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Altenin. I. The Isolation of Altenin from *Alternaria Kikuchiana* Tanaka

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Alternaria Kikuchiana Tanaka is a fungus which produces a black spot disease in pears. The metabolite of this fungus contains a phytopathogenic substance for which the name "Altenin" has been proposed. A drop of an aqueous solution of altenin (2×10^{-5} mg./ml.) produces a black spot on pear leaves. The stability of altenin at 80, 60, and 40°C, and the behavior of altenin in relation to some adsorbents and solvents, were studied. Its isolation from the culture filtrate was found to be properly accomplished by adsorption with active charcoal, elution with acetone, and chromatography with silica gel and alumina columns. By these procedures, besides altenin, six metabolites, diheptyl phthalate, myristic acid, succinic acid, and the ester of C_{15}-carboxylic acid, C_{19}-steroid and trihydroxy anthraquinone, were obtained.

Alternaria Kikuchiana Tanaka is a fungus which is phytopathogenic to pears especially to the improved sort of *Pyrus Ussuriensis var. Aromatica* Rehder (Japanese name *Nijusseiki*).[1] On the leaves and fruits of the pears, it produces a black spot disease.[2] When the culture broth of this fungus, or ethyl ether or an ethyl acetate extract of the culture, is put on the plucked leaves of the pear, a similar black spot grows along the veins of the leaf. These phenomena suggest that there is some pathogenic substance in the metabolite of this fungus.[3] This substance was called "Altenin."[4] This paper will deal with the isolation of altenin and with some of its chemical properties.

Results and Discussion

As the first step, the stability of altenin was examined.[3] Its aqueous solution (pH 7.5) lost its pathogenic activity in 10 min. at 80°C, and in an hour at 60°C, but kept it for several hours at 40°C, as is shown in Table I. When the acetone solution with silica gel or alumina was left standing at room temperature, no change in activity was observed over a period of several hours. Further, the activities of the solutions in organic solvents, such as benzene, acetone, ethyl ether, methanol, ethanol or ethyl acetate, did not change at room temperature over a period of several hours.

Table I. The stability of altenin in aqueous solution (pH 7.5)

Time min.	40°C	60°C	80°C
0	18.6	18.6	7.4 (mm²)
5	17.8	19.8	2.3
10	19.2	21.2	0.5
20	9.1	11.0	0
40	12.7	4.8	0
80	17.9	3.2	0

The isolation and purification of altenin was attemped by two routes. In the first, it was extracted with ethyl acetate from the culture filtrate and then purified through silica gel-column chromatography. By this procedure, besides the active substance, five inactive metabolites, diheptyl phthalate, myristic acid, the ester of C_{15}-carboxylic acid, C_{19}-steroid and trihydroxy anthraquinone were obtained.

In the second route, the adsoption, extraction and chromatography were employed. Several adsorbents and eluting solvents were tested. The

Table II. Adsorption power of adsorbents

Adsorbent (g./l.)	Time hr.	Activity, % Remained in solution	Adsorbed on adsorbent
Calcium carbonate (40)	1	55	45
Animal charcoal (10)	1	22	78
Hyflosupercel (20)	1	23	77
Active charcoal (10)	1	20	80
Active charcoal (20)	1	1	99
Active charcoal (20)	5	0	100

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2) R. Mohri, *The Liberal Arts Journal of Natural Science of Tottori University* (*Tottori Daigaku Gakugei Gakubu Kenkyu Hokoku*), **13**, 55 (1962).
3) I. Hiroe, S. Nishimura and M. Sato, *Transactions of Tottori Society of Agriculture Science* (*Tottori Nogaku Kaiho*), **11**, 291 (1958).
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TABLE III. ELUTION POWER OF SOLVENTS

Adsorbent (g./l.)		Elution (%) by solvent				
		Methanol	Ethanol	Acetone	Ethyl ether	Benzene
Calcium carbonate	40	2	1.6	2.5	1	0.1
Animal charcoal	10	0.4	0.4	0.2	0.1	0.2
Hyflosupercel	20	17	16	22	2	6
Lead acetate	40	1.2	1.6	6.2	1.6	2.8
Active charcoal	10	23	40	40	28	6

The yield of pathogenic substance through the adsorption for 1 hr. and elution for 1 hr.

