CHROM. 3507

Paper chromatographic evaluation of the contribution to polarity of hydroxyl and other groups at the C-17, C-20 and C-21 positions of the pregnane side chain

Considerable information is available concerning the contribution to polarity of hydroxyl and other groups at various sites on the pregnane nucleus[1,2], but little is known relative to the activity of such groups at the three possible side chain positions*, particularly at C-20. In an effort to meet this need we attempted first to utilize some of the C-20-epimeric 17α,20-glycols, 20,21-glycols and 17α,20,21-glycerols which we had prepared previously from the corresponding 20-ketones[3–5]. But this led to difficulties in interpretation since many of the ketones designated compounds of reference were themselves subject to 1:2 or other interactions. We therefore prepared a number of 20-deoxysteroids[6] of which the more important members constitute the series 5β-pregnan-3α-ol (I), 5β-pregnane-3α-21-diol (II), 5β-pregnane-3α, 17α-diol (III) and 5β-pregnane-3α, 17α,21-triol (IV, Fig. 1). These compounds, together with some of those previously prepared, gave unambiguous values. The purpose of this paper is to present these results.

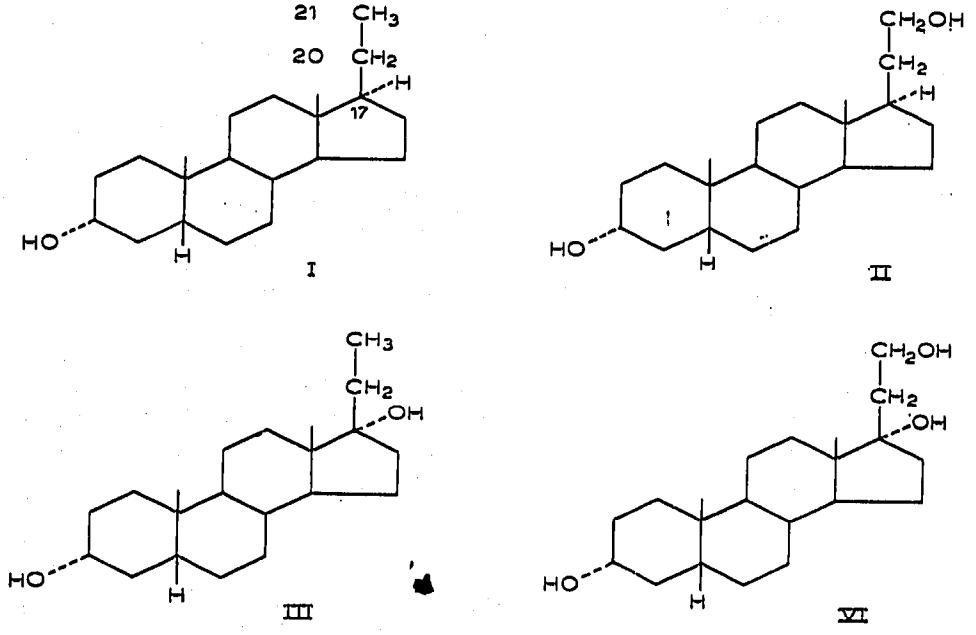

Fig. 1. Structures of the 20-deoxysteroids. The arabic numbers, in I, denote the side chain positions*. I = 5β-Pregnan-3α-ol; II = 5β-pregnan-3α,21-diol; III = 5β-pregnane-3α,17α-diol; IV = 5β-pregnane-3α,17α,21-triol.

Methods

Chromatography was carried out in the descending fashion on unwashed 19 × 60 cm sheets of Whatman No. 1 paper. Following application of the steroids in the minimally detectable amounts, the sheets were equilibrated for 8 to 12 h in paper-lined glass

* Carbon-17 is part of the ring system, but it is convenient for the purposes of this study to consider the 17α-hydroxyl group a substituent of the side chain.

jars containing both phases of the system. The temperature, usually 24° at the beginning of equilibration, was allowed to fall to around 22° during the equilibration and chromatography periods; this appeared to give better defined solvent fronts, especially with those systems containing a large proportion of isooctane. The steroids were detected at the conclusion of chromatography by heating the sheets at 70–80° following dipping in a 10 % solution of phosphomolybdic acid in ethanol.

