16α-HYDROXY STEROIDS

III. RECOGNITION OF THE 16α,17α-DIOL FEATURE OF TRIAMCINCOLONE BY CYCLIC KETAL FORMATION ON PAPERGRAMS

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INTRODUCTION

In studies of microbiological 16α-hydroxylation of steroids bearing the dihydroxyacetone side chain it was necessary to establish the presence of the anticipated 16α,17α-diol feature by definitive means with very small amounts of sample. Although mobility behavior in several paper chromatographic systems serves adequately for recognition of known steroids, other evidence is required when dealing with steroids not previously characterized. Of the auxiliary techniques commonly employed to support papergram identifications—derivative formation (acetylation, oxidation), elution and spectral studies, bioassay, etc.—none was considered sufficiently specific for our purposes. Of the specific reactions of 16α,17α,21-trihydroxy-20-ketones the formation of 16α,17α-cyclic ketals[1,2] was selected as being both specific for the 16α,17α-diol feature and as being adaptable to reaction on the very paper which later serves for papergram analysis.

This report deals with the development and application of the method of forming 16α,17α-cyclic ketals and acetals *in situ* on papergrams for the early recognition of the 16α,17α-diol feature of triamcinolone (9α-fluoro-11β,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20-dione) and related 16α-hydroxylated steroids.

EXPERIMENTAL

Paper chromatographic separations of the steroids and steroid ketal (acetal) derivatives formed in the reaction studies were made using the solvent systems already described[3]. Systems V and VI were used for analysis of the ketals and acetals formed; System II was used for the analysis of unaltered steroid alcohols. The usual precautions and techniques for operation of Bush-type systems were carefully observed[4]. The finished chromatograms were examined for ultraviolet absorbing zones, for unsaturated 3-ketosteroid zones (*via* isonicotinic acid hydrazide) and for reducing α-ketol zones (*via* alkaline tetrazolium blue) in the manner already described[3,5].

The steroids used were of high purity as evidenced by papergram behavior, ultraviolet absorption spectra, and infrared absorption spectra. 16α,17α-Cyclic

ketal and acetal derivatives used as reference materials were prepared by the method of FRIED[1]. All steroids were applied at 20 μg levels to sheets of unwashed Whatman No. 1 filter paper as 1 mg/ml solutions in absolute methanol.

Cyclic ketal formation on paper was promoted by spotting a suitable volume (20–40 μl) of diluted perchloric acid (0.3 ml of reagent 70 % perchloric acid diluted to 50 ml with either acetone or with methanol) directly over the dried steroid spot previously applied. The area covered by the diluted acid was purposely made larger than that of the steroid zone so as to assure complete reaction of all of the steroid. The acetone-diluted reagent was used for acetonide formation, the methanol-diluted reagent for other ketals and for acetals. The treated paper was then hung in a closed chamber saturated with vapors of the carbonyl compound to be used. Saturation was assured by a layer of the liquid ketone in the bottom of the chamber. The chamber was kept at 29°, the same temperature at which the chromatogram was developed. After a suitable time interval of exposure to the carbonyl compound, the paper was removed, dried momentarily in air and developed chromatographically.

A standard reaction condition was established using 20 μg of steroid, 30 μl of diluted perchloric acid and exposure to acetone for 16 h. These conditions are sufficient for complete conversion of the 16α-hydroxy steroids studied to their respective 16α,17α-acetonides.

The treated paper is equilibrated and developed in the system of choice and the products formed of the reaction are visualized and marked. Suitable reference compounds of the anticipated cyclic ketal are run along with the experimental sample. By inspection the identification of a reaction product ketal with a reference known ketal can be made, and thus the presence of the 16α,17α-diol feature surmised. In no case was the mobility of the derivative formed on paper different from that of an authentic reference derivative.

Control experiments were run with each steroid used. Each sample was:
(a) treated with acid, held for 16 h in air, but not exposed to acetone,
(b) not treated with acid but exposed to acetone for 16 h, and
(c) treated with acid and exposed to acetone for 16 h.

These control experiments were performed on both 16α-hydroxy and non-16α-hydroxy steroids. Each experiment was run in at least two different chromatographic systems.

