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184. Toshio Nambara and Motohiko Katō : Analytical Chemical Studies on Steroids. VII. The Zimmermann Complexes Derived from *i*-Androstanolone and Its Related 17-Oxosteroids.*[1]

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In the investigation of 17-oxosteroid levels in blood plasma the amount of the individual steroid is usually too small to permit characterization as well as quantitative determination. In 1956, Kellie, *et al.*[1)] proposed a valuable method for identification of 17-oxosteroid by means of paper chromatography employing so-called Zimmermann complex which is produced by acidification of the colored solution. This technique enables us to further characterize a trace of oxosteroid, which has previously been estimated. In contrast with this study, Francis, *et al.*[2)] reported that of several 17-oxosteroids dehydroisoandrosterone (Ia) and *i*-androstanolone (6β-hydroxy-3α,5α-cycloandrostan-17-one) (IIa) gave two chromogenic derivatives on chromatogram respectively, which seemed to be a characteristic feature for these two oxosteroids. On the other hand the mechanism of Zimmermann reaction has been investigated by several workers[3)~7)] and in consequence, was postulated as shown in Chart 1.

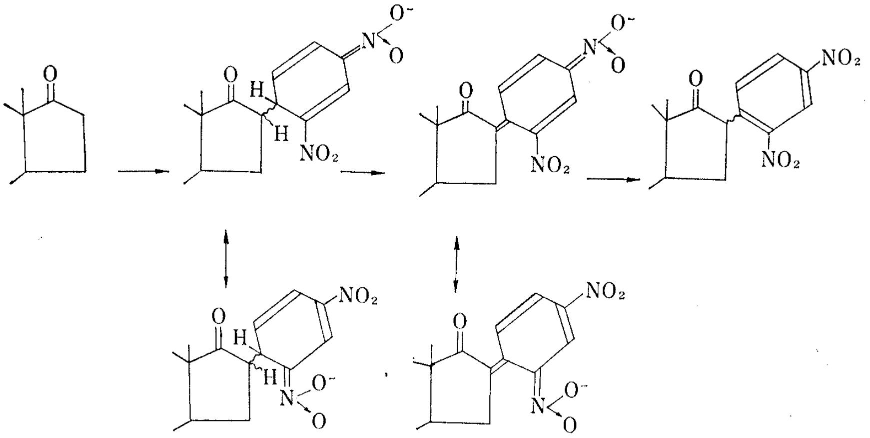

Chart 1. The Postulated Mechanism of Zimmermann Reaction

The authors have much interest in these findings and attempted to clarify this exceptional situation. First, the reaction was carried out using *i*-androstanolone and the resultant product was subjected to paper chromatography following the procedure of Francis, *et al.* In actuality, the result observed by the authors coincided with that of the above-mentioned paper. Namely the reaction product was found to consist of two Zimmermann chromophores, of which the more polar one was major, the less

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*[1] Part Ⅵ : This Bulletin, **13**, 1004 (1965).

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1) A.E. Kellie, E.R. Smith : Nature, **178**, 323 (1956).

2) F.E. Francis, N.C. Shen, R.A. Kinsella, Jr. : Biochim. et Biophys. Acta, **58**, 604 (1962).

3) O. Neunhoeffer, K. Thewalt, W. Zimmermann : Z. physiol. Chem., Hoppe-Seyler's, **323**, 116 (1961).

4) T.J. King, C.E. Newall : J. Chem. Soc., **1962**, 367.

5) C.S. Corker, J.K. Norymberski, R. Thow : Biochem. J., **83**, 583 (1962).

6) A.B. Roy : Austral. J. exp. Biol., **40**, 415 (1962).