The systems were based where possible on the components toluene–isooctane–aqueous methanol since this type of system had the widest useful range in polarity of the several tested. In order to standardize the procedure further, the composition of individual systems was adjusted so that the R_F value of the most polar (least mobile) steroid was around 0.1. The composition of the systems referred to by number in Tables II-IV appears in Table I.

TABLE I

COMPOSITION OF PAPER CHROMATOGRAPHIC SYSTEMS

System No.	*Composition*[a] *(ml)*
1	Tol, 20; Iso, 180; AM, 160; HOH, 40
2	Tol, 80; Iso, 120; AM, 160; HOH, 40
3	Tol, 120; Iso, 80; AM, 160; HOH, 40
4	Tol, 170; Iso, 30; AM, 160; HOH, 40
5	Iso, 200; AM, 150; HOH, 50
6	Iso, 200; AM, 170; HOH, 30
7	Tol, 70; Iso, 130; AM, 160; HOH, 40
8	Tol, 40; Iso, 160; AM, 160; HOH, 40
9	Tol, 30; Iso, 170; AM, 160; HOH, 40
10	Tol, 105; Iso, 95; AM, 160; HOH, 40
11	IPE, 30; Hep, 170; AM, 160; HOH, 40

[a] Tol = toluene; Iso = isooctane (2,2,4-trimethylpentane); AM = methanol; IPE = isopropyl ether; Hep = *n*-heptane.

The experimental method consisted of simultaneously chromatographing pairs of steroids, one lacking (the "stem compound") and the other possessing the hydroxyl or other group in question. The results are expressed in terms of the ΔR_{M0} convention of BUSH[1], which is based on the original ΔR_M concept of BATE-SMITH AND WESTALL[7].

Results and discussion

We have considered our results in terms of rather arbitrarily defined ΔR_{M0} limits: values over 1.9 seem appropriate for unhindered primary hydroxyl groups, while the range 0.9 to 1.9 would include unhindered to somewhat hindered secondary and tertiary hydroxyl groups*. We recognize that observed ΔR_{M0} values express the net effect of a number of influences, but for the purposes of this discussion we have limited ourselves to a consideration of those factors which can be predicted on the basis of the structure and conformation of the side chain. Considered in these terms, decreases in ΔR_{M0} values from "base" levels (*vide infra*) are ascribed chiefly to 1:2 (vicinal) or 1:3 interactions between the various substituents of the side chain.

* These limits are derived from our earlier results and from the compilations of BUSH[1] and from GLICK[8]. Too few values are available to permit more than an arbitrary placing of the limits.

Table II gives $\Delta R_{M\theta}$ values for hydroxyl groups at the C-17, C-20 and C-21 positions both in the absence and in the presence of other hydroxyl groups. Values for examples 1, 2 and 3 are for hydroxyl groups at C-17, C-20 and C-21, respectively, under circumstances where the side chain is otherwise unsubstituted. They may be regarded as "base" values, useful as standards of reference. Opportunity for 1:2 interaction is provided in examples 4 and 5 where a hydroxyl group is introduced at C-20 in a 17α-ol (example 4) and in a 21-ol (example 5). Moderate to marked interaction occurs in both cases as judged by the diminished $\Delta R_{M\theta}$ values. That C-17 : C-21 (1:3) interaction also can occur is shown by the low values in example 6 (introduction of a hydroxyl group at C-21 in a 17α-ol) and in example 7 (the reverse). Example 8 illustrates that where both 1:2 and 1:3 interactions are possible, additive effects need not result.

