Chem. Pharm. Bull. 36(11)4261—4268(1988)

On the Rearrangement Reactions of Pregnanediol Disulfate to Δ^{13}-Steroid, and Its 20-Isomeric Sulfate to D-Homosteroids (Clinical Analysis on Steroids. XLII[1])

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(Received February 20, 1988)

17α-Ethyl-17β-methyl-5β-pregn-13-en-3α-ol, a major hydrolysis product of pregnanediol disulfate in 3 N hydrochloric acid at 95 °C, was shown to be formed *via* two reaction pathways, a concerted mechanism and a stepwise mechanism involving the C_{20}-carbocation, in an approximate ratio of 75 : 25.

D-Homoannulation of the isomeric sulfate, 5β-pregnane-3α,20β-diol disulfate, giving 17α-methyl-D-homo-5β-androstane-3α-17aβ-diol as a predominant product under the same hydrolysis conditions, was shown to occur mainly (*ca.* 90%) by the concerted mechanism.

Keywords——pregnanediol disulfate; 5β-pregnane-3α,20β-diol disulfate; hydrolysis; D-homosteroid; steroidal carbocation; Δ^{13}-steroid; rearrangement reaction

Pregnanediol disulfate (**2**) and its C_{20}-isomer, 5β-pregnane-3α,20β-diol disulfate (**4**), when hydrolyzed in refluxing 3 N hydrochloric acid, gave numerous kinds of common degradation products.[2,3] The main product from **2** was 17α-ethyl-17β-methyl-5β-androst-13-en-3α-ol (**5**),[2,4] whereas that from **4** was 17α-methyl-D-homo-5β-androstane-3α,17aβ-diol (**6**).[3] Other products from both sulfates were such compounds as pregnanediol (**1**), 5β-pregnane-3α,20β-diol (**3**), 17α-methyl-17aβ-chloro-D-homo-5β-androstan-3α-ol (**7**), steroidal mono-olefins including 5β-pregn-20-en-3α-ol (**8**), and steroidal dienes.[2,3b] From the above results and further detailed studies,[5,6] the following conclusions (a)—(e) were reached (see also Chart 2).

(a) Both sulfates (**2** and **4**) formed the C_{20}-carbocation (I) during the hydrolyses.

(b) This cation could act as a precursor not only to the Δ^{13}-steroid (**5**), but also to D-homosteroids (**6** and **7**). This was confirmed by the hydrolysis of 5β-pregn-20-en-3α-ol sulfate (**9**), which corresponds to a sulfate of the conjugate base of a C_{20}-cation (I). Such a rearrangement reaction passing through I was defined as a stepwise mechanism (pathways B and C).[7]

(c) D-Homoannulation of **2**, and Δ^{13}-steroid formation from **4** proceed *via* cation I.

(d) The difference of the main reactions between the two sulfates (**2** and **4**) led us to speculate, in addition to the stepwise mechanism, an involvement of another mechanism in the hydrolyses, *i.e.*, the concerted mechanisms illustrated by pathways A and D (Chart 2).

(e) D-Homoannulation of both sulfates arose by a shift of C_{16}–C_{17} bond to C_{20}, regardless of the nature of the mechanism involved.

In the present study, we planned to clarify the relative importance of the concerted and the stepwise mechanisms which might be involved in the formation of **5** from **2**, and also in the D-homoannulation of **4**. Although the stepwise mechanism can be confirmed by trapping the

C_{20}-cation (I),[5] there is no direct procedure to demonstrate concerted mechanisms. Fortunately, rearrangement reactions of **9** arise only by the stepwise mechanism, so comparison of the hydrolysis of **2** or **4** with that of **9** is expected to reveal the involvement of the concerted mechanism, and this was done in the following way.

