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52. Kyosuke Tsuda, Nobuo Ikekawa,*1 Yoshihiro Sato, Sayoko Tanaka, and Hiroko Hasegawa : Steroid Studies. XXXV.*2 Gas Chromatography of Androstane and Pregnane Derivatives.

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Although several recent communications[1~6)] have reported gas chromatographic separation of androstane and pregnane derivatives, the behavior of thermally unstable steroids, except the adrenal cortical steroids[7)] and vitamin D,[8)] have not been reported. In order to obtain the correlation between the structure of steroids and their retention time, and to subsequently utilize these results in the studies of microbiological transformation of steroids, gas chromatograms of hydroxy derivatives of pregrane and androstane were carried out.

Sample——The samples used in these studies were androstane and pregnane derivatives obtained either by synthesis or by microbiological transformation in this laboratory and are as follows :

Androstane series : Δ^1, Δ^6, or Δ^{15} derivatives of 4-androstene-3,17-dione and 6α-, 6β-, 11α-, 11β-, 14α-, and 15α-monohydroxy or monoacetoxy derivative.

Pregnane series : Δ^7, 7-oxo, 7α-hydroxy, and 7β-hydroxy derivative of 5α-pregnane-3,20-dione, 7α-, 7β-, 11α-, 14α-, 14β-, 15α-, and 15β-hydroxy (or acetoxy) and 14,15-epoxide derivative of 4-pregnene-3,20-dione.

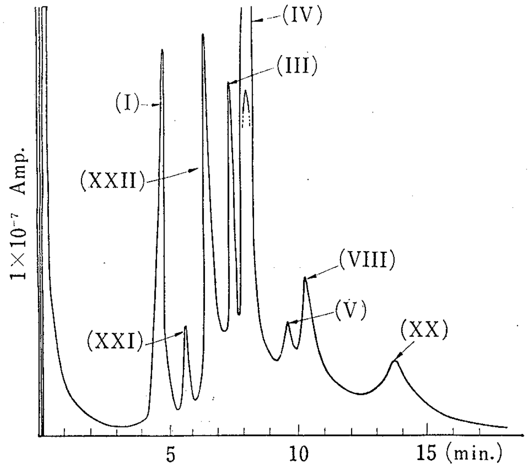

Fig. 1. Separation of a Mixture of Eight Steroids
(I, Ⅲ, Ⅳ, Ⅴ, Ⅷ, XX, XXI and XXⅡ)
(Cholestane 10.2 min.)

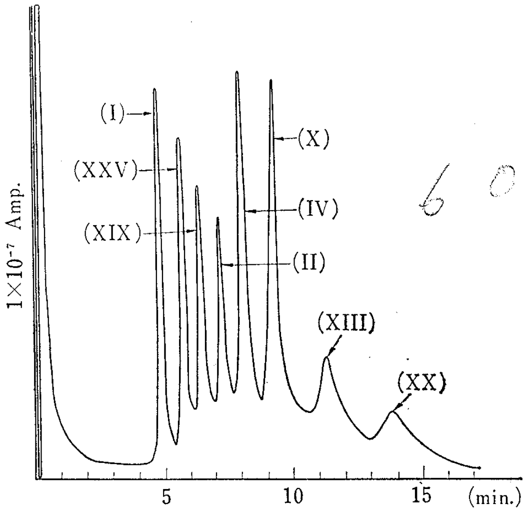

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Fig. 2. Separation of a Mixture of Eight Steroids
(I, Ⅱ, Ⅳ, Ⅹ, XⅢ, XIX, XX and XXV)
(Cholestane 10.2 min.)

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Apparatus and Method——A packed column was used in conjunction with a Barber-Colman Model 10 chromatographic unit containing an argon ionization detector. The U-shaped column, of 6 feet × 8 mm. (int. diam.), was packed with 1% SE-30 (G. E.) silicone polymer on Chromosorb W (60～80 mesh). Column temperature was 220°, cell temp. 170°, flash heater temp. 295°, and argon inlet pressure was 2.5 kg./cm^2.

