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Chromogenic Reactions of Steroids with Strong Acids. II.[1] On the Kägi–Miescher Reaction of Epitestosterone[2]

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When epitestosterone (17α-hydroxyandrost-4-en-3-one) (I) was heated with formic acid, 17-methyl-18-norandrosta-4,13(17)-dien-3-one (III) and 17-methyl-18-norandrosta-4,13(14)-dien-3-one (IV) were formed, besides the 17α-formate of I. Kägi–Miescher reaction of I, III, and IV gave almost identical absorption spectra (Fig. 3). The olefinic products, III and IV, were, contrary to the original substrate (I), so sensitive that the bathochromic absorption was noticeable already at the first stage of this reaction without any heating. Although any increase in intensity was never observed on boiling in this case, a remarkable hyperchromic change at the wave-length (580 nm) of the maximum absorption was noticed at the second stage of the reaction, in which bromine molecules are the regular additive. It seems in the Kägi–Miescher chromogenic reaction that the possible intermediates, III and IV, including their protonated carbonium ions are oxidized mainly by bromine molecule to produce the characteristic chromophore in an acid medium.

Formerly, Kägi and Miescher[4] reported that epitestosterone (17α-hydroxyandrost-4-en-3-one) (I) can easily show the bathochromic light absorption when it was treated with strong mineral acid containing halogen molecules, in contrast to its 17β-isomer, testosterone (17β-hydroxyandrost-4-en-3-one) (II). The color reaction of II was studied by Koenig, *et al.*[5] with sulphuric acid containing guaiacolsulphonate and cupric ions. The specificity of the Koenig reaction was examined[6] and the colorimetric method based on this reaction was presented for the estimation of I, II including its propionate as well as its oxime, and androst-4-ene-3,17-dione in the biological samples.[5–7] As to the products which could skillfully be isolated from the chromogenic reaction mixture of this kind and might offer some information on the mechanism of the reaction, little has ever been investigated. From the hydrogenated 5α-derivative (androstane-3β,17α-diol) of I, 3β-formyloxy pseudo androstene (17-methyl-3β-formyloxy-18-nor-5α-androst-13(17)-ene) was derived when this diol was refluxed with anhydrous formic acid.[8] The mixture of the isomeric 17-methyl-3-methoxy-18-norestra-1,3,5(10)-trienes and the highly dehydrogenated 17-methyl-18-norsteroids such as 3′-methyl-7-methoxy-1,2-cyclopenteno-9,10-dihydrophenanthrene and its 6-sulphonate were isolated from the solution of Kober reaction,[9] where methyl ether of estradiol (3-methoxy-estra-1,3,5(10)-trien-17β-ol) was heated with 78% (w/w) sulphuric acid for 20 minutes at 100°.[10] Sadée, *et al.*[11] were also successful in isolating the respective 17β-methyl-18-nor-4,6,8(14)-trienones from the reaction mixture of aldadienic acid (3-(3-oxo-17β-hydroxy-andro-

1) Part I: M. Kimura and K. Harita, *Chem. Pharm. Bull.* (Tokyo), **21**, 1205 (1973).
2) This work was presented at the Meeting of the Hokkaido Branch of the Japan Society for Analytical Chemistry, Feb. 1968.
3) Location: *Nishi-6-chome, Kita-12-jo, Kita-ku, Sapporo, 060, Japan.*
4) H. Kägi and K. Miescher, *Helv. Chim. Acta*, **22**, 683 (1939).
5) V.L. Koenig, F. Melzer, C.M. Szego, and L.T. Samuels, *J. Biol. Chem.*, **141**, 487 (1941).
6) R.P. Martin, *Acta, Endocrinol.*, **40**, 263 (1962).
7) G.W. Oertel, *Acta Endocrinol.*, **37**, 237 (1961).
8) K. Miescher and H. Kägi, *Helv. Chim. Acta*, **32**, 761 (1949).
9) S. Kober, *Biochem. Z.*, **239**, 209 (1931); *idem*, *Biochem. J.*, **32**, 357 (1938).
10) M. Kimura, K. Akiyama, K. Harita, T. Miura, and M. Kawata, *Tetrahedron Letters*, **1970**, 377.
11) W. Sadée, S. Riegelman, and L.F. Johnson, *Steroids*, **17**, 595 (1971).

