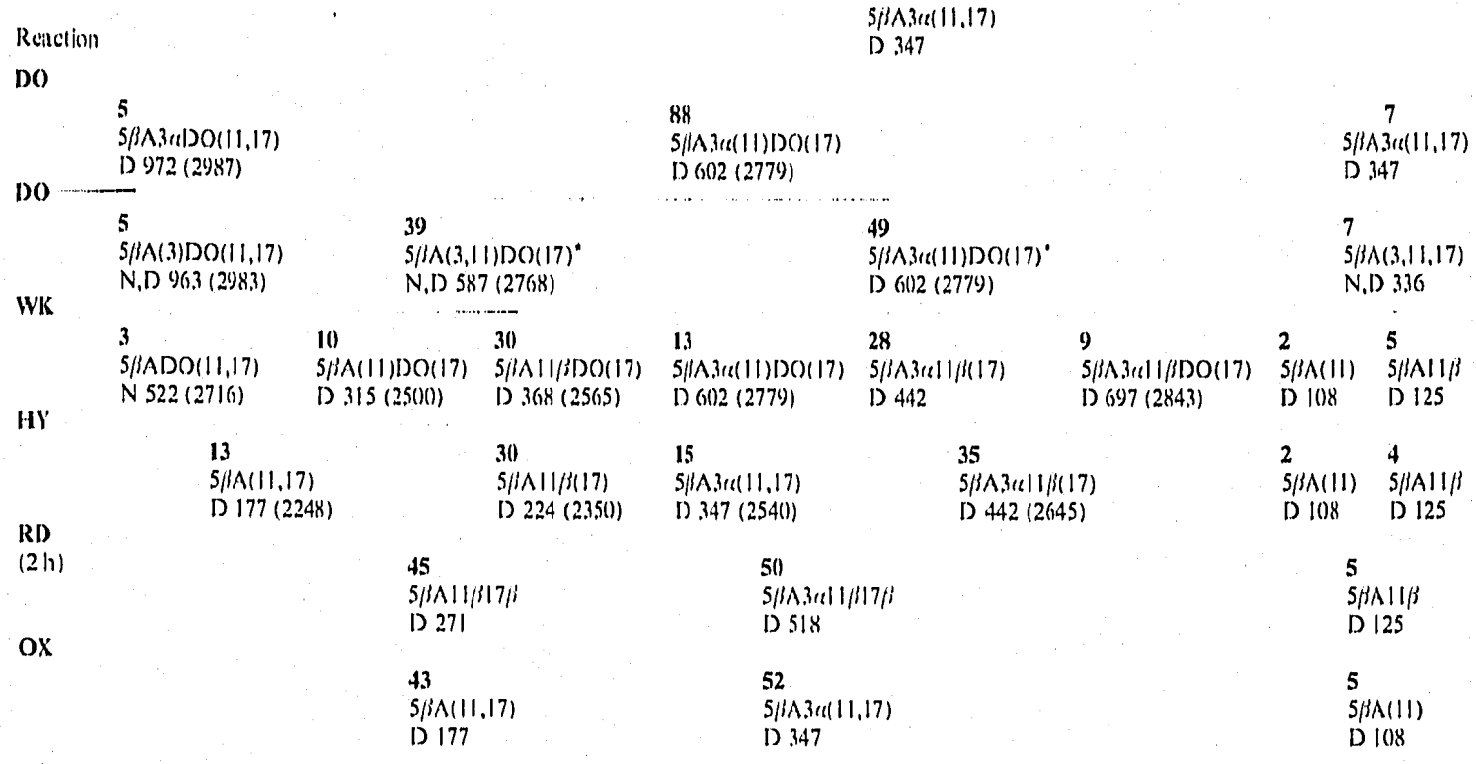

Diagram 16. Synthesis of 5β*A*(11), 5β*A*11β, 5β*A*(11,17), 5β*A*11β(17) and 5β*A*11β17β. * Separable by TLC.

