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66. Shoji Shibata*1 and Tetsuro Ikekawa*2 : Metabolic Products of Fungi. XX.*3 The Biosynthesis of Rugulosin.

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The biosyntheses of several phenolic compounds were elucidated by the acetate theory. This scheme of biosynthesis was extended to the polynuclear phenolic compounds, such as polyhydroxy-anthraquinones and their derivatives, by Birch, *et al.*[1] and Gatenbeck, *et al.*[2]

Recently a role of malonate in the biosynthesis of simple phenolic compounds has been postulated by Mosbach,[3] Bentley, *et al.*[4] and Bu'Lock, *et al.*[5]

The present study has been pursued to investigate the incorporation of malonate into the polynuclear anthraquinone series compounds.

Rugulosin which has been used in the present study was first isolated from *Penicillium rugulosum* THOM and *P. wortmanii* KLÖCKER[6] and then has been found widely in some other fungi of *Penicillium*,[7] *Endothia*[8] and other species[9] accompanying skyrin.

Recently rugulosin has been found to be produced by *P. brunneum* UDAGAWA[10] in a very high yield, which has been employed for the present biosynthetical study.

Malonate [2-^{14}C] with or without competition of acetate [1-^{14}C] was administered to the cultivation of *P. brunneum* UDAGAWA, and the labeled rugulosin isolated from the mycelium was degraded to determine the location of the radioactivity.

Experimental

Cultivation——*Penicillium brunneum* UDAGAWA (NHL 6054) was incubated stationarily at 25~27° on the Czapek-Dox medium (containing 5% glucose). Three 1 L.-flasks containing 350 cc. of medium were employed for 1 group of experiment.

On the 12th day of inoculation, the isotopic labeled precursor was administered to the medium. The experiment was designed to carry out in 3 different series. (Exp. I~III).

Experiment I——A solution of 0.1 mc. of Ca malonate [2-^{14}C] (0.12 mc./m mole) dissolved in distilled water (50 cc.) was divided into 3 equal portions to add to each flask of the mold culture.

Experiment II——A solution of 0.07 mc. of AcONa [1-^{14}C] (1 mc./m mole) dissolved in distilled water (50 cc.) was divided into 3 equal portions to add to each flask of the mold culture.

A solution of non-labeled malonic acid (1 m mole/L.) was subsequently added to the medium.

Experiment III——An aqueous solution of 0.07 mc. of AcONa [1-^{14}C] (1 mc./m mole) was divided into 3 equal portions, which was added to each flask of mold culture.

Reference Experiment using Mevalonate [2-^{14}C] and Acetate[1-^{14}C]——N,N'-Dibenzylethylenediamine salt of mevalonic acid [2-^{14}C] (11.91 μc.) or AcONa [1-^{14}C] (11.06 μc.) was added to the mold culture at the 11th day of inoculation.

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1) A. J. Birch, A. J. Ryan, H. Smith : J. Chem. Soc., **1958**, 4773.
2) S. Gatenbeck : Acta Chem. Scand., **12**, 1211, 1985 (1958); *Ibid.*, **14**, 102, 296 (1960); S. Gatenbeck, P. Barbersgard : *Ibid.*, **14**, 230 (1960).
3) K. Mosbach : Naturwiss., **48**, 525 (1961).
4) R. Bentley, J. G. Keil : Proc. Chem. Soc., **1961**, 111.
5) J. D. Bu'Lock, H. M. Smalley : *Ibid.*, **1961**, 209.
6) J. Breen, J. C. Dacre, H. Raistrick, G. Smith : Biochem. J., **60**, 618 (1955).
7) Y. Yamamoto, A. Hamaguchi, I. Yamamoto, S. Imai : Yakugaku Zasshi, **76**, 1428 (1956).
8) S. Shibata, T. Murakami, O. Tanaka, G. Chihara, M. Sumimoto : This Bulletin, **3**, 274 (1955); S. Shibata, T. Murakami, M. Takido : *Ibid.*, **4**, 303 (1956).
9) S. Shibata, J. Shoji, A. Ohta, M. Watanabe : *Ibid.*, **5**, 380 (1957).
10) S. Udagawa : This Bulletin, **11**, 402 (1963).

The radioactivities of all the samples in this study were measured by Nuclear Chicago Co. Model D 47 Gas Flow Counter, and Model 186 Decade Scaler.

Isolation of ^{14}C-Labeled Rugulosin——Seventy two hours (76 hr. in the case of using mevalonate [2-^{14}C]) after the administration of ^{14}C-labeled precursors, the mycelia were harvested. The radioactivity of 1 cc. of the culture filtrate was determined, from which the total activity was calculated. The dried mycelia were extracted first with petr. ether, and the radioactivity of the extract was measured.

