The extract was washed with 5% HCl, H_2O, dried over Na_2SO_4 and evaporated to give 150 mg. of prisms. The combined prisms were recrystallized from MeOH to obtain 580 mg. of acetate (XI), m.p. 202~203°. IR : $\nu_{\max}^{\text{KBr}}$ 1648 cm^{-1} ($NCOCH_3$). *Anal.* Calcd. for $C_{27}H_{37}ON$: C, 82.81; H, 9.61; N, 3.58. Found : C,82.57; H, 9.52; N, 3.44.

The N-Acetyl Carboxylic Ester (XII) from the N-Acetyl Styrene Derivative (XI)——To a solution of 540 mg. of the styrene derivative (XI) and 100 mg. of K_2CO_3 in 200 ml. of dioxane and 80 ml. of H_2O was added a solution of 2.1 g. of $NaIO_4$ and 50 mg. of $KMnO_4$ in 80 ml. of dioxane and 80 ml. of H_2O. The reaction mixture was stirred for 20 hr. at room temperature. After the excess oxidation reagent was decomposed with $NaHSO_3$, the solvent was concentrated in reduced pressure. The residue was poured into H_2O, extracted with Et_2O. The ethereal extract was dried over Na_2SO_4 and evaporated to give 550 mg. of an amorphous product.

The nonpurified carboxylic acid was dissolved in 50 ml. of Et_2O and a solution of CH_2N_2 in 20 ml. of Et_2O, prepared from 2 ml. of nitrosomethylurethane, was gradually added to the solution under ice-cooling. The reaction mixture was allowed to stand overnight and the excess CH_2N_2 and solvent were concentrated to dryness. The residual oil was chromatographed on alumina (grade I, 30 g.). Benzene elutes gave 80 mg. of methyl benzoate, and Et_2O elutes gave 430 mg. of N-acetyl carboxylic ester (XII) which was recrystallized from Me_2CO to yield 300 mg. of colorless prisms, m.p. 158~159°. IR $\nu_{\max}^{\text{CHCl}_3}$ cm^{-1} : 1715 (CO_2CH_3), 1612 ($NCOCH_3$). *Anal.* Calcd. for $C_{21}H_{33}O_3N$: C, 72.58; H, 9.57; N, 4.03. Found : C, 72.85; H, 9.55; N, 3.59.

We thank Mr. M. Matsui, the Director of this Laboratory for his encouragement and Dr. S. W. Pelletier, Professor of the University of Georgia for kindly sending us the infrared spectra of the degradation product of atisine and veatchine and for his valuable discussion.

Summary

The tetracyclic anisole compound (I) was converted into the N-acetyl carboxylic ester (XII). The infrared spectrum of the latter was coincided with that of a degradation product of atisine and veatchine.

(Received February 25, 1964)

[Chem. Pharm. Bull. 12 (7) 824 ~ 827] *UDC 543.41[547.92]-4*

118. **Kazukichi Kato** : A New Color Reaction of Steroid with Anhydrous Aluminum Chloride and Anisaldehyde. III.*1 A Method for the Colorimetric Determination of Allylestrenol.

(*Shinagawa Factory, Sankyo Co., Ltd.**2)

In the first paper of this series,[1] a colorimetric determination of ethylestrenol(17α-ethyl-17β-hydroxyestr-4-ene) with anhydrous aluminum chloride and anisaldehyde was reported. Since several derivatives of estrenol are used for various therapeutic purposes, a method of assay which is selective and generally applicable to them is urged to be established.

The present work deals with a method for the colorimetric determination of allylestrenol(17α-allyl-17β-hydroxyestr-4-ene) which is used as a pure gestagen. There was no report on the assay of this steroid, and for lack of the selective color reaction the

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*1 Part II : This Bulletin, **12**, 582 (1964).

*2 Nishi-shinagawa, Shinagawa-ku, Tokyo (加藤寿吉).

1) K. Kato : This Bulletin, **12**, 578 (1964).

determination of phenolic steroids has been performed by the usual color reaction with concentrated sulfuric acid.[2] While the new color reaction mentioned above was expected to be applicable here, some modifications of procedure were anticipated to be necessary, because allylestrenol has a double bond in the side chain in addition to another one in the ring A. The results from an investigation on the selectivity of this color reaction revealed that a double bond in a steroidal molecule may be responsible for the development of color, as described in the second paper of this series.