TABLE XI

RETENTION TIMES (t'_{SR}), L_R, G_R AND R_b VALUES, AND SOURCES OF STANDARD STEROIDS

Mixture	*Abbreviation*	t'_{SR} (*min* $\times 10^{-2}$)	L_R	G_R	R_b*	*Source***
A	5α*A*3β(11)	270	2431.3	152.6	0.693	Prepared: OX-WK-5α*A*3β11β(17); *cf.*, Diagram 10
	5α*A*3β11β	318	2502.4	223.7	0.512	Prepared: WK-5α*A*3β11β(17); *cf.*, Diagram 10
	5α*A*3β(17)	348	2541.5	262.8	0.720	A 2490
	14*A*3β17β	401	2603.1	347.1	0.480	A 5600
	5α*A*3β(11,17)	440	2643.4	365.0	0.615	Prepared: OX (1 h) 5α*A*3β11β(17)***
	5α*A*3β11β(17)	571	2756.6	477.9	0.405	A 1500
	5α*A*3β17β(11)	612	2786.7	508.0	0.190	Prepared: 0.5 h RD-5α*A*3β(11,17)
	15*A*3β11β17β	688	2837.5	568.5	0.090	Prepared: 2 h RD-15*A*3β(11,17)
B	5β*A*11β	125	2097.0		0.975	Prepared: WK-5β*A*(3,11,17); *cf.*, Diagram 13
	5β*A*(3,11,17)	336	2526.1		0.875	A 4010
	5α*A*(3,11,17)	378	2578.0		0.918	Prepared: OX-5α*A*3α(11,17)§; also Diagram 12
	5α*A*3α11β(17)	431	2634.4		0.485	A 1330
	14*A*17β(3,11)	597	2776.0		0.437	SRC
	14*A*3β11β17β	647	2811.0		0.092	Prepared: 0.5 h RD-14*A*(3,11,17); *cf.*, Diagram 6
C	5β*A*3α(11)	212	2326.3		0.745	Prepared: WK-5β*A*3α(11,17); *cf.*, Diagram 13
	5β*A*3α11β	247	2392.7		0.650	Prepared: WK-5β*A*3α11β(17); *cf.*, Diagram 14
	5β*A*3α17β	337	2527.0		0.405	A 3030
	5β*A*3α11β17β	517	2713.5		0.093	A 3940
	5β*A*17β(3)	333	2522.4		0.734	A 3730

* All compounds obtained through preparations were purified by TLC; all preparations were completed in 8 working days. Concentrations of mixture components were adjusted proportionally to retention time so as to obtain approximately uniform peak heights. Amount injected was such as to obtain *CMt* (see *GLC techniques*) of unsaturated steroids.

** For source of starting material in preparations, see Table V or VI.

*** 95% 5α*A*3β(11,17), 5% 5α*A*(3,11,17).

§ 95% 5α*A*(3,11,17), 5% 5α*A*3β(11,17).

DISCUSSION

Steroid preparation

The simplicity of the preparative procedures described under Methods allows a number of samples to be handled serially; identification by GLC of products obtainable in a few hours is facilitated by their high yields and predictability.

Most of the observations recorded below on effects of the classical reactions upon functional groups at positions 3, 11 and 17 were made in the course of a preliminary standardization of the preparative procedures with the help of structurally simple steroids. Application of the standardized procedures to the more complex

TABLE XII

G_R AND ΔG_R VALUES

G_R*	Group	ΔG_R**						
		(11)	(11,17)	17β(11)	11β	11β(17)	11β17β	ΣG_R***
(150.5)	(11)							
152.6								
(361.0)	(11,17)	(210.5)						413.2
365.0		213.5						
(507.0)	17β(11)	(356.5)	(146.0)					498.6
508.0		355.4	143.0					
(222.0)	11β	(71.5)	(139.0)	(285.0)				
223.7		71.1	141.3	284.3				
(477.0)	11β(17)	(326.5)	(116.0)	(30.0)	(254.2)			484.7
477.9		325.3	112.9	30.1	255.0			
(568.0)	11β17β	(418.5)	(207.0)	(61.0)	(346.0)	(91.0)		567.5
568.5		415.9	203.5	60.5	344.8	90.6		
(262.7)	(17)	(112.2)	(99.3)	(244.3)	(40.7)	(215.3)	(305.3)	
262.8		110.2	103.2	245.2	39.1	215.1	305.7	
(346.5)	17β	(196.0)	(14.5)	(160.5)	(124.5)	(130.5)	(221.5)	
347.1		194.5	13.9	160.9	125.4	130.9	221.4	

* G_R values in parentheses, column 1, are averages of G_R-normal values for each group indicated in column 2, as shown in footnote * in Tables III–X; G_R values just below these were derived from standard compounds in Mixture A (*cf.*, Table XI, column 4 and text).

** ΔG_R values were obtained for all possible group pairs by using the G_R values from column 1 (*cf.*, footnote *); the ΔG_R values in parentheses correspond to the G_R values in parentheses. Each of the two framed sets of ΔG_R values correspond to related groups: the (11)-featuring and 11β-featuring groups, respectively.

*** For definition of ΣG_R see text.

TABLE XIII

ERRORS IN SECONDS OF RETENTION TIME OBSERVED IN L_R VALUES CALCULATED FROM GLC DATA OBTAINED WITH STANDARD STEROIDS LISTED IN TABLE XI

Differences observed between calculated L_R values and L_R values listed in Tables III–X were obtained in L_R units. One L_R unit corresponds to 0.233% of the retention time, t'_{NR}, or 0.14 · t'_{NR} sec when t'_{NR} is expressed in minutes. No error is given for nineteen compounds used as standards, indicated by S, nor for six 5β-A-3β-steroids not calculated from the G_R data in Table XI (see text).