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TABLE I. The Rt Values of Zimmermann Complexes Derived from Some 17-Oxosteroids

Compounds	Distance (cm.) from origin[a]	
	Spot 1	Spot 2
Zimmermann complexes of		
dehydroisoandrosterone	7.7	
i-androstanolone	8.9	1.5
16ξ-(2,4-Dinitrophenyl) derivative of		
dehydroisoandrosterone	7.5	
i-androstanolone	8.3	
3β-chloroandrost-5-en-17-one	1.3	
androsta-3,5-dien-17-one	0	
3α,5α-cycloandrost-6-en-17-one	0	

Filter paper: Tōyō-Roshi No. 51A (2×40)
Developing solvent: Methylcyclohexane-propylene glycol
Developing time: 46 hr.
Detection: 10% Methanolic KOH
a) Distance between origin and center of intensity of the purple color

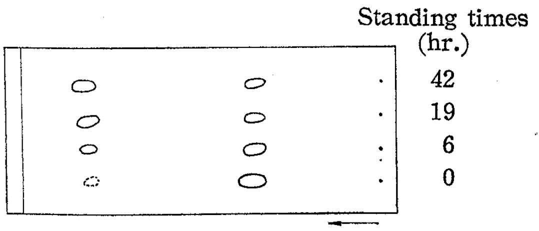

Fig. 1. Thin-layer Chromatogram of Zimmermann Complexes Produced at Various Standing Time after Acidification of Colored Solution of *i*-Androstanolone

Adsorbent: Silica gel G (E. Merck Co.) Layer thickness 0.3 mm.
Developing solvent: benzene-ether (1:1)
Developing time: 20 min. (at room temperature)
Detection: 10% methanolic KOH

polar one being minor, by purple stain on chromatogram upon exposure to sodium hydroxide solution (Table I).

Separation of these two Zimmermann complexes was also achieved by thin-layer chromatography using silica gel G as adsorbent. In addition, it was revealed that the ratio of the less polar chromophore to the more polar one increased along with the standing time after acidification with hydrochloric acid as illustrated in Fig. 1.

Accordingly, of two chromophore products the former seemed to be an artifact yielded from the latter during the course of separation. It is sufficiently substantiated that 6β-hydroxy-3α,5α-cyclo-structure is vulnerable with acid. Hence it seemed very likely that the steroidal moiety of the complex initially produced would be modified only by contact with hydrochloric acid yielding an apparently different Zimmermann complex.

In order to clarify this point, preparation of Zimmermann complexes was attempted employing some 17-oxosteroids *i.e.* 3β-chloroandrost-5-en-17-one (Ⅲa), androsta-3,5-dien-17-one (Ⅳa) and 3α,5α-cycloandrost-6-en-17-one (Ⅴa), which would be possible products derived from *i*-androstanolone. Of these compounds (Ⅳa) and (Ⅴa) were synthesized in the different manners from those which have already been reported. The crude product obtained from 17-oxosteroid by Zimmermann reaction on the preparative scale was chromatographed on acid-washed alumina, followed by recrystallization to give Zimmermann complexes, Ⅲb, m.p. 192~194°, Ⅳb, m.p. 190~192°, and Ⅴb, m.p. 185~187°, respectively.

Isolation of two complexes produced from *i*-androstanolone was attained with success by preparative thin-layer chromatography using silica gel G. The adsorbent corresponding to the upper spot detected by coloration with sodium hydroxide was collected and eluted to furnish a crystalline compound, m.p. 188~190°. The chromatographic behavior as illustrated in Fig. 2 and the infrared spectrum exhibiting no

