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Biosynthesis of Natural Products. IV.[1] Biosynthesis of Itaconitin. (1). Degradation Studies on Itaconitin

Ushio Sankawa and Shoji Shibata

Faculty of Pharmaceutical Sciences, University of Tokyo[2]

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The degradation reaction to identify the individual carbon atoms of itaconitin were established as shown in Chart 1.

Aspergillus itaconicus Kinoshita was first reported as a fungus producing itaconic acid (I).[3] Thereafter some strains of this fungus were found to produce a yellow pigment, itaconitin, in a good yield along with itaconic acid (I).[4] The structure of itaconitin (II) was established by Nakajima as being a derivative of disubstituted maleic anhydride.[5] The biosynthesis of I was thoroughly investigated by Bentley, *et al.*,[6] and showed that I is derived from citric acid *via cis*-aconitic acid. The acid anhydride part of II reveals a remarkable structural resemblance to I. This fact and the unique structure of II promoted us to study the biogenetical relation of these two fungal metabolites. Although a number of degradation reactions have been described by Nakajima in his paper on structural studies,[5] some new investigations of the reactions were required for the biosynthetical experiment to identify the individual carbon atoms of II. This paper mainly deals with a work to find out suitable degradation reactions, and, in addition to this, some characteristic reactions of itaconitin (II) are also discussed.

Anhydroitaconitin (III:R=H) is a key compound in the degradation of II, because it is easily obtained in a high purity and retains all the carbon atoms which exist in II itself. III (R=H) is usually prepared *via* acetylanhydroitaconitin (III: R=Ac) which is formed by heating II in acetic anhydride. When itaconitin-^{14}C obtained by the feeding experiment of sodium acetate (1-^{14}C)[7] was heated in acetic anhydride for 5 min. under the presence of large amount of non-labelled III (R=H); recovered III (R=H) showed much higher specific activity than that of III (R=Ac) obtained from the same reaction mixture. This result indicates that III (R=H) was actually formed in the reaction mixture. According to this result, some attempts were made to obtain III (R=H) directly from II. However no

Table I

Itaconitin	Acetylanhydroitaconitin	Anhydroitaconitin
1.4×10^5 dpm/mM	1.4×10^4dpm/mM	5.4×10^4 dpm/mM

1) Part III: S. Shibata, U. Sankawa, H. Taguchi and K. Yamasaki, *Chem. Pharm. Bull.* (Tokyo), **14**, 474 (1966).
2) Location: *Hongo, Bunkyo-ku, Tokyo.*
3) K. Kinoshita, *Acta Phytochemica*, **5**, 271 (1931).
4) K. Kinoshita, *Shigen Kagaku Kenkyusho Iho*, **17—18**, 78 (1950).
5) *a*) K. Kinoshita and S. Nakajima, *Chem. Pharm. Bull.* (Tokyo), **6**, 31 (1958); *b*) S. Nakajima, *Chem. Pharm. Bull.* (Tokyo), **8**, 56 (1960); *c*) *Ibid.*, **8**, 1051 (1960); *d*) S. Nakajima, K. Kinoshita and S. Shibata, *Chem. Pharm. Bull.* (Tokyo), **13**, 58 (1965); *e*) S. Nakajima, *Chem. Pharm. Bull.* (Tokyo), **13**, 64, 69, and 73 (1965).
6) R. Bentley and C.P. Thiessen, *J. Biol. Chem.*, **226**, 673, 689 and 703 (1957).
7) *cf.* U. Sankawa and S. Shibata, *Chem. Pharm. Bull.* (Tokyo), **17**, 2025 (1969).