M	Abbreviation	(11)-Featuring groups			11β-Featuring groups			Position 17	
		11	(11,17)	17β(11)	11β	11β(17)	11β17β	(17)	17β
I	5β-A	- 0.9	0	0	S*	- 0.6*	- 3*	- 0.2	- 0.8
II	5α-A	- 0.2	- 0.5	0	- 0.5	- 1.0	- 2.5	0	- 0.2
III	5β-3β				*	*	*	- 0.6	- 0.4
IV	5α-3α	0	- 0.5	- 0.7	- 1.9*	S*	2.4*	- 0.2	- 0.8
V	5β-A(3)	0*	S*	- 2.0*	- 1.4*	- 2.5*	- 3.5*	- 0.6	S*
VI	5β-A-3α	S*	- 1.0*	- 3.0*	S*	- 1.4*	S*	- 2.0	S*
VII	5α-A(3)	- 1.4*	S*	0*	- 0.5	- 2.4	- 1.4	- 0.8	- 0.8
VIII	-14-A-3β	- 1.5*	- 3.0*	- 2.9*	- 0.4*	- 0.3*	S*	0	S
IX	-15-A-3β	- 0.5	- 3.5	- 1.5	- 0.8	- 0.8	S	- 1.6	- 1.0
X	5α-A-3β	S	S	S	S	S	- 2.5	S	- 1.5
XI	-14-A(3)	0*	- 2.0*	S*	- 1.6	- 3.0	- 2.9	- 1.0	0

* Indicates G_R-odd steroid.

cases described in Diagrams 1–16 confirmed and completed these observations, which can be summarized as follows.

RD. The reduction with sodium borohydride of (3) and (17) to hydroxyl groups was completed in 0.5 h; the complete reduction of (11) required 2 h. This unique peculiarity of (11) was used to synthesize (11)-containing steroids; it was also useful in the identification of these steroids[2]. RD reduction favoured almost exclusively the formation of one stereoisomer, as follows: 5α(3) → 5α3β; 5β(3) → 5β3α; Δ14(3) → Δ14 3β; (11) → 11β; (17) → 17β.

With the sole exception of (3) in 5β(3), all carbonyl groups were reduced to β-hydroxyl groups. The proportion of 17-α isomer formed was very small, being 0.8, 1.5 and 0.8% for 5βA3α(17), 5αA3α(17) and Δ15A3β(17), respectively. In the reduction of (3) and (11), the proportion of minor isomer was even smaller. A significant fraction of conjugated Δ14(3) was usually reduced to 5α3β and 5β3α, as exemplified in Diagram 6; neither Δ14A3β nor Δ15A3β were affected.

WK. In the one-step procedure used for the Wolff–Kishner reaction[8–10], the hydrazone was formed and decomposed in a single operation. The usual side-reaction leading from the carbonyl to the hydroxyl group was limited by the use of excess of hydrazine[11]. While over 90% removal of (3) and (17) was observed, that fraction of (11) which reacted did so entirely through the side-reaction, which in all instances followed the pattern of the RD reduction, as follows: 5α(3) → 5α3β; 5β(3) → 5β3α; (11) → 11β; (17) → 17β.

Double bonds in Δ14A3β and Δ15A3β were unaffected; the abnormal behaviour of Δ14A(3) is not discussed in this paper.

DO. Conversion of carbonyl groups to dioxolone (ketal) derivatives proceeded smoothly in excellent yield for (3) and (17). A small proportion of (11) reacted (*cf*., Diagram 16). The abnormal conversion of Δ14(3) into Δ15 ketal derivatives was not used in the present preparations. Replacement of *p*-toluenesulphonic acid by 1 mg of adipic acid[12] led to poor yields in all instances, although a maximum yield of 75% was obtained with 10 mg of adipic acid.

DO derivatives that are unaffected in RD, WK, OX and TMS reactions were used to protect carbonyl groups in reaction sequences. Their usefulness in steroid identification is briefly discussed below.

OX. Oxidation of hydroxyl groups at positions 3, 11 and 17 with chromium trioxide usually proceeded smoothly to completion in 5 h; occasionally, 3α and 3β hydroxyl groups were not completely oxidized (Diagrams 10, 13, 14 and 16). The extent of oxidation was readily adjustable by modification of the reaction time (*cf*., footnote§, Table V, and footnote ***, Table XI).

TMS. The formation of TMS derivatives was complete under the present conditions for all hydroxyl groups except 11β, which remained unaffected (*cf*., D/N retention time data in Diagrams 1–16).

While the retention time shift due to TMS derivatization was positive in all instances relevant to this paper, the extent of this shift was not precisely predictable even for closely related compounds. This is undoubtedly due to the fact that complex intramolecular interactions in non-derivatized hydroxylated steroids lead to subtle structural changes that affect retention times. Complex steroid–polar column interactions similarly explain why steroid retention times on polar columns cannot be predicted accurately.

The favourable effect of TMS derivatization on the thermal stability of steroids has been repeatedly confirmed throughout this work. Many non-derivatized steroids were altered or decomposed to a variable extent on both polar and non-polar columns.

HY. Hydrolysis of TMS and DO derivatives always proceeded smoothly to completion.