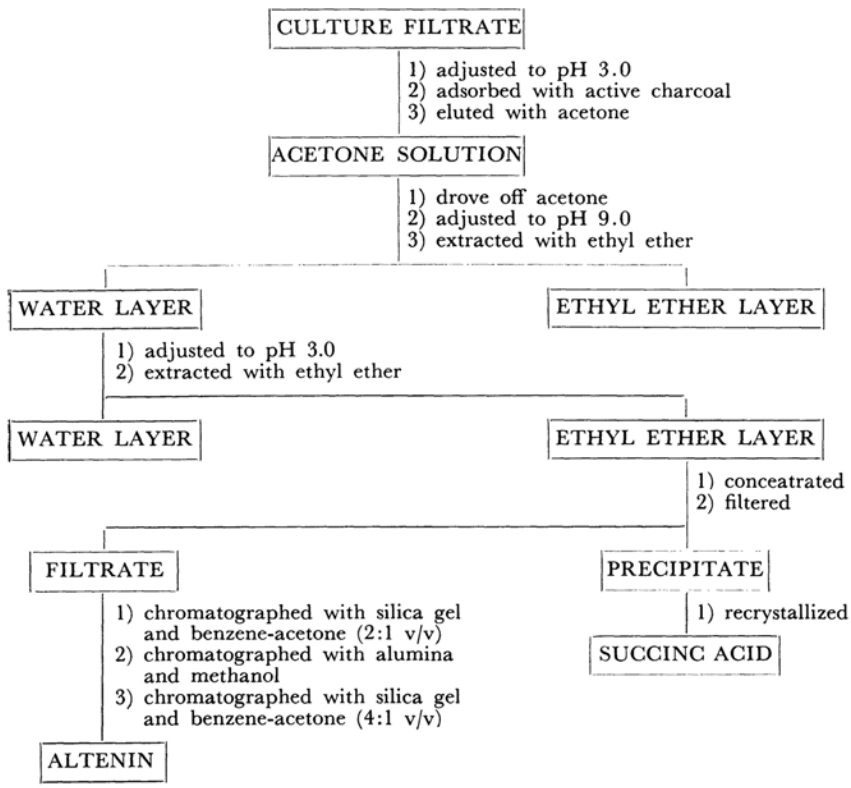

Fig. 1. Isolation of altenin.

results of these experiments are shown in Tables II and III. It was found that the most effective adsorbent was the active charcoal; from it acetone could elute altenin most effectively. By this procedure, besides altenin, succinic acid was obtained.

Altenin was also extracted with ethyl ether from the culture filtrate, which had been adjusted to pH 3 with hydrochloric acid. The distribution coefficients, K, of altenin at 20°C between ethyl ether and water were measured by the counter-current distribution method; they were found to be as follows: >10 (pH 3.5), 5 (pH 5.0), 2 (pH 5.5), 1 (pH 6.0) and <0.1 (pH 9.0).

By applying these results, altenin was isolated by the process illutsrated schematically in Fig. 1. One milligram of altenin was obtained from about 4 l. of the culture filtrate. One drop of an aquoeus solution of altenin (2×10^{-5} mg./ml.) exhibited a distinct phytopathogenic effect.

TABLE IV. THE DISTRIBUTION COEFFICIENT, K, OF ALTENIN AT 20°C

pH	3.5	5.0	5.5	6.0	9.0
Tube No.*	10	9	7	5	1
K	10	5	2	1	0.1

* The tube number of maximum activity.

Altenin is a yellow liquid which gives only one spot on silica-gel thin-layer chromatography with a benzene-acetone (1 : 1 v/v) mixture; its R_f value was 0.76, while that of methyl red, a pilot dye, was 0.37. On alumina thin-layer chromatography with a methanol-water (5 : 1 v/v) mixture, its R_f value was 0.87, while that of methyl red was 0.41. The spot of the thin-layer chromatogram was developed by heating it after it had been

sprayed with concentrated sulfuric acid or with a *o*-dianisidine solution. By this method, it was demonstrated that altenin was the unique phytopathologically active substance in the metabolites of the fungus *Alternaria Kikuchiana* Tanaka.

The details of the cultivation of this fungus and the chemical studies of altenin will be reported on in the near future.

Experimental

The Cultivation of *Alternaria Kikuchiana* Tanaka (No. 5).—Though this fungus had previously been cultivated in stationary cultivation,[5] altenin enough for chemical study could not be obtained by this method. After many trial experiments, the following method was found to be effective.

