[Chem. Pharm. Bull. 24(10)2373—2376(1976)]

UDC 547.922.04.08 : 543.41.066

Dimeric Steroid Formation in the Color Reaction of Cholesterol

Yoshihisa Kurasawa, Atsushi Takada, and Takeo Ueda

School of Pharmaceutical Sciences, Kitasato University[1]

(Received January 12, 1976)

The color reaction of cholesterol with trichloroacetic acid and hydrochloric acid (TCA–HCl) (1 : 1) was carried out in the presence of oxygen and/or hydrogen peroxide at a room temperature, and 3,5-cholestadiene, 3,3′-bis(3,5-cholestadiene), cholesteryl chloride and cholesteryl trichloroacetate were isolated from the colored reaction mixture. From the observation of the absorption spectra, it was inferred that the absorption maximum at 560 or 563 nm should be ascribed to the carbocation formed by the protonation of 3,3′-bis(3,5-cholestadiene) in TCA–HCl (1 : 1). It was found that the dimerization was the main oxidative reaction in the present investigation, although it was suggested by many investigators that the formation of the monomeric cholestapolyenes should be the primary oxidation in the Liebermann-Burchard and the Zak reactions. From the result that the chlorinated steroids other than cholesteryl chloride were not acquired, it was assumed that cholesteryl chloride should be produced through the intermediate resulting from the dehydration of cholesterol in the acidic solution, and that 3,3′-bis(3,5-cholestadiene) also should be formed through this intermediate by an oxidative mechanism.

It has been reported by many investigators that the dimeric steroids such as 3,3′-bis(3,5-cholestadiene) (I) and 3,3′-bis(2,4-cholestadiene) (II) were isolated in the various color reactions of cholesterol with the Brønsted and/or the Lewis acids. The dimer (I) was obtained in the reaction with trichloroacetic acid and hydrochloric acid (TCA–HCl) (10 : 1)[2]; the dimer (II) in the reactions with zinc chloride and acetyl chloride (Tschugaeff reaction),[3] ferric chloride, perchloric acid and phosphoric acid,[4] ferric chloride and sulfuric acid (Zak-Henly reaction),[5] antimony trichloride and acetyl chloride[6]; the dimers (I and II) in the reactions with sulfuric acid and acetic anhydride (Liebermann-Burchard reaction),[7] sulfuric acid (Salkowski reaction)[8] and trichloroacetic acid and antimony trichloride.[9] In the study of the Liebermann-Burchard reaction of cholesterol on the reaction mechanism, Watanabe[7] claimed that cholesterol should be dehydrated to 3,5-cholestadiene (III) and 2,4-cholestadiene (IV), and the corresponding dimers (I and II) were simultaneously formed from III and IV. On the survey of the Liebermann-Burchard and the Zak reactions of cholesterol, Burke, *et al.*[10] quantitatively measured the formation of sulfur dioxide and ferrous ion to suggest that the oxidation yielded the conjugated cholestapolyenes such as V (in the Zak reaction) and VI (in the Liebermann-Burchard reaction). Dulou, *et al.*[8] also observed the formation of sulfur dioxide in the Liebermann-Burchard reaction to offer the suggestion similar to that of Burke, *et al.* and Watanabe. According these findings, it seems that the above dimeric steroids were produced by the oxidation in the presence of a certain hydrogen acceptor.

1) Location: *Shirokane, Minato-ku, Tokyo, 108, Japan.*
2) Y. Kurasawa, A. Takada, and T. Ueda, *Chem. Pharm. Bull.* (Tokyo), **24**, 859 (1976).
3) T. Watanabe, *Eisei Shikenjo Hokoku*, **80**, 442 (1960); *idem, ibid.*, **80**, 445 (1960).
4) T. Momose, Y. Ueda, M. Kageura, T. Masumura, and K. Ohta, *Chem. Pharm. Bull.* (Tokyo), **12**, 1415 (1964).
5) T. Momose, Y. Ueda, M. Kageura, T. Masumura, and K. Ohta, *Chem. Pharm. Bull.* (Tokyo), **12**, 1500 (1964).
6) Y. Kurasawa, A. Takada, and T. Ueda, *Chem. Pharm. Bull.* (Tokyo), **24**, 487 (1976).
7) T. Watanabe, *Eisei Shikenjo Hokoku*, **77**, 87 (1959).
8) R. Dulou, J. Chopin, and Y. Raoul, *Bull. Soc. Chim. France*, **1951**, 616; J. Chopin, *ibid.*, **1956**, 258.
9) Y. Kurasawa, A. Takada, and T. Ueda, unpublished data.
10) R.W. Burke, B.I. Diamondstone, R.A. Velapoldi, and O. Menis, *Clin. Chem.*, **20**, 794 (1974).