Product identification

Diagrams 1–16 demonstrate the general effectiveness of simple procedures in generating predictable steroids from compounds of known structure. Most important, they also indicate many possible ways in which these procedures can produce structural evidence complementary to that afforded by direct GLC analysis of natural steroids in fractions obtained by preliminary TLC separation. Indeed, the strategy adopted in this case parallels that used in identifying products in preparations described by Diagrams 1–16.

In one-step preparations, the major and minor products could be presumed from the above observations on the reaction course. Each of the presumed structures had corresponding M_R and G_R values from which L_R values could be calculated through eqn. 8. A comparison of calculated and observed values readily confirmed the presumed identity. Additional evidence could often be obtained from a comparison with L_R values calculated through eqn. 15: in this case, L_R values of M-corresponding members in related groups and ΔG_R values of the relevant groups were used.

In multi-step preparations, positively identified products of the first reaction each led to similarly predictable and identifiable products in the second step, and so on to the last step. Corresponding percentages in consecutive reaction mixtures were important clues to the correct derivation of products from those in the preceding reaction. In addition, the fact that many minor products in a given sequence had retention times identical with those of steroid standards or those of positively identified major products in one-step preparations provided further evidence of identity.

Further information was derived from a number of tests, including a comparison of retention times in non-derivatized (N) mixtures with those in derivatized (D) mixtures: for instance, a retention time shift following TMS derivatization always characterized the presence of hydroxyl groups other than 11β. A comparison of TMS-derivatized with DO-derivatized material revealed large retention time shifts, the magnitude of which indicated specific structures: the (17) → DO(17) conversion, for instance, corresponded to an increase of about 250 L_R units when (11) was present in the molecule, but only 210 L_R units with 11β (Diagrams 12 and 16).

L_R, M_R and G_R values

Table I gives M_R values of the androstane series, all but two of which were derived from authentic specimens. Values for Δ14A3β and Δ14A(3) were calculated from the L_R values of Δ14A3β(17) and Δ14A(3,17), respectively, using the G_R value of group (17) and a variant of eqn. 8, namely,

$$M_R = L_R - G_R \qquad (16)$$

Table II demonstrates the perfect correspondance of M_R values in the androstane and pregnane series, expressed by

$$M_R\text{P} = M_R\text{A} + 226 \qquad (17)$$

In fact, the two groups can be regarded as related, with $\Delta IG_R = 226$; in the present system, 226 is also the L_R increment for two additional methylene groups.

Each of the groups in Tables III–X features a number of G_R-odd steroids characterized by a G_R value considerably below that of G_R-normal compounds. This fact is clearly apparent in *LM* plots (Fig. 2).

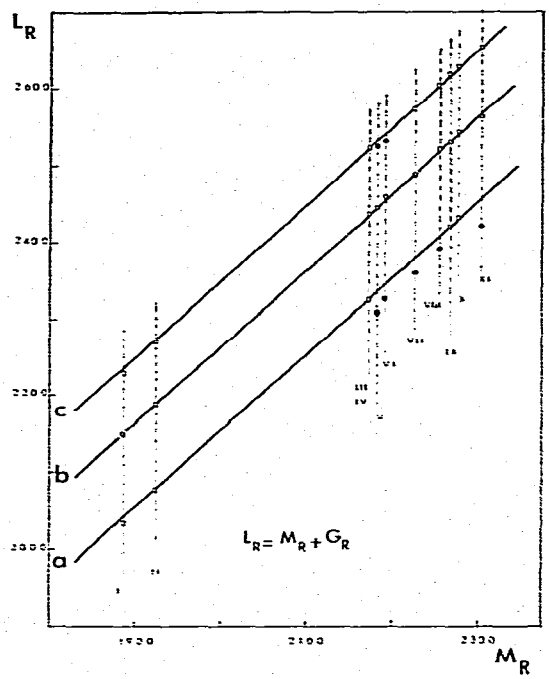

Fig. 2. Examples of *LM* plots. ○, G_R-normal steroids; ●, G_R-odd steroids. Radius of circles is approximately 2 L_R units. Group (11), plot a, includes five G_R-odd steroids; *cf.*, Table III. Group (17), plot b, includes G_R-normal steroids only; *cf.*, Table IX. Group 17β, plot c, includes two G_R-odd steroids; *cf.*, Table X.

The number of G_R-odd steroids varies from group to group. While group (17) has none, group 17β includes two; each of the three (11)-featuring groups includes five, and each of the 11β-featuring groups includes six G_R-odd steroids. As shown in Table XIII, G_R-odd steroids in all three (11)-featuring groups correspond to the same M-features; similarly, the G_R-odd steroids in the three 11β-featuring groups correspond to the same M-features; 5β(3), 5β3α and 14A3β are the only M-features that correspond to G_R-odd steroids in both sets of groups. The only M-features that correspond to G_R-normal steroids in all groups are 5αA, 15A3β and 5αA3β.

