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Biosynthesis of Natural Products. V.[1] Biosynthesis of Itaconitin. (2). Tracer Studies on Itaconitin

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The biosynthetical studies of itaconitin were carried out with labelled compounds. C_{10}-chain (C-1–9,13) and methyl carbon (C-14) were found to be derived from acetate–malonate and C_1-unit. C_3-unit (C-10–12) was most probably introduced from Phosphoenolpyruvate (PEP) or oxaloacetate which is formed by the carboxylation of PEP.

As described in the previous paper,[1] itaconitin (I) is a derivative of disubstituted maleic anhydride, and some similarity of a part of its structure with itaconic acid (II) has been suggested. Nonadrides in which three fungal metabolites, glauconic, glaucanic and byssochlamic acids are belonged have been shown to possess disubstituted maleic anhydride portion in their molecules.[3]

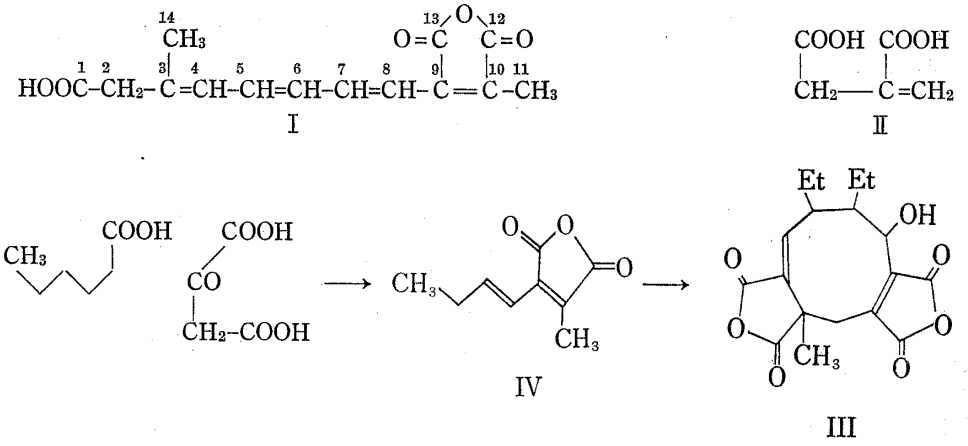


Chart 1

The biogenesis of nonadride was suggested by Barton, *et al.* in their paper on the structural studies.[3] Glauconic acid (III) has been proved as beeing formed from two moieties of C_9-intermediate (IV), which has been shown to be derived from hexanoic acid and oxaloacetate.[4] It would be worthwhile to note that in *Penicillium purpurogenum*, which was used for the biosynthetic studies of glauconic acid (III), oxaloacetate is supplied not only from TCA cycle, but also from malate formed by the carboxylation of pyruvate. In the present paper the biosynthesis of itaconitin (I) is discussed.

According to the fact that itaconic acid (II) is derived from citric acid *via cis*-aconitic acid,[5] citric acid (1,5-^{14}C) was fed to *Aspergillus itaconicus* Kinoshita. Sodium acetate (1-^{14}C), (2-^{14}C), diethyl malonate (1,3-^{14}C), (2-^{14}C) and sodium formate (^{14}C) were administered separately to prove the origin of C–1—8 and 14 of itaconitin (I). Succinic acid (2,3-^{14}C) and glucose (6-^{14}C) were finally used to investigate the origin of the anhydride part.

1) Part IV: U. Sankawa and S. Shibata, *Chem. Pharm. Bull.* (Tokyo), **17**, 2020 (1969).
2) Location: *Hongo, Bunkyo-ku, Tokyo.*
3) J.E. Baldwin, D.H.R. Barton and J.K. Sutherland, *J. Chem. Soc.*, **1965**, 1767.
4) J.L. Bloomer, C.E. Moppet and J.K. Sutherland, *Chem. Comm.*, **1965**, 619; *ibid.*, **1966**, 772.
5) R. Bentley and C.P. Thiessen, *J. Biol. Chem.*, **226**, 673, 689 and 703 (1957).

Materials and Methods

Cultivation——*Aspergillus itaconicus* Kinoshita was incubated stationarily at 28—30° on Tabuchi's medium.[6] Two to three flasks containg 150 ml medium were employed for each experiment.