As the yield of each hydrolyzate should be constant under fixed reaction conditions, the yields are considered to be conditional constants. Thus, the ratio of the yield of Δ^{13}-steroid (**5**) to that of D-homosteroids (**6** and **7**) should be constant. When the ratio obtained from the sulfate **9** is substituted as R_1 (Eq. 1), the conditional constant R_1 represents the proportion of the two reaction pathways accounted for by the stepwise mechanism. Another conditional constant R_2 (Eq. 2) obtained by similar treatment for the sulfate **2**, should represent the ratio of the yield of Δ^{13}-steroid (**5**) formed by the two mechanisms to that of D-homosteroids by the stepwise mechanism. The difference of the two conditional constants (R_2-R_1), thus, corresponds to the involvement of the concerted mechanism in the formation of **5**. Participation of the concerted mechanism in the formation of **5** from **2**, therefore, can be obtained from Eq. 3.

$$R_1=\frac{\text{the yield of } \Delta^{13}\text{-steroid (5) from 9}}{\text{the yield of D-homosteroids (6 and 7) from 9}} \tag{1}$$

$$R_2=\frac{\text{the yield of } \Delta^{13}\text{-steroid (5) from 2}}{\text{the yield of D-homosteroids (6 and 7) from 2}} \tag{2}$$

$$\left[\begin{array}{l}\text{participation (\%) of concerted mechanism}\\ \text{in the formation of 5 from 2}\end{array}\right]=\frac{R_2-R_1}{R_2}\times 100 \tag{3}$$

Application of similar treatment to D-homoannulation of **4** was carried out. When the ratios of the yield of D-homosteroids to that of Δ^{13}-steroid from **9** and from **4** are defined as conditional constants, R_3 and R_4, respectively, these values should represent the ratios of the yields of both products generated only by the stepwise mechanism, and by both mechanisms, respectively. The equations are as follows.

$$R_3=\frac{\text{the yield of D-homosteroids (6 and 7) from 9}}{\text{the yield of } \Delta^{13}\text{-steroid (5) from 9}} \tag{4}$$

$$R_4=\frac{\text{the yield of D-homosteroids (6 and 7) from 4}}{\text{the yield of } \Delta^{13}\text{-steroid (5) from 4}} \tag{5}$$

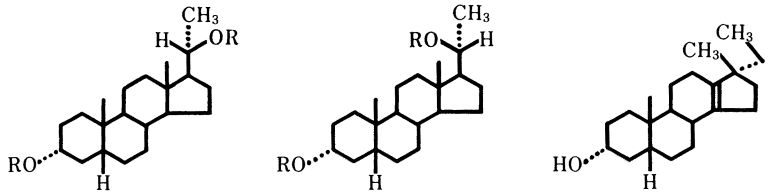
$$\left[\begin{array}{l}\text{participation (\%) of concerted mechanism}\\ \text{in the D-homoannulation of 4}\end{array}\right]=\frac{R_4-R_3}{R_4}\times 100 \tag{6}$$

To utilize Eq. 3 or 6, it is necessary to establish an analytical procedure for exact estimation of the products (**5**, **6** and **7**), and this was carried out by using gas chromatography (GC).

Experimental

Materials——Steroidal sulfates, **2**,[9] **4**,[3b] and **9**[5] (as potassium salts) were obtained by the reported methods. Standard compounds (**5**, **6**, **7** and other degradation compounds) were synthesized by the known method.[4] Estradiol 3-methylether was prepared in this laboratory from estradiol (Steraloids, N.H., U.S.A.) by treatment with diazomethane. Trifluoroacetic anhydride (TFAA) and TMSI-H (a pyridine solution of hexamethyldisilazane and trimethylchlorosilane) were purchased from Gasukuro Kogyo, Co., Ltd. (Tokyo, Japan). All other chemicals used were of reagent grade, and were used without further purification.

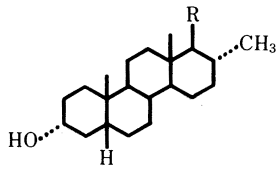
GC——GC was carried out on a 4CM gas chromatograph (Shimadzu, Kyoto, Japan) using a glass column (2 m × 3 mm, i.d.) packed with 1.5% OV-1 on Shimalite W (80—100 mesh) with nitrogen as a carrier gas at the flow rate of 30 ml/min. The column temperatures were 200 and 220 °C for the analyses of trifluoroacetate and trimethylsilylether, respectively.