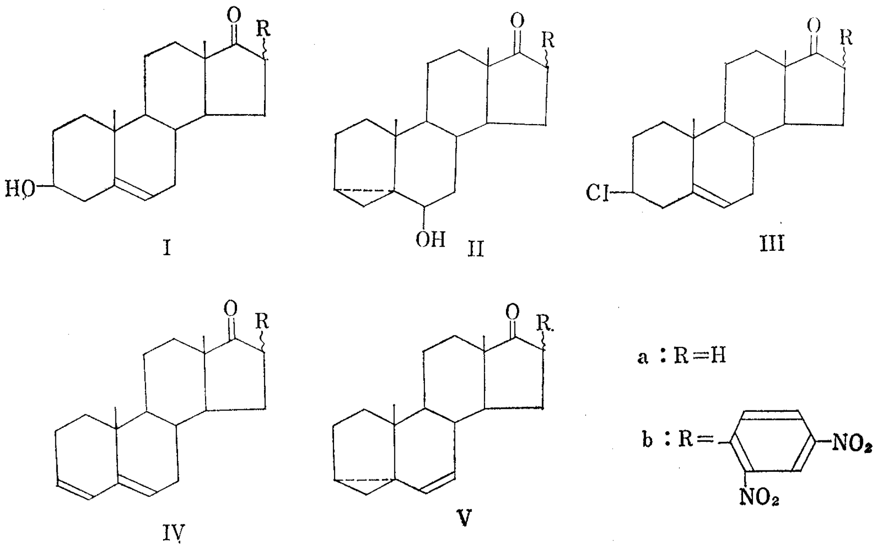

Chart 2.

skeletal deformation vibrations characteristic for 3,5-cyclo-structure[8] indicated that the compound would presumably be 3β-chloro-16ξ-(2,4-dinitrophenyl)androst-5-en-17-one (IIIb). In fact, mixed melting point measurement and infrared spectra comparison proved it to be entirely identical with the authentic sample (Fig. 3).

In addition, another Zimmermann complex, m.p. 113~116°, which could be isolated from the lower spot on thin-layer chromatogram, still remained 3,5-cyclo-structure

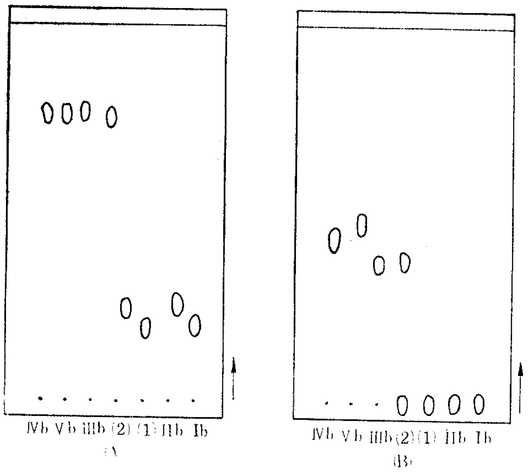

Fig. 2. Thin-layer Chromatogram of Zimmermann Complexes Produced from *i*-Androstanolone and its Related 17-Oxosteroids

(1) Zimmermann complex produced from dehydroisoandrosterone (Ia)
(2) Zimmermann complexes produced from *i*-androstanolone (IIa)
Adsorbent : Silica gel G (E. Merck Co.) Layer thickness 0.3 mm.
Developing solvent : (A) benzene-ether (4:1) (B) benzene
Developing time : 25 min. (at room temperature)
Detection : 10% methanolic KOH

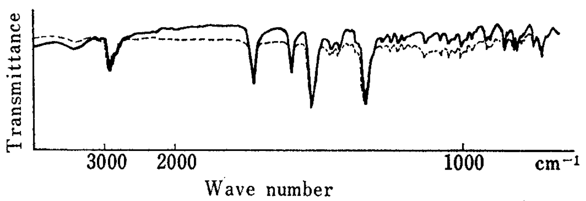

Fig. 3. The Infrared Spectra of the less Polar Zimmermann Complex Produced from *i*-Androstanolone (------) and 3β-Chloro-16ξ-(2,4-dinitrophenyl)androst-5-en-17-one (——)

