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64. Shoji Shibata, Ushio Sankawa, Heihachiro Taguchi, and Kazuo Yamasaki: Biosynthesis of Natural Products. III.*2 Biosynthesis of Erythroskyrine, A Coloring Matter of *Penicillium islandicum* Sopp.

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The structure of erythroskyrine (I), a nitrogen-containing orange red pigment of *Penicillium islandicum* Sopp,[1] was put forward by Shoji and Shibata.[2] The proposed structure (I) has suggested that it would be biosynthesized by condensation of L-valine and a polyketide intermediate which is formed by the acetate-malonate biosynthetical pathway. The present study has been designed to prove the distribution of ^{14}C in the molecule of erythroskyrine which was obtained by the cultivation of the mold on Czapek-Dox media containing sodium acetate ^{14}C-labeled in 1 or 2, and diethyl malonate ^{14}C-labeled in 1 or 2, respectively.

In the case of feeding of DL-valine-1-^{14}C, the ^{14}C-labeled erythroskyrine isolated from mycelia was decomposed into N-methylvaline (III) by ozonization. The product (III) was characterized as the crystalline 2,4-dinitrophenyl derivative, which was then decomposed by UV-illumination[3] into isobutyraldehyde (IV) (2,4-dinitrophenylhydrazone:

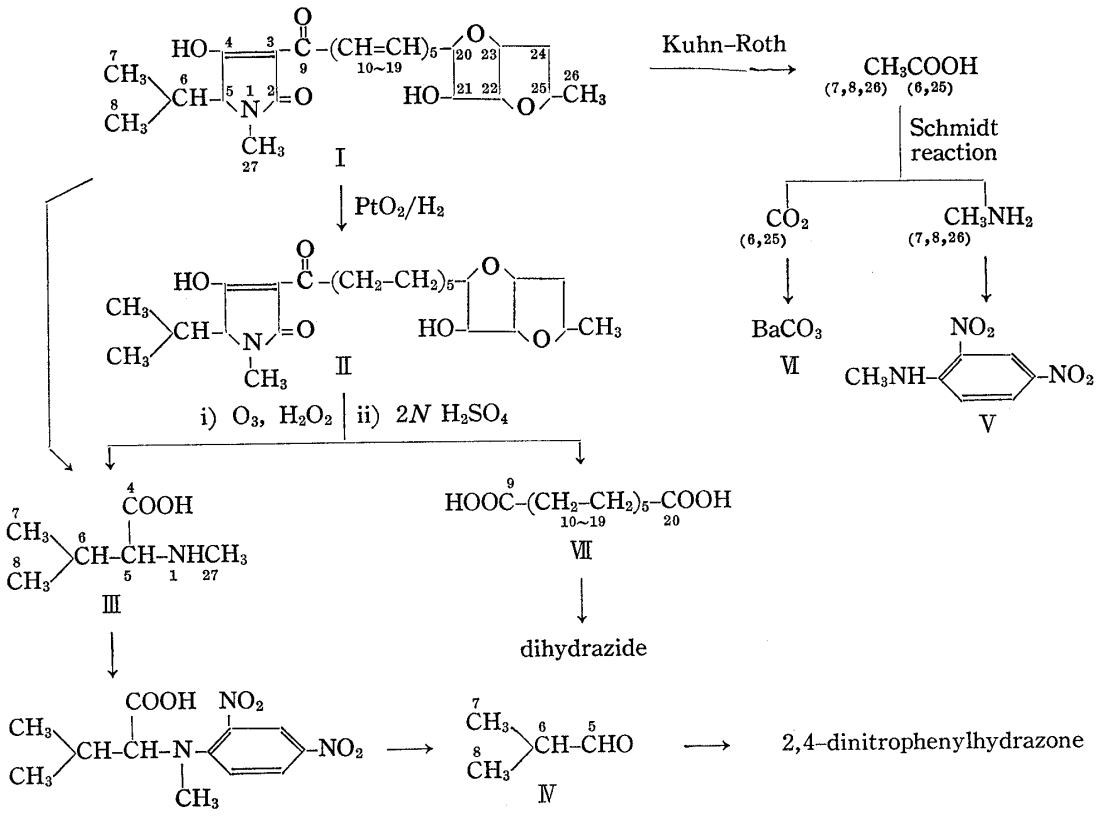

Chart 1.