Feeding of Labelled Compounds——All the labelled compounds were purchased from Dai-ichi Chem. Co., Ltd. The labelled compounds were dissolved in sterilized water, acetone or dimethyl sulfoxide and the solution was added to the flask on the day indicated in Table I.

Isolation of ^{14}C-Labelled Itaconitin——The mycelium was filtered off to separate the culture filtrate, which was made acidic by the addition of conc. hydrochloric acid and left to stand overnight to obtain the precipitate of itaconitin. The clear supernatant solution was removed by decantation completely as possible and the yellow precipitate was collected on a filter, washed with water and dried. The yellow powder was treated with hot acetone to filter off insoluble substances. On evaporating acetone *in vacuo* crude ^{14}C-labelled itaconitin (200—300 mg) was obtained. In some cases non-labelled itaconitin in 0.1N NaOH solution was added as a carrier to the culture filtrate prior to the addition of conc. hydrochloric acid. When the purification of ^{14}C-labelled itaconitin was required, crude itaconitin was recrystallized repeatedly from EtOH or $CHCl_3$. Thus obtained itaconitin showed mp 169°.

The Procedure of Determination of Radioactivity——The same procedure was used as described previously.[8]

Degradation of ^{14}C-Labelled Itaconitin——The various samples of ^{14}C-labelled itaconitin obtained by present experiments were degradated as described in the previous paper.[1]

Results and Discussion

The incorporation ratios of ^{14}C-labelled compounds into itaconitin of *A. itaconicus* are shown in Table I.

Table I

Compound (50 μ ci)	Day of administration after inoculation	Period of cultivation after administration of conpound	dpm/mM itaconitin[a]	Incorporation %
CH_3COONa (1-^{14}C)	11	3	6.9×10^6	4.2
CH_3COONa (2-^{14}C)	11	3	5.2×10^6	3.5
Diethyl malonate (1,3-^{14}C)	11	3	2.0×10^6	1.3
Diethyl malonate (2-^{14}C)	11	2	1.2×10^7	15
HCOONa(^{14}C)	11	2	3.1×10^7	31
Citric acid (1,5-^{14}C)	11	2	6.7×10^3	0.012
$CH_3COCOONa$ (1-^{14}C)	11	3	1.6×10^4	0.01
Glucose (6-^{14}C)[b]	7	10	7.0×10^4	0.17
Succinic acid (2,3-^{14}C)[b]	7	14	1.2×10^5	0.24
CH_3COONa (2-^{14}C)[b]	7	14	1.0×10^6	1.0

a) In some cases specific activity was measured as anhydroitaconitin.
b) The mould strain used in these experiment was different from the strain used in the other experiment and the production of itaconitin by this strain was less than the other strain.

Sodium acetate (1-^{14}C), (2-^{14}C), diethyl malonate (1,3-^{14}C), (2-^{14}C) and sodium formate (^{14}C) showed high incorporation ratios, whereas citric acid (1,5-^{14}C) and sodium pyruvate (1-^{14}C) gave poor incorporation ratios. The radioactivities of the degradation products of itaconitin are shown in Tables II—IX.

The results of the degradation of ^{14}C-labelled itaconitin obtained from sodium acetate-^{14}C and diethyl malonate-^{14}C (Tables II, III, V and VI) clearly indicate that ten carbon atoms, C–1—9 and 13, is derived from acetate-malonate as usually shown in polyketide or fatty acid.

6) T. Tabuchi, *Tokyo Kyoiku Daigaku Nogakubu Kiyô*, **9**, 245 (1963).
7) S. Shibata, U. Sankawa, H. Taguchi and K. Yamasaki, *Chem. Pharm. Bull.* (Tokyo), **14**, 474 (1966).
8) K. Kinoshita and K. Nakajima, *Chem. Pharm. Bull.* (Tokyo), **6**, 31 (1958).