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sta-4,6-dien-17α-yl)propionic acid) and 6-dehydro-17α-methyl derivative of II with 62% sulphuric acid at room temperature. In the preceding paper of this series,[1] it was clarified that 17-methyl-18-norandrosta-4,13(17)-dien-3-one (III)[12] and 17-methyl-18-norandrosta-4,13(14)-dien-3-one (IV)[13] were produced on treatment of II with hot perchloric acid and was reported briefly that the epitestosterone (I) gave also these products besides 17α-formate of I when it was refluxed in formic acid. The present paper deals with the formation of these products and with their possible part taken in the Kägi–Miescher chromogenic reaction of epitestosterone (I).

Result and Discussion

Products from the Reaction of Epitestosterone (I) with Formic Acid

A mixture of I and 98% formic acid turned red-violet when it was heated at 100°. The colorless oil obtained from the first fraction in column chromatography of the reaction-products revealed two spots in thin-layer chromatography (TLC) and was found from the results of gas–liquid chromatography (GLC) to be the mixture of several components as shown in Fig. 1. Although the individual separation of the two components having highly adjecent retention times (t_R) around 6 minutes was unfortunately in no success, the peak of t_R=6.1 was identified on admixture with the authentic IV in GLC and it was also recognized from the nuclear magnetic resonance spectrum (NMR) that another one was III as shown in Fig. 2. The second fraction in the column chromatography gave 17α-formate (t_R=14.0) of I.

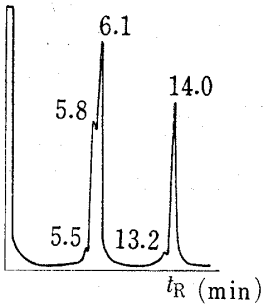

Fig. 1. Gas–Liquid Chromatography of the Products from the Reaction of Epitestosterone (I) with Formic Acid

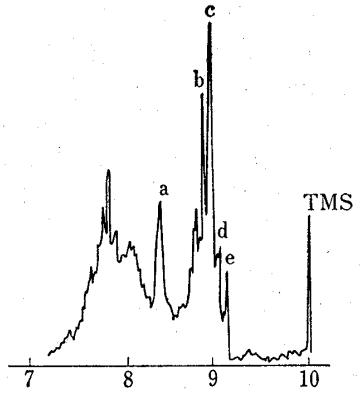

Fig. 2. NMR Spectrum of the Colorless Oil obtained from the Reaction of Epitestosterone (I) with Formic Acid

a : 17-CH_3 in III *b* : 19-CH_3 in IV
c : 19-CH_3 in III *d* and *e* : 17-CH_3 in IV

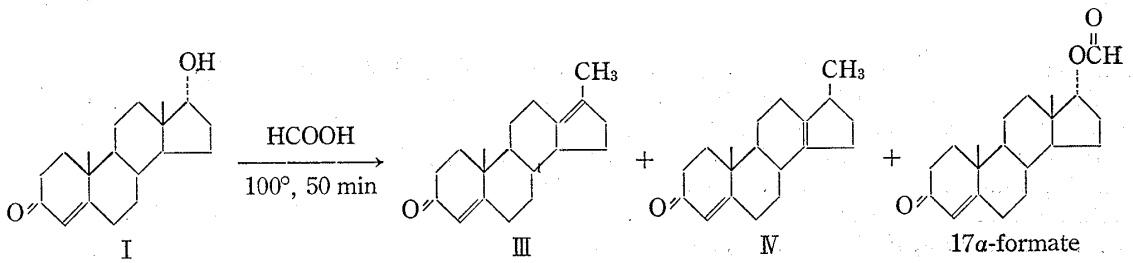

Chart 1

12) F. Sondheimer, O. Mancera, M. Urquiza, and G. Rosenkranz, *J. Am. Chem. Soc.*, **77**, 4145 (1955).
13) L.H. Knox, E. Velarde, S. Berger, D. Cuardriello, and A.D. Cross, *J. Org. Chem.*, **29**, 2187 (1964).