G_R values of multi-functional steroids (Table XII, first and last columns) do not necessarily equal the sum, ΣG_R, of G_R values of the component functions when present alone. Only G_R11β17β equals ΣG_R11β17β = G_R11β + G_R17β; G_R11β(17) is smaller and G_R17β(11) larger than the corresponding ΣG_R value; G_R(11,17) is much smaller than ΣG_R(11,17).

Ignorance of the above complexities, which is understandable in view of the

paucity of steroid standards, made the prediction of retention times on the basis of structure very hazardous prior to the present study. The finding of group-specific G_R-normal and G_R-odd steroid patterns immediately afforded a simple procedure for the accurate calculation of L_R values for G_R-normal steroids. Because the G_R value of all G_R-normal steroids in a group is very close to the G_R value of the group, the G_R value of a single G_R-normal steroid in the group could be used in eqn. 8 with M_R values taken from Table I in order to calculate the G_R value of all G_R-normal steroids in the group. One way to obtain quickly G_R values for all groups was to chromatograph simultaneously the TMS derivatives of G_R-normal steroids representative of all groups, *e.g.*, Mixture A in Table XI. As the internal standard S1, 5αA3β, is included in all test solutions (*cf. GLC techniques*), it was convenient to include in Mixture A as many 5αA3β compounds as possible so as to find the corresponding G_R values through eqn. 10, by the method of relative retention time (*cf. GLC data analysis*). The G_R values of components .14A3β17β and .15A3β11β17β were determined through eqn. 9, using M_R values taken from Table I. The calculated G_R values are given in Table XII, column 1, under the corresponding experimentally determined G_R values of the groups shown in parentheses.

Application of eqn. 8 using the calculated G_R values and M_R values taken from Table I afforded calculated L_R values for 45 G_R-normal steroids. The calculated values were compared with the experimental values listed in Tables III–X. Differences or calculation errors expressed in seconds of retention time are listed in Table XIII.

The .1G_R value of related groups

LL plots (Fig. 3) clearly indicated a close relationship between groups featuring (11), and similarly, between groups featuring 11β; such relationships were, moreover, suggested by the two sets of groups each having a characteristic pattern of G_R-odd and G_R-normal steroids (Table XIII). The key to such relationships and the basis for accurate calculations of L_R values is the .1G_R value of related groups. Eqns. 11, 13 and 14, as well as *LL* plots (see *GLC data analysis*) afforded several ways of calculating .1G_R values; eqn. 15 enabled accurate calculations of L_R values to be made for both G_R-odd and G_R-normal steroids.

The first column in Table XII shows G_R values derived from Mixture A as described above. The values in parentheses are the G_R values of groups shown in Tables III–X. The two sets of .1G_R values shown in Table XII were obtained by using eqn. 11 and pairs of G_R values in all possible combinations: .1G_R values in parentheses are derived from experimental G_R values. As seen from Table XII, discrepancies between the two sets of .1G_R values are generally small. The reason for this is that departures from strict normalcy such as oddity and, in the case of G_R-normal steroids, small deviations from the G_R value of the group, are similar for M-corresponding members of related groups; as .1G_R values are obtained as differences between L_R values (eqn. 13) of M-corresponding steroids or, as in the present case, as differences between G_R values (eqn. 11), deviations that affect L_R and G_R values are cancelled to a large extent in .1G_R values of related groups.

Although a single chromatogram of Mixture A gives all G_R and .1G_R values required for the calculations, calculation of L_R values through eqn. 15 still requires the L_R values of M-corresponding steroids. The six compounds in Mixture B, Table XI, and the first two compounds listed in Mixture C served this purpose. L_R values

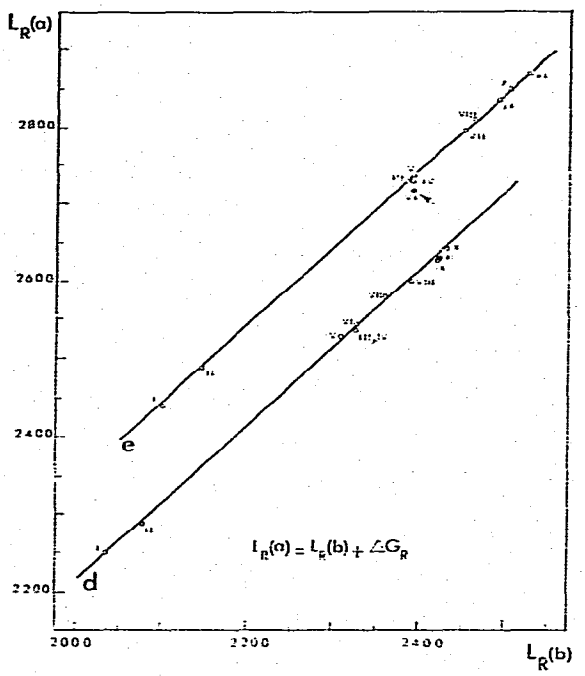

Fig. 3. Examples of L_iL plots. Plot d: Groups (11) and (11,17), *cf.*, Tables III and V, are related. Plot e, Groups 11β and 11β17β: *cf.*, Tables IV and VIII, are also related. However, point VI (arrow) fits poorly owing to the unique oddness of 5βA3α11β17β (*cf.*, Discussion).

