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Chromogenic Reactions of Steroids with Strong Acids. III.[1] Comparative Study on the Reactions initiated by Brøнsted and Lewis Acids for the Chemical Analysis of Testosterone[2]

Michiya Kimura and Kazuaki Harita

Faculty of Pharmaceutical Sciences, Hokkaido University[3]

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Spectroscopic and gas-chromatographic observations were done on the chromogenic reactions of testosterone (I) with Brønsted acids such as sulphuric acid, phosphoric acid, and perchloric acid and with Lewis acids such as $SbCl_3$, $SbCl_5$, $ZnCl_2$, and $AlCl_3$. Although the spectra due to the reactions with various Brønsted acids were different in each of the colorimetric procedures for I, the residues obtained from the reaction mixtures gave a similar spectrum with each other, when they were submitted again to the colour development at room temperature in the dichloromethane solution saturated with 70% $HClO_4$ (Fig. 1). From this and the results of gas chromatography (Fig. 2) of these residues, 17-methyl-18-norandrosta-4,13(17)-dien-3-one (II) and/or 17-methyl-18-norandrosta-4,-13(14)-dien-3-one (III) were/was likely to be the intermediary chromogen(s) commonly present in these colorimetric reaction mixtures.

Lewis acids in the reactions with I, on the contrary, gave entirely different results as shown in Fig. 2 and 3.

In contrast to I, the dienones, II and III, showed readily at room temperature the bathochromic absorption with $HClO_4$ as well as $SbCl_5$ in dichloromethane, which was markedly intensified in the presence of oxidizing agent (Fig. 4).

The chromogenic and fluorogenic reactions of steroids have widely been used for the qualitative and quantitative analysis with various Brønsted as well as Lewis acids. In spite of numerous reports on the colorimetric methods based on these reactions, little has been known on the detailed chemistry in the reaction mixtures. In the preceding papers of this series, the chromogenic reactions of testosterone (17β-hydroxyandrost-4-en-3-one) (I) with perchloric acid in dichloromethane[4] and of epitestosterone (17α-hydroxyandrost-4-en-3-one) with sulphuric acid containing bromine molecules in acetic acid[1] (Kägi-Miescher reaction[5])

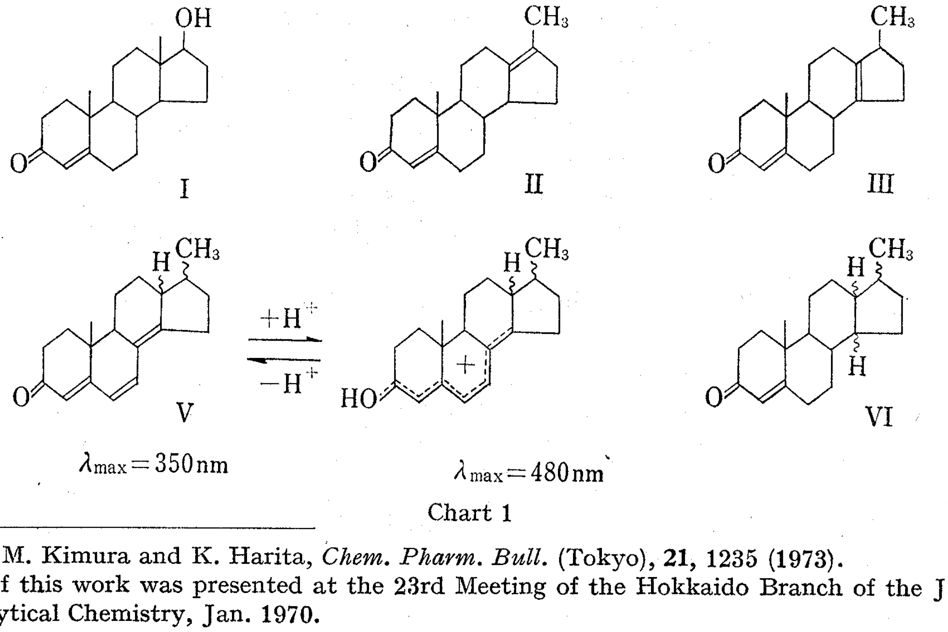

Chart 1

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[2] A part of this work was presented at the 23rd Meeting of the Hokkaido Branch of the Japan Society for Analytical Chemistry, Jan. 1970.
[3] Location: *Nishi-6-chome, Kita-12-jo, Kita-ku, Sapporo, 060, Japan.*
[4] M. Kimura and K. Harita, *Chem. Pharm. Bull.* (Tokyo), 21, 1205 (1973).
[5] H. Kägi and K. Miescher, *Helv. Chim. Acta*, 22, 683 (1939); K. Miescher and H. Kägi, *ibid.*, 32, 761 (1949).

