652. *Gas Chromatography of Steroids. ΔR_{Mg} Values for Hydroxy- and Acyloxy-groups.*

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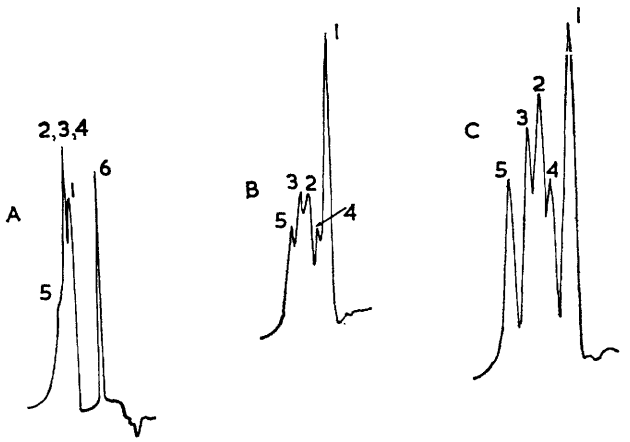
The ΔR_{Mg} parameters for hydroxy-, acetoxy-, and propionyloxy-groups at C-3 and C-20 of the steroid nucleus have been determined for the fluoroalkyl-silicone stationary phase QF-1-0065.

THE fluoroalkyl-silicone polymer QF-1-0065 [1] is a particularly suitable stationary phase for use in gas chromatography of steroids. It exhibits a greater affinity for ketones than for alcohols.[1] Further, the relative retention times for steroid alcohols can be altered by their conversion into simple derivatives such as methyl ethers,[1] trimethylsilyl ethers,[2] acetates,[1,3] propionates,[3] and trifluoroacetates.[1] The selective retention behaviour of different functional groups on QF-1-0065 has been considered from a quantitative standpoint [4] and it has been shown that the logarithm of the relative retention time (r) of a compound is made up of the additive contributions of the individual groups (g) together with the log r contribution of the nucleus (N) to which they are attached:

$$\log r = \Sigma\Delta R_{Mg} + \log r_N$$

ΔR_{Mg} and the related term ΔR_{Mr} have the same chromatographic meaning as in paper chromatography and are used here to denote changes in log r resulting from differences in structure of the type defined by Bush.[5] This paper reports the ΔR_{Mg} parameters for hydroxyl groups at C-3 and C-20 of the steroid nucleus, and also the ΔR_{Mr} values resulting from esterification of these groups.



Separation of a mixture of five pregnanediols (A), their diacetates (B), and their dipropionates (C).

(1) 5β-Pregnane-3α,20β-diol; (2) 5α-pregnane-3α,20α-diol; (3) 5α-pregnane-3β,20β-diol; (4) 5β-pregnane-3α,20α-diol; (5) 5α-pregnane-3β,20α-diol; (6) cholestane standard.

The retention times (relative to cholestane) for unsubstituted steroids chromatographed on 6% QF-1-0065 at 250° have been reported.[4] In Table 1 are given the relative retention times of some mono- and di-substituted steroids, and their fully acylated derivatives,

[1] VandenHeuvel, Haahti, and Horning, *J. Amer. Chem. Soc.*, 1961, **83**, 1513; Haahti, VandenHeuvel, and Horning, *Analyt. Biochem.*, 1961, **2**, 344.
[2] Luukkainen, Haahti, and Horning, *Biochim. Biophys. Acta*, 1961, **52**, 599.
[3] Knights and Thomas, *J. Endocrinol.*, 1962, **24**, iii.
[4] Knights and Thomas, *Analyt. Chem.*, 1962, **34**, 1046; *Nature*, 1962, **194**, 833; *Chem. and Ind.*, 1963, 43.
[5] Bush, *Biochem. Soc. Symp.*, 1960, **18**, 1; "Chromatography of Steroids," Pergamon Press, 1961.

chromatographed under these conditions. The ΔR_{Mg} values were obtained by subtracting, from the log r of the compounds listed, the log r values of the appropriate steroids devoid of the designated functional group. The results for these non-interacting substituents are sufficiently characteristic that generalizations can be made about the relative elution rates of steroid epimers. For epimers which differ only in respect of the configuration of the C-3 hydroxyl group, the axial compound is eluted ahead of its equatorial isomer. Similarly, the 20β-alcohol would be the more mobile of any pair of steroids differing only in configuration at C-20. The relative selectivity towards isomers differing in configuration at more than one position is also predictable. Thus a chromatogram of a mixture of five pregnanediols (Fig. 1A) shows only two peaks (with a single inflection), the main peak corresponding to an unresolved mixture of 5α-pregnane-3α,20α-

TABLE 1.