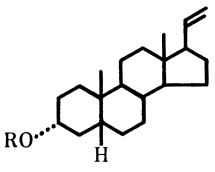
1 : R=H
2 : R=SO_3H

3 : R=H
4 : R=SO_3H

5



6 : R=OH
7 : R=Cl



8 : R=H
9 : R=SO_3H

Chart 1

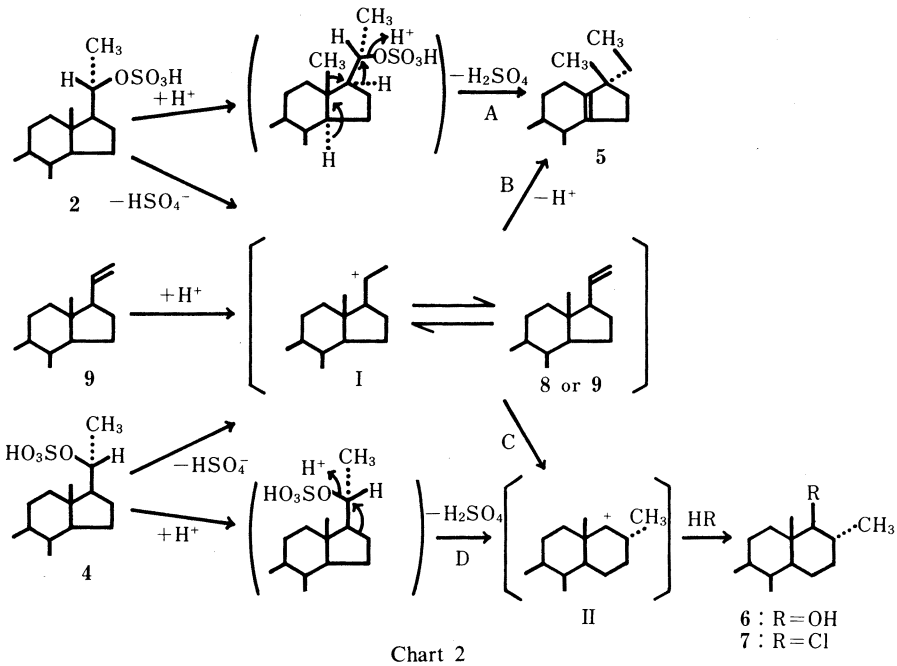


Chart 2

Hydrolysis (General Procedure)——Aliquots of 1 ml of aqueous solutions containing each sulfate (0.1, 0.5, 1.0, 10, 20 50 and 100 μM) were heated on a water bath at 85, 90, 95 and 100 °C (controlled within $\pm$0.2 °C). These solutions were combined with the same volume of hydrochloric acid (1—12 N) at the same temperature. After a definite time of heating, the reaction was terminated by cooling the solutions on ice, followed by neutralization with the same volume of sodium hydroxide solution (4 ml final volume). After addition of a known amount of internal standard, each solution was extracted (3 ml × 3) with a mixture of chloroform and ethyl acetate (3 : 1, v/v). The combined organic phases were washed with water (3 ml × 3), dried on anhydrous Na_2SO_4, and concentrated under a nitrogen stream to give the residue.

Quantitative Analysis——GC: Steroidal materials, **1**, **3**, **5**, **6**, **7**, **8** and other degradation products,[4] were derivatized by the following procedures for GC.

Trimethylsilylation: TMSI-H solution (100 μl) was added to test tubes containing 20 μg of each steroid. The solutions were warmed at 60 °C for 5 min, then dried under a nitrogen stream. The residues obtained were taken up in

n-hexane (500 μl), and the supernatants were submitted to GC. Relative retention times of **5**, **6** and **7** were 0.51, 1.15 and 1.67, respectively, based on that of the internal standard as 1.00 (13.9 min).

Trifluoroacetylation: TFAA (5 μl) was added to tetrahydrofuran solutions (100 μl) containing each steroid (20 μg), and the mixtures were kept at 50 °C for 10 min. The solutions were concentrated under a nitrogen stream to give residues, which were subjected to GC as acetone solutions. Relative retention times; **5**=0.52, **6**=1.55, **7**=1.53 and internal standard=1.00 (10.6 min).