for 5αA3α11β and 5αA3α11β17β, for example, were obtained from L_R 5αA3α11β(17) = 2634.2 (Table XI) with ΔG_R 11β(17)/11β = 255 and ΔG_R 11β(17)/11β17β = 90.6 (Table XII), as follows:

$$L_R\ 5\alpha A3\alpha 11\beta = L_R\ 5\alpha A3\alpha 11\beta(17) - \Delta G_R\ 11\beta(17)/11\beta$$
$$= 2634.2 - 255$$
$$= 2379 \text{ (found 2385, Table IV)}$$

and

$$L_R\ 5\alpha A3\alpha 11\beta 17\beta = L_R\ 5\alpha A3\alpha 11\beta(17) + \Delta G_R\ 11\beta(17)/11\beta 17\beta$$
$$= 2634.2 + 90.6$$
$$\approx 2725 \text{ (found 2729, Table VIII).}$$

Usually, one G_R-odd steroid will give the L_R value of two others. In some instances, which are readily discovered from *LL* plots, M-corresponding steroids that do not belong to related groups have approximately the same degree of oddity. This occurs for the 5βA(3)-compounds featuring (11) or 11β and the 14A3β-compounds of the same six groups. In each instance, a single M-corresponding steroid suffices to calculate the L_R values of five others. In all, the L_R values of 21 G_R-odd steroids were calculated by the above method. The differences with L_R values listed in Tables III–VIII, expressed in seconds of retention time, were entered in Table XIII.

The L_R value of three compounds could not be calculated by the above methods: G_R-odd steroids $5\beta A3\alpha 17\beta$ and $5\beta A17\beta(3)$, which have no correspondence in other groups, must be available as standards (Table XI). The case of $5\beta A3\alpha 11\beta 17\beta$ is unique: as shown by LL plots (*e.g.*, plot e, Fig. 3), the oddity of this compound far exceeds that of parent compounds in related groups. Consequently, it also must be available as a standard (Table XI).

The unavailability of standards for $5\beta A3\beta$-steroids featuring either (11) or 11β did not prevent a reliable calculation of L_R values to be made for these compounds. As will be described in a later paper, the following sets of groups in the pregnane (P) and androstane (A) series

P (11): (11,20): $20\beta(11)$	P 11β: $11\beta(20)$: $11\beta 20\beta$
A (11): (11,17): $17\beta(11)$	A 11β: $11\beta(17)$: $11\beta 17\beta$

are closely related (*cf.*, Fig. 4). As in all comparable instances the patterns of odd-steroids and the degree of oddity were identical, it was possible to establish that $5\beta A3\beta$-steroids featuring (11) in the androstane series were G_R-normal, and those featuring 11β were G_R-odd steroids (Table XIII). Calculations of L_R values for the $5\beta A3\beta$-steroids of the androstane series could then be made through eqn. 15 using the L_R values of the pregnane counterparts and appropriate ΔG_R values (Tables III–

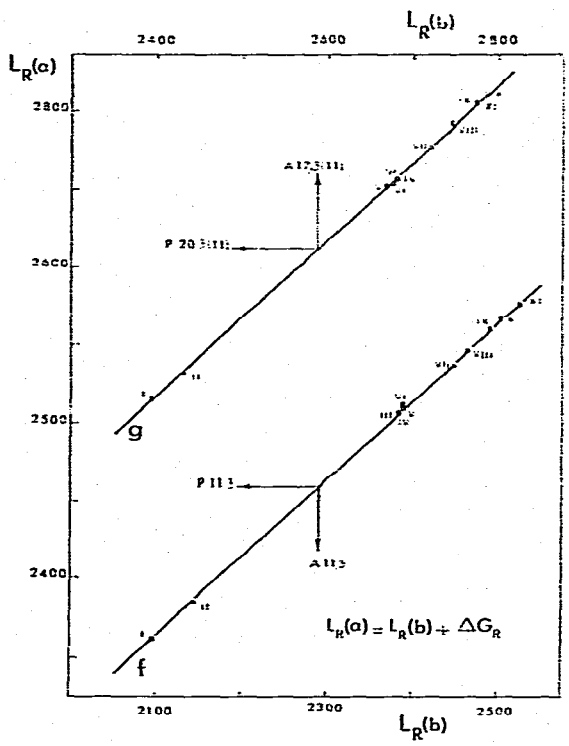

Fig. 4. Plot f illustrates relatedness of groups of the androstane and pregnane series containing (11). Groups featuring 11β are also related, *cf.*, plot g and Discussion.

VIII). Thus, given a single G_R-normal steroid standard for each of the six groups in the pregnane series, ΔIG_R values relating corresponding groups of the androstane and pregnane series can be derived; hence, all L_R values for 66 compounds of the pregnane groups can be calculated through eqn. 15 from the L_R values of M-corresponding androstane steroids. As other groups, in both the androstane and the pregnane series, are related to the above, the relatively small number of standards listed in Table XI will give calculated L_R values for a very large number of steroids.