The relative retention times in Tables I～VIII were calculated from the retention time of cholestane, 10.2 min., used as a reference compound. The relative retention time marked with an asterisk in Tables V and VII was obtained by using the column packed with 1% SE-30 on Gas Chrom P (100～140 mesh). In this case, the retention time of the reference compound, cholestane, was 3.86 min. A typical sample was 2～5 μl of a 0.5% solution of steroid in acetone. Separations of some mixtures of several compounds under these conditions are shown in Figs. 1, 2, and 3.

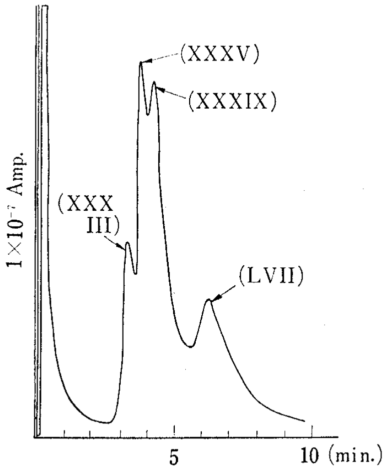

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Fig. 3. Separation of a Mixture of Four Steroids (XXXIII, XXXV, XXXIX and LVII) (Cholestane 3.86 min.)

Androstane Series

11- and 14-Substituted Compounds——Seven kinds of 14α-hydroxy compounds (II～VIII) and six kinds of 11-substituted compounds (IX～XIV) gave a sharp single peak without decomposition, having relative retention time as shown in Table I. However, 14-hydroxyandrost-4-ene-3,17-dione (V) does not always show a sharp peak but at times a broad peak with a slightly longer retention time and low intensity, indicating decomposion of the compound.

Lipsky and Landowne[6] have shown that the 5α-series have a lower retention time than the 5β-series in the C_{19}-steroids, but in the case of 14α-hydroxy derivatives (III and IV), the 5β-compound (III) has a lower retention time than (IV) as in the case of C_{27}-steroid.[1]

Table I. Relative Retention Time of 11- or 14-Substituted Compounds

Dehydroepiandrosterone (I)	0.49
3β,14-Dihydroxyandrost-5-en-17-one (II)	0.74
14-Hydroxy-5β-androstane-3,17-dione (III)	0.76
14-Hydroxy-5α-androstane-3,17-dione (IV)	0.83
14-Hydroxyandrost-4-ene-3,17-dione (V)	0.96
14-Hydroxyandrosta-4,6-diene-3,17-dione (VI)	0.99
3β,14-Dihydroxyandrost-5-ene-17-one-3-monoacetate (VII)	1.00
14-Hydroxyandrosta-1,4-diene-3,17-dione (VIII)	1.05
Androst-4-ene-3,11,17-trione (IX)	0.70
11α-Hydroxyandrost-4-ene-3,17-dione (X)	0.96
11β-Hydroxyandrost-4-ene-3,17-dione (XI)	1.02
11β-Hydroxyandrosta-1,4-diene-3,17-dione (XII)	1.02
11β-Acetoxyandrost-4-ene-3,17-dione (XIII)	1.13
11β-Acetoxyandrosta-1,4-diene-3,17-dione (XIV)	1.16

1-, 6-, or 14-Unsaturated 3,17-Diketones and A-Ring Aromatic Compounds——Eight compounds (XV~XXII) shown in Table II gave a sharp peak, consistent with the absence of decomposition. There was no difference in the retention time between 14β-androsta-4,15-diene-3,17-dione (XV) (C/D *cis*) and androsta-4,15-diene-3,17-dione (XVI) (C/D *trans*) under these conditions. The fact that the 1,4-dienone[*4] (XIX) was unchanged in the column during chromatography was indicated by the ultraviolet spectrum of the product (244 mμ). 1-Dehydrotestololactone (XX) showed a considerably longer retention time than (XIX).