were studied and several products were isolated. 17-Methyl-18-norandrosta-4,13(17)-dien-3-one (II) and 17-methyl-18-norandrosta-4,13(14)-dien-3-one (III) were thus found to be the essential intermediates common in these chromogenic reactions. It was also demonstrated that the chromophore responsible for the absorption maximum at 372 nm in concentrated sulphuric acid is the carbonium ion (IV) present at the early period in the Kober reaction[6] of estradiol (estra-1,3,5(10)-triene-3,17β-diol).[7] In relation to the chromogenic reaction of I in concentrated sulphuric acid, 17-methyl-18-norandrosta-4,6,8(14)-trien-3-one (V) was likely to be derived from the intermediary dienones, II and III, formed in advance and to be the chromophore responsible for the absorption maximum at 480 nm.[8] In the present paper, the spectroscopic as well as gas chromatographic observations are presented and discussed on the reaction mixtures given in several well-known qualitative and colorimetric procedures, in which various Brønsted and Lewis acids are employed for the analysis of I.

Result and Discussion

Numerous methods for the estimation of testosterone (I) have extensively been developed by using various acids and particularly sulphuric acid has often been employed in these colorimetric reactions. The absorption spectra were observed on the reaction mixtures of I obtained under the directions of the several standard procedures reported and those due to the reaction initiated with sulphuric acid (a, b, c, d, and e)[9–13] are shown in Fig. 1 (A). Colorimetric methods employing phosphoric acid[14] and perchloric acid [15] have also been presented and the spectra taken under the respective directions reported are also shown in Fig. 1 (A). No bathochromic absorption in the visible region was observed in the reaction of I with hydrochloric acid,[12] nitric acid,[12] formic acid,[16] or trichloroacetic acid.[17] The colorimetric reactions of this kind are likely to be affected with the factors such as acidity, temperature, time, solvent, light, as well as additive reagent and the resultant absorption spectrum would also be variable even if the intermediary chromogen once formed is identical. In order to have some informations on such chromogen (s), absorption spectrum was observed on the respective colorimetric reaction mixture as follows. After extracting the reaction mixture with organic solvent, the extract was submitted to the colour development with the dichloromethane solution saturated with 70% $HClO_4$ at room temperature. Similar absorption spectra were thus obtained as shown in Fig. 1 (B), whereas no substrate (I) was detectable in thin-layer (TLC) as well as gas-liquid chromatography (GLC) of the extracts except the case with phosphoric acid. It may, therefore, be assumed that the common chromogen(s) would be produced in most of these colorimetric reactions. Since the dienones, II and III, have been assumed as the intermediary chromogens in the reaction of I with single use of perchloric acid in chloroform medium[4] and readily gave the similar spectra as shown in Fig. 1 (B), they were likely to be formed also in these reactions employing the mixture of Brønsted acids and the additives.

Rather drastic conditions required in these colorimetric reactions were expected to produce

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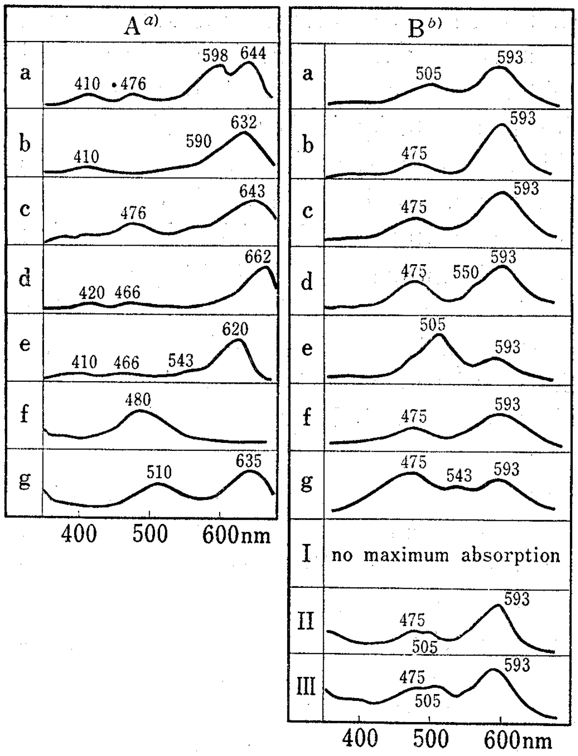