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Formic acid (98—100%) is a mild dehydrating reagent for steroids and can differentiate pairs of isomers and closely related steroids by development of color or fluorescence from only those compounds with sterically hindered OH or tertiary OH group; I is readily detected even in admixture with the isomeric II.[14] During course of the studies on mechanism of the new color reaction, Kägi and Miescher found that androstane-3β,17α-diol gave 3β-formyloxy-pseudo-androstene (17-methyl-3β-formyloxy-18-norandrost-13(17)-ene) when it was refluxed with anhydrous formic acid.[8] Methyl ester of α-estradiol also formed various 17-methyl-18-nor-homologues in the same condition.[15] The formation of III and IV from I by the same procedure could thus be reasonable in common with the acid-catalyzed reaction of 17α-hydroxy steroids; migration of 18-methyl group to the adjecent 17-position has long been known to occur with the preceding retropinacolic elimination of 17-hydroxyl group.[4,16] The 17β-hydroxy isomer (II) gave, on the other hand, no visible light absorption but merely its formate as a main product when it was refluxed in formic acid and the preliminary dehydration of II by boiling with phosphoroxychloride in quinoline was necessary for the positive Kägi–Miescher chromogenic reaction.[4] The positive Kober reaction[9] was observed when α-estradiol was heated with 40% (v/v) sulphuric acid containing quinol for five minutes, in contrast to the β-isomer for which higher concentration (52%) of the acid and longer period (40 min) were indispensable.[17] In connection with that the 17-methyl-18-norsteroidal olefins are the essential intermediates in such chromogenic reactions,[1] these results would indicate that 17α-OH in I is, contrary to 17β-OH in II, intended to be attacked and eliminated by the acid molecules far more readily than anticipated, in particular relation to the steric effect due to the neighbouring 18-methyl group.

Kägi-Miescher Chromogenic Reaction of Epitestosterone (I) and Its Related Substances

Kägi–Miescher test is specific for 17α-OH function in various steroids[18] and consists of two stages: (1) acetic acid solution (1—2 ml) of 17α-hydroxy steroid (1—2 mg) is boiled with one drop of conc. sulphuric acid for a few seconds and (2) 1% acetic acid solution of bromine is added dropwise to the cold mixture; blue-violet color being immediately developed.[4] At the merely first stage of the reaction, I gave blue color (λ_{max}=580 nm) with red fluorescence, though entirely inert before boiling. A remarkable hyperchromic change was noticed at the second stage without any shift of the wave length of maximum absorption as shown in Fig. 3. The olefinic products, III and IV, on the other hand, were so highly sensitive that the bathochromic absorption was already noticeable at the first stage without any heating. Although any increase in intensity was never observed on boiling at this stage, the absorption spectra entirely identical with that given by I and the similar hyperchromic change were also noticed at the second stage. The simply dehydrated homologue of I, androsta-4,16(17)-dien-3-one (V),[19] was, on the contrary, insensitive at the first stage but a light absorp-

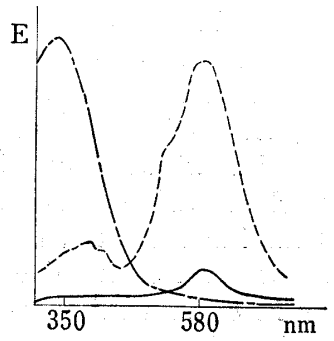

Fig. 3. Absorption Curves of the Kägi–Miescher Reaction Mixture of Epitestosterone (I)

——: at the first stage
·····: at the second stage
— · —: after irradiation[a]

a) The reaction mixture was irradiated for 3 min by using 100W Hg lamp (Toshiba) under N_2 stream at room temperature.