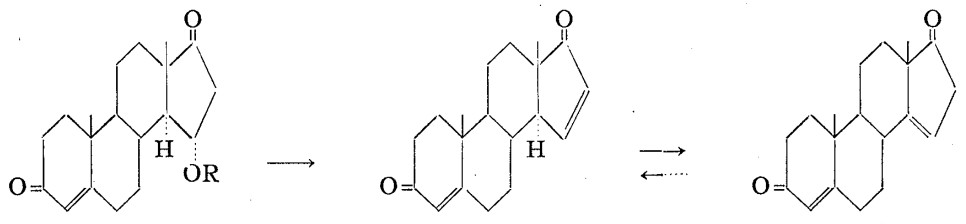
Table II. Relative Retention Time of 1-, 6-, or 14-Unsaturated 3,17-Diketones and A-Ring Aromatic Compounds

14β-Androsta-4,15-diene-3,17-dione (XV)	0.55
Androsta-4,15-diene-3,17-dione (XVI)	0.55
Androst-4-ene-3,17-dione (XVII)	0.63
Androsta-4,6-diene-3,17-dione (XVIII)	0.63
Androsta-1,4-diene-3,17-dione (XIX)	0.67
1-Dehydrotestololactone (XX)	1.34
3-Hydroxyestra-1,3,5(10)-trien-17-one (XXI)	0.57
1-Hydroxy-4-methylestra-1,3,5(10)-trien-17-one (XXII)	0.67

15-Substituted Compounds——15-Hydroxy (XXIII) and acetoxy (XXIV) derivatives gave a single sharp peak possessing the same retention time as that of the 15-ene derivative (XVI) (Table III). The ultraviolet spectrum ($\lambda_{\max}^{\mathrm{EtOH}}$ 236 mμ) of the product from runs with (XXIII) was found to be identical with that of (XVI). However, the infrared spectrum of thesubstance shows that the product is mixture (ca. 1:1) of androsta-4,14-diene-3,17-dione (LXVIII) and (XVI) ($\lambda_{\max}^{\mathrm{CHCl_3}}$: isolated 17-ketone, 1758 cm^{-1}; 15-en-17-one, 1716 cm^{-1}; 4-en-3-one, 1670 cm^{-1}). To collect a sufficient sample for the infrared spectrum, distillates were collected successively at ca. 170° for 2 hr. The fact that (XXIII) was transformed into (XVI) in the flash heating step and that isomerization of the double bond had occurred after the chromatography was found by the following experiment. The 14β-derivative (XV) was unchanged by heating at 160~165° for 2 hr. However, the heating of (XVI) under these same conditions resulted in the formation of a mixture of (XVI) and (LXVIII). This was indicated by the ultraviolet (238 mμ) and infrared spectra (1758, 1716, 1670 cm^{-1}) of the pyrolytic product.

Table III. Relative Retention Time of 15-Substituted Compounds

15α-Hydroxyandrost-4-ene-3,17-dione (XXIII)	0.55
15α-Acetoxyandrost-4-ene-3,17-dione (XXIV)	0.55
15α-Hydroxyandrosta-1,4-diene-3,17-dione (XXV)	0.57
Androst-4-ene-3,15,17-trione (XXVI)	—



(XXIII) R=H (XXIV) R=Ac (XVI) (LXVIII)

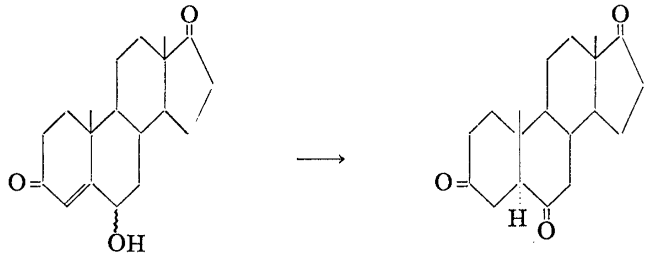
In the case of the 15α-acetoxy derivative (XXIV) and 15α-hydroxyandrosta-1,4-diene-3,17-dione (XXV), apparently the same transformation might have occurred in the flash heater step as judged from their retention time. The 15,17-diketone (XXVI) gave no recognizable peak.