Calibration Curves: Mixtures containing an exact amount of each steroid (**5**, **6** and **7**) and a known amount of internal standard were derivatized by the above procedure and subjected to GC. Calibration curves were constructed by plotting the peak height of each steroid to that of internal standard against the amount of the former.

Recovery Test: An exact amount (1, 5 and 20 μg) of each steroid and a known amount of internal standard were made up to 4 ml (final volume) with water. Finally, each solution was hydrolyzed and treated in the same way as described above.

Stability of **5**, **6** and **7**: Methanolic solutions (100 μl) containing authentic steroids (**5**, **6** and **7**) were warmed to remove methanol. To the wet residues, 3 N HCl (1 ml) was added to give final concentrations of 10, 50 and 100 μM. These solutions were heated at 95 °C. After appropriate time intervals, the reaction was stopped by adding ice, followed by neutralization with 0.3 N NaOH. The mixtures were extracted with ether (3 ml × 3) containing an internal standard. The combined organic phases were washed once with water (1 ml), and dried over anhydrous Na_2SO_4. Evaporation of the ether gave a residue, which was analyzed by GC.

Results and Discussion

Quantification of the Degradation Products

Degradation products of sulfates (**2**, **4** and **9**) were measured by GC. As these sulfates gave many kinds of isomeric products on hot acid hydrolysis, simultaneous separation of all the products by GC is very difficult.[4] Derivatization of these hydrolyzates with TFAA or TMSI-H, however, made it possible to separate **5**, **6** and **7** from other products. Quantification of these products were carried out by the internal standard method, and their calibration curves were linear in appropriate ranges of the steroids tested. In order to confirm the validity of the present method for the determination of these steroids, a recovery test was carried out using authentic samples. It was evident that the steroids were recovered to a satisfactory extent. The analytical procedure for **5**, **6** and **7** by GC was, thus, established.

Examination of Reaction Conditions

As reported previously,[10,11] hydrolyses of steroidal sulfates by mineral acids are influenced by such factors as the concentration of acid, reaction temperature and reaction time. In order to obtain the exact conditional constants, it is necessary to choose reaction conditions where each product is generated linearly.

Influence of the Acid Concentration: The effect of hydrochloric acid concentration upon the degradation was examined using **4** at 95 °C for 10 min, and the results are shown in Fig. 1.

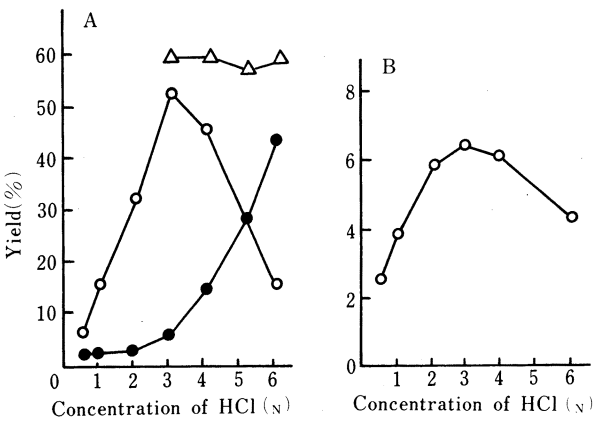


Fig. 1. Effect of Hydrochloric Acid Concentration upon the Yields of Products (**5**, **6** and **7**) Obtained by the Hydrolysis of 5β-Pregnane-3α,20β-diol Disulfate (**4**)

A, —○—, **6**; —●—, **7**; —△—, **6**+**7**. B, —○—; **5**. Hydrolyses were carried out using 10 μM sulfate at 95 °C for 10 min. Each point represents the mean value of 5 experiments (C.V., 2.2—5.0).