A 200-l. tank fermenter was employed; it was charged with 120 l. of an aqueous solution of 4% sucrose, 0.2% dibasic ammonium phosphate, 1.1% monobasic potassium phosphate, 0.05% magnesium sulfate and 0.05% potassium chloride.

During cultivation, rotary stirring was effected, the temperature was maintained at 30°C, and air (20 l./min.) was bubbled in continuously. On the third day, a small quantity of silicon oil was added as an antifoam agent. On the ninth day, the culture broth exhibited a pH value of 3.0 and the maximum altenin activity. At this point the cultivation was stopped.

The Black-spots Test.—Fresh, young leaves of *Pyrus Ussuriensis var. Aromatica* Rehder (Japanes name *Nijusseiki*) were employed for this test. When an ethyl ether extract of the culture filtrate (270 ml.) was concentrated under a diminished pressure, a residue (85 mg.) was obtained. This residue was dissolved in an ammonium acetate-buffered (pH 8.0) solution, the concentrations of which were 85, 17, 3.4 and 0.7 mg./ml. A drop of each of these four solutions was put on the pear leaf. After 17 hr. at 23°C, the areas of the black spots were measured (Fig. 2).

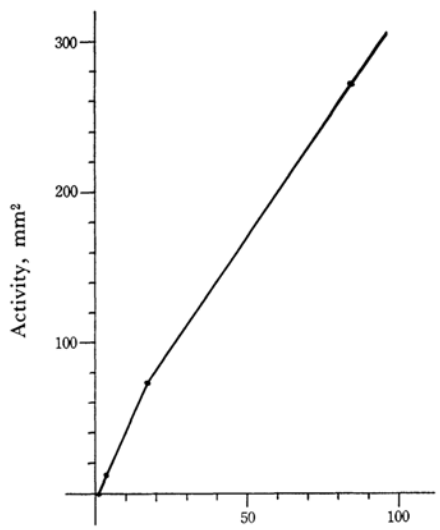

Fig. 2.

The Stability of Altenin.—The culture filtrate was heated to 40, 60, and 80°C for 80 min. and then the change in altenin activity was measured by the black spots test. At 40°C, the activity was observed for several hours. At 60°C, however the activity was lost in 40 min. and at 80°C in 10 min. The results are listed in Table I. However, altenin was stable for 2 days at room temperature, even when air was passed through the altenin aqueous solution.

The Isolation of Altenin (Route 1.)—The culture filtrate (130 l.) was extracted with ethyl acetate (13 l.). After the ethyl acetate had been removed under reduced pressure, the residue (40 g.) was passed through the silica gel (Wakogel C-2) column with benzene (fraction A) and with acetone (fraction B). After the evaporation of the benzene, the residue of fraction A (12.3 g.) was passed through the silica gel (Wakogel C-2) column with a benzene-acetone (20 : 1 v/v) mixture (fraction C) and with acetone (fraction D). The evaporation residue from the fraction C was chromatographed with silica gel (Merck 7729) and with a benzene-acetone (2 : 1 v/v) mixture; a yellow fluorescent elution contained 7 mg. of crude altenin, which showed spots with R_f values of 0.60, 0.70 and 0.78 on silica-gel (Wakogel B-5) thin-layer chromatography with a benzene-acetone (1 : 1 v/v) mixture.

During the above mentioned procedures, the following five metabolites were isolated.

Diheptyl Phthalate.—The residue from the fraction D was chromatographed with silica gel (Wakogel C-2) and with a benzene-acetone (1 : 1 v/v) mixture. A colorless liquid was thus obtained, b. p. 332—337°C.

Found: C, 72.89; H, 9.45; mol. wt. (Rast), 360. Calcd. for $C_{22}H_{34}O_4$: C, 73.23; H, 8.95%; mol. wt., 362.5.

From the infrared absorption spectrum, this substance was presumed to be an ester of some aromatic carboxylic acid. Liquid-gas chromatography suggested that the hydrolysate of this substance consisted of one alcohol and one aromatic acid. This acid was sublimed to a crystalline substance, m. p. 129°C, which was identified with phthalic anhydride. From these results alcohol was proved to be a heptanol. Therefore, the original substance was diheptyl phthalate.

Myristic Acid.—The fraction C was chromatographed through the silica gel (Merck 7729) column with a benzene-acetone (2 : 1 v/v) mixture. The solid substance which remained after the solvent had been removed from the first elution was recrystallized from aqueous acetone, m. p. 51.5°C; its infrared absorption spectrum showed absorptions at 1700 ($\nu_{C=O}$), 1290 (δ_{C-O}) and 940 cm^{-1} (δ_{C-O}). This substance with myristic acid.