Experimental

Material——Allylestrenol : Allylestrenol (N. V. Organon, Holland) was dried at 30° over P_2O_5 under reduced pressure (5 mm. Hg) to constant weight, m.p. 81°. *Anal.* Calcd. for $C_{21}H_{32}O$: C, 84.00; H, 10.67. Found : C, 83.80; H, 10.74.

Sample Solution——An accurately weighed quantity of allylestrenol was dissolved in $CHCl_3$ to prepare a sample solution. Unless otherwise described, the sample solutions in the following experiments contained 50 μg. of allylestrenol per 1 ml.

The test solutions were prepared according to the same procedure, and the measurements were carried out with the same apparatus, as described in the preceding paper.

Procedure——Take 1 ml. of the sample solution containing 20~100 μg. of allylestrenol in a 10 ml. volumetric flask, add 1 ml. of 6.6% anhyd. $AlCl_3$ solution to it, and shake thoroughly. Add 3 ml. of 4.6% anisaldehyde solution, shake and mix. Stopper the flask, and allow to stand at 25° for 15 min. Then add sufficient amount of nitrobenzene-benzene (1:1) solution to make exactly 10 ml. Determine the absorption of the solution at a wave length of 590 mμ within 10 min. As a blank, the solution is used which is prepared by treating 1 ml. of $CHCl_3$ in the same manner. A calibration curve is prepared from a series of the standard solutions containing 10~120 μg. of allylestrenol per 1 ml. respectively by the above procedure.

Results

The colored solution obtained from 1 ml. of the sample solution by this procedure gave an absorption spectrum, as shown in Fig. 1, and absorption maximum existed at 590 mμ. The absorbance of the blank was about 0.05 at this wave length.

An example of the calibration curve is shown in Fig. 2.

In order to establish the procedure for determination, reaction conditions and stability of the produced color were examined as follows.

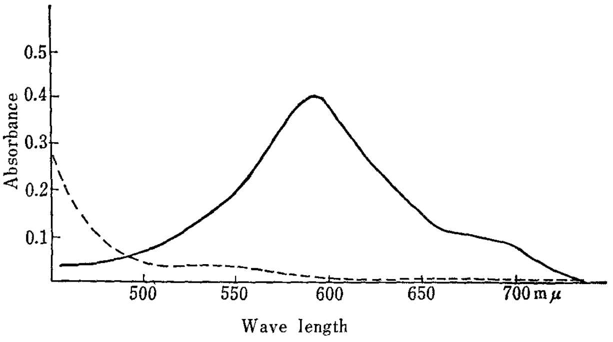

Fig. 1. Absorption Spectrum of Colored Solution

----- Blank solution
——— Colored solution obtained from 50 μg. of allylestrenol

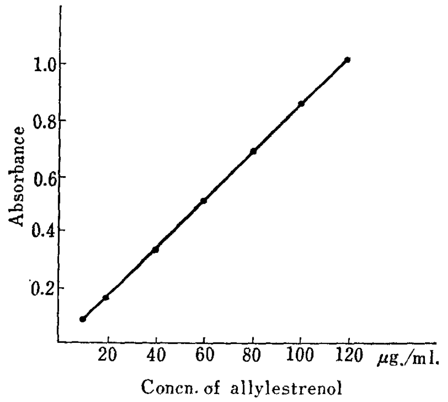

Fig. 2. Calibration Curve

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[2] S. Bernstein, R. H. Lenhard : J. Org. Chem., 18, 1146 (1953).

Effect of the Reaction Time and Temperature

Results were obtained as shown in Table I, when the reaction time and temperature were varied, while other conditions were left as described in this procedure. In the range of 20~30° of the reaction temperature and of 10~30 minutes of the reaction time, absorbance of the colored solution was constant. Therefore, 25° and 15 minutes were chosen for the determination.

Table I. Effect of Reaction Time and Temperature on Absorbance of Colored Solution

Reaction time (min.) Reaction temp. (°C)	5	10	15	20	25	30
20	0.380	0.409	0.410	0.414	0.415	0.409
25	0.408	0.415	0.413	0.409	0.412	0.413
30	0.410	0.410	0.410	0.410	0.410	0.413
35	0.396	0.395	0.389	0.351	0.329	0.325

Effect of the Concentration of the Reagents

The concentrations of reagents adopted for the determination of ethylestrenol could not be applied in this case, because the color produced therewith from allylestrenol tended to change rapidly. Transition of the absorption curve is given in Fig. 3. As the quantity of anisaldehyde increased, color produced was stabilized for a while with improving sensitivity (Fig. 4). In order to minimize error, however, 3 ml. of anisaldehyde solution was used here, instead of 1 ml. of a higher concentration.