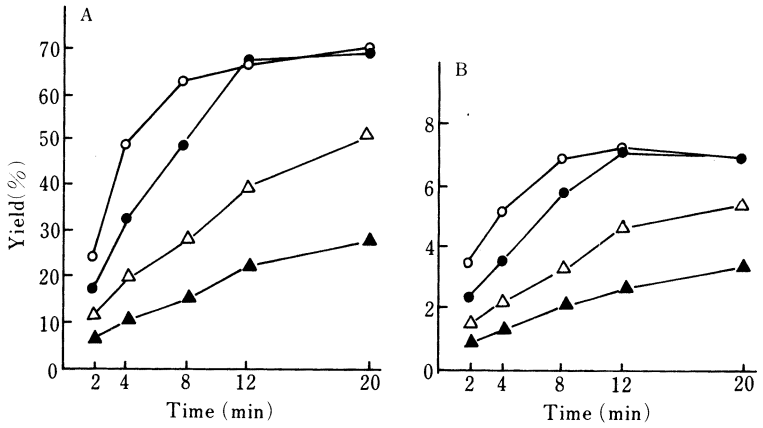


Fig. 2. Time-Courses of Product Formation Obtained by the Hydrolysis of 5β-Pregnane-3α,20β-diol Disulfate (**4**) at Different Reaction Temperatures

A, **6** and **7**; B, **5**. ○—○, 100 °C; ●—●, 95 °C; △—△, 90 °C; ▲—▲, 85 °C. Hydrolyses were carried out using 10 μM sulfate in 3 N hydrochloric acid. Each point represents the mean value of five experiments (C.V., 1.6—4.4).

The yields of **5** and **6** increased up to the acid concentration of 3 N, and decreased rapidly thereafter with a rapidly increasing formation of **7**. The decrease of **5** is considered to involve further decomposition to steroidal dienes, because significant amounts of dienes are produced at acid concentrations over 3 N. Although the yield of **6** decreased rapidly over 3 N concentration, the total amounts of D-homosteroids produced were almost constant as shown in Fig. 1, which suggests an increase of nucleophilic attack of chloride ion on the cation (II) with increasing acid concentration. Analogous tendencies were observed in the experiments using **2** and **9**: formations of **5** and **6** became maximum when the hydrolyses were carried out in 3 N HCl.

Influence of the Reaction Temperature and Time: The effects of the reaction temperature and time on the productions of Δ^{13}-steroid and D-homosteroids were examined. Figure 2 shows time-courses of the production of **5** and two D-homosteroids from **4** in 3 N HCl at different temperatures. At 85 and 90 °C, compound **5** was produced linearly in proportion to the reaction time up to 20 min, but in low yields. At 95 and 100 °C, the yield increased rapidly but retained linearity up to 12 and 8 min, respectively. Similar tendencies were observed in the production of D-homosteroids (**6** and **7**).

Analogous results were obtained from the hydrolyses when **2** and **9** were used. The yields of **5** and D-homosteroids at 95 °C were linear up to about 12 and 16 min for the sulfates **2** and **9**, respectively. From these results, the reaction temperature 95 °C is considered to be suitable for obtaining the exact conditional constants.

Influence of the Substrate Concentration: To examine whether the ratios of the yields are affected by the substrate concentration, hydrolysis of **4** was carried out in 3 N HCl at 95 °C for 8 and 12 min. Ratios of the yields of **5** and **6** were almost constant at substrate concentrations from 0.5 to 50 μM for both periods, but became gradually smaller at concentrations over 100 μM. Under 0.5 μM, the products were not detectable because of their low yields. As similar results were obtained from the hydrolyses of **2** and **9**, the experiments were carired out at the substrate concentration of 10 μM, which corresponds to the normal urinary pregnanediol value in nonpregnant and pregnant women.[12]

Conclusion: From the above experiments, the reaction conditions for obtaining the exact conditional constants were chosen to be as follows: substrate concentration = 10 μM, acid (HCl) concentration = 3 N, reaction temperature = 95 °C, and reaction time = up to 12 min.

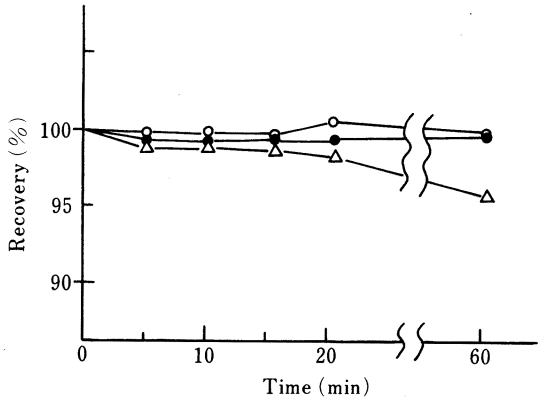


Fig. 3. Time-Courses of the Recoveries of 17α-Ethyl-17β-methyl-5β-Pregn-13-en-3α-ol (**5**), 17α-Methyl-D-homo-5β-androstane-3α,17aβ-diol (**6**) and 17α-Methyl-17aβ-chloro-D-homo-5β-androstan-3α-ol (**7**), during Heating in 3 N Hydrochloric Acid at 95 °C

—△—, **5**; —●—, **6**; —○—, **7**. Each point represents the mean value of 5 experiments (C.V., 2.0—3.3).