The defatted mycelia were extracted with Et_2O exhaustively, and the ethereal extract was dissolved in Me_2CO by warming.

On standing in a refrigerator, crude crystals of rugulosin were separated out, which were purified by chromatography on a $CaHPO_4$ column using a mixture of hexane-Me_2CO-H_2O (4:1:0.1) as the developing solvent.

The fine yellow crystals of pure rugulosin, m.p. 290°(decomp.), $[\alpha]_D$ +492°(dioxane), were finally obtained by recrystallization from MeOH.

Two or three milligrams of the purified rugulosin was weighed accurately and dissolved in Me_2CO to 25 cc. A small portions of the solution were pipetted (0.5, 1.0, 1.5 cc.) into sample disks, and the radioactivity of each sample was determined at the infinite thinness level to give the specific activity.

The acetonic mother liquor from which the crude rugulosin was separated contained skyrin and emodin.

From the mixture, emodin was removed by chromatography on $CaHPO_4$, and skyrin was purified repeatedly by chromatography using the same adsorbent and solvent system employed as above.

Skyrin was treated with alkaline $Na_2S_2O_4$ to be cleaved into 2 moles of emodin which was purified by column chromatography on $CaHPO_4$ and by sublimation *in vacuo* to give m.p. 254～255°. It gave no depression of melting point on admixture with the authentic sample.

The radioactivity of emodin was measured at the infinite thinness level.

Degradation of ^{14}C-Labeled Rugulosin——The purified radioactive rugulosin was diluted to 10～30 times with nonlabeled rugulosin, and recrystallized again. The specific activity of the diluted sample was measured by the thin layer method as described above. a) The Kuhn-Roth Oxidation : The diluted ^{14}C-labeled rugulosin (100～140 mg.) was weighed and added with the Kuhn-Roth reagent (40 cc.).

The mixture was heated at 130° under N_2-stream for about 2 hr. CO_2 evolved during the reaction was trapped into $Ba(OH)_2$ solution to separate as $BaCO_3$ (yield, 80%) whose radioactivity was measured. Unless otherwise mentioned, the measurement of radioactivities of the samples obtained by the degradation of rugulosin were made at the infinite thinness level.

The cooler was rinsed with distilled water, and anhyd. $MgSO_4$ (10～15 g.) was added to the flask. The contents of the flask were steam distilled using a declining cooler to separate AcOH.

The distillate containing AcOH was made alkaline by the addition of dil. NaOH solution, and then concentrated under N_2-stream. The residual substance was placed in a flask for the Schmidt reaction and dried in a desiccator.

b) The Schmidt Degradation : AcONa obtained by the Kuhn-Roth oxidation of labeled rugulosin was dried and added with NaN_3 (20 mg.) and conc. H_2SO_4 (3 cc.) under ice-cooling. The mixture was heated at 40～60° for 2 hr. CO_2 liberated during the reaction was trapped into $Ba(OH)_2$ to form $BaCO_3$ (yield, 80～90%), which was placed in a plate to measure the radioactivity. The contents of the reaction flask were diluted with H_2O under ice-cooling and made alkaline by the addition of conc. NaOH. The mixture was steam-distilled under N_2 stream and CH_3NH_2 evolved was collected in dil. HCl. Methylamine hydrochloride was obtained on evaporation of the distillate to determine the radioactivity. Methylamine chloroplatinate : m.p. 230～231°(decomp.).

Table I.

Experiment	I	II	III
Tracer (and competitor)	Ca malonate [2-^{14}C]	Na acetate [1-^{14}C] +non-labeled malonic acid	Na acetate [1-^{14}C]
Total radioactivity administered (c.p.m.)	5.32×10^7	4.27×10^7	4.27×10^7
Total radioactivity of the culture filtrate (c.p.m.)	3.95×10^6	1.83×10^5	2.70×10^5
Percentage of radioactivity remained in the culture filtrate (%)	7.43	0.11	0.16
Total radioactivity of petroleum ether extract (c.p.m.)	9.59×10^4	2.86×10^5	3.71×10^5
Incorporation ratio of radioactivity into petroleum extract (%)	0.18	0.67	0.87
Yield of rugulosin (mg.)	237	177	182
Specific activity of rugulosin (c.p.m./m mole)	4.95×10^6	3.55×10^6	3.73×10^6
Incorporation of radioactivity into rugulosin (%)	4.07	2.71	2.93
Specific activity of emodin obtained from skyrin (c.p.m./m mole)	3.09×10^6	1.49×10^6	1.63×10^6

Results

The radioactivities of the precursors administered, the culture filtrate, the petroleum ether extract of mycelia and rugulosin isolated are given in the Table I.