Fig. 1. Absorption Spectra due to the Reactions of Testosterone (I) with Brønsted Acids

a) spectrum due to the reaction of I with H_2SO_4 (a—e), H_3PO_4 (f), or $HClO_4$ (g)

b) spectrum due to the reaction of dichloromethane-$HClO_4$ reagent with the residue obtained from the corresponding reaction mixture in A

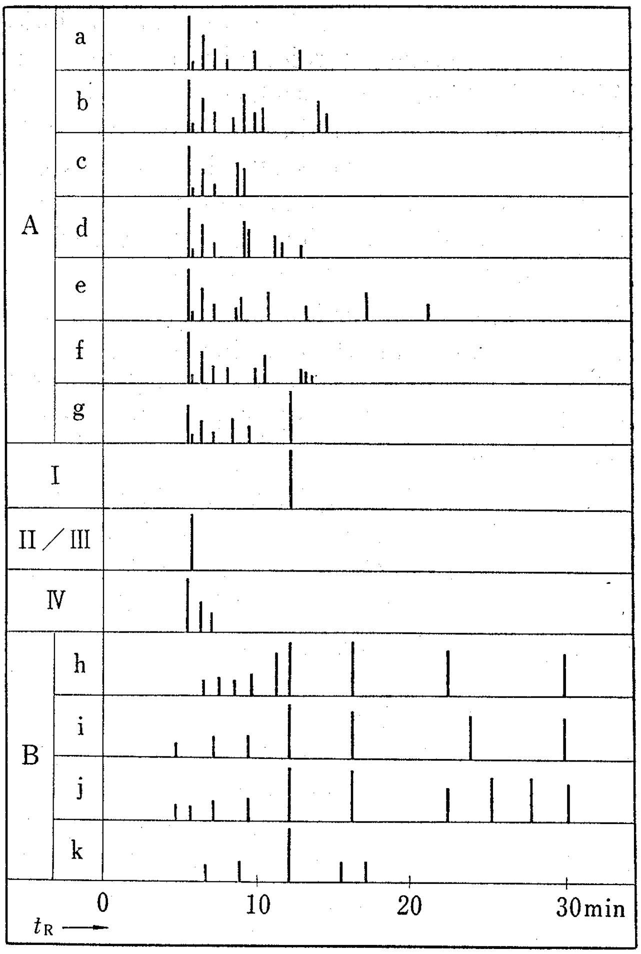

Fig. 2. Gas Chromatograms of the Products from the Reaction of Testosterone (I) with Brønsted Acids and Lewis Acids

A: residue from the reaction of I with H_2SO_4 (a—e), H_3PO_4 (f), or $HClO_4$ (g)

B: residue from the reaction of I with $SbCl_3$ (h), $SbCl_5$ (i), $ZnCl_2$ (j), or $AlCl_3$ (k)

different derivatives from I, and GLC was of particular use for the comparative inspection of them. The above-mentioned extracts from the respective colorimetric reaction mixture were thus submitted to GLC and the results are summarized in Fig. 2. Although various compounds were found to be differently formed in each of the colorimetric reactions and were not necessarily identified exactly by their retention times alone, it may be suggested also from the result shown in Fig. 2 that the chromogenic dienone(s),4) II and/or III, and the steric isomers (VI)4) of non-chromogenic 17-methyl-18-norandrost-4-en-3-one would be the common products. Thus, these dienones are likely to be the intermediary chromogens commonly present in a series of colorimetric reactions of I with the mixture of vatious Brønsted acids and additive reagents. While the light absorption at the region of 470—480 nm was observable in most cases as shown in Fig. 1 (B), identification of the trienone (V)8) in GLC was in no success and further examination is now in progress.