On the other hand, any of the dimeric steroid was not isolated from the red colored solution of cholesterol with TCA–HCl (1: 1), while III, cholesteryl chloride (VII) and cholesteryl trichloroacetate (VIII) were obtained.[2] Furthermore, this colored solution showed the absorption maxima at 420 and 500 nm, lacking the absorption maximum at 560 nm which was ascribed to the coloration of the dimer (I).[2] However, it was assumed that the dimer (I) should be produced in the reaction of cholesterol with TCA–HCl (1: 1) in the presence of a hydrogen acceptor. Thus, the reaction of cholesterol with TCA–HCl (1: 1) in the presence of oxygen and/or hydrogen peroxide was investigated to confirm this assumption.

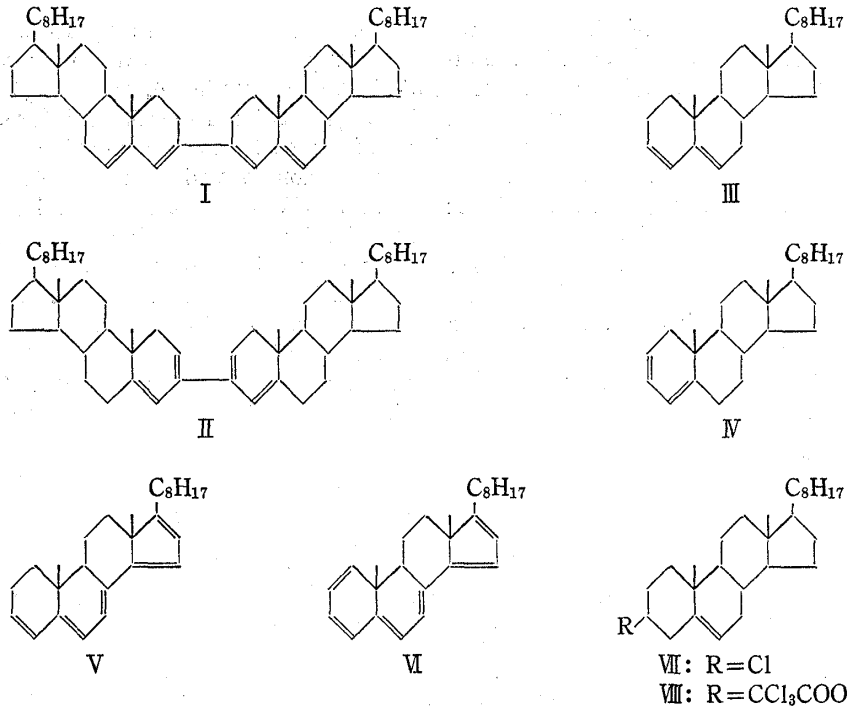

Chart 1

A solution of cholesterol with TCA–HCl (1: 1) was colored red initially and then purplish red gradually in the presence of oxygen at a room temperature. The absorption spectrum of the colored reaction mixture is shown in Fig. 1, and the absorption maxima are observed at 408 and 563 nm. The colored reaction mixture was worked up in the similar procedure to that described in the preceding paper,[2] giving four reaction products.

From the inspection of the spectral data, they were identified as I, III, VII and VIII respectively. This finding coincided with the results in the reaction of cholesterol with TCA–HCl (10: 1). The monomeric cholestapolyenes were not isolated, which were reported by Burke, *et al.*[10] I and III were colored red immediately on shaking with TCA–HCl (1: 1). VII was colored red gradually, and VIII did not exhibit any coloration on shaking with TCA–HCl (1: 1) at a room temperature. The absorption spectra of the color developed by I, III and VII with TCA–HCl (1: 1) are shown in Fig. 2. III and VII showed the quite similar absorption maxima at 420 and 500 nm in the absorption spectra, while I did at 500 and 560 nm. The same reaction products (I, III, VII and VIII) were isolated in the reaction of cholesterol with TCA–HCl (1: 1) in the presence of hydrogen peroxide.

In the previous paper,[2] it was reported that the absorption maximum at 560 nm was observed in the colored solution of the dimer (I), as well as in the reaction mixture which involved the dimer (I) as a reaction product. In the present investigation, the similar results were ascertained by the comparison of Fig. 1 with Fig. 2. The formation of the dimer (I) is supported by the observation of the absorption maximum at 563 nm in Fig. 1. Therefore, it may be said that the absorption maximum at 560 nm was ascribed to the coloration of the dimer (I)

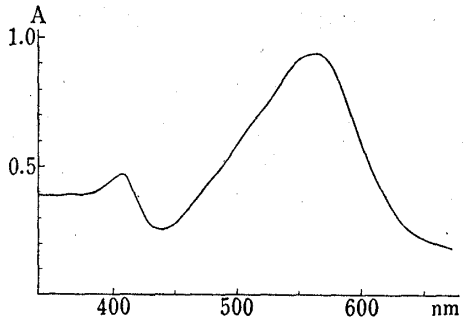

Fig. 1. Absorption Spectrum of Cholesterol with TCA–HCl (1:1) in the Presence of Oxygen

To a solution of 19.10 mg of cholesterol in 1 ml of $CHCl_3$ was added 200 ml of TCA–HCl (1:1) under the conduction of oxygen with stirring for 90 min at a room temperature. Spectrum was recorded at 2 hr.