TABLE I. Time-Courses of the Yields of Δ^{13}-Steroid (**5**) and D-Homosteroids (**6** and **7**) from Pregnanediol Disulfate (**2**), 5β-Pregnane-3α,20β-diol Disulfate (**4**) and 5β-Pregn-20-en-3α-ol Sulfate (**9**) in 3 N Hydrochloric Acid at 95°C, and Ratios of the Yields of Both Steroids

Substrates	Reaction time (min)	Yield (%)		Ratio yield of **5** / yield of **6** and **7**	
		Δ^{13}-Steroid (**5**)	D-Homosteroids (**6** and **7**)		
2	2	9.6	1.8	5.3	
	4	17.1	3.9	4.4	4.5 (mean)
	8	34.6	8.3	4.2	
	12	51.2	12.6	4.1	
	20	52.1	13.6	—	
4	2	2.4	17.4	0.14	
	4	3.4	31.3	0.11	0.12 (mean)
	8	5.8	48.1	0.12	
	12	7.1	66.7	0.10	
	20	7.0	68.7	—	
9	2	4.6	3.9	1.2	
	4	10.1	9.7	1.0	1.1 (mean)
	8	16.7	14.1	1.2	
	12	22.3	21.3	1.0	
	20	25.1	26.4	—	

Stability of the Rearranged Products under the Reaction Conditions

For the exact determination of the steroids (**5**, **6** and **7**), it is necessary to confirm whether they are stable or not. To examine this, each steroid (**5**, **6** and **7**, at 50 μM concentration) was heated in 3 N HCl at 95 °C. Figure 3 shows the time-courses of the recoveries. Up to 20 min, no degradation of the steroids was observed except **5**, which decomposed very slowly but without any detectable formation of **6** or **7**. Similar results were obtained when 10 and 100 μM solutions of each steroid were used. Quantitative determination of **5**, **6** and **7** produced under the above conditions, thus, should be possible without any correction.

Ratio of Concerted and Stepwise Mechanisms

Hydrolyses of **2**, **4** and **9** in 3 N HCl at 95 °C were carried out, and their products were measured at appropriate time intervals. The ratios of the yield of Δ^{13}-steroid (**5**) and that of D-homosteroids (**6** and **7**) were obtained as summarized in Table I, where the ratios for the sulfate **9** are approximately 1. In contrast, the ratios for the sulfates **2** and **4** are about 4.5 and

TABLE II. Time-Courses of the Proportion (%) of the Concerted Mechanism Involved in the Rearrangement Reaction of Pregnanediol Disulfate (**2**) to Δ^{13}-Steroid (**5**) and of 5β-Pregnane-3α,20β-diol Disulfate (**4**) to D-Homosteroids (**6** and **7**) in 3 N Hydrochloric Acid at 95 °C

Type of reaction	Participation (%) of the concerted mechanism in the rearrangement reactions at different reaction times (min)				
	2	4	8	12	Mean
Δ^{13}-Steroid (**5**) from **2**	77	77	71	76	75
D-Homoannulation of **4**	88	90	90	91	90

0.12, respectively. These results suggest that: (a) The two rearrangement reactions (pathways B and C) *via* the C_{20}-cation (I) occur to almost the same extent. (b) The large difference of the ratios between **2** and **4** implies participation of two mechanisms in different ratios. (c) The stepwise mechanism is considerably involved in the formation of **5** from **2**, but only slightly in the D-homoannulation of **4**.