The effect of a large substituent at C-21 (carbomethoxyl or tosyloxyl) on the contributions of hydroxyl groups at C-17 and C-20 is indicated in Table III. Comparison of the $\Delta R_{M\theta}$ values for examples 9, 10 and 11 with, respectively, those for examples 2, 4 and 1 of Table II, shows that: (a) the carbomethoxyl group can interact with hydroxyl

TABLE II

CONTRIBUTION OF HYDROXYL GROUPS AT C-17, C-20, AND C-21 POSITIONS

Example No.	*Stem compound*	*System No.*	R_F *values*[a]		$\Delta R_{M\theta}$[b]			
					C-17	*C-20*		*C-21*
						α	β	
1	5β-Pregnan-3α-ol	1	0.91	0.38	1.21			
2	5β-Pregnan-3α-ol	1	0.91	0.13, 0.20		1.82	1.59	
3	5β-Pregnan-3α-ol	1	0.91	0.09				1.98
4	5β-Pregnane-3α,17α-diol	2	0.62	0.11, 0.15		1.12	0.96	
5	5β-Pregnane-3α,21-diol	3	0.55	0.11, 0.15		0.98	0.83	
6	5β-Pregnane-3α,17α-diol	3	0.74	0.32				0.77
7	5β-Pregnane-3α,21-diol	3	0.55	0.32	0.41			
8	5β-Pregnane-3α,17α,21-triol	4	0.47	0.11, 0.13		0.85	0.76	

[a] In this and in the remaining tables, values in the left column are derived from the stem compounds, and those in the right column from the substituted stem compounds. In the 20-ol series, the order is α,β.
[b] All values are positive.

TABLE III

CONTRIBUTION OF HYDROXYL GROUPS AT C-17 AND C-20 IN C-21 METHYL ESTERS AND TOSYLATES

Example No.	*Stem compound*	*System No.*	R_F *values*		$\Delta R_{M\theta}$		
					C-17	*C-20*	
						α	β
9	Methyl 5β-pregnan-3α-ol-21-oate	5	0.76	0.09, 0.12		1.49	1.37
10	Methyl 5β-pregnane-3α, 17α-diol-21-oate	2	0.66	0.15, 0.20		1.04	0.89
11	Methyl 5β-pregnan-3α-ol-21-oate	6	0.58	0.16	0.85		
12	21-Tosyloxy-5β-pregnane-3α,17α-diol	7	0.46	0.11, 0.17		0.84	0.62
13	21-Tosyloxy-5β-pregnan-3α-ol	8	0.78	0.16	1.26		

groups at C-20 and that this effect is diminished (or obscured) if a hydroxyl group also is present at C-17, and (b) in the absence of a functional group at C-20, the carbomethoxyl group interacts strongly with a 17α-hydroxyl group. When the values for examples 12 and 13 are compared with those for examples 4 and 1 in Table II, it can be concluded that : (a) substitution of the large tosyloxyl for the small methyl group at C-21 serves further to depress the activity of the 17α,20-diol system, and (b) in contrast to the carbomethoxyl group, 1:3 interaction between the tosyloxyl and the 17α-hydroxyl groups does not occur.

TABLE IV

CONTRIBUTION OF THE CARBONYL GROUP AT C-20

Example No.	*Stem compound*	*System No.*	R_F *values*		ΔR_{Mg} *C-20*
14	5β-Pregnan-3α-ol	6	0.88	0.31	1.23
15	5β-Pregnane-3α,21-diol	9	0.14	0.13	0.03
16	5β-Pregnane-3α,17α-diol	8	0.51	0.13	0.84
17	5β-Pregnane-3α,17α,21-triol	10	0.27	0.10	0.51
18	Methyl 5β-pregnan-3α-ol-21-oate	6	0.58	0.33	0.43

Table IV gives the contribution of the C-20 carbonyl group both in the absence and in the presence of hydroxyl or other groups. Example 14 gives the "base" value in the absence of other substituents on the side chain. The remaining values show that interaction between the C-20 carbonyl and adjacent groups is moderate (17α-hydroxyl, example 16), marked (21-carbomethoxyl, example 18) and extreme (21-hydroxyl, example 15). The value for example 17 suggests that where the carbonyl group at C-20 is flanked by two hydroxyl groups, the above 20:21 interaction is diminished.