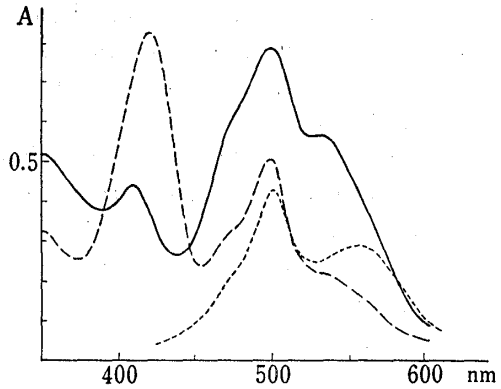

Fig. 2. Absorption Spectra of I, III, and VII with TCA–HCl (1:1)

------: I, ——: III, ----: VII

To solutions of 0.52 mg of I in 1 ml of $CHCl_3$, 1.16 mg of III in 1 ml of $CHCl_3$, and 19.52 mg of VII in 1 ml of $CHCl_3$ was added 10 ml of TCA–HCl (1:1), and the respective colored solutions were kept at a room temperature. Spectrum I was recorded at 16 min, spectrum 3 at 8 min and spectrum VII at 11 min.

having the conjugated tetraene system in the color reactions of cholesterol with TCA–HCl (1:1) and (10:1). In the study by Burke,[10] it was suggested that the absorption maxima at 563 and 478 nm were attributed to the coloration of the steroids with the conjugated pentaene system and the conjugated tetraene system respectively. This suggestion is inconsistent with the authors' result that the colored solution of the dimer (I), which has the conjugated tetraene system, showed the absorption maximum at 560 or 563 nm.

It has been indicated by Brieskorn and Hofmann[11] that the formation of the dimeric steroids was not primary in the Liebermann-Burchard reaction, since the dimeric steroids could not be the end product giving rise to the blue-green coloration which was observed at the final stage in the Liebermann-Burchard reaction, and that the monomeric cholestapentaene exhibiting the absorption maximum at 610 to 620 nm was produced by the oxidative mechanism. In TCA–HCl (1:1) in the presence of the above-mentioned hydrogen acceptors, however, it is evident that the dimer (I) was obtained by the oxidation instead of the monomeric steroids possessing the conjugated double bonds more than three double bonds. Thus, it may be

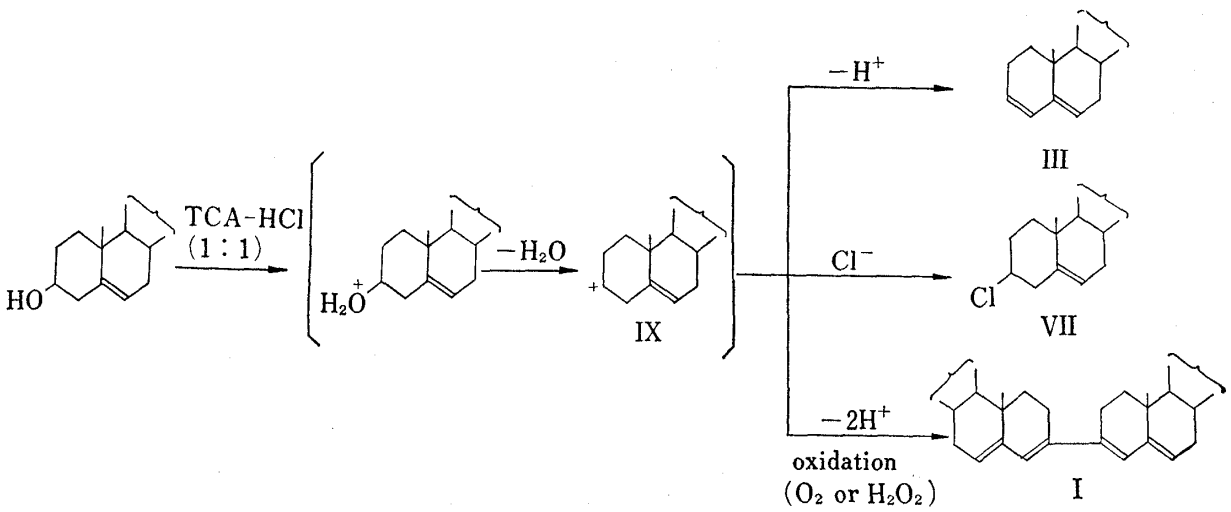

Chart 1

11) C.H. Brieskorn and H. Hofmann, *Arch. Pharm.*, **297**, 577 (1964).