ΔR_{Mg} parameters of some hydroxy- and acyloxy-substituents for compounds chromatographed on QF-1-0065.

		Alcohol		Acetate		Propionate	
Group	Steroid	r	ΔR_{Mg} (OH)	r	ΔR_{Mg} (OAc)	r	ΔR_{Mg} (OPr)
3-OH (equatorial)	5α-Androstan-3β-ol	0·54	0·48	0·79	0·65	0·98	0·74
	5α-Pregnan-3β-ol	0·83	0·47	1·27	0·66	1·51	0·73
	5α-Pregnane-3β,20α-diol	2·27	0·46	5·18	0·62	7·18	0·71
	5α-Pregnane-3β,20β-diol	2·02	0·46	4·99	0·64	6·52	0·72
	5α-Cholestan-3β-ol	3·03	0·48	4·36	0·64	5·16	0·71
	Average		0·47		0·64		0·72
	5β-Androstan-3α-ol	0·50	0·48	0·67	0·61	0·78	0·67
	3α-Hydroxy-5β-androstan-17-one	2·23	0·49	3·04	0·62	3·42	0·68
	5β-Pregnan-3α-ol	0·77	0·50	0·996	0·61	1·16	0·67
	3α-Hydroxy-5β-pregnan-20-one	2·76	0·48	3·63	0·60	4·13	0·65
	5β-Pregnane-3α,20β-diol	1·87	0·47	3·97	0·62	5·18	0·70
	5β-Cholestan-3α-ol	2·88	0·50	3·64	0·61	4·19	0·67
	Average		0·49		0·61		0·67
3-OH (axial)	5α-Androstan-3α-ol	0·48	0·43	0·72	0·61	0·80	0·65
	3α-Hydroxy-5α-androstan-17-one	2·19	0·43	3·29	0·62	3·58	0·65
	5α-Pregnan-3α-ol	0·75	0·43	1·13	0·61	1·25	0·65
	5α-Pregnane-3α,20α-diol	2·08	0·43	4·54	0·57	6·17	0·65
	Average		0·43		0·60		0·65
	5β-Androstan-3β-ol	0·47	0·43	0·70	0·63	0·86	0·71
	3β-Hydroxy-5β-pregnan-20-one	2·66	0·46	3·94	0·63	4·37	0·68
	5β-Cholestan-3β-ol	2·40	0·43	3·80	0·63	4·40	0·69
	Average		0·44		0·63		0·69
20α-OH	5α-Pregnan-20α-ol	0·78	0·44	1·23	0·63	1·38	0·69
	5α-Pregnane-3α,20α-diol	2·08	0·44	4·54	0·60	6·17	0·69
	5α-Pregnane-3β,20α-diol	2·27	0·45	5·18	0·61	7·18	0·68
	5β-Pregnane-3α,20α-diol	2·17	0·45	4·26	0·63	5·82	0·70
	Average		0·45		0·62		0·69
20β-OH	5α-Pregnane-20β-ol	0·71	0·40	1·15	0·61	1·25	0·65
	5α-Pregnane-3β,20β-diol	2·02	0·40	4·99	0·58	6·52	0·64
	5β-Pregnan-20β-ol	0·63	0·41	0·95	0·59	1·04	0·63
	5β-Pregnane-3α,20β-diol	1·87	0·39	3·97	0·60	5·18	0·65
	Average		0·40		0·60		0·64

diol, 5α-pregnane-3β,20β-diol, and 5β-pregnane-3α,20α-diol. For the first two of these diols, ΔR_{Mg} for an equatorial hydroxyl group is greater than for an axial hydroxyl group by 0·04 but this is counteracted by the smaller ΔR_{Mg} of the 20β-alcohol than the 20α-alcohol (difference 0·05). Similarly, 5α-pregnane-3α,20α-diol and 5β-pregnane-3α,20α-diol

TABLE 2.

ΔR_{Mr} acylation values for 3-hydroxy-steroids.

Series	Group	ΔR_{Mr} (acetyln.)	ΔR_{Mr} (propionyln.)
5β	3-Equatorial OH (α)	0·12	0·18
	3-Axial OH (β)	0·19	0·25
5α	3-Equatorial OH (β)	0·17	0·25
	3-Axial OH (α)	0·17	0·22
Δ^5	3β-OH	0·18	0·24

are not resolved because the axial–equatorial difference is in this case balanced by the opposing log r N resulting from a change in configuration at C-5 (α→β = −0·06).