A multiplicity of related groups implies a multiplicity of M-corresponding members. Given the appropriate ΔIG_R values, any M-corresponding steroid will lead to the L_R values of all others. In this flexible system, one can use whatever available standard is suitable, check the result of a calculation by using a different approach, and obtain a refined value from the average of two or three solutions. In this regard, it should be noted that the L_R value of a G_R-normal steroid can be calculated not only by the G_R method (eqns. 9 and 10), but also by the ΔIG_R method when the L_R value of an M-corresponding member of a related group is available.

Conditions for effectiveness

The number of steroid hormones and metabolites in extracts from animal tissues and body fluids is very large. Gas–liquid chromatograms of plasma neutral steroids, for example, contain so many peaks that most of them overlap to form a heavy background from which a few emerge. This situation obtains regardless of the type of column used. However, a detailed, precise measurement of peak retention times becomes possible when a preliminary fractionation, by TLC for instance, is performed[1,2].

Non-polar columns yield predictable retention times (see above). Optimal separation on such columns is achieved when the preliminary TLC fractionation is carried out on the basis of differences in polarity[2–5]. Under these conditions, a sufficient degree of peak separation is reached for the effective application of the present method when thin-layer chromatograms of neutral steroids are divided in 8–10 zones and the corresponding extracts are chromatographed on high-efficiency non-polar columns of 4,500–6,000 theoretical plates[2,4].

With few exceptions, steroid concentrations in plasma are low, yet many have important biological significance. Therefore, it is necessary that fractionation steps be designed in order to avoid interfering contamination by non-steroidal material originating from plasma as well as from solvents, reagents, glassware, etc., and by steroidal and other material derived from the experimenter's skin. Blank assays carried through the complete procedure used in this laboratory have shown that interfering contamination can be reduced to insignificant proportions by the application of simple precautionary measures, and this will be described in a later paper.

Understandably, when the number of structures that may possibly correspond to a given peak is small, subsequent peak identification is faster and simpler. On the other hand, this number increases with increasing uncertainty as regards the accuracy of calculated L_R values. Low calculation errors and a knowledge of the limits of error are therefore desirable.

Of the 63 errors observed for calculations (Table XIII) based on data listed in Table XI, 80% were $\leqslant$ 2 sec, 97% were $\leqslant$ 3 sec; the two largest errors, 3.5 sec, were approximately 1% of the retention time in each instance.

The relatively large errors corresponding to 5βA(3)-compounds of 11β-featuring groups, and to 14A3β-compounds of (11)-featuring groups, should be noted in Table XIII. In each instance, the L_R value of a single standard steroid was used to calculate L_R values in both sets of groups. Because the two sets of steroids have oddities which differ by about 3 L_R units, errors were larger in the set not related to the standard; indeed, the accuracy of calculations by the ΔG_R method depends on the reference steroids being closely related to those for which L_R values are sought. Note also the large error corresponding to G_R-normal 15A3β(11,17); the G_R value of this steroid, 358, is 6 L_R units smaller than that of 5αA3β(11,17) which was used as a standard (*cf.* Table V). It is evident that a bias can be introduced in calculations made through the G_R method by the standard selected for this purpose. In this example, the preferred standard would be 5βA(11,17), as the G_R value of this steroid, 361, is also the average G_R-normal value of the group.

Calculations by the present method amount essentially to simple algebraic summations of experimental L_R values. Errors on these values will affect the accuracy of calculations; they can be minimized by a strict adherence to normal conditions and the application of retention time corrections described under *GLC techniques*.

It is possible to apply the present method of calculation to data obtained with different internal standards, temperatures and flow-rates. Internal standards S1 and S2 used in the present study were selected because peaks of most naturally occurring steroids appeared between those of S1 and S2; the TMS derivatives of these standards were chromatographically very stable; their solutions in carbon disulphide had excellent keeping properties (ref. 4, p. 456), and the ratio R_{tX} was sensitive to changes in temperature. By bringing the retention times of S1 and S2 to 1.90 and 12.68 min, respectively, most chromatograms were completed within 15 min. However, modifications to the above conditions which did bring changes to M_R, L_R, G_R and ΔG_R values did not alter the patterns of G_R-odd steroids and related groups. Hence the basic equations and calculation methods still applied.

Data obtained with other methylpolysiloxane stationary phases on Gas-Chrom Q, such as SE-30 and OV-1, displayed the same patterns. Although L_R and G_R values were numerically different, the present calculation methods remained valid.

Practical aspects of GLC techniques

Normal conditions are readily maintained within limits defined under *GLC techniques* with GLC equipment featuring precision control of oven temperatures and carrier gas flow-rates. In this laboratory, temperature and flow-rate re-adjustments were seldom required for periods of up to 2 weeks of uninterrupted operation with only the detector turned off overnight.