Several metal chlorides are known to give the chromogenic reactions with steroidal compounds. Cholesterol in glacial acetic acid when treated with acetyl chloride and zinc chloride develops a bright red18) and other steroids such as corticoids,19) progesterone,19) estrogens20) can produce pigments when benzoyl chloride, phthalic acid anhydride21) or other acid anhy-

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drides[22] is employed instead of acetyl chloride. In the Pincus reaction, androsterone and many other 17-ketosteroids give a blue colour with antimony trichloride in acetic acid.[23] While this chloride can produce colour with non-steroidal compounds such as triterpenes, pyrrole, thiophene, furan and their derivatives and its chloroform solution is the Carr-Price reagent for vitamin A,[24] various steroids such as cardenolides, hormones, and sapogenins are also known to give colour reactions with antimony trichloride. Although stannic chloride in thionyl chloride is a color reagent for various triterpenes[25] and arsenic trichloride solution can be used as the modified Carr-price reagent,[26] no report is known at present of their application for steroid analysis. As for testosterone (I) derivatives, the sulphate and 17-methyl homologue were found to produce colour with antimony pentachloride in the mixture of acetic acid and acetic anhydride[27] and antimony trichloride in nitrobenzene,[28] respectively. The colorimetric method employing antimony trichloride was presented by Muller[29] for the determination of I.

The absorption spectra were taken of the reaction mixtures of I with each of $SbCl_3$, $SbCl_5$, $ZnCl_2$, and $AlCl_3$, reagents as described in the experimental part and are shown in Fig. 3. The extracts from the respective reaction mixture were then treated with the dichloromethane solution saturated with 70% $HClO_4$ as described above and the absorption spectra thus obtained are also shown in Fig. 3. No dienones, II and III, were noticed in this case as similarly indicated in GLC of the respective extracts (Fig. 2). Since the presence of intact substrate (t_R=about 12 min) and the formation of its acetate (t_R=about 16 min) were noticeable in GLC, the reactions of I with these Lewis acids seemed to proceed incompletely; I is negative for the characteristic bathochromic absorption (λ_{max}=593 nm) in the second reactions with perchloric acid (Fig. 1-B). The chromogenic reactions of I with Brønsted acids are thus considerably different from those with Lewis acids where no intermediary dienone, II or III, was produced at least in the conditions examined.

As for the bathochromic maximum absorption at the region of 550—650 nm shown in Fig. 1, it has been designated for all of the colorimetric measurements using sulphuric acid examined[9–13] and was given also by the dienones, II and III, as mentioned above. It is of particular interest as shown in Fig. 4 that these dienones can, in contrast to I, give such bathochromic absorption also with antimony pentachloride in dichloromethane, which was remarkably intensified by bromine molecules as has been shown in the second stage of the Kägi-Miescher reaction.[5] A sulphuric acid solution of methyltestosterone (17β-hydroxy-17α-methylandrost-4-en-3-one) shows visible light absorption when heated and aluminium chloride (a green colour) or ferric chloride (λ_{max}=430 nm) is added after cooling.[28] From the colour reaction mixture of cholesterol with anisaldehyde and aluminium chloride, the highly unsaturated condensation product having benzal function at C-4 was isolated and the colour development was explained to be due to a coupling of the empty orbital of aluminium chloride with the terminal carbanions in the conjugated system of this intermediate.[30] While 1,1-diphenyl-4-methylpentene-(1) was slow in reacting with $SbCl_3$ in the mixture of chloroform and acetic acid, 1,1-diphenyl-4-methylpentadiene-(1,3) was so sensitive as to give a cherry-red colour immediately, though entirely negative when the terminal methyl group was replaced

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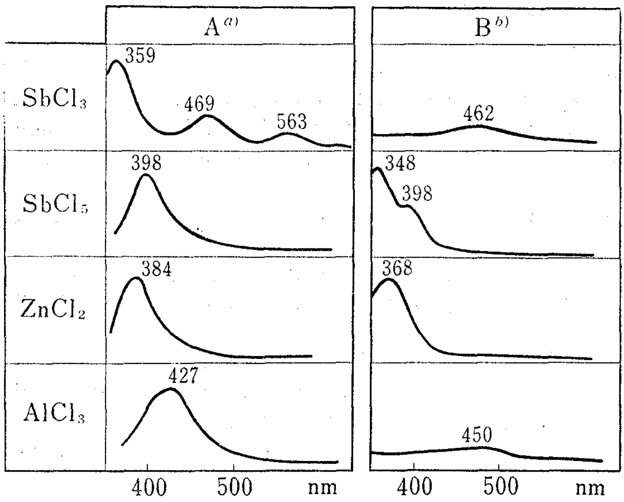

Fig. 3. Absorption Spectra due to the Reactions of Testosterone (I) with Lewis Acids

a) spectrum due to the reaction of I with Lewis acid
b) spectrum due to the reaction of dichloromethane-$HClO_4$ reagent with the residue obtained from the corresponding reaction mixture in A