14) R.J. Boscott, *Nature*, **164**, 140 (1949).
15) to be published.
16) A. Cohen, J.W. Cook, and C.L. Hewett, *J. Chem. Soc.*, **1935**, 445.
17) T. Lunaas, *Acta Chem. Scand.*, **18**, 321 (1964).
18) L.F. Fieser and M. Fieser, "Steroid," Reinhold Publ. Co., New York, 1959, p. 468, 529.
19) H.B. Henbest and W.R. Jackson, *J. Chem. Soc.*, **1962**, 954.

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Table I. Kägi–Miescher Test for Epitestosterone (I) and Related Compounds

Compound	First stage (AcOH–conc.H_2SO_4)		Second stage (1% Br_2 in AcOH)
	cold	boiled	
17-Methyl-18-norandrosta-4,13(17)-dien-3-one (III)	+	+	++
17-Methyl-18-norandrosta-4,13(14)-dien-3-one (IV)	+	+	++
Epitestosterone (I)	−	+	++
Androsta-4,16-dien-3-one (V)	−	−	+
Androst-4-en-3-one (VI)	−	−	−
Testosterone (II)	−	−	−

tion at 580 nm was observed at the second one. These results and those obtained from the same reactions of some other related compounds are collected in Table I. Although most of the substrate remained intact in the reaction mixture of V at the first stage, the presence of III and/or IV was noticed by GLC. The weakly positive reaction of V at the second stage is, therefore, reasonably understandable to be due to the trace of 17-methyl-18-nor-derivative(s) formed at the first stage of the Kägi–Miescher reaction. Since androst-4-en-3-one (VI)[20] has no leaving group at 17-position and thus 18-methyl group cannot be transfered, it was entirely negative in this reaction as expected.

The blue color produced at the second stage of the reaction of I, III, or IV was as stable as for about ten days long at room temperature. However, the highly colored reaction mixture turned yellow (λ_{max}=350 nm) as shown in Fig. 3 when it was irradiated for a few minutes under nitrogen stream and the characteristic blue-color was never regenerated by the further addition of bromine solution. This indicates some photosensitive character[4] of the chemical species responsible for such bathochromic absorption and examination of the irradiated products are now in progress.

Kägi and Miescher assumed that the 17-androstanol was initially dehydrated in an acid medium to form pseudo-androstene which was then dehydrogenated further by addition of bromine with subsequent elimination of hydrobromic acid and by oxidation with sulphuric acid or photocatalysis to an increase in double bond so as to be enough to show the so-called halochromism with strong acid.[4] Results of the study on the chromogenic reaction of II with perchloric acid[1] and of the present investigation are also likely to indicate the essential role of the intermediates, III and IV, in producing the chromophore. The remarkable hyperchromic effect of bromine molecules as shown in Fig. 3 seemed to bring about the mere increase in concentration of the identical chromophore through some uncertified fashion. This might offer some direction on investigating the chemical structure of this chromophore. It is interesting, in this respect, that other halogens were similarly as effective as bromine in the Kägi–Miescher reaction and that the intermediary 17-methyl-18-norsteroidal olefins, III and IV, were found to be sensitive to light and air.[1] Olefins can produce alkenyl cations (λ_{max}=300 nm) in sulphuric acid and a trace of strong oxidant (selenic acid) enhances markedly the reaction, indicating that an oxidation process is involved in formation of the chromophore.[21a] Diphenylethylene in silica-alumina or acetic acid–sulphuric acid system produces radical cation (λ_{max}=607 nm) and this is also enhanced by selenic acid.[21b] The oxidative role of bromine molecules in the Kägi–Miescher reaction is thus likely to be reasonable, though the similar effect of aliphatic acid anhydrides[4] and aromatic aldehydes[22] may not necessarily

20) R.H. Shapiro and C. Djerassi, *J. Am. Chem. Soc.*, **86**, 2825 (1964); *idem*, *J. Chem. Soc.*, **1962**, 470.
21) *a*) H.P. Leftin, "Carbonium Ions," Ed. G.A. Olah and P. von R. Schleyer, Vol. I, Intersci. Publ., New York, 1968, p. 389; *b*) *Ibid.*, p. 402.
22) K. Miescher, *Helv. Chim. Acta*, **29**, 743 (1946).