Confirmation of acetonide formation with triamcinolone was made by streaking 200 μg of triamcinolone across a 7-inch sheet of paper. Seven such sheets were prepared. Over each steroid zone was streaked 150 μl of a solution of 0.30 ml of 70 % perchloric acid diluted with 25 ml of acetone. The sheets were exposed to acetone vapors for 16 h, then equilibrated and run in System VI[3]. The steroid zone was located by ultraviolet light absorption, eluted with hot acetone, the eluted material concentrated and chromatographed over Florisil (adsorbed from benzene solution), reconcentrated and rechromatographed on two 7-inch sheets of filter paper using System V[3]. The steroid zone was located by cutting a small strip from the center of the sheets and visualizing with isonicotinic acid hydrazide and tetrazolium blue. The

steroid zone was eluted with hot acetone, concentrated *in vacuo*, and the crystalline precipitate was recrystallized from methanol and then from acetone. Comparison of infrared absorption spectra of *ca.* 100 μg of the sample in a potassium bromide disk over the range 2–15 μ and of sulfuric acid absorption spectra[6] over the range 220–600 mμ established the identity of the sample with that of authentic triamcinolone 16α,17α-acetonide.

RESULTS

The reaction on papergrams of a variety of 16α,17α,21-trihydroxy-20-ketosteroids with a variety of carbonyl compounds to form the 16α,17α-cyclic ketal or acetal is evidenced in Tables I and II. Each 16α-hydroxy steroid examined afforded its respective 16α,17α-cyclic derivative under reproducible conditions and with no detectable other products. Although ketal (acetal) formation was not complete in all cases, only the anticipated product was found together with unaltered parent steroid. Using the standard conditions of 16 h of exposure to acetone, complete reaction with 6 representative 16α-hydroxy steroids was accomplished, with no artifacts of degradation, rearrangement, dehydration, etc. encountered. A high order of confidence was established for the reaction of 16α-hydroxy steroids with acetone under the standard conditions.

Under control condition (a) both 16α-hydroxyhydrocortisone and 9α-fluoro-16α-hydroxyhydrocortisone formed traces of artifacts of an undetermined nature. These artifacts were not present in the pure untreated steroid sample and they were not found under the standard reaction conditions. Their presence under control condition (a) was not of further concern. The papergram mobilities of steroids treated under control condition (a) were not affected by the treatment, the same R_F values being obtained for reference steroids and for derivatives formed on paper.

Control condition (b) did not have any effect on migration, color tests, etc. of any steroid studied.

Long exposure (64 h) of other 16α-hydroxy steroids to control condition (a) gave the same indications of traces of artifact formation, as did exposure of non-16α-hydroxylated steroids to these conditions. It is important to limit the exposure of treated steroids on paper to 16 h and even then it may be necessary to discount traces of artifacts in certain cases. In no instance did artifact formation appear except in trace amounts, and it is only with the very sensitive isonicotinic acid hydrazide and tetrazolium blue reagents that they may be detected at all.

Whereas the reaction between 16α,17α-diols and acetone is complete in a relatively short time, other ketones and acetaldehyde do not react completely in short times, and in some cases not at all (Table II). Although 9α-fluoro-16α-hydroxyhydrocortisone formed a ketal (acetal) in each case, its 1-dehydro analog, triamcinolone, failed to form a derivative with methyl isobutyl ketone even after 64 h of exposure. Throughout the study the impression was obtained that 16α,17α-diol Δ^4-ketosteroids reacted more readily than their respective 1-dehydro analogs. No experiments were run at elevated temperature to promote reaction.

TABLE I

COMPARISON OF RESULTS FROM REACTION WITH ACETONE (STANDARD CONDITIONS) OF 16α-HYDROXYLATED AND NON-16α-HYDROXYLATED STEROIDS

Steroid parent	*Parent non-16α-hydroxylated steroid*			*16α-Hydroxylated steroid analog*		
	System	R_F	*Identity*,***	*System*	R_F	*Identity*,****
Hydrocortisone (F)	V	0.07	F	V	0.58	F 16 IP
	VI	0.01	F	VI	0.40	F 16 IP
Prednisolone (Δ^1-F)	II	0.77	Δ^1-F	V	0.44	Δ^1-F 16 IP
	VI	0.0	Δ^1-F	VI	0.29	Δ^1-F 16 IP
9α-Fluorohydrocortisone (9αFF)	II	0.80	9αFF	II	0.95	9αFF 16 IP
	V	0.02	9αFF	V	0.49	9αFF 16 IP
9α-Fluoroprednisolone (9αFΔ^1-F)	II	0.75	9αFΔ^1-F	II	0.88	9αFΔ^1-F 16 IP
	VI	0.0	9αFΔ^1-F	V	0.34	9αFΔ^1-F 16 IP
				VI	0.15	9αFΔ^1-F 16 IP
Reichstein's Substance S (S)	II	0.93	S	V	0.94	S 16 IP
	V	0.44	S	VI	0.90	S 16 IP
9(11)-Dehydro Substance S ($\Delta^{9(11)}$-S)	V	0.40	$\Delta^{9(11)}$-S	V	0.92	$\Delta^{9(11)}$-S 16 IP
	VI	0.18	$\Delta^{9(11)}$-S	VI	0.91	$\Delta^{9(11)}$-S 16 IP

* Abbreviations used throughout the tables are: F, hydrocortisone; F 16, 16α-hydroxyhydrocortisone; 9αFF, 9α-fluorohydrocortisone; 9αFF 16, 9α-fluoro-16α-hydroxyhydrocortisone; Δ^1-F, prednisolone; S, Reichstein's Substance S; S 16, 16α-hydroxy Substance S; IP, 16α,17α-cyclic acetonide (isopropylidenedioxy); etc.