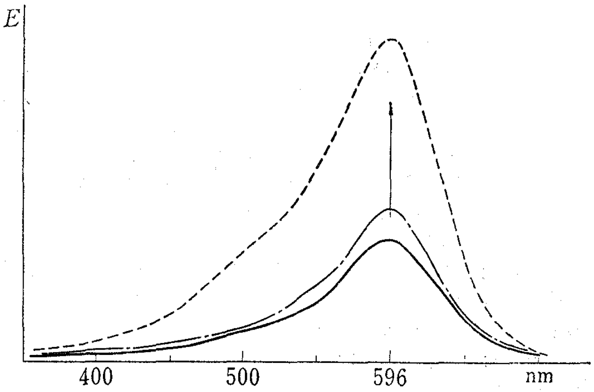

Fig. 4. Absorption Spectra due to the Reaction of Dienones (II and III) with Antimony Pentachloride in Dichloromethane

dienone: 294 μg, $SbCl_5$: 11.6 mg, CH_2Cl_2: 5 ml, at room temperature
1. without Br_2
———: 28 min (ε′: 1350) ———: 40 min (ε′: 1760)
2. added one drop of 1% Br_2–AcOH at 42 min after the reaction
- - - - -: 45 min (ε′: 3650)

by steroidal moiety.[31] The colour reactions (λ_{max}=510—520 nm) with $SbCl_3$ of the steroids having conjugated system such as ergosterol (ergosta-5,7,22-trien-3β-ol) and 7-dehydrocholesterol (cholesta-5,7-dien-3β-ol) which were treated in advance with peroxide,[32] were assumed to be due to the formation of 1:1 complex of ($SbCl_5^-$) with the positively charged intermediate.[33] Although hexachloroantimonate salts of several stable carbonium ions have been reported to be prepared by the reaction of $SbCl_5$ with certain aromatic hydrocarbons,[34] the ability of this Lewis acid to abstract hydride ion may find its explanation in traces of proton impurities in the reaction medium.[35] Charge transfer reaction has, on the other hand, been known to occur between tetracene and $SbCl_5$ in nitrobenzene.[36] These varieties of reported fact may, at least, suggest that highly conjugated system is necessary for the bathochromic absorption also in this kind of chromogenic reaction. In the present study, it was noteworthy in this respect that the colour development of the dienone, II or III, with antimony pentachloride mentioned above was rather slow, when no oxidizing agent such as bromine molecules is present (Fig. 4). Some oxidation processes involving hydride abstraction may, therefore, be assumed in this chromogenic reaction. In order to obtain information on the chemical species responsible for this characteristic light absorption, further investigations are now in progress.

Experimental

Testosterone (I) was obtained from the commercial sources and purified in an usual manner, mp 155°. The dienones, II and III, were prepared as has been reported.[4] Absorption spectral measurements were run on Hitachi Model 3T recording spectrometer. TLC was carried out on silica gel (Wakogel B-5 or B-5-F) plate by the solvent system of AcOEt–cyclohexane (1:1 or 2:1) and the spots were stained by spraying H_2SO_4 and heating on a hot-plate. GLC was run on Shimadzu Model GC-4A gas chromatograph at 200°

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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.

Colour Reaction with Brønsted Acid (Fig. 1-A)——a)[9]: Chloroform solution (1 ml) of I (10 mg) was added to the reagent (2 ml)—70% H_2SO_4 containing 0.5% iron alum was made up by mixing 7 volumes of conc. H_2SO_4 with 1 volume of water and 2 volumes of 2.5% iron alum solution-in a tube. The mixture obtained was gently heated in a sand-bath reaching a temperature of 60—70° after 10 min. After appropriate dilution with 60% H_2SO_4, was taken absorption spectrum.

The role of the ferric ion was assumed that of an oxidizing agent, since ferrous ion can not replace it in the production of a blue-green colour.[9]

b)[10]: I (10 mg) was dissolved in the reagent (2 ml)—2,4-dinitrophenol (0.25 g) in conc. H_2SO_4 (50 ml)—and the solution was heated in a water-bath for 1 min. After cooling the solution, was added water (4 ml) to it and then heated again at 100° for 5 min.

c)[11]: I (10 mg) was added to conc. H_2SO_4 (2 ml) with stirring and the mixture was heated in a water-bath for 2 min without stirring. The reaction mixture was then cooled in an ice-water for 5 min, saturated aqueous guaiacol sulphonats (2 ml) and 1% aqueous $CuSO_4$ (0.3 ml) were added with stirring, and re-heated at 100° for 2 min; during this period the solution was stirred 3 times. After re-cooled in the ice-water, the reaction mixture was diluted to 10 ml mark with 50% H_2SO_4.

d)[12]: Mohr's Salt Reagent: To an aqueous solution (20 ml) of Mohr's salt (1 g) was added conc. H_2SO_4 (1 ml), then 30% H_2O_2 (1 ml), and the mixture was boiled for 5 min. After cooling and adding water (50 ml), 3 ml of the solution thus obtained was made up to 100 ml with conc. H_2SO_4.