Spectrophotometer : Koken Model DS-402G
State : KBr micro disk

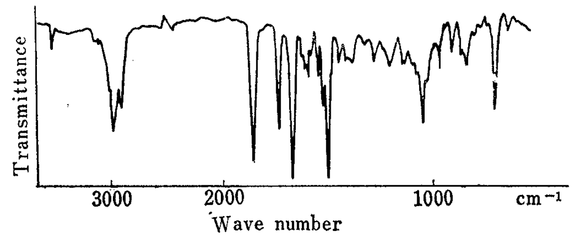

Fig. 4. The Infrared Spectrum of 6β-Hydroxy-16ξ-(2,4-Dinitrophenyl)-3α,5α-cycloandrostan 17-one

Spectrophotometer : Koken Model DS-301
State : $CHCl_3$ solution

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[8] M.L. Josien, N. Fuson, A.S. Cary : J. Am. Chem. Soc., **73**, 4445 (1951); M.L. Josien : Compt. rend., **231**, 131 (1950).

according to the inspection of the infrared spectrum (Fig. 4). On the basis of usual criteria (mixed melting point, infrared spectra, rotation and thin-layer chromatography), this compound proved to be identical with the complex (IIb), which was prepared from *i*-androstanolone by means of acid-washed alumina chromatography described above.

On the other hand, concerning Zimmermann complex of dehydroisoandrosterone, even when the standing time after acidification was prolonged, only a single complex was afforded, and it was elucidated as 3β-hydroxy-16ξ-(2,4-dinitrophenyl)androst-5-en-17-one (Ia), m.p. 186~188°, reported in the previous paper.7)

In conclusion, of two Zimmermann chromophores produced from *i*-androstanolone the less polar one has proved to be an artifact yielded on the exposure to hydrochloric acid, and the result observed by Francis, *et al.* concerning Zimmermann complex of dehydroisoandrosterone should be corrected.

Experimental[*3]

Androsta-3,5-dien-17-one (IVa)——A solution of dehydroisoandrosterone *p*-tosylate (2.25 g.) dissolved in dimethylsulfoxide (75 g.) was heated at 80~90° for 4 hr. The reaction mixture was diluted with H_2O, and extracted with ether. The ether extract was washed with 5% $NaHCO_3$, H_2O and dried over anhyd. Na_2SO_4. After evaporation of solvent the residue obtained was chromatographed on Al_2O_3 (50 g.). Elution with hexane-benzene (1:2) and recrystallization of the eluate from MeOH gave IVa as colorless leaflets. Yield, 435 mg. m.p. 86~88°. $[\alpha]_D^{28}$ −88°(c=0.57). UV λ_{max}^{EtOH} mμ (log ε): 234 (4.21). *Anal.* Calcd. for $C_{19}H_{26}O$: C, 84.39; H, 9.69. Found: C, 83.97; H, 9.85. Djerassi, *et al.*9) prepared this compound by the different method and reported it m.p. 80~82°.

3α,5α-Cycloandrost-6-en-17-one (Va)——A solution of dehydroisoandrosterone *p*-tosylate (3.7 g.) in MeOH (180 ml.) containing Na acetate (4 g.) was refluxed for 2 hr. After evaporation of solvent the residue was poured into H_2O, extracted with ether, dried over anhyd. Na_2SO_4 and concentrated. The residue thus obtained was chromatographed on Al_2O_3 (50 g.) and eluted with hexane and hexane-benzene (4:1) to give 6β-methoxy-3α,5α-cycloandrostan-17-one as oily product (2.16 g.). This product was dissolved in xylene (70 ml.) containing Al_2O_3 (7 g.), which had been previously dehydrated, and refluxed for 20 hr. Then, another portion of Al_2O_3 (0.5 g.) was added and the reaction mixture was further refluxed for 10 hr. After removal of Al_2O_3 by filtration the filtrate was concentrated *in vacuo* and chromatographed on Al_2O_3 (40 g.). Elution with hexane-benzene (9:1 to 8:2) and recrystallization of the eluate from MeOH gave Va as colorless needles. Yield. 378 mg. m.p. 135~138°. $[\alpha]_D^{28}$ +13°(c=0.46). *Anal.* Calcd. for $C_{19}H_{26}O$: C, 84.39; H, 9.69. Found: C, 84.20; H, 9.61. Romes, *et al.*10) prepared this compound by the different method and reported it m.p. 137~138°, $[\alpha]_D^{20}$ +13°(c=0.5, MeOH).