** Only the parent steroid was detected, with no trace of any other component.

*** Only the anticipated 16α,17α-acetonide was detected, with no trace of any other component.

TABLE II

REACTION OF 16α-HYDROXYLATED STEROIDS WITH CARBONYL COMPOUNDS OTHER THAN ACETONE

Carbonyl compound	*Steroid*	*Reaction conditions*		*Components found*			*Comments*
		Acid volume, µl	*Exposure time, h*	*System*	R_F	*Identity**	
Acetaldehyde	9αFF 16	30	16	II	0.92	9αFF 16 ED	Incomplete reaction under standard conditions
					0.57	9αFF 16	
				VI	0.22	9αFF 16 ED	
					0.0	9αFF 16	
	9αFΔ^1-F 16	30	16	II	0.93	9αFΔ^1-F 16 ED	Incomplete reaction under standard conditions
					0.45	9αFΔ^1-F 16	
				VI	0.12	9αFΔ^1-F 16 ED	
					0.0	9αFΔ^1-F 16	
Methyl ethyl ketone	9αFF 16	20	16	V	0.58	9αFF 16 MEK	Complete reaction; no other products
				VI	0.42	9αFF 16 MEK	
	9αFΔ^1-F 16	30	64	V	0.45	9αFΔ^1-F 16 MEK	Incomplete reaction at 16 h
				VI	0.28	9αFΔ^1-F 16 MEK	Complete at 64 h
Methyl isobutyl ketone	9αFF 16	40	40	VI	0.64	9αFF 16 MIBK	Incomplete reaction under several conditions. Very faint other components formed
					0.0	9αFF 16	
	9αFΔ^1-F 16	40	64	II	0.47	9αFΔ^1-F 16	No reaction observed between steroid and MIBK; some isomerization observed
					0.25	Triamcinolone isomer	
				VI	0.0	9αFΔ^1-F 16	

* Abbreviations: ED, 16α,17α-ethylidenedioxy; MEK, 1-methylpropylidenedioxy; MIBK, 1,2,2-trimethylpropylidenedioxy. Other abbreviations listed in footnote* of Table I.

The reactions of some other steroid 1,2-diols and/or 1,3-diols are listed in Table III.

Treatment of the filter paper with the dilute perchloric acid reagent caused no variation in mobility of steroid components in the three Bush-type systems used. The paper is altered in its properties only where the acid is spotted, and a large whitish spot is seen at the origin after color development on the finished chromatogram. The perchloric acid spot does not interfere with detection by ultraviolet absorption or by isonicotinic acid hydrazide fluorescence. Detection of immobile reducing

TABLE III

REACTION WITH ACETONE (STANDARD CONDITIONS) OF OTHER STEROID DIOLS

Steroid diol	*Components found*			*Comments*
	System	R_F	*Assigned identity**	
Triamcinolone isomer	II	0.88	IP derivative	Partial reaction; reaction also incomplete after 64 h
		0.25	Isomer	
	VI	0.02	Isomer and IP derivative	
1,2-Dihydrotriamcinolone isomer	II	0.89	Isomer IP	Partial reaction
		0.30	Isomer	
20,20-Dihydro-9α-fluorohydrocortisone	II	1.0	IP derivative	Partial reaction. No reference samples used
		0.42	20-$H_2$9αFF	
	VI	0.25	IP derivative	
		0.0	20-$H_2$9αFF	
16α,17α-Dihydroxyprogesterone	V	1.0	IP derivative	Complete reaction indicated, with no unaltered diol (at R_F 0.61 and 0.46 in Systems V and VI)
	VI	1.0	IP derivative	
14α-Hydroxy Substance S	VI	0.30	14-Dehydro S	Dehydration rather than acetonide formation
		0.12	14α-Hydroxy S	

* Abbreviations: IP, cyclic acetonide; 20-H_2, 20,20-dihydro-.

steroids with tetrazolium blue is not possible. In no case did the acid treatment interfere with visualization procedure once the steroid had migrated off the origin position.

Where the acetone-diluted perchloric acid reagent was compared with the methanol-diluted reagent, no effect was noticed with 16α,17α-diols for acetonide formation. However, use of the acetone-diluted reagent for some other ketal reactions gave some traces of what would appear to be the cyclic acetonide (from relative migration measurements) together with the major ketal product formed from reaction with ketone vapors. These trace artifacts were not examined further.