postulated that the dimerization should be the main oxidative reaction in TCA–HCl (1: 1) in the presence of the hydrogen acceptors, while the formation of the monomeric cholestapolyenes should be due to the primary oxidation in the Liebermann-Burchard reaction according to Brieskorn and Hofmann.[11]

On the other hand, any of the chlorinated steroid at any position of C_4 to C_6 was not acquired, and VII was an only chloride isolated. Therefore, it was assumed that VII should be produced through the intermediate (IX) resulting from the dehydration of cholesterol in the acidic solution, and that the dimer (I) also should be brought about through IX by an oxidative mechanism. The inferable steps of this reaction considered by the authors are shown in Chart 2.

Experimental[12]

Reaction of Cholesterol with TCA–HCl (1: 1) in the Presence of Oxygen——To a solution of 10 g of cholesterol in 50 ml of chloroform was added 400 ml of TCA–HCl (1: 1) under the conduction of oxygen, stirring for 90 minutes at a room temperature. The reaction mixture was poured into ice water, and the reaction product was extracted with chloroform. The combined chloroform layer was washed with water, saturated $NaHCO_3$ solution and water successively, and dried over Na_2SO_4. The evaporation of chloroform gave an oily residue. The oily residue obtained was dissolved in a small amount of hexane and submitted to column chromatography on silica gel eluting with hexane.

From the first fraction, an oily mixture was obtained. The oily mixture was dissolved in a small amount of petroleum ether with heating, and cooled in an ice bath to give insoluble crystalline powder. Rechromatography on alumina and then recrystallization from chloroform gave analytically pure 3,3′-bis(3,5-cholestadiene) as colorless needles, mp 244—246°. Yield, 25 mg. It showed no melting point depression on admixture with the authentic sample prepared by the Squire's method.[13] Mass Spectrum *m/e*: 734 (M^+). *Anal.* Calcd. for $C_{54}H_{86}$: C, 88.21; H, 11.79. Found: C, 87.94; H, 11.85.

The above filtrate was evaporated *in vacuo* and rechromatographed on alumina eluting with hexane to give colorless crystals. Recrystallization from ethanol–acetone gave analytically pure 3,5-cholestadiene as colorless prisms, mp 78—79°. Yield, 1.46 g. It showed no melting point depression on admixture with the authentic sample prepared by the Mauthner's method.[14] Mass Spectrum *m/e*: 368 (M^+). *Anal.* Calcd. for $C_{27}H_{44}$: C, 87.97; H, 12.03. Found: C, 87.87; H, 12.13.

Successive elution of the original silica gel column gave colorless crystals. Recrystallization from acetone gave analytically pure cholesteryl chloride as colorless needles, mp 95—96°. Yield, 195 mg. Mass Spectrum *m/e*: 405 (M^+). It showed no melting point depression on admixture with the authentic sample. *Anal.* Calcd. for $C_{27}H_{45}Cl$: C, 80.05; H, 11.20. Found: C, 80.21; H, 11.14.

Further elution of the original silica gel column gave crude cholesteryl trichloroacetate. Recrystallization from ligroin gave colorless prisms, mp 147—148°. Yield, 203 mg. Mass Spectrum *m/e*: 368 (M^+-164). IR ν_{max}^{KBr} cm^{-1}: 1760 (C=O). *Anal.* Calcd. for $C_{29}H_{45}O_2Cl_3$: C, 65.47; H, 8.53. Found: C, 65.40; H, 8.43.

Reaction of Cholesterol with TCA–HCl (1: 1) in the Presence of Hydrogen Peroxide——To a solution of 10 g of cholesterol in 50 ml of chloroform was added 400 ml of TCA–HCl (1: 1). The solution was colored red. To the red colored solution was added 12 ml of 3% hydrogen peroxide. The reaction mixture was stirred for 90 minutes at a room temperature. Reaction products were obtained by the same procedure as above. Yield was as follows: 3,5-cholestadiene, 1.04 g; 3,3′-bis(3,5-cholestadiene), 55 mg; cholesteryl chloride, 173 mg; cholesteryl trichloroacetate, 185 mg.

12) Absorption spectra were measured by Shimazu MPS-50L Spectrophotometer in a cell of 10 mm optical length, infrared (IR) spectra by JASCO IRA-1 Spectrophotometer and MS by JEOL JNS-01S Mass Spectrometer.

13) E.N. Squire, *J. Am. Chem. Soc.*, **73**, 2586 (1951).

14) J. Mauthner and W. Suida, *Monatsh.*, **17**, 29 (1896).