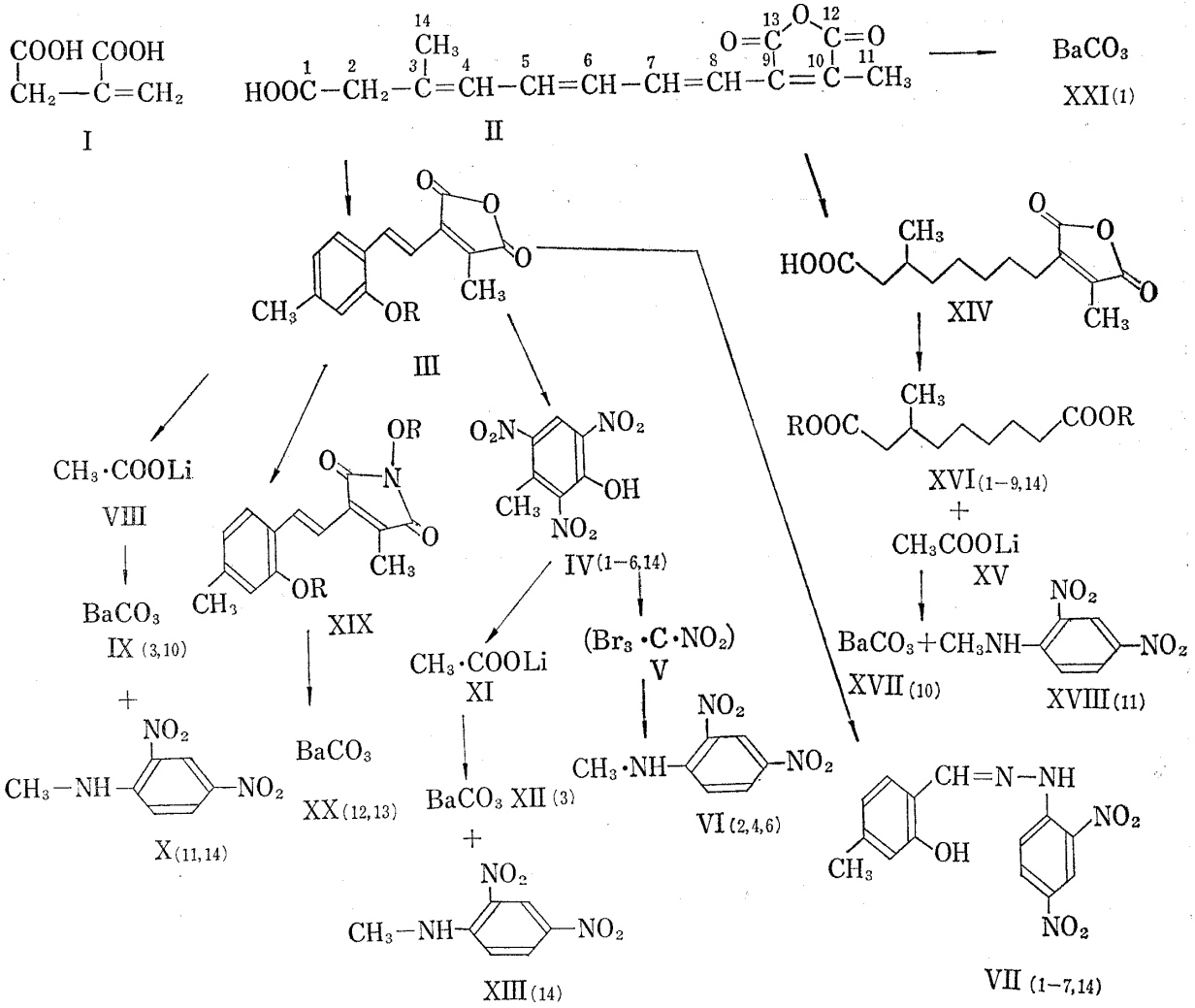

Chart 1

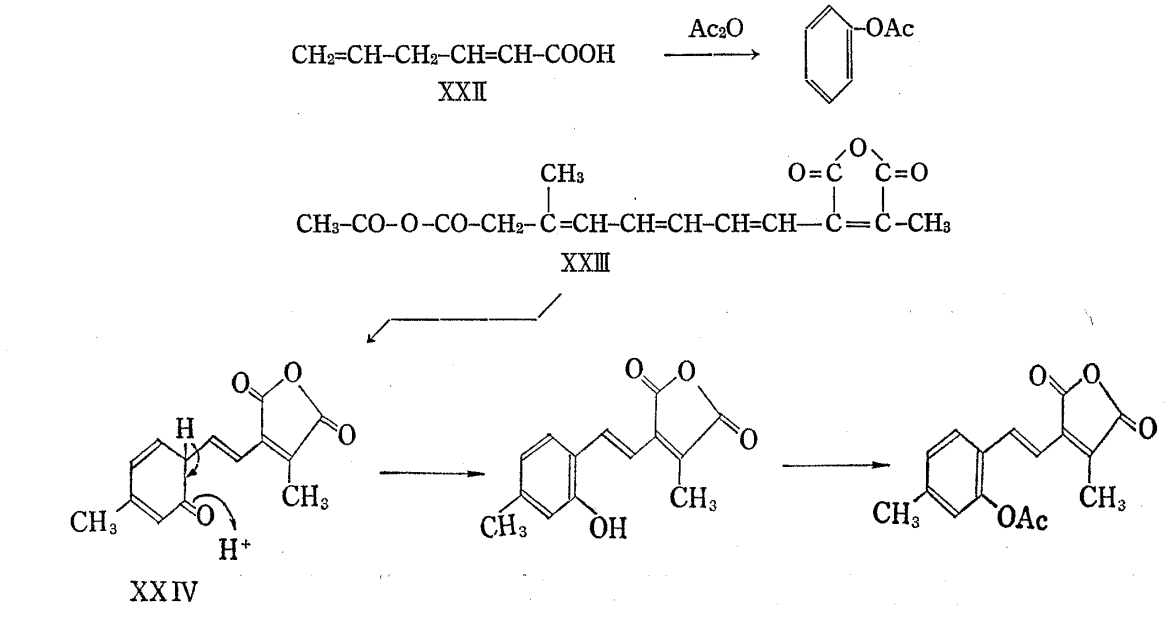

Chart 2

successful result was obtained by the action of conc. sulphuric acid, boron trifluoride, polyphosphoric acid or aluminium chloride on II instead of acetic anhydride. Similar observation was reported on the aromatization of 2,5-hexadienoic acid (XXII).[8] These observation suggest that mixed anhydride such as XXIII would be involved in the aromatization of itaconitin. The reaction seems to follow a sequence shown in Chart 2.

Trinitro-*m*-cresol (IV) which was obtained by nitric acid oxidation of III (R=H) was further submitted to bromopicrin reaction to give bromopicrin (V).[9] It was reduced with iron powder and dil. hydrochloric acid to methylamine (VI), which originates from C-2, 4 and 6 of II. 4-Hydroxy-2-methylbenzaldehyde obtained by the ozonolysis of III (R=H) was purified and characterized as its 2,4-dinitrophenylhydrazone (VII).[5d] The difference of specific activities between IV and VII would give the radioactivity at C-7 of II.

The Kuhn–Roth oxidation followed by the Schmidt degradation was carried out on III (R=H) and VI. In the latter case methyl group, C-14, of II would be obtained as N-methyl-2,4-dinitroaniline (XIII), and it would be useful to determine the origin of the methyl group.