6β-Hydroxy-16ξ-(2,4-dinitrophenyl)-3α,5α-cycloandrostan-17-one(IIb)——To a solution of 6β-hydroxy-3α,5α-cycloandrostan-17-one (230 mg.) and *m*-dinitrobenzene (134 mg.) dissolved in EtOH (14 ml.) was added 2.5*N* KOH (0.32 ml.) and the mixture was allowed to stand in refrigerator overnight. After evaporation of solvent a dark black solid product obtained was extracted with benzene (100 ml.) to remove the unchanged starting material. The filtrate was discarded, and the residue was dissolved in EtOH and passed through a column packed with acid-washed Al_2O_3 (7 g.). The yellow colored fraction, which showed distinct Zimmermann color promptly upon addition of NaOH solution, was collected and concentrated to dryness *in vacuo* below 25° to provide yellow solid product (85 mg.). The crude product was dissolved in hexane-benzene (1:2) and rechromatographed on acid-washed Al_2O_3 (2.5 g.). Elution with benzene and benzene-ether (4:1) afforded a pale yellow crystalline product. Recrystallization from MeOH gave IIb as colorless needles. Yield, 31 mg. m.p. 113~116°. $[\alpha]_D^{20.2}$ −44°(c=0.29). IR $\lambda_{max}^{CHCl_3}$ μ: 11.96. *Anal.* Calcd. for $C_{25}H_{30}O_6N_2$: C, 66.05; H, 6.65; N, 6.16. Found: C, 65.86; H, 6.92; N, 5.89.

3β-Chloro-16ξ-(2,4-dinitrophenyl)androst-5-en-17-one (IIIb)——Employing 3β-chloroandrost-5-en-17-one (IIIa) (250 mg.) Zimmermann reaction was carried out and the reaction product was treated in the same manner as described above. Elution with hexane-benzene (3:2 to 2:3) and recrystallization of the eluate from MeOH gave IIIb as colorless needles. Yield, 22 mg. m.p. 192~194°. $[\alpha]_D^{18.2}$ −100°(c=0.30). *Anal.* Calcd. for $C_{25}H_{29}O_5N_2Cl$: C, 63.49; H, 6.18; N, 5.92. Found: C, 63.65; H, 5.84; N, 6.05.

[*3] All melting points were taken on the micro hot-stage apparatus and are uncorrected. All rotations were measured in $CHCl_3$ unless otherwise stated.

9) C. Djerassi, G. Rosenkranz, T. Romeo: U.S. Pat. 2,698,854 (Jan. 4, 1955); C.A., **50**, 13105 (1956).

10) A. Romes, R. Villote: Ann. Chim. (Rome), **47**, 684 (1957).

16ξ-(2,4-Dinitrophenyl)androsta-3,5-dien-17-one (IVb)——Employing androsta-3,5-dien-17-one (IVa) (240 mg.) Zimmermann reaction was carried out and the reaction product was treated in the same manner as described above. Elution with hexane-benzene (3:1 to 1:1) and recrystallization of the eluate from EtOH gave IVb as colorless needles. Yield, 34 mg. m.p. 190~192°. $[\alpha]_D^{19.3}$ $-198°$ (c=0.73). *Anal.* Calcd. for $C_{25}H_{28}O_5N_2$: C, 68.79; H, 6.46; N, 6.42. Found : C, 68.51; H, 6.45; N, 6.24.