An Ester of C_{15}-Carboxylic Acid.—By the chromatography of the fraction C with silica gel (Merck 7729) and chloroform, a liquid metabolite was isolated. Its infrared absorption spectrum showed the ester carbonyl group at 1740 cm^{-1}. By hydrolysis, a free carboxylic acid, m. p. 50.5°C, was obtained. The reaction of this substance with benzyl amine produced the *N*-benzyl amide, m. p. 88.5°C.

Found: C, 79.64; H, 11.17; N, 3.83; mol. wt.

5) R. Mohri and S. Ikeda, *The Liberal Arts Journal of Natural Science of Tottori University (Tottori Daigaku Gakugei Gakubu Kenkyu Hokoku)*, **10**, 38 (1960).

(Rast), 330. Calcd. for $C_{22}H_{37}NO$: C, 79.70; H, 11.25; N, 4.23%; mol. wt., 331.5.

It was suggested that this metabolite was an ester of C_{15}-saturated carboxylic acid.

C_{19}-Steroid.—After chromatography with silica gel (Wakogel C-2) and with a benzene-acetone (20 : 1 v/v) mixture, fraction A gave a solid material, which was then recrystallized from aqueous methanol. Colorless needles (11 mg.; m. p. 180°C) were obtained from 1.2 l. of the culture filtrate.

Found: C, 78.64; H, 10.35; mol. wt. (Rast), 301. Calcd. for $C_{19}H_{30}O_2$: C, 78.57; H, 10.41%; mol. wt., 290.4.

Its infrared absorption spectrum showed absorptions at 3450 (νOH), 1630 ($\nu_{C=C}$), 2950 and 2850 cm^{-1} (νCH), while the ultraviolet absorption spectrum showed a maximum at 230 mμ (ε 690) in ethanol, and at 257 (ε 20000), 253 (ε 18000), 262 (ε 14000), 375 (ε 2000) and 522 mμ (ε 9600) in concentrated sulfuric acid.[6] The color reactions of this substance were as follows: Liebermann-Burchard's reaction,[7] green; Salkowski's reaction,[8] red; Rosenheim's reaction,[9] green; Kägi-Miescher's reaction,[10] violet. These color reactions suggested the presence of an unsaturated steroid. By acetylation with acetic anhydride and pyridine, a mono acetate, m. p. 201°C, was obtained.

Found: C, 75.70; H, 9.66. Calcd. for $C_{21}H_{32}O_3$: C, 75.86; H, 9.70%.

2, 8-Dihydroxy-1-hydroxymethyl-9, 10-anthraquinone.—The solvent of the blue fluorescent elution from the silica gel (Wakogel C-2) chromatography of the fraction A with a benzene-acetone (20 : 1 v/v) mixture was removed. When the residue was then sublimed, pale yellow needles, m. p. 269°C (decomp.), were obtained.

Found: C, 64.78; H, 4.60; mol. wt. (Rast), 230. Calcd. for $C_{15}H_{10}O_5$: C, 66.67; H, 3.73%; mol. wt., 270.2.

The infrared absorption spectrum showed absorptions at 3420, 3350, 1665, 1620, 1590, 980, 850, 830 and 740 cm^{-1},[11] while the ultraviolet absorption spectrum showed its maxima at 257 (ε 49700), 289 (ε 10400), 300 (ε 10200) and 340 mμ (ε 11500) in ethanol,[3,12,13] these values shifted in an alkaline solution to 265 (ε 37600), 314 (ε 11300) and 370 mμ (ε 16600), and a pK_a value of 9.6 was obtained by a spectrometric method. This substance showed a negative color reaction with zirconium nitrate. This substance was methylated with diazomethane to a methylated substance, m. p. 177°C. These data suggested that this substance was possibly 2, 8-dihydroxy-1-hydroxymethyl-9, 10-anthraquinone.