DISCUSSION

Although many chemical reactions of steroids have been run on filter paper, their use has been confined to purposes of visualization of resolved steroids after chromatographic irrigation. That this restriction need not be the case is established by the results of the present study, as it is readily apparent that select chemical reactions of steroids may be accomplished on paper prior to chromatography.

The formation of 16α,17α-cyclic ketals on filter paper and subsequent analysis is an especially favorable situation, both from a viewpoint of use of volatile ketones to afford simple reaction conditions and from the viewpoint of the particularly reactive 16α,17α-diol feature of the steroids studied.

The further application of the volatile reactant-reaction on filter paper principle should be considered in such cases as acetylations, hydrolyses, brominations, and catalytic reductions. Catalytic reduction of unsaturated fatty acids on filter paper has already been reported; the saturated fatty acids so produced were then resolved chromatographically on the same paper[7].

The procedure is reliable for the early recognition of the *cis*-diol feature, particularly of the especially reactive 16α,17α-diol feature, in routine microbiological screening programs. It is currently accepted that *cis*-1,2-diols and 1,3-diaxial diols may form cyclic acetonides[8,9]. Thus other steroid diols which form cyclic acetonides could be mistakenly recognized as 16α,17α-diols using the procedure in unknown situations.

Under the standard conditions with acetone certain other steroids diols (see Table III) do indeed form more mobile derivatives on papergrams, such derivatives being tentatively identified as the respective cyclic acetonide. In the few cases studied complete reaction was not attained, and considerable unaltered diol was found, in distinction to the complete reaction encountered with established 16α,17α-diols.

20,20-Dihydro-9α-fluorohydrocortisone[10] would be expected to form a 20,21-cyclic acetonide[11,12], and 16α,17α-dihydroxyprogesterone should also form a 16α,17α-acetonide. In both cases more mobile components were noted, but no effort was made to establish the precise nature of the product formed. Mobility and color test behavior of the products is consistent with a cyclic acetonide formulation. It should be pointed out that 16α,17α-dihydroxyprogesterone was completely converted to more mobile material whereas 20,20-dihydro-9α-fluorohydrocortisone was but partially converted.

In the instance of triamcinolone isomer and 1,2-dihydrotriamcinolone isomer[13], partial reaction to form the acetonide occured. Comparison of the product formed from triamcinolone isomer with authentic triamcinolone isomer cyclic acetonide[14] indicated identity. These rearrangement products of the parent steroids also form derivatives with acetaldehyde and methyl ethyl ketone on paper, and again the $\Delta^{1,4}$-3-ketone, triamcinolone isomer, is more sluggish in derivative formation than its Δ^{4}-3-ketone analog, 1,2-dihydrotriamcinolone isomer. These isomeric steroids are suspected of being 16α-hydroxylated D-homoannulated structures[14] and as such should form cyclic acetonides[15,16].

The one case of a known 1,3-diaxial diol in Table III involves 14α-hydroxy Reichstein's Substance S (14α,17α,21-trihydroxy-4-pregnene-3,20-dione). The product obtained, together with apparently unaltered steroid, was slightly less mobile than Substance S, and therefore cannot be the postulated 14α,17α-cyclic acetonide, but rather is more likely the 14-dehydro derivative, 17α,21-dihydroxy-4,14-pregnadiene-3,20-dione. Substance S and the 16α,17α-cyclic acetonide of 9α-fluoro-16α-hydroxy-hydrocortisone have approximately the same mobility in the system used, and the

anticipated mobility for the postulated 14α,17α-cyclic acetonide should be substantially greater than that of Substance S, rather than slightly inferior. The known acid dehydration of 14α-hydroxy Substance S to the 14-dehydro derivative would also support this concept[17, 18].

Despite the possible confusion of potential acid-induced rearrangements with or without subsequent derivative formation, of partial reaction effects, of dehydration effects, reaction at other diol sites, etc. all of which tend to compromise the full reliability of the procedure in unknown situations, strict adherence to standard conditions, together with the proper use of related, known reference compounds, will permit differentiation of 16α,17α-diols from other types. In certain cases some modification of the standard conditions might permit a similar specific application to other microbiological screening programs where other diols capable of cyclic acetonide formation are sought, as in recent microbiological 12β-hydroxylation of 11β-hydroxy steroids[19].

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SUMMARY

A procedure for the preparation of 16α,17α-cyclic acetonide derivatives of steroid 16α,17α-diols directly on paper chromatograms is described. After reaction on paper under standard conditions the acetonides formed are resolved by chromatographic development of the paper and the separated components are visualized. The procedure is suggested for early recognition of the 16α,17α-diol feature of certain steroid molecules.

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