16ξ-(2,4-Dinitrophenyl)-3α,5α-cycloandrost-6-en-17-one (Vb)——Employing 3α,5α-cycloandrost-6-en-17-one (Va) (250 mg.) Zimmermann reaction was carried out and the reaction product was treated in the same manner as described above. Elution with hexane-benzene (3:1 to 3:2) and recrystallization of the eluate from MeOH gave Vb as colorless needles. Yield, 22 mg. m.p. 185~187°. $[\alpha]_D^{26}$ $-73°$ (c=0.44). *Anal.* Calcd. for $C_{25}H_{28}O_5N_2$: C, 68.79; H, 6.46; N, 6.42. Found : C, 68.83; H, 6.55; N, 6.60.

Paper Chromatography of Zimmermann Complexes Derived from Some 17-Oxosteroids——The Zimmermann reactions were carried out using dehydroisoandrosterone and *i*-androstanolone under the conditions described by Callow & Callow.[11] The colored solution was diluted with H_2O (80 ml.), acidified with conc. HCl (10 ml.), extracted with benzene (10 ml. × 3) and dried over anhyd. Na_2SO_4. After evaporation of solvent the crude product obtained was chromatographed on Tōyō-Roshi No. 51A filter paper (2 × 40), which had previously been washed with 95% EtOH and then with ether, dried and impregnated with propylene glycol. After equilibriation period of 2 hr. chromatography was carried out at room temp. using methylcyclohexane as developing solvent for 46 hr. The paper strip was dried overnight and the Zimmermann complex was detected by purple spot appeared immediately when 10% methanolic KOH was sprayed. The result of typical chromatographic separation is shown in Table I.

Isolation of the Zimmermann Complexes Derived from *i*-Androstanolone——According to the procedure of Callow & Callow[11] Zimmermann reaction was carried out in EtOH (4 ml.) using *i*-androstanolone (100 mg.), *m*-dinitrobenzene (60 mg.) and 2.5*N* KOH (2 ml.). The colored solution was diluted with H_2O (48 ml.), acidified with conc. HCl (6 ml.), allowed to stand at room temp. for 2 days and extracted with benzene. The benzene extract was submitted to preparative thin-layer chromatography employing silica gel G as adsorbent and benzene-ether (1:1) as developing solvent. Upon spraying 10% methanolic KOH solution purple spots appeared immediately in two areas (Rf 0.35 and 0.82) occupied by the Zimmermann complexes, respectively. The adsorbent corresponding to the each spot was collected and eluted with ether. Eluate from the upper spot gave colorless needles (1.8 mg.), m.p. 188~190°. Mixed melting point and IR spectra comparison with the authentic sample proved it to be 3β-chloro-16ξ-(2,4-dinitrophenyl)-androst-5-en-17-one. Elution from the lower spot and recrystallization of the eluate from MeOH gave colorless needles, m.p. 113~116°. Mixed melting point and IR spectra comparison proved it to be 6β-hydroxy-16ξ-(2,4-dinitrophenyl)-3α,5α-cycloandrostan-17-one.

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Summary

The structure of Zimmermann complexes produced from *i*-androstanolone (6β-hydroxy-3α,5α-cycloandrostan-17-one) was investigated. By means of preparative thin-layer chromatography two complexes were successfully isolated and were elucidated as 6β-hydroxy-16ξ-(2,4-dinitrophenyl)-3α,5α-cycloandrostan-17-one, m.p. 113~116°, and 3β-chloro-16ξ-(2,4-dinitrophenyl)androst-5-en-17-one, m.p. 192~194°, respectively. These results revealed that the former complex would be transformed into the latter one by contact with hydrochloric acid. On the other hand dehydroisoandrosterone gave Zimmermann complex, 3β-hydroxy-16ξ-(2,4-dinitrophenyl)androst-5-en-17-one, m.p. 186~188°, as a single product.

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[11] N.H. Callow, R.K. Callow, C.W. Emmens : Biochem. J., 32, 1312 (1938).