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*2 Part II (Biogenesis of Plant Products): This Bulletin, **11**, 545 (1963).

1) R. H. Howard, H. Raistrick: Biochem. J. (London), **56**, 216 (1954).
2) J. Shoji, S. Shibata: Chem. & Ind. (London), **1964**, 419; J. Shoji, S. Shibata, U. Sankawa, H. Taguchi, Y. Shibanuma: This Bulletin, **13**, 1240 (1965).
3) D. W. Russell: J. Chem. Soc., **1963**, 894.

m.p. 184°). In the case of feeding of ^{14}C-labeled acetate and malonate, the ^{14}C-labeled erythroskyrine was subjected to the Kuhn-Roth reaction to obtain acetic acid which was decomposed by the Schmidt reaction to give carbon dioxide and methylamine. On the other hand, the ^{14}C-labeled erythroskyrine was converted into decahydro derivative (II) and treated with ozone to afford N-methylvaline (III) and dodecanedioic acid (VII). The radioactivities of all the fragments obtained by the above reactions were determined to prove the distribution of ^{14}C.

Experimental

The Procedure of Determination of Radioactivities——Radio-active erythroskyrine and its degradation products except $BaCO_3$ was degraded into CO_2 by the van Slyke-Folch oxidation[4] using Barker's apparatus. The carbon dioxide was absorbed into Hyamine 10-X hydroxide (Packard) (1 ml.), which was dissolved into toluene scintilator (0.4% PPO, 0.01% POPOP) (15 ml.) and measured by a liquid scintilation counter (Packard).

The result was corrected referring the correction curve which was made using benzoic acid-^{14}C (Packard) as the standard. Carbon dioxide was liberated from $BaCO_3$ by the addition of conc. H_2SO_4 in Barker's apparatus, and the activity was measured as above.

Preparation of ^{14}C-Labeled Erythroskyrine——*Penicillium islandicum* Sopp, strain S was inoculated to the Czapek-Dox solution (150 ml. in each flask), and after 4~6 days cultivation the solution of ^{14}C-labeled precursors in sterilized distilled water was added (1 ml. for 1 flask each) (Table I).

Table I.

Compounds	Day of addition of the compound after inoculation	^{14}C-Labeled compound added (μc.)	Period of cultivation after addition of the compound (day)	Incorporation ratio (%)
DL-Valine-1-^{14}C	4	100	3	0.19
Na Acetate-1-^{14}C	6	50	9	0.46
Na Acetate-2-^{14}C	4	50	4	0.96
Diethyl malonate-1-^{14}C	4	100	6	0.26
Diethyl malonate-2-^{14}C	6	100	9	0.12

The mycelia harvested were moistened with 2*N* HCl and extracted 3 times with benzene using 800 ml. each. The benzene extract was washed with water, dried over anhydrous Na_2SO_4 and concentrated. The precipitates which were formed on addition of petroleum ether were mixed with anhydrous Na_2SO_4 (6 g.), or in the case of forming no precipitation, the concentrated solution was mixed with Na_2SO_4, and then chromatographed on a $CaHPO_4$ column using hexane-acetone (10:1) as the developing solvent. The orange red third band from the bottom of the column was eluted, and the residue obtained on evaporation was recrystallized from EtOH.

The labeled erythroskyrine thus obtained was diluted with non-labeled erythroskyrine and purified to get the crystals of a definite radio-activity by repeated recrystallization.

Degradation of ^{14}C-Labeled Erythroskyrine——^{14}C-Labeled erythroskyrine which was obtained using Na acetate-1-^{14}C or -2-^{14}C and diethyl malonate-1-^{14}C or -2-^{14}C as the precursors was degraded by the Kuhn-Roth oxidation followed by the Schmidt reaction.