[*4] Lipsky and Randowne6) have reported that the decomposition of the 1,4-dienone derivatives occurred when the sample contained in the microsyringe needle remained in the zone of the flash heater for 5 seconds.

6-Substituted Compounds——The relative retention times of six kinds of 6-substituted derivatives (XXVII～XXXII) are shown in Table IV. The 6α-acetoxy (equatorial) compound (XXXII) gave a single sharp peak but the 6β-acetoxy (axial) compound (XXXI) gave three peaks. The 3,6-diketone (XXVII), 6β-hydroxy-4-en-3-one (XXVIII) and 6α-hydroxy-4-en-3-one (XXIX) gave the same retention time. The ultraviolet spectrum of the eluted product of (XXIX) exhibited no absorption of an α,β-unsaturated ketone but possessed a low broad peak at 253 mμ. After allowing to stand overnight in an alkaline solution of tetramethylammonium hydroxide (TMAH), the spectrum changed to a typical absorption of a standard 3,6-diketone (XXVII) (257, 383 mμ).9) This can be interpreted as the transformation of (XXIX) during chromatography to 3,6-diketone. On the other hand, the ultraviolet spectrum of the 6β-hydroxy derivatives (XXVIII) after chromatography possesses a maximum at 305 mμ (low) and a shoulder at ca. 230 mμ. In an alkaline solution of TMAH, the maximum shifted to 383 and 254 mμ (shoulder). From the above results, it can be assumed that some of (XXVIII) was changed to a 3,6-diketone on chromatography.

Table IV. Relative Retention Time of 6-Substituted Compounds

5α-Androstane-3,6,17-trione (XXVII)			0.79
6β-Hydroxyandrost-4-ene-3,17-dione (XXVIII)			0.79
6α-Hydroxyandrost-4-ene-3,17-dione (XXIX)			0.79
Androst-4-ene-3,6,17-trione (XXX)			0.91
6β-Acetoxyandrost-4-ene-3,17-dione (XXXI)	0.64	0.81	0.94
6α-Acetoxyandrost-4-ene-3,17-dione (XXXII)			0.99



(XXVIII, XXIX) (XXVII)

Pregnane Series

Unsaturated 3,20-Diones and Epoxides——5α-Pregnane-3,20-dione (XXXIII), fivekinds of pregn-4-ene-3,20-dione (XXXIV～XXXVIII), and two kinds of 14,15-epoxide (XXXIX and XL) listed in Table V gave one sharp peak without decomposition. The gas chromatography of (XXXVII) usually affords one sharp peak but occasionally resulted in a lower peak and a longer retention time due to decomposition.

Table V. Relative Retention Time of Unsaturated 3,20-Diketones and Epoxides

5α-Pregnane-3,20-dione (XXXIII)	0.72	
5α-Pregn-7-ene-3,20-dione (XXXIV)	0.79	0.88*
Pregn-4-ene-3,20-dione (XXXV)	0.86	1.00*
Pregna-4,6-diene-3,20-dione (XXXVI)	0.94	1.06*
Pregna-1,4-diene-3,20-dione (XXXVII)	0.95	
Pregna-4,14-diene-3,20-dione (XXXVIII)		0.94*
Pregn-4-ene-3,20-dione 14β,15β-oxide (XXXIX)		1.18*
Pregn-4-ene-3,20-dione 14α,15α-oxide (XL)		1.27*

Effect of a double bond on retention time has alreay reported with respect to C_{27}-,

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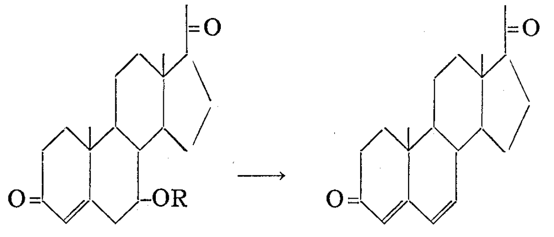
9) A. S. Meyer: J. Org. Chem., **20**, 1240 (1955).