No successful result was obtained so far in the attempts to obtain C-10, 11 and 12 of II as a derivative of pyruvate.[10] However, lithium acetate (XV) was obtained by the ozonolysis of hexahydroitaconitin (XIV). It is obvious that lithium acetate (XV) was derived from C-10 and 11 of II. It was further degradated into barium carbonate (XVII) and N-methyl-2,4-dinitroaniline (XVIII). At the same time 3-methylnonandioic acid (XII: R=H) was obtained and it was characterized as di-*p*-bromophenacyl ester (XII: R=*p*-bromophenacyl).[5c]

The carbonyl carbon atoms of anhydride ring, C-12 and 13, were liberated as carbon dioxide by the hydrolysis of *p*-toluenesulphonyl hydroxyimide (XIX: R=Ts). This reaction had been employed by Barton, *et al.* in their structural studies on nonadrides.[11] III (R=H) was first converted into its N-hydroxyimide (XIX: R=H) with hydroxylamine. XIX (R=H) obtained as deep red crystals was further treated with *p*-toluenesulphonyl chloride and sodium hydroxide in acetone–water to give pale yellow ditosylate (XIX: R=Ts). The hydrolysis of XIX (R=Ts) gave 1.7 mole equivalent carbon dioxide, which was collected as barium carbonate (XX).