The radioactivities of the degradation products of rugulosin obtained by the Experiments I. II, and III are shown in the Table II.

Table II.

Experiment	I	II	III
Tracer (and competitor)	Ca malonate [2-^{14}C]	Na acetate [1-^{14}C] +non-labeled malonic acid	Na acetate [1-^{14}C]
Specific activity of diluted rugulosin used for degradation (c.p.m./m mole)	7.20×10^5	1.10×10^5	1.48×10^5
Specific activity (corr.[a]) of $BaCO_3$ derived by Kuhn-Roth reaction (c.p.m./m mole)	2.76×10^4	3.59×10^3	4.84×10^3
Specific activity (corr.[a]) of $CH_3NH_2\cdot HCl$ derived by Schmidt reaction (c.p.m./m mole)	6.48×10^2	nil	nil
Specific activity (corr.[a]) of $BaCO_3$ derived by Schmidt reaction (c.p.m./m mole)	1.08×10^2	8.37×10^3	1.11×10^4

a) Correction was made on the basis of radioactivity of rugulosin used for degradation.

The incorporation of mevalonate [2-^{14}C] into rugulosin was examined in comparing with that of acetate [1-^{14}C].

Table III.

Tracer	Na acetate [1-^{14}C]	N,N'-Dibenzylethylene diamine salt of mevalonic acid [2-^{14}C]
Total activity administered (μc.)	11.06	11.91
Total activity of the culture filtrate (c.p.m.)	4.17×10^5	7.50×10^5
Total activity of petroleum ether extract (c.p.m.)	1.36×10^5	3.75×10^4
Specific activity of rugulosin (c.p.m./m mole)	2.16×10^6	3.78×10^4

Discussion

The incorporation of ^{14}C in rugulosin obtained by the Experiments I, II, and III was illustrated in the following scheme of degradation reactions :

In regard to the Experiment I an insignificant incorporation of malonate-^{14}C to the methylamine which was derived from ^{14}C-labeled rugulosin by the two-steps degradation reactions showed that the terminal methyl groups and their neighboring C(7,7') are not derived from the malonate unit. On the other hand, in the same experiment, a remarkable incorporation of malonate-^{14}C to the carbon atoms of remaining part of rugulosin molecule was revealed by the high specific activity of carbon dioxide liberated by the Kuhn-Roth oxidation of ^{14}C-labeled rugulosin. According to the Experiment II using acetate [1-^{14}C] in the presence of non-labeled malonic acid and the Experiment III using acetate [1-^{14}C] only as the precursor, a higher incorporation of ^{14}C into C(7) and C(7') was shown in comparison with the activity of the other remaining active carbons in the rugulosin molecule (7.6% excess in the Experiment II, and 5.8% excess in the Experiment III).

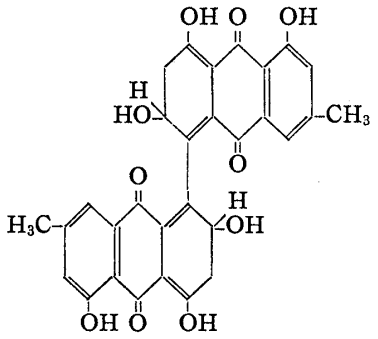
Compatible with the above result, a higher incorporation of ^{14}C at the terminal CH_3*-C unit of 6-methyl salicylic acid, griseofulvin and curvularin was also shown by Birch, *et al.*[11] in their experiment using acetate [1-^{14}C] as the tracer with or without competition of malonate.

11) A. J. Birch, A. Cassera, R. W. Rickards : Chem. & Ind. (London), **1961**, 792; A. J. Birch, R. A. Massy-Westropp, R. W. Rickards, H. Smith : J. Chem. Soc., **1958**, 360; A. J. Birch, O. C. Musgrave, R. W. Rickards, H. Smith : *Ibid.*, **1959**, 3146.