Table II. Incorporation of ^{14}C from CH_3COONa (1-^{14}C) into Itaconitin

	dpm/mM	Observed %	Theoretical %
Anhydroitaconitin	7.4×10^4	—	100
$BaCO_3$ (C-3,10)	0	0	0
N-Me-2,4-dinitroaniline (C-11,14)	0	0	0
Anhydroitaconitin	2.7×10^5	—	100
Trinitro-*m*-cresol (C-1—6,14)	1.7×10^5	63	60
N-Me-2,4-dinitroaniline (C-2,4,6)	5.1×10^4	19	20
Anhydroitaconitin-N-hydroxyimide	4.8×10^5	—	100
$BaCO_3$ (C-12,13)	4.7×10^4	9.8	10

Table III. Incorporation of ^{14}C from CH_3COONa (2-^{14}C) into Itaconitin

	dpm/mM	Observed %	Theoretical %
Anhydroitaconitin	3.1×10^5	—	100
Trinitro-*m*-cresol (C-1—6,14)	1.9×10^5	61	60
4-Hydroxy-2-methylbenzaldehyde 2,4-dinitrophenylhydrazone (C-17, 14)	2.4×10^5	77	80
Itaconitin	2.1×10^5	—	100
$BaCO_3$ (C-1)	2.9×10^4	14	20
Itaconitin	6.8×10^4	—	100
$BaCO_3$ (C-1)	6.9×10^3	10	20
Anhydroitaconitin-N-hydroxyimide	1.7×10^5	—	100
$BaCO_3$ (C-12,13)	1.4×10^3	0.8	0
Itaconitin	9.9×10^4	—	100
Di-*p*-bromophenacyl 3-Me-nonandioate (C-1—9,14)	8.2×10^4	86	100
N-Me-2,4-dinitroaniline (C-11)	6.5×10^2	0.7	0
$BaCO_3$ (C-10)	1.1×10^3	1.1	0

Table IV. Incorporation of ^{14}C from HCOONa (^{14}C) into Itaconitin

	dpm/mM	Observed %	Theoretical %
Anhydroitaconitin	1.1×10^6	—	100
Trinitro-*m*-cresol (C-1—7,14)	9.2×10^5	84	100
Trinitro-*m*-cresol	3.9×10^5	—	100
$BaCO_3$ (C-3)	0	0	0
N-Me-2,4-dinitroaniline (C-14)	7.4×10^5	88	100

Table V. Incorporation of ^{14}C from Diethyl Malonate (1,3-^{14}C) into Itaconitin

	dpm/mM	Observed %	Theoretical %
Anhydroitaconitin	6.4×10^4	—	100
Trinitro-*m*-cresol (C-1—6,14)	3.3×10^4	52	50
4-Hydroxy-2-methylbenzaldehyde 2,4-dinitrophenylhydrazone (C-17,14)	2.4×10^5	77	80
Anhydroitaconitin-N-hydroxyimide	5.4×10^4	—	100
$BaCO_3$ (C-12, 13)	5.8×10^3	11	12.5
Itaconitin	3.4×10^4	—	100
Di-*p*-bromophenacyl 3-Me-nonandioate (C-1—9, 14)	2.8×10^4	82	75
Itaconitin	1.4×10^5	—	100
$BaCO_3$ (C-1)	4.3×10^3	3.1	0

Table VI. Incorporation of ^{14}C from Diethyl Malonate (2-^{14}C) into Itaconitin

	dpm/mM	Observed %	Theoretical %
Anhydroitaconitin	4.6×10^5	—	100
Trinitro-*m*-cresol (C-1—6,14)	2.1×10^5	48	50
N-Me-2,4-dinitroaniline (C-2,4,6)	6.1×10^2	0.2	0
4-Hydroxy-2-methylbenzaldehyde 2,4-dinitrophenylhydrazone (C-1—7,14)	2.4×10^5	77	75
Anhydroitaconitin	4.3×10^5	—	100
Trinitro-*m*-cresol (C-1—6,14)	2.3×10^5	54	50
Itaconitin	7.4×10^5	—	100
$BaCO_3$ (C-1)	1.3×10^4	1.7	0
Itaconitin	3.7×10^4	—	100
Di-*p*-bromophenacyl-3-Me-nonandioate (C-1—9,14)	3.6×10^4	98	100