A mixture of this reagent (4 ml) and I (several μg) was heated in a water-bath for 4 min, then cooled in an ice-water for 2 min, and water (2 ml) was added gently to it. The solution thus obtained was shaken vigorously for 15 sec.

e)[13]: A mixture of I (several μg) and 70% (v/v) H_2SO_4 (1 ml) was heated in a water-bath for 4 min with occasional stirring and was then cooled in a running water. After the solution was mixed with 0.1% (w/v) solution (1 ml) of $FeCl_3$ in 3% (v/v) aqueous. AcOH and stood at room temperature for 5 min, it was diluted with 50% (v/v) H_2SO_4 (5 ml).

f)[14]: I (10 mg) was dissolved in redistilled absolute EtOH (0.2 ml) and incubated for 10 min at 80° with 85% H_3PO_4 (5 ml).

g)[15]: I (several μg) was dissolved in 10% (w/v) solution of vanillin in glacial acetic acid (0.1 ml) and 72% $HClO_4$ (0.2 ml) was then added. The mixture was stirred well and incubated in a water-bath at 70° for 15 min. After cooling in a running water, glacial acetic acid (10 ml) was added to the reaction mixture.

Colour Reaction with Lewis Acid (Fig. 3-A)——a) ($SbCl_3$)[29]: To a $CHCl_3$ solution (1 ml) of I (10 mg) was added $SbCl_3$ reagent (10 ml)—AcCl (1 ml) was added to the filtrate of the mixture of $CHCl_3$ (50 ml) and $SbCl_3$ (9 g)—and the mixture was stirred well at 20°.

b) ($SbCl_5$)[27]: To the $SbCl_5$ reagent ($SbCl_5$: gl. AcOH: Ac_2O=1: 5: 2) (5 ml) was added I (10 mg) and the solution was stood at 24° for 7 min.

c) ($ZnCl_2$)[18c]: To a $CHCl_3$ solution (1 ml) of I (10 mg) was added the $ZnCl_2$ reagent (2 ml)—AcCl (1 ml) was mixed with 20% gl. AcOH solution (2 ml) of $ZnCl_2$—and the mixture was heated at 65° for 6 min. After the reaction mixture was cooled in a running water, it was made up to 10 ml with $CHCl_3$.

d) ($AlCl_3$)[37]: To a nitromethane solution (5 ml) of I (10 mg) was added the $AlCl_3$ reagent—$AlCl_3$ (4 g) was dissolved in CH_3NO_2 (5 ml)—and the mixture was incubated at 80° for 10 min.

Pretreatment of the Samples for TLC, GLC, and Spectroscopic Inspection with Perchloric Acid——The respective reaction mixture of I with Brønsted acid was diluted with water, neutralized with alkali, and extracted with $CHCl_3$. As for the reaction mixture with Lewis acid, organic solvent was evaporated *in vacuo* to the resinous residue, to which was then added appropriate amounts of water and $CHCl_3$ for extracting the products. The organic layers thus obtained were washed with water, dried over anhyd. Na_2SO_4, and evaporated to dryness *in vacuo* to afford yellow-brown oil. These oily samples were submitted to TLC as well as GLC (Fig. 2) and examined with $HClO_4$ in dichloromethane (Fig. 1- and 3-B).

Colour Development in the Dichloromethane Solution Saturated with 70% Perchloric Acid (Fig. 1- and 3-B)——The mixture of dichloromethane (10 ml) and 70% $HClO_4$ (2 ml) was shaken and stood at room temperature for 10 min. The organic layer involving 0.024% (w/v) of $HClO_4$ was employed for the colour development. This system is highly advantageous in that the samples to be inspected are readily soluble, the conditions are milder, the reaction is faster—instantaneous in most cases—, and that the results are favourably reproducible.

A few milligrammes of the sample was dissolved in this dichloromethane-$HClO_4$ reagent (5 ml) at room temperature and the absorption spectrum was observed after several minutes.

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