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be explained in terms of the same mechanism. In fact, the bathochromic absorptions (λ_{max}= 480 and 596 nm) of the olefins, III and IV, in sulphuric acid medium were remarkably intensified in the presence of selenic acid.[23] Through disproportionation process and/or oxidation with sulphuric acid, these intermediary olefins were found to give 17-methyl-18-norandrosta-4,6,8(14)-trien-3-one (VII) which is responsible to the absorption maximum at 480 nm in the chromogenic reaction of II with strong mineral acid, as briefly reported.[23] Under the conditions of Kägi–Miescher reaction, however, it seems to be no doubt from Fig. 3 that the substrate (I) cannot remain in stage of the trienone (VII). The above-mentioned positive reaction of the intermediary dienones (III and IV) already noticeable at the first stage without any heating, may be due to the results of their multiple disproportionations, oxidation with sulphuric acid and/or dissolved oxygen. Details of the chemical species responsible for the maximum light absorption at 580 nm are now under investigation.

Experimental[24]

Materials——Epitestosterone (I), mp 219—222° ($CHCl_3$–*n*-hexane), acetate, mp 114—116° (ether–*n*-hexane), was prepared by the method of Sondheimer, *et al.*[12] from testosterone tosylate. Testosterone (II) was obtained from the commercial sources and purified in an usual manner, mp 155°. 17-Methyl-18-norandrosta-4,13(17)-dien-3-one (III), 17-methyl-18-norandrosta-4,13(14)-dien-3-one (IV), androsta-4,16(17)-dien-3-one (V) and androst-4-en-3-one (VI) were prepared and purified as reported at the preceding paper.[1]

Formation and Isolation of III, IV, and 17α-Formate of I——A solution of I (108 mg) in 98% HCOOH (3 ml) was heated on water-bath for 50 min under nitrogen in a vessel covered with aluminium foil. The red-violet solution was poured into ice-water and extracted with benzene. After usual work-up, evaporation of the solvent *in vacuo* left yellow residue (115 mg) which gave two spots in TLC and multiplet peaks in GLC (Fig. 1). The solid residue was then submitted to column chromatography on neutral alumina in a same way as described in the preceding paper.[1] The colorless oil (43 mg) from the eluate with *n*-hexane–benzene (4: 6) gave one spot in TLC but it was found to be a mixture of III and IV from the analysis by GLC as well as NMR. GLC: t_R=6.1 with shoulder at 5.8. NMR ($CDCl_3$) τ: 4.25 (s, 4-H), 8.40 (s, 17-CH_3 in III), 8.84 (s, 19-CH_3 in IV), 8.87 (s, 19-CH_3 in III), 8.96 and 9.09 (d, 17-CH_3 in IV). In the column chromatography of the reaction products, the next fraction eluted with the same solvent system gave the formate of I (31 mg), colorless flakes, mp 180—181° (EtOH). Mass Spectrum: 316 (M^+). NMR ($CDCl_3$) τ: 1.98 (s, 17α-formate), 4.25 (s, 4-H), 5.05 (d, 17-H), 8.8 (s, 19-CH_3), 9.20 (H, 18-CH_3). GLC: t_R=14.0.

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23) M. Kimura, K. Harita, and T. Miura, *Chem. Pharm. Bull.* (Tokyo), **20**, 1829 (1972).

24) Melting points were taken on a micro hot-stage apparatus and are uncorrected. Absorption spectral measurements were run on Hitachi Model 3T recording spectrometer. NMR spectra were obtained by Hitachi Model H-6013 spectrometer at 60 Mc. Mass spectra were measured by Hitachi Model RMU-6E. TLC was carried on silica gel (WACOGEL B-5) plate by the solvent system of AcOEt–benzene (1: 1). GLC was run on Shimadzu Model GC-4A gas chromatograph at 200° using a stainless steel column (3 m×3 mm i.d.) of 1.5% SE-30 on Shimalite W (60—80 mesh) with a N_2 flow of 45 ml/min and a hydrogen flame ionization detector.

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