Table VII. Incorporation of ^{14}C from Glucose (6-^{14}C) into Itaconitin

	dpm/mM	Observed %
Itaconitin	7.0×10^4	(100)
$BaCO_3$ (C-10)	2.4×10^3	3.5
N-Me-2,4-dinitroaniline (C-11)	1.0×10^4	14
$BaCO_3$ (C-10)	2.1×10^3	3
N-Me-2,4-dinitroaniline (C-11)	1.3×10^4	19

Table VIII. Incorporation of ^{14}C from Succinic Acid (2,3-^{14}C) into Itaconitin

	dpm/mM	Observed %
Itaconitin	1.2×10^5	(100)
$BaCO_3$ (C-10)	7.8×10^3	6.4
N-Me-2,4-dinitroaniline (C-11)	7.5×10^3	6.1

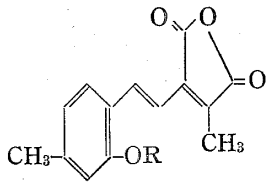
Table IX. Incorporation of ^{14}C from CH_3COONa (2-^{14}C) in longer feeding Experiment

	dpm/mM	Observed %
Itaconitin	1.0×10^6	(100)
$BaCO_3$ (C-10)	1.2×10^4	1.2
N-Me-2,4-dinitroaniline (C-11)	1.6×10^4	1.6

The failure of the incorporation of citric acid (1,5-^{14}C) (Table I) excludes the possibility that the acid anhydride portion of itaconitin is derived from citric acid. This results consistent with that obtained in glauconic acid.[4]

Barium carbonate obtained on the decarboxylation of ^{14}C-labelled itaconitin from sodium acetate (2-^{14}C) showed much lower specific activity than that expected (Table III). Moreover, barium carbonate obtained by the decarboxylation of ^{14}C-labelled itaconitin from diethyl malonate (1,3-^{14}C), showed much higher activity than it had been expected (Table V). This result suggests that not only carboxyl carbon, C–1, but also carbonyl carbons, C–12 and 13, in anhydride ring were liberated to some extent as carbon dioxide. As it has already mentioned, the liberation of carbon dioxide from anhydroitaconitin (V: R=H) was not observed under the same condition.[1] The different behavior between itaconitin (I) and anhydroita-

conitin (V: R=H) to the decarboxylation reaction would be attributed to the difference in the reactivities of the anhydride ring of these compounds. Itaconitin (I) shows λ_{max} 338 mμ in ethanol, whereas in chloroform λ_{max} shift to 403 mμ which is also observed in ethanol as a shoulder.[8] Same type of shift is observed in acetylanhydroitaconitin (V: R=Ac). On the other hand, neither anhydroitaconitin (V: R=H) nor methylanhydroitaconitin (V: R=Me) shows such remarkable shift of UV-maximum under the same condition. In solution, disubstituted maleic anhydride exists as an equillibrium mixture of the anhydride form and probably the hemiester form. If itaconitin (II) is an equillibrium mixture in solution, it would be reasonable to assume that the hemiester form is dominant in protic solvent such as ethanol and the anhydride form is dominant in nonprotic solvent such as chloroform. In the case of anhydroitaconitin (V: R=H) and methylanhydroitaconitin (V: R=Me), an electron supply from hydroxyl or methoxyl group acts to increase the electron density in anhydride ring, and consequently the anhydride forms of these compounds would be stabilized. The observation on the UV absorption shift in protic and nonprotic solvents is fully consistent with the result of decarboxylation.



V

If we assume that the both carbonyl carbon, C–12 and 13, in anhydride ring of itaconitin (II) are decarboxylated in an equal probability, carbon dioxide evolved would be calculated to contain approximately 75% carbon dioxide being originated from C–1 and 25% from C–12 and 13. Barium carbonate obtained by the decarboxylation of ^{14}C–labelled itaconitin from diethyl malonate (2-^{14}C) showed less than one tenth radioactivity of C–3, C–5, C–7, and C–9. This result indicates that the carboxyl group, C–1, is formed by the oxidation of starting methyl of C_{10}–polyketide or fatty acid.

Concerning with the origin of the C–14 methyl of itaconitin, sodium formate (^{14}C) was shown to be incorporated efficiently into it. No doubt it is derived from C_1–unit. As far as this paper concerns, we cannot conclude, whether C–1—9 and 13 is derived from polyketide of fatty acid.