The Test of an Adsorbent and an Eluting Solvent.—To each 1 l. of the culture filtrate, adsorbents, calcium carbonate, animal charcoal, hyflosupercel and active charcoal, in the amounts shown in Table II, were added; they were then stirred for one hour at room temperature. After the adsorbent had been filtered off with a suction pump, the altenin activity of the filtrate was examined. The activity was then compared with that of the original culture filtrate. The results are listed in Table II. Each filtered adsorbent in the preceding experiments was air dried and divided into ten equal parts. Each of these parts was added to a test solvent (10 ml.) and stirred for one hour at room temperature. After the adsorbent had been filtered off, the solvent was evaporated off under reduced pressure. To each residue there was added 100 ml. of a buffer solution of ammonium acetate adjusted to pH 7.8. The altenin activity of each solution was tested on the leaves of the pear, and it was compared with the altenin activity of the original culture filtrate. The results are expressed in percentages in Table III. Because of its greater volatility and greater eluting power, acetone was the most suitable eluting solvent.

The Distribution Coefficient of Altenin.—The ethyl ether extract of the culture filtrate was subjected to the counter-current distribution at 20°C with ethyl ether and aqueous solutions of various pH values. For the sake of simplicity, only ten tubes were used. From the altenin activity of each layer, the distribution coefficient, K, was calculated to be as shown in Table IV.

The Isolation of Altenin (Route 2).—A process for the isolation of altenin from the culture filtrate is schematically showed in Fig. 1. The culture filtate (101.) was adjusted to pH 3.0; then active charcoal (100 g.) was added, and the mixture was stirred for five hours. The active charcoal was filtered, and on it the pathogenic substance was adsorbed. After it had been dried in the air, the active charcoal was eluted with acetone (1 l.), and the acetone solution was concentrated to ca. 200 ml. under reduced pressure at 35—40°C in a nitrogen atomsphere. The residual solution was adjusted to pH 9.0 with aqueous ammonia, and the dusty substance was filtered off. This alkaline solution was extracted with ethyl ether (100 ml.). The aqueous layer was adjusted to pH 3.0 with dilute hydrochloric acid, and extracted with three 70 ml. portions of ethyl ether. The combined ethyl ether extracts were then dried over anhydrous sodium sulfate. On the removal of the solvent under reduced pressure at 35—40°C, the residue (170 mg.) was obtained. It was dissolved in a cold mixture of benzene and acetone (2 : 1 v/v) and filtered. The precipitate was recrystallized from ethyl ether, m. p. 183°C, which was identified with succinic acid. The filtrate was passed through a silica-gel (Merck 7729) column with a benzene-acetone (2 : 1 v/v) mixture. The blue fluorescent elution which was obtained was again passed through an alumina (Wako alumina) column with methanol. (The alumina had been deactivated with 6.7 per cent of water before use.) The methanol elution was then again passed through a silica-gel (Merck 7729) column with a benzene-actone (4 : 1 v/v) mixture. Altenin (2.5 mg.) was thus obtained as a yellow liquid.

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Altenin showed a spot on silica-gel (Wakogel B-5) thin-layer chromatography with a benzene-acetone (1 : 1 v/v) mixture; the R_f value was 0.76, while that of methyl red, a pilot dye, was 0.37. On alumina (Merck 1090) thin-layer chromatography with a methanol-water (5 : 1 v/v) mixture, the R_f value was 0.87, while that of methyl red was 0.41. Altenin was developed on thin-layer chromatography by heating it over 80°C for one hour after it had been sprayed with concentrated sulfuric acid. Altenin was developed to yellow by heating it after it had been sprayed with an *o*-dianisidine solution.[14]

The Activity of Altenin.—The phytopathogenic activity of aqueous solutions of altenin with concentrations of 1×10^{-4}, 2×10^{-5}, 5×10^{-6} mg./ml. were examined by the black-spots test; it was found that the lower limit of the pathogenic activity was about 2×10^{-5} mg./ml.

The Test of the Pathogenic Activity of Other Metabolites.—The ethyl ether extract of the culture filtrate was developed by silica-gel (Wakogel B-5) thin-layer chromatography with a benzene-acetone (1 : 1 v/v) mixture; the chromatogram was divided into ten parts: R_f value, 0—0.1; 0.1—0.2; ··· ; 0.8—0.9; 0.9—1.0. Each part of the silica gel was eluted with acetone (5 ml.), and the elution was concentrated under reduced pressure. The residues obtained were each diluted to 0.1 ml. with an aqueous buffer solution of ammonium acetate (pH 7.4). The pathogenic activity of these solutions were then examined by the black-spots test. Only the solution from the R_f 0.5—0.6 division, which contained altenin, exhibited the black spot on a pear leaf.

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