The carboxyl carbon atom of II had been expected to be decarboxylated easily. Actually, 0.6 mole equivalent carbon dioxide was collected as barium carbonate (XXI) when II was heated at 120—180° with copper chromate in quinoline, whereas carbon dioxide was not evolved from III (R=H) under the same condition. This reaction would be useful to determine the origin of the carboxyl carbon atom, C-1 of II.

Experimental

Itaconitin (II)——Itaconitin (II) was isolated from the culture medium of *Aspergillus itaconicus* Kinoshita by the method described by Kinoshita and Nakajima.[5a]

Anhydroitaconitin (III: R=H)——Crude itaconitin (320 mg) in Ac_2O was heated under reflux for 5 min. Ac_2O was removed *in vacuo* to obtain a brown solid, which was disolved in a small volume of $CHCl_3$. The solution was loaded on the top of chromatographic column packed with silicic acid (or $CaHPo_4$). A pale yellow band resulted by developing with benzene was eluted and the residue obtained on evaporation was refluxed with 10% NaOH (20 ml) for 30 min. Yellow precipitates separated from acidified solution changed to orange on heating. The precipitate was collected by filtration, washed with water and dried in H_2SO_4 disicator. Orange powder was recrystallized from $CHCl_3$ to give anhydroitaconitin (III: R=H) (140 mg) of mp 180.5—182.5°.[11]

Trinitro-*m*-cresol (IV)——A suspension of anhydroitaconitin (500 mg) in dil. HNO_3 (10 ml) (9 ml conc. HNO_3 and 1 ml H_2O) was heated on a boiling water bath for 3 hr, and the mixture was kept in refrigerator

8) G.P. Chiusoli and G. Agnes, *Zeitschr. Naturforschung*, **17b**, 852 (1962).
9) A.J. Birch, C.J. Moyl, R.W. Rickards and Z. Vanek, *J. Chem. Soc.*, **1962**, 3586.
10) N. Wilkmann, *Ann.*, **530**, 20 (1937).
11) All the mp's were measured with Yanagimoto heating block.

overnight to precipitate trinitro-*m*-cresol as pale yellow crystals. It was recrystallized from $CHCl_3$–*n*-hexane (or dil. hydrochloric acid) to give IV, mp 103—105° (120 mg).

Bromopicrin Reaction of Trinitro-*m*-cresol——A mixture of trinitro-*m*-cresol (IV) (200 mg), $Ba(OH)_2$ (4 g) and H_2O (80 ml) was added under stirring dropwise to a cold mixture of $Ba(OH)_2$ (30 mg), Br_2 (4 ml) and H_2O (500 ml). After the addition had been completed, the reaction mixture was stirred further for 1 hr, and then submitted to steam distillation to collect 300 ml of distillate, from which bromopicrin was separated as an oil. Bromopicrin was extracted with ether and the solution was dried over anhydrous Na_2SO_4. Ether was removed and oily bromopicrin was mixed with 0.1N HCl (30 ml) and Fe powder (1 g). The mixture was left at room temperature overnight, diluted with appropriate volume of H_2O and made alkaline with conc. NaOH solution. The alkaline solution was submitted to steam distillation again, and the distillate was introduced into dil. HCl. $MeNH_2$ HCl was obtained on evaporation of the distillate, which was treated with 2,4-dinitrofluorobenzene (80 mg) in a mixture of EtOH (4 ml) and $NaHCO_3$ saturated H_2O (4 ml) for 1 hr. Water was added to precipitate N-Me-2,4-dinitroaniline, which was collected by filtration, washed with water and dried. A yellow powder obtained was recrysatllized from $CHCl_3$–*n*-henane (or EtOH) to give yellow crystals of mp 177.5—180.5° (50 mg).