Conditional constants, R_1—R_4, were calculated by using the yields in Table I, followed by their substitution into Eqs. 3 and 6. Participation of the concerted mechanism in the Δ^{13}-steroid formation and in the D-homoannulation was concluded to be as summarized in Table II. Regardless of the reaction time, all values appear to be constant. In the case of the sulfate **2**, about 75% of **5** was produced by the concerted mechanism (pathway A), and thus the rest (about 25%) must be formed *via* cation I (pathway B). In the case of the sulfate **4**, on the other hand, about 90% of the D-homoannulation proceeded by the concerted mechanism (pathway D), and the stepwise mechanism (like pathway C) only accounted for approximately 10%.

References and Notes

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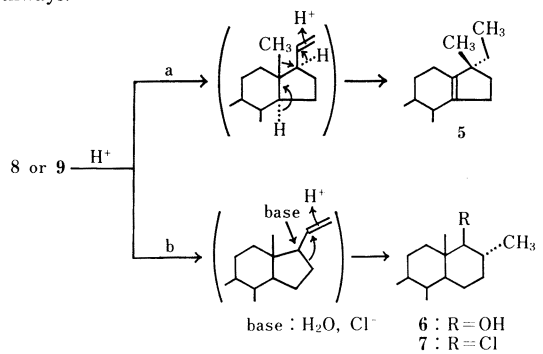
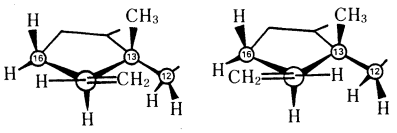
7) In this paper, we define "stepwise mechanism" as any pathway *via* the carbocation (I) as an intermediate in the rearrangement reactions from the sulfates to the decomposed products. For productions of Δ^{13}-olefin and D-homosteroids, however, the following proton-catalyzed reaction pathways (a and b) passing through not cation I but the Δ^{20}-olefin **8** (and **9**, if the sulfate group remains intact) are considered. In the following, we consider the feasibility of these pathways.

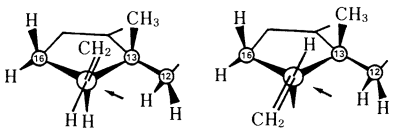


(a) Δ^{13}-Steroid formation: For occurrence of this reaction (pathway a), the conformation of the side chain must be restricted to give antiparallel relationships among the protonation site, the $C_{17\alpha}$-H, and the 18-methyl group. Two rotational conformers satisfying this steric condition are possible: Va and Vb, which are shown by Newman's projection along the C_{17}–C_{20} bond. However, the reaction can not be expected to occur for the following reasons. First, these rotamers are considered to be highly unstable because of the steric interaction between two hydrogens at C_{21} and $C_{12\beta}$ for Va, and those at C_{21} and $C_{16\beta}$ for Vb.[8] Secondly, the reactions through these rotamers have thermodynamic disadvantages in the transition states. In both rotamers, protonation and methyl migration have to occur simultaneously at the β-site which is crowded by the $C_{16\beta}$-hydrogen and C_{18}-methyl group.



Va Vb

(b) D-Homoannulation: D-Homoannulation of Δ^{20}-steroid by a proton-catalyzed mechanism also requires restricted steric environments as illustrated by VIa and VIb: for occurrence of the reaction, the protonation site, the C_{16}–C_{17} bond, and the direction of attack at C_{17} by a base (shown by an arrow) have to be arranged in an antiparallel relationship. As was reported previously,[8] rotamer VIb is the most stable rotational conformer, whereas VIa is the most unstable one. However, D-homoannulation through these rotamers would have to proceed by simultaneous attack by a proton (H_3O^+) and a base (H_2O or Cl^-) at the sterically crowded α-site (shown by an arrow). Such a simultaneous attack by two different reacting species at two closed carbons at the crowded site is extremely unlikely, because even the attack of base (H_2O) on the C_{20}-carbocation from the α-site was not observed.[6]



VIa VIb

In summary, rearrangement reactions of Δ^{20}-steroid to **5**, **6** and **7** could proceed *via* the C_{20}-carbocation (I), a form of the conjugate base. In the present paper, any other possible pathways if they exist, after the formation of cation I, were included in the stepwise mechanism, and were differentiated from a concerted mechanism such as pathway A or D, shown in Chart 2.

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