The very strong interaction within the 20-keto-21-ol system was unexpected. Unlike other pairs, where ΔR_{Mg} values were easily duplicated and where different types of systems gave very similar valuesr, echromatography of the example 15 pair (system 9) gave a slightly negative ΔR_{Mg} value and chromatography with system 11 provided a value of + 0.28.

Our observed ΔR_{Mg} values for isolated hydroxyl groups at C-21, C-20 and C-17 are of the magnitude expected for unhindered primary, secondary and tertiary hydroxyl groups, respectively. We wish to point out that the values in Table II for the contribution of hydroxyl groups at C-20 are distinctly larger, with but one exception, than those obtained in this laboratory for what appear to be unhindered secondary nuclear hydroxyl groups (average values of 1.44 and 1.38 in two series). It seems reasonable to conclude that such nuclear alcohols are more hindered than generally supposed.

Since the chemical activity of a particular oxygen function on the side chain is largely uninfluenced by the introduction of additional substituents in its vicinity, it is evident that the chromatographic effects which we observed are not due primarily to steric interference between groups. A more likely explanation lies in the occurrence of hydrogen bonding, particularly of the intramolecular type. The possibility of free

rotation around both the C-17: C-20 and C-20: C-21 bonds can readily result in conformations which favor the generation of five- and six-membered rings. Formulas for eight possible ring systems are indicated in Fig. 2. Structures of this type could account for the observed deviations in ΔR_{Mg} values by limiting the association of hydroxyl or carbonyl groups with solvent molecules, particularly of the stationary phase. The ability of a third polar group to diminish the effect of an existing interaction (as in examples 8, 12 and 17) may be due to a sterically induced restriction

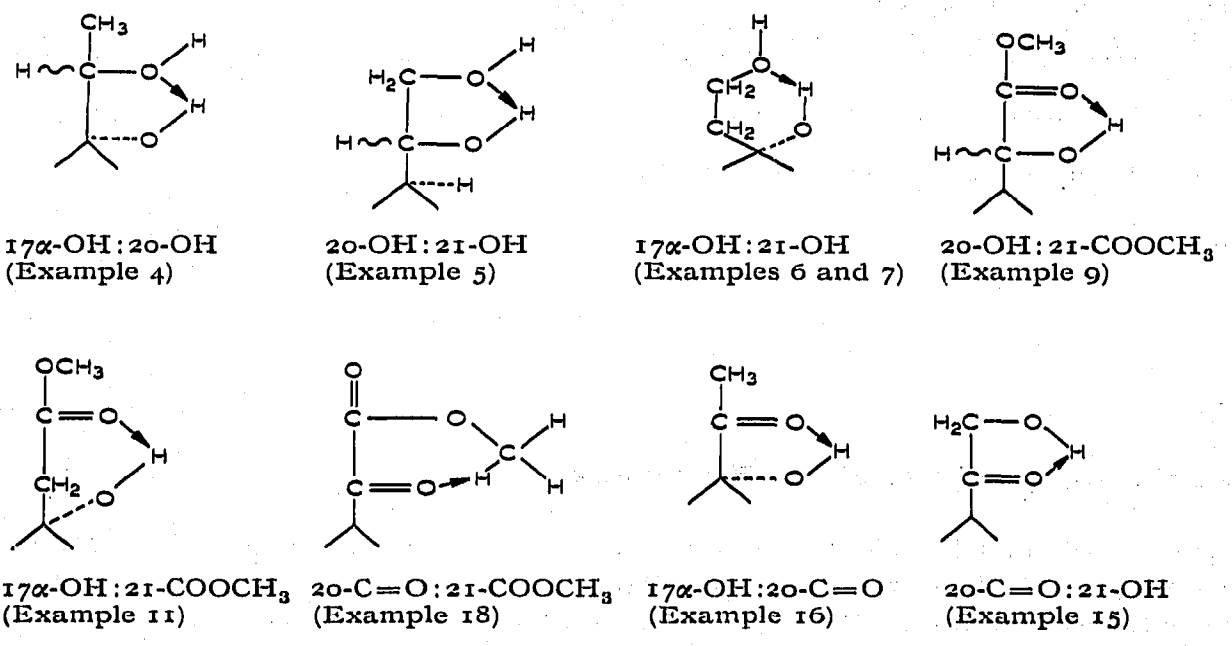

Fig. 2. Representations of possible intramolecular hydrogen-bonded ring systems. The interacting groups are indicated in each case.