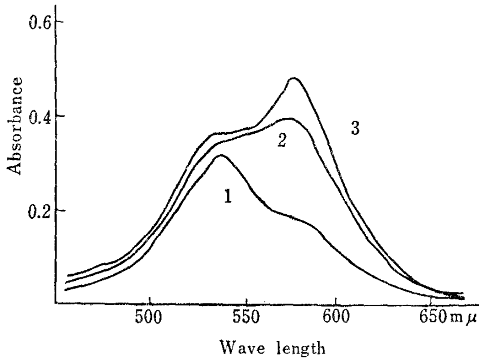

Fig. 3. Transition of Absorption Spectrum of Colored Solution obtained from 80 μg. of Allylestrenol using 1 ml. of 4.6% Anisaldehyde Solution

1: Just after addition of anisaldehyde solution
2: 5 min. after addition
3: 10 min. after addition

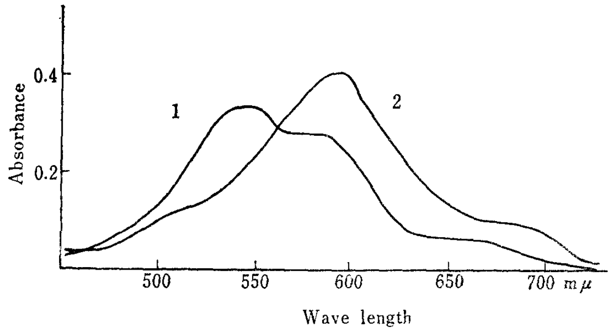

Fig. 4. Transition of Absorption Spectrum of Colored Solution obtained from 50 μg. of Allylestrenol using 3 ml. of 4.6% Anisaldehyde Solution

1: Just after addition of anisaldehyde solution
2: 5 min. after addition

Table II. Effect of Concentration of Anhydrous Aluminum Chloride Solution and of Anisaldehyde Solution on Absorbance of Colored Solution

Anisaldehyde (%) $AlCl_3$ (%)	4.0	4.2	4.4	4.6	4.8	5.0
6.0	0.334	0.334	0.338	0.342	0.341	0.351
6.2	0.409	0.401	0.401	0.398	0.388	0.376
6.4	0.415	0.411	0.409	0.409	0.412	0.412
6.6	0.411	0.412	0.409	0.415	0.409	0.412
6.8	0.415	0.412	0.412	0.415	0.412	0.412
7.0	0.411	0.409	0.409	0.409	0.409	0.410

Results were obtained as shown in Table II, when the concentrations of both reagents were varied, while other conditions were left as described in this procedure. In the range of 6.4~7.0% of anhyd. $AlCl_3$ solution and of 4.0~5.0% of anisaldehyde solution, absorbance of the colored solution was found to be constant. Therefore, 1 ml. of 6.6% anhyd. $AlCl_3$ solution and 3 ml. of 4.6% anisaldehyde solution were adopted for the determination.

Stability of the Produced Color

When the colored solution was allowed to stand for 30 minutes after diluting with nitrobenzene-benzene (1:1) solution, results were obtained as shown in Table III. Absorbance of the colored solution was constant within 15 minute.

Table III. Stability of Colored Solution

Elapsed time after dilution (min.)	0	5	10	15	20	25	30
Absorbance	0.410	0.410	0.408	0.408	0.400	0.395	0.391

Accuracy of the Method

σ was 0.96% ($n=6$), calculated on the data which were obtained from a sample solution containing 50 μg. of allylestrenol per 1 ml., as shown in Table IV.

Table IV. Absorbances of Colored Solution obtained from 50 μg. of Allylestrenol

	1	2	3	4	5	6	$\bar{x}$
Absorbance	0.413	0.408	0.415	0.413	0.410	0.418	0.413

The author expresses his deep gratitude to Professor Z. Tamura and Assist. Professor T. Nambara, Faculty of Pharmaceutical Sciences, University of Tokyo, for their kind guidance, and Mr. J. Nakahira, Director of this Factory, Dr. H. Negoro and Dr. H. Sakurai for their constant encouragement.

Thanks are also due to the members of the Elementary Analysis Laboratory, Sankyo Research Laboratories.

Summary

A method for the colorimetric determination of allylestrenol with anhydrous aluminum chloride and anisaldehyde is proposed. The procedure for the determination of ethylestrenol has been considerably modified for application to this steroid which has another double bond in the side chain. This fact is consistent with the previous observation that a double bond in the steroidal molecule may be a fundamental factor for the coloration.

(Received March 7, 1964)