It is necessary to discuss the origin of C–10—12, C_3–unit. Sodium pyruvate (1–^{14}C) gave a quite poor incorporation into itaconitin and the ^{14}C distribution could not be determined. Although sodium acetate (2–^{14}C) was an extremely efficient precursor of C–1—9, it showed poor incorporation into the C_3–unit. Longer feeding did not show any effective increase of the incorporation into the C_3–unit (Table IX). These results suggest that TCA cycle and pyruvate are rather poor source of the C_3–unit. The incorporation into C–10 and 11 from succinic acid (2,3–^{14}C) was 6.1 and 6.4% respectively (% to the total radioactivity of itaconitin). This result indicates succinic acid (2,3–^{14}C) was extensively degraded into acetate, presumably, *via* malate, and reincorporated into the part originating from acetate-malonate. On the other hand, the incorporation into C–10 and 11 from glucose (6–^{14}C) was 3.3 and 16.5%, respectively (Table VII). This result suggests again the supply of the C_3–unit from TCA cycle is less important, because C–10 and C–11 should be labelled equally if ^{14}C from glucose (6–^{14}C) had been introduced through TCA cycle. It would be probable to assume that the C_3–unit is introduced from C_3-intermediate of glycolysis, such as phosphoenolpyruvate (PEP). A recent report on the biosynthesis of protolichesterinic acid gave nearly the same conclusion on the origin of similar C_3–unit.[9] However, there is another possibility, since PEP carboxylase was found in animals,[10] plants,[11] and microorganisms,[12] and if we assume oxaloacetate supplied

9) J.L. Bloomer, W.R. Eder and U.F. Hoffman, *Chem. Comm.*, **1968**, 354.
10) M.F. Utter and K. Kurosawa, "Methods in Enzymology," Vol. I, ed. by S.P. Colowick and N.O. Kaplan, Academic Press, New York, 1955, p. 758.
11) B. Vennesland, "Methods in Enzymology," Vol. V, ed. by S.P. Colowick and N.O. Kaplan, Academic Press, New York, 1962, p. 617.
12) *cf.* B.D. Sanwal and P. Maeba, *J. Biol. Chem.*, **241**, 4557 (1966).

from PEP by the carboxylation is the direct origin of the C_3–unit, the ^{14}C distribution in itaconitin would be compatible with the results mentioned above. As far as the present study concerns, it is still difficult to conclude which case should be adopted for the biosynthesis of itaconitin. The presumable course is illustrated in Chart 2.

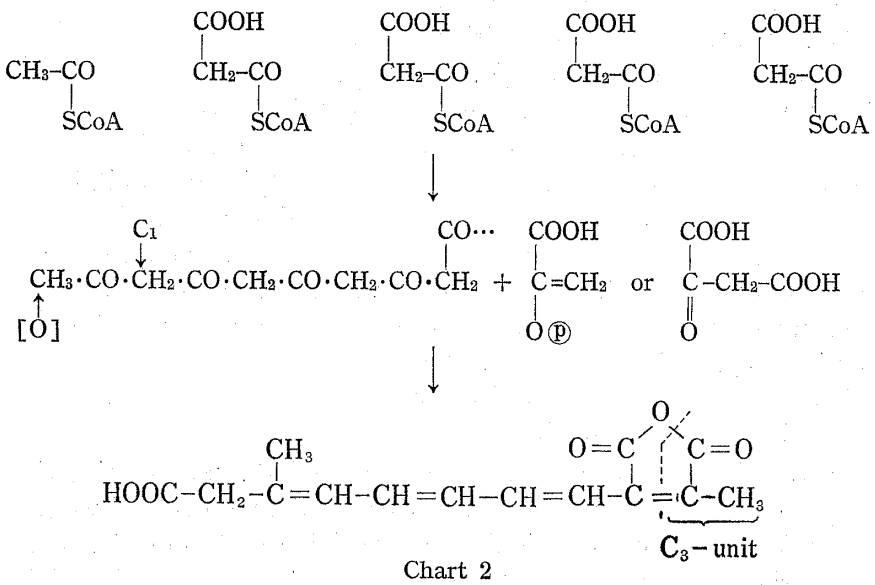


Chart 2

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