The Kuhn-Roth Oxidation of Anhydroitaconitin (III: R=H) followed by the Schmidt Reaction——A mixture of anhydroitaconitin (III: R=H) (C-Me value 1.61) (40 mg) and the Kuhn–Roth regent[1] (25 ml) was refluxed for 2 hr. After the addition of $MgSO_4$ (10 g) and an appropriate volume of H_2O, distillation was continued to obtain *ca.* 100 ml. of distillate. Water was added occasionally to maintain the volume in the flask at *ca.* 50 ml. The distillate was neutralized with 1/2N LiOH solution (pH meter) and evaporated to dryness (0.5 ml 1/2N LiOH was required for neutralization). The residue was treated with a small volume of EtOH (anhydrous) and insoluble inorganic compounds were removed by filtration. On removing EtOH *in vacuo*, CH_3COOLi was obtained as a state of foam, which was disolved in conc. H_2SO_4 (2 ml) and NaN_3 (200 mg) was added. The mixture heated at 65—80° and CO_2 evolved was introduced into the solution of $Ba(OH)_2$. $BaCO_3$ (IX) (24.5 mg) was obtained. The residual solution in the flask was made alkaline with 30% NaOH and submitted to steam distillation. The distillate was directly introduced into dil. HCl. On evaporation of H_2O, $MeNH_2$ HCl was obtained. It was disolved again in H_2O (1 ml) and saturated with $NaHCO_3$. Ethanolic solution (1 ml) of 2,4-dinitrofluorobenzene (1 g 2,4-dinitrofluorobenzene in 25 ml EtOH) was added to the solution, and the mixture was stirred for 2 hr. A yellow solid precipitated by the addition of H_2O (20 ml) was collected by filtration, washed with H_2O and dried. Thus obtained yellow powder was submitted to chromatography on florisil using ether as solvent. Recrystallization from $CHCl_3$–*n*-hexane (or EtOH) gave N-Me-2,4-dinitroaniline (X) (7 mg) of mp 177—180°. *Anal.* Calcd. for $C_7H_7O_4N_3$ (N-Me-2,4-dinitroaniline): C, 42.64; H, 3.58; N, 21.32; Found: C, 42.64; H, 3.58; N, 21.12.

The Kuhn-Roth Oxidation of Trinitro-*m*-cresol (IV) followed by the Schmidt Reaction——Trinitro-*m*-cresol (IV) (250 mg) was submitted to Kuhn–Roth oxidation followed by Schmidt reaction. $BaCO_3$ (XII) (150 mg) and N-Me-2,4-dinitroaniline (XIII) (17 mg) of mp 178—179° were obtained.

Anhydroitaconitin-N-hydroxyimide (XIX: R=H)——Anhydroitaconitin (III: R=H) (150 mg), H_2NOH HCl (210 mg) and AcONa (250 mg) were disolved in EtOH–H_2O, and the reaction mixture was boiled for 20 min. Deep red precipitates separted from the solution on diluting with H_2O, were filtered and dried. On recrystallization from EtOH–H_2O XIX (R=H) (100 mg) of mp 218—219° was obtained. *Anal.* Calcd. for $C_{14}H_{13}O_4N$: C, 64.86; H, 5.05; N, 5.40; Found: C, 65.06; H, 5.03; N, 5.45.

Ditosylanhydroitaconitin-N-hydroximide (XIX: R=Ts)——XIX (R=H) (90 mg) and TsCl (450 mg) were disolved in acetone (10 ml) and 1.75N NaOH (2 ml) was added dropwise to the solution under stirring. Stirring was continued further for 30 min at room temperature, and the reaction mixture was diluted with H_2O. Acetone was removed *in vacuo* and the residual solution was extracted with $CHCl_3$. The extract was dried over anhydrous $MgSO_4$. Solvent was removed *in vacuo* to leave a pale yellow solid. The solid was recrystallized from acetone–H_2O to give pale yellow ditosylate (XIX: R=Ts) of mp 167—168°. *Anal.* Calcd. for $C_{28}H_{25}O_8NS_2$: C, 59.24; H, 4.43; N, 2.47. Found: C, 59.54; H, 4.46; N, 3.01.

Hydrolysis of Ditosylate (XIX: R=Ts)——Ditosylate (XIX: R=Ts) was hydrolyzed without recrystallization. Crude ditosylate obtained from 90 mg. of XIX (R=H) and 1.72 N NaOH (CO_2 free) (20 ml) was refluxed for 20 min. A sodalime–tube was fitted on the top of condensor to prevent the contamination of atomspheric CO_2. The reaction mixture was made acidic with dil. HCl and CO_2 evolved was swept into $Ba(OH)_2$ solution by the stream of N_2 to obtain $BaCO_3$ (61 mg), which was collected by filtration (1.7 mole equivalent to XIX (R=H)).