C_{28}-, and C_{29}-sterols.[10] The double bond effect listed in Tables I, II, and V shows that the double bond conjugated with the 3-ketone increases the retention time (III, IV → V), but the one conjugated with the 17-ketone decrease decreases (XVII→XVI). The retention time of pregna-4,14-diene-3,20-dione (XXXVIII) was shorter than that of the parent compound (XXXV). The introduction of a double bond at position 1 or 6 increasesthe retention time slightly. The order of increasing the retention time is 3-keto-4-ene, 3-keto-4,6 diene, and 3-keto-1,4-diene.

7-Hydroxy- or 7-Acetoxy-3-oxo-4-ene Derivatives——The derivatives of 7*α*- or 7*β*-hydroxy- or acetoxy-3-oxo-4-ene, as shown in Table VI, gave a sharp peak, but it was found that they were dehydrated or deacetylated in the flash heating step. This was indicated by the fact that the 7*β*-hydroxy compound (XLI) and 7*β*-acetoxy compound (XLII) showed the same retention time as pregna-4,6-diene-3,20-dione (XXXVI) with the products having an ultraviolet maximum at 283 mμ. The same results were obtained from 7*β*,15*β*-dihydroxy compound (XLIII), 7*α*,15*β*-dihydroxy compound (XLIV), and 7*β*-monoacetoxy-15*β*-hydroxy compound (XLV) which possess the same retention time and ultraviolet spectrum as 15*β*-hydroxypregna-4,6-diene-3,20-dione (XLVI). The product from chromatography of 7*α*,15*β*-diacetoxy compound (XLVII) has an ultraviolet maximum at 283 mμ, from which it can be assumed that the compound (XLVII) was transformed to 15*β*-acetoxypregna-4,6-diene-3,20-dione (XLVIII).

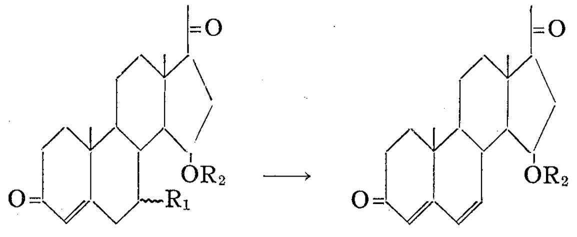
Table VI. Relative Retention Time of 7-Hydroxy- or Acetoxy-3-keto-4-ene Derivatives

7β-Hydroxypregn-4-ene-3,20-dione (XLI)	0.94
7β-Acetoxypregn-4-ene-3,20-dione (XLII)	0.94
7β,15β-Dihydroxypregn-4-ene-3,20-dione (XLIII)	1.55
7α,15β-Dihydroxypregn-4-ene-3,20-dione (XXLIV)	1.55
7β,15β-Dihydroxypregn-4-ene-3,20-dione 7-monoacetate (XLV)	1.55
15β-Hydroxypregna-4,6-diene-3,20-dione (XLVI)	1.55
7α,15β-Diacetoxypregn-4-ene-3,20-dione (XLVII)	1.57



(XLI) R=H
(XLII) R=Ac

(XXXVI)



(XLIII) R_1=7β-OH, R_2=H
(XLIV) R_1=7α-OH, R_2=H
(XLV) R_1=7β-OAc, R_2=H
(XLVII) R_1=7α-OAc, R_2=Ac

(XLVI) R_2=H
(XLVIII) R_2=Ac

7-, 11-, and 15-Substituted Derivatives——7-Oxo, and 7*α*- and 7*β*-hydroxy or acetoxy derivatives of 5*α*-pregnane (XLIX~LIII) all have a sharp peak (Table VII), but the 7*α*-hydroxy compound (LI) frequently shows a low peak (relative retention time, 1.25) by decomposition. The 11*α*-, 15*α*-, and 15*β*-hydroxy or -acetoxy compounds (LIV~LIX) also exhibit a sharp peak.