Degradation of ^{14}C-Labeled Rugulosin

Exp. I $※CH_2(COOH)_2$
Exp. II $CH_3*COOH + CH_2(COOH)_2$
Exp. III CH_3*COOH

↓ *P. brunneum*



Rugulosin

Exp. I 7.20×10^5 c.p.m./mmole
Exp. II 1.10×10^5 c.p.m./mmole
Exp. III 1.48×10^5 c.p.m./mmole

Kuhn-Roth reaction →

$※*CO_2 \longrightarrow Ba※*CO_3$

Exp. I 2.76×10^4 c.p.m./mmole
Exp. II 3.59×10^3 c.p.m./mmole
Exp. III 4.84×10^3 c.p.m./mmole

CH_3*COOH

↓ Schmidt reaction

CH_3NH_2 → $CH_3NH_2\cdot HCl$

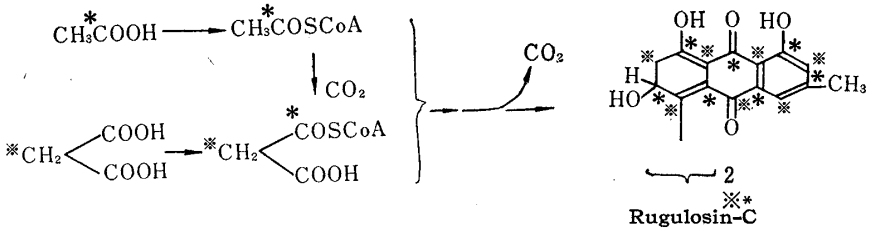
Exp. I 6.48×10^2 c.p.m./mmole
Exp. II nil
Exp. III nil

$*CO_2$ → $Ba*CO_3$

Exp. I 1.08×10^2 c.p.m./mmole
Exp. II 8.37×10^3 c.p.m./mmole
Exp. III 1.11×10^4 c.p.m./mmole

It has now been proved that the fungal anthraquinones and their related compounds having methyl or corresponding group in the β-position, as being represented by rugulosin in the present study, are biosynthesized mainly from malonyl units combining with terminal acetyl unit.

By the generally accepted biochemical sequence, malonyl CoA and acetyl CoA would take part as the actual biosynthetical units in the living cells of fungi.



Rugulosin-C

The specific activity of rugulosin as well as the incorporation ratio of ^{14}C to it were observed as being highest in the Experiment I, and the lower values were obtained in the sequence of Experiment III and II.

This fact would indicate that malonate is the precursor which forms directly the main part of anthraquinone nucleus.

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Summary

Penicillum brunneum UDAGAWA was fed with malonate [2-^{14}C], and rugulosin-^{14}C isolated from the mycelia was degraded to prove that the terminal C-CH_3 group was not labelled with ^{14}C.

Using acetate [1-^{14}C] with or without competition of inactive malonic acid, a predominant incorporation of acetate unit into the terminal C-CH_3 unit was revealed.

It has been established that the fungal anthraquinone series compounds are biosynthesized by the malonate-acetate condensation.

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67. Shoji Shibata*1 and Motoko Nakahara*2 : Studies on the Constituents of Japanese and Chinese Crude Drugs. VIII.*3 Paeoniflorin, A Glucoside of Chinese Paeony Root. (1).

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The Chinese Paeony root (the root of *Paeonia albiflora* PALLAS (: *P. lactiflora* PALLAS)) is well-known as one of the important medicaments in the traditional old Chinese medicine.

However, none of the especial principle, except benzoic acid,[1] has been isolated from the Paeony root. Recently, Ohta, *et al.*[2] suggested that benzoic acid must exist combined with some principle in the Paeony root, on which, however, they gave no further evidence.

From the methanolic extracts of the Paeony root a colorless hygroscopic amorphous substance has now been isolated, which, so far, has not been obtained in a crystalline form in spite of many efforts in purification using chromatography and counter current distribution.

The uniformity of this principle has almost been established as it gave a single spot on paper chromatogram and single peak of fractionation in the counter current distribution.

The principle has now been named paeoniflorin, with which the present paper chiefly concerns.

Accompanying with paeoniflorin, a high content of sucrose in Paeony root is noted, the yield of which is variable depending on the sources of material.

Paeoniflorin is a neutral glucoside in which benzoic acid exists as a benzoyl grouping. It gives no coloration with ferric chloride and does not reduce Fehling's reagent.

The glucoside was affected neither by emulsin nor snail enzyme, whereas it was hydrolyzed readily by dilute mineral acid to give D-glucose and benzoic acid quantitatively, but the aglycone was failed to be obtained in a pure state due to its instability

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1) Y. Asahina, S. Okuno : Yakugaku Zasshi, **27**, 1237 (1907).
2) T. Ohta, T. Miyazaki : Tokyo Yakka-Daigaku Nenpo (Ann. Rep. Tokyo Coll. of Pharmacy), **1954**, 266.