of rotation which lessens the formation of the preferred conformer. The fact that ΔR_{Mg} differences between 20α- and 20β-ols remain relatively constant regardless of the nature of adjacent substituents suggests that the degree of association of both epimers with polar groups is similar even though considerable steric barriers have to be overcome in some cases in order to achieve this state. Support for this view may be derived from the case of ethylene glycol, in which strong hydrogen bonding occurs despite the steric and dipolar repulsion of the hydroxyl groups[9]. One must conclude that the energy gained in hydrogen bonding outweighs the repulsive forces.

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1 I. E. BUSH, *The Chromatography of Steroids*, Pergamon, New York, 1961.
2 R. NEHER, *Steroid Chromatography*, Elsevier, Amsterdam, 1964.
3 M. L. LEWBART AND J. J. SCHNEIDER, *J. Org. Chem.*, 29 (1964) 2559.
4 J. J. SCHNEIDER AND M. L. LEWBART, *Tetrahedron*, 20 (1964) 943.
5 M. L. LEWBART AND J. J. SCHNEIDER, *J. Biol. Chem.*, 241 (1966) 5325.
6 M. L. LEWBART AND J. J. SCHNEIDER, *J. Org. Chem.*, 33 (1968).
7 E. C. BATE-SMITH AND R. G. WESTALL, *Biochim. Biophys. Acta*, 4 (1950) 427.
8 D. GLICK (Editor), *Methods of Biochemical Analysis*, Vol. 13, Interscience, New York, 1965, pp. 357–433.
9 E. L. ELIEL, *Stereochemistry of Carbon Compounds*, McGraw-Hill, New York, 1962, pp. 131–132.

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CHROM. 3453

Estimation of the products of DNA alkylation

A variety of nucleic acid bases are methylated both *in vivo* and *in vitro* by chemical mutagens such as N-methyl-N-nitro-N-nitrosoguanidine and N-methyl-N-nitroso-*p*-toluenesulfonamide (MNTS)[1]. As no simple convenient assay for the commonly alkylated bases (7-methylguanine, 1-methyladenine, and 3-methyladenine) was available, a thin-layer chromatographic procedure was developed.

Methylated DNA was prepared by adding 0.5 mg of ^{14}C-MNTS (New England Nuclear Corporation) and 9.5 mg of MNTS (Aldrich Chemical Co.) in 2 ml of ethanol to 10 ml of water containing 10 mg of highly polymerized DNA (Nutritional Biochemicals Corporation) and reacting for 7 days in the dark at 26°. The products were separated by two extractions with chloroform–octanol (1:1, v/v) (in which MNTS is soluble), followed by chromatography on Sephadex G-25 (Pharmacia Fine Chemicals, Ltd.). After concentration using a rotary evaporator, the DNA was hydrolyzed in 2 ml of 1 *N* HCl at 100° for 1 h to release purine bases and pyrimidine nucleotides.

Adenine, guanine, 7-methylguanosine, thymidine, and deoxycytidine monophosphoric acid were obtained from Sigma Chemical Co. The 7-methylguanosine was hydrolyzed to the free base using 1 *N* HCl at 100° for 1 h. 1-Methyladenine and 3-methyladenine were prepared according to BROOKES AND LAWLEY[2].

Thin-layer plates were prepared in the standard manner with MN cellulose (300 G containing binder, Canadian Laboratories Ltd.). The samples were applied to the plates (activated at 120° for 30 min) using 5 μl capillary pipettes. Five micrograms each of solutions of authentic samples of the methylated purines were also applied as markers. After development with methanol–HCl–H_2O (7:2:1, v/v) for a