4-Hydroxy-2-methylbenzaldehyde 2,4-Dinitrophenylhydrazone——The solution of anhydroitaconitin III (R=H) (100 mg) in EtOH (5 ml) was cooled with ice–salt mixture treated with the stream of O_3 until the orange yellow colour of III (R=H) had disappeared. After removing the solvent *in vacuo*, ozonide was treated with H_2O on a boiling water bath. The reaction product was extracted with ether and dried over $MgSO_4$. 4-Hydroxy-3-methylbenzaldehyde obtained on evaporation of ether was treated with 2,4-dinitrophenylhydrazine reagent to precipitate 2,4-dinitrophenylhydrazone which was purified by chromatography on silica gel using benzene as solvent. VII (35 mg) was obtained as deep red crystals of mp 265—267.5°.

Ozonolysis of Hexahydroitaconitin (XIV)——II (400 mg) was hydrogenated using Pd–C (10%) as a catalyst in EtOAc (50 ml). A hundred and one ml H_2 (theoretical 115 ml) was absorbed within 10 min. Oily

XIV obtained on removing the solvent *in vacuo*, was dissolved in CH_2Cl_2 and treated with the stream of O_3 at a temperature of −60—70° for 3 hr. The solvent was removed *in vacuo* at room temperature, and the residue was mixed with 5% NaOH containing 15% H_2O_2 (10 ml) and the mixture was left overnight in a refrigerator. An excess H_2O_2 was decomposed with Pd black, and the reaction mixture was made acidic with dil. H_2SO_4. The solution was steamdistilled, and the distillate, *ca.* 200 ml, was neutralized with 1/2 N LiOH (pH meter). CH_3COOLi (XV) was obtained as a foam when the solvent was distilled off *in vacuo*. XV was futher degradated by Schmidt reaction into $BaCO_3$ (XVII) (170 mg) and $MeNH_2$, and latter was characterized as N-Me-2,4-dinitroaniline (XVIII) (22 mg). The solution remaining in the steam distillation flask was extracted with ether, and the ethereal solution was washed with water and dried over anhydrous $MgSO_4$. Ether was removed and the oily residue was disolved in EtOH. The solution was neutralized with 1/2 N NaOH (BTB was used as an indicator) and 2.5 mole equivalent *p*-bromophenacyl bromide was added to the neutrlized solution. The mixture was refluxed for 4 hr. Solvent was removed *in vacuo* to leave a solid, which was treated with benzene. The benzene soluble product was chromatographed on silica gel using benzene as an eluate to obtain di-*p*-bromophnacyl 3-methylnonandioate (XVI R=*p*-bromophenacyl), which was recrystallized twice from MeOH and once from EtOH. XVI was obtained as colourless crystals of mp 98—101° (30 mg). *Anal.* Calcd. for $C_{26}H_{28}O_6Br_2$ (di-*p*-bromophenacyl 3-methylnonandioate): C, 52.38; H, 4.74; Found: C, 51.79; H, 4.60.

Decarboxylation of Itaconitin (II)——A mixture of II (100 mg), copper chromate (100 mg) and quinoline (2 ml) was heated 120—180° under the stream of N_2. Evolved CO_2 was collected as $BaCO_3$ (48 mg). Anhydroitaconitin (III R=H) showed no evidence of liberation of CO_2 under the same condition.

Aromatization of Itaconitin (^{14}C)——A mixture of itaconitin (^{14}C) (1.4×10^5 dpm/mM) (100 mg), anhydroitaconitin (500 mg) and Ac_2O (20 ml) was refluxed for 2 min. The excess Ac_2O was decomposed with water. A precipitated brown solid was collected by filtration and submitted to chromatography on silicic acid to separate III (R=Ac) and III (R=H), which showed 1.4×10^4 dpm/mM and 5.4×10^4 dpm/mM respectively.[12]

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12) The measurement of radioactivity was carried out with the same method reported in ref. 1)