On the other hand, a few hours were usually required in order to establish normal conditions from the moment the chromatograph was switched on; first, to allow the system to reach complete thermal equilibrium, then to make appropriate adjustments.

To bring the R_{tX} value within the required limits, changes as small as 0.2°, corresponding to very small angular displacements of the temperature adjustment knob, were necessary. This was best accomplished by tapping the knob tangentially on the appropriate side while observing the thermometer. The proposed retention time correc-

tions were valid only within the prescribed R_{tN} limits (eqn. 4), *i.e.*, within a small temperature interval, because the retention times of different steroids varied differently with the temperature (*cf.*, ref. 2, p. 6).

The maintenance of normal conditions within the prescribed limits, and the application of retention time corrections designed to compensate for small fluctuations of temperature and flow-rate within these limits, afforded the reproduction of retention times of all steroids within $\pm 1 \cdot 10^{-2}$ min for the last 8 years. After 4 years of continuous use, the first column pair still yielded the same L_R values although the number of theoretical plates, and the temperature corresponding to normal conditions, had decreased slowly by 30% and 2°, respectively.

Columns packed with 2–6% stationary phase on Gas-Chrom Q yielded identical L_R values when used under the same normal conditions.

The above observations clearly suggest that the adoption by laboratories of a common combination of normal conditions and column type would make possible the production and general use of GLC data of unprecedented precision.

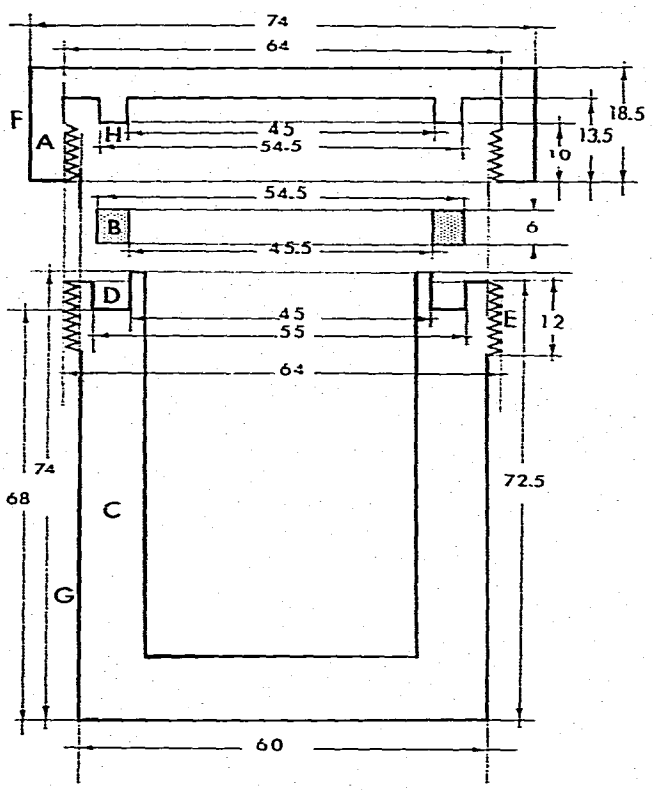

Fig. 5. Stainless-steel pressure vessel. Section, dimensions in millimetres. Lid A screws on to body (C); tightness is ensured by ring B, machined from a PTFE bar, seated in groove D and pressed by ridge H when the lid is tightened. The sides (F) of the lid and (G) of the body are knurled for a better grip when tightening, which must be done by hand. The thread (E) (1.5 mm per turn, 2 mm deep) must be worked-in with dry Molykote, Type Z (Alpha Molykote Corp., Stamford, Conn., U.S.A.) so as to ensure smooth, seizure-free operation.

APPENDIX

Pressure vessel for Wolff–Kishner reduction

The small stainless-steel vessel shown in Fig. 5 allowed the heating at 190° for several hours, under nitrogen, of very small volumes of reaction mixture without appreciable loss of ethanol. The system was found to be much more convenient than the use of sealed tubes. The micro-reaction tubes were simply filled with the required amounts of reagents (*cf.*, *Steroid preparation*) and placed in the vessel around a tube containing 4 ml of ethanol, which maintained a solvent-saturated atmosphere at all times during the heating period. During the cooling period, most of this ethanol condensed on the vessel walls and was found at the bottom. No significant loss of solvent occurred from reaction mixtures either from heating or during the preliminary filling with nitrogen. This was done by connecting the desiccator in which the metal vessel was placed (*cf.*, *Steroid preparation*), through a three-way vacuum valve alternately to a rotary vacuum pump (protected by a dry ice–ethanol-cooled trap), and to a nitrogen line under 30 lb. pressure. The desiccator was directly connected to a 800-mm long, mercury-filled, U-tube manometer whereby either vacuum ($\leq$ 3 mm) or pressure ($\leq$ 1 atm) could be monitored at all times. Each cycle of evacuating and filling took about 4 min; two consecutive cycles were applied.

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