It will be seen in Table 1 that the ΔR_{Mg} value for a 3-hydroxyl group is dependant only on its axial–equatorial nature and is not significantly influenced by the configuration at

TABLE 3.

ΔR_{Mr} (acylation) values for 3β-hydroxy-Δ^5-steroids.

Steroid	r	ΔR_{Mr} (acetyln.)	ΔR_{Mr} (propionyln.)
3β-Hydroxyandrost-5-en-17-one	2·15	0·18	0·26
3β-Hydroxypregn-5-en-20-one	2·78	0·19	0·22
3β,17α-Dihydroxypregn-5-en-20-one	4·19	0·17	0·24
Cholest-5-en-3β-ol	2·77	0·17	0·25

C-5. On the other hand, ΔR_{Mg} parameters for acetates and, to a more marked extent, propionates appear to be more sensitive to differences in configuration both at C-3 and C-5.

In practice these differences give rise to the improved resolution pattern seen on comparison of a chromatogram of the five diols (Fig. 1A) with the chromatograms of the same mixture after acetylation (Fig. 1B) or propionylation (Fig. 1C). An important consequence of the greater variation of ΔR_{Mg} values for acetates and propionates, as compared with those of the free alcohols, is that ΔR_{Mr} (acylation) increments become characteristic of the functional group involved. This can be seen from the ΔR_{Mr} (acylation) values for 3-hydroxy-steroids summarized in Table 2. Also included in this Table is the acylation increment for a 3β-hydroxy-Δ^5-compound averaged from the values given in Table 3. The ΔR_{Mr} (acetylation) values for most of the substituents lie within the range 0·17—0·19. The low acetylation increment for an equatorial hydroxyl group of the 5β-series is noteworthy, because it is a property by which this group can be distinguished from the others listed in the Table. A similar effect is noted for its ΔR_{Mr} (propionylation) value. A practical consequence of this low acylation increment is that whereas 5α- and 5β-pregnane-3α,20α-diol are inseparable under the chromatographic conditions used in this work, the two compounds can be resolved as their diacetates or dipropionates. The 5β-compound is eluted ahead of its 5α-isomer, and the difference in log r of the resolved esters is of the order expected from the difference in ΔR_{Mr} (acylation) values of a 3α-hydroxy-group in the 5α- and 5β-series.

The application of this method of chromatographic analysis to the identification of steroids in natural extracts is being studied.[6]

EXPERIMENTAL

A Pye argon chromatograph having a strontium-90 ionization detector was used. Acid-washed Celite (545) was graded to 100—120 mesh and then coated with 6% QF-1-0065 (Dow

[6] Chamberlain and Thomas, *Biochem. J.*, 1963, **86**, 3P; Chamberlain, Knights, and Thomas, *J. Endocrinol.*, in the press.

Corning Corpn.; British supplier, Midland Silicones Ltd.). A modified Swoboda back-flush column [7] (Pye catalogue No. 12540) was used in order to dispense with the need for an argon preheater. The column (effective length 3 ft. 3 in.) was filled with packing in 6 in. steps, each load being consolidated by bouncing the column on the floor. A glass-yarn plug was then inserted to within 2 in. of the top of the column. The column was inverted and tapped until the packing had been completely displaced from the bottom. It was then turned right way up and bounced until the packing was fully consolidated. The glass yarn was pushed down on to the top of the packing. The precolumn (effective length 6 in.) was also packed with Celite coated with 6% QF-1-0065. The column was preconditioned at 250° for 18 hr. before use.

Operating Conditions.—The temperature of the column was maintained at 250°. Commercially available 99·95% argon was used without further purification. The inlet pressure was adjusted to 20—22 lb/sq. in., giving a flow rate of 50—55 ml./min.

Sample Injections.—Samples (*ca.* 10 μg.) were introduced on to rolls of 50-mesh stainless-steel gauze either from solution (5—10 μl) or as solid. The gauzes were dropped on to the column after the argon had been turned off and the precolumn removed. About 10 gauzes could be accommodated before the column required emptying. In view of the low heat capacity of the gauzes, no time was needed to allow them to reach working temperature.

Steroids.—The monofunctional steroid alcohols were kindly provided by Professor W. Klyne from the Medical Research Council Steroid Reference Collection, and the pregnanediols by Dr. E. Forchielli (The Worcester Foundation). The remaining steroids were obtained from commercial sources. Acyl derivatives were prepared by treating a solution of the steroid in pyridine with the appropriate anhydride at room temperature overnight. The excess of reagent was removed in a stream of nitrogen, and the product chromatographed without further purification.

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[7] Swoboda, *Chem. and Ind.*, 1960, 1262.