By acetylation of the hydroxyl groups, it was found that the axial 7*α*-, 11*β*-, and 15*β*-acetoxy derivatives (L, XIII, XIV, and LVIII) show slightly longer retention time than the free hydroxyl compouns (LI, XI, XII, and LVII), while the equatorial 3*β*-, 7*β*-, and 15*α*-acetoxy compounds (VII, LIII, and LIX) have considerably longer retention time than the corresponding hydroxyl derivatives (II, LII, and LVI). As an exception, 11*α*-acetoxy compound (LIV) has a shorter retention time than (LV).

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[10] K. Tsuda, K. Sakai, N. Ikekawa: This Bulletin, **9**, 835 (1961).

Table VII. Relative Retention Time of 7-, 11-, or 15-Substituted Compounds

5α-Pregnane-3,7,20-trione (XLIX)	1.08	
7α-Acetoxy-5α-pregnane-3,20-dione (L)	1.14	
7α-Hydroxy-5α-pregnane-3,20-dione (LI)	1.19	
7β-Hydroxy-5α-pregnane-3,20-dione (LII)	1.22	
7ξ-Acetoxy-5α-pregnane-3,20-dione (LIII)	1.47	
11α-Acetoxypregn-4-ene-3,20-dione (LIV)		1.45*
11α-Hydroxypregn-4-ene-3,20-dione (LV)		1.65*
15α-Hydroxypregn-4-ene-3,20-dione (LVI)	1.42	1.66*
15ξ-Hydroxypregn-4-ene-3,20-dione (LVII)	1.43	1.56*
15β-Acetoxypregn-4-ene-3,20-dione (LVIII)	1.50	
15α-Acetoxypregn-4-ene-3,20-dione (LIX)	1.64	

Axial 7α- and 15β-hydroxy compounds (LI, LVII) were eluted before their corresponding equatorial hydroxy compounds (LII, LVI), as in the case of the 3-position.[11,12] However, in the epimeric isomers of 11-hydroxyandrost-4-ene-3,20-dione (X, XI) and 14,15-dihydroxy-14β-pregn-4-ene-3,20-dione (LX, LXI), the reverse effect was observed in retention time.

Table VIII. Relative Retention Time of 14,15-Dihydroxy Compounds and Others

14,15β-Dihydroxy-14β-pregn-4-ene-3,20-dione (LX)		1.70
14,15α-Dihydroxy-14β-pregn-4-ene-3,20-dione (LXI)		1.80
14,15α-Dihydroxy-14β,17-isopregn-4-ene-3,20-dione (LXII)		1.91
14-Hydroxypregn-4-ene-3,20-dione (LXIII)	…	1.36
14,15α-Dihydroxypregn-4-ene-3,20-dione (LXIV)	0.91	1.80
14,15β-Dihydroxypregn-4-ene-3,20-dione (LXV)	0.93	2.09
6β,11α-Dihydroxypregn-4-ene-3,20-dione (LXVI)	1.93	1.98
6ξ,15β-Dihydroxypregn-4-ene-3,20-dione (LXVII)	…	2.08

14,15-Dihydroxy Isomers and Others——The results of the chromatography of six kinds of 14,15-dihydroxy derivative (LX～LXV) are shown in Table VIII. Of these compounds, the 14β-hydroxy derivatives (LX～LXII) showed one sharp peak, but the 14α-hydroxy derivatives (LXIII～LXV) and two other dihydroxy compounds (LXVI, LXVII) have the main peak with minor peaks of shorter retention time, which correspond to the decomposition products.

The authors are deeply indebted to Sankyo & Co., Ltd. for enabling them to use their gas chromatography apparatus. They also thank Dr. K. Sakai, Takamine Research Laboratory, Sankyo & Co., Ltd., for assisting them in running the gas chromatograms, and also Dr. R. Hayashi, of the same laboratory, for providing the compound (LXIII).

Summary

Gas chromatographic behaviors of 32 kinds of androstane derivatives and 34 kinds of pregnane derivatives are demonstrated. The correlation between the structure and retention time is discussed.

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