The radioactive erythroskyrine which was formed from ^{14}C-labeled precursors other than Na acetate-2-^{14}C was hydrogenated and then decomposed into dodecanedioic acid by ozonization.

The Kuhn-Roth Reaction : A mixture of erythroskyrine (100~150 mg.) and Kuhn-Roth reagent (35 ml.) was heated in a reaction apparatus at 130° for 2.5 hr. under N_2-stream. $MgSO_4$ (20 g.) was added, and the reaction mixture was steam-distilled to collect distillate (250 ml.) whose pH was adjusted to 9.0 with *N*/50 NaOH. The residue obtained on evaporation was treated with warm EtOH, and filtered to remove insoluble substance. Sodium acetate obtained on evaporation was treated with conc. H_2SO_4 (2~3 ml.) and NaN_3 (20 mg.), and CO_2 generated was absorbed into $Ba(OH)_2$ solution to obtain $BaCO_3$. The reaction mixture in the flask was poured into water, and steam-distilled after making the mixture alkaline to liberate CH_3NH_2 which was absorbed into dil. HCl. The residue obtained on evaporation was dissolved into a small amount of H_2O. To the solution a few drops of a solution of 2,4-dinitrofluorobenzene (1 g.) in EtOH (25 ml.) and saturated aq. $NaHCO_3$ (2 ml.) were added, and the mixture was stirred for 2 hr. 2,4-Dinitrophenylmethylamine (=N-methyl-2,4-dinitroaniline) was separated which was recrystallized from $CHCl_3$-*n*-hexane.

4) Van Slyke, *et al.* : J. Biol. Chem., **191**, 299 (1951).

Ozonolysis of Decahydroerythroskyrine——Erythroskyrine (500 mg.) dissolved in EtOH (30 ml.) was hydrogenated using PtO_2 (100 mg.) as a catalyst to absorb 5 moles of H_2 for 30 min. The almost colorless reaction mixture was evaporated to remain lemon yellow oil (decahydroerythroskyrine), which was dissolved in HOAc (15 ml.) and ozonized for 20 hr. at room temperature. The reaction mixture which was added with H_2O_2 (3.75 ml.) was allowed to stand overnight, and then the solvent was distilled off *in vacuo*.

The residue which was mixed with a small amount of water was distilled *in vacuo*, and this process was repeated twice. 2*N* H_2SO_4 (15 ml.) was added to the residue, and the mixture was heated in a boiling water bath for 8 hr. The syrupy insoluble portion was collected on a filter which was washed with water, then extracted with ether. The ether-soluble portion was treated with CH_2N_2 in ether overnight to form methyl ester.

The methyl ester was dissolved in EtOH (2 ml.) and treated with $NH_2NH_2 \cdot H_2O$ (1～2 ml.) on a boiling water bath for 10 min., to yield crystals of dodecanedioic acid dihydrazide on cooling, which were recrystallized from MeOH to give m.p. 189～190.5° (yield 3.6～2.0%). The H_2SO_4-soluble portion was extracted continuously for 12 hr. using an extractor, and the aqueous layer was passed first through an ion-exchange resin column IR 4B (treated with 5% NaOH) to remove H_2SO_4, and then passed through a column of IRA 120 (treated with 10% HCl) to absorb amino acid which was eluted with 5% NH_4OH. After evaporation of water, the residue which was dissolved in a small amount of water was mixed with 4% alcoholic solution of 2,4-dinitrofluorobenzene (2 ml.) and saturated aq. $NaHCO_3$. The mixture was stirred for 2.5 hr. and alcohol was distilled off. The excess of dinitrofluorobenzene was removed by shaking with ether, and the aqueous layer was acidified with 2*N* HCl and extracted with ether. The ethereal portion was evaporated, and the residue which was dissolved in $CHCl_3$ was chromatographed over a column (50 cm. × 4 cm.) of Celite 545 (90 g.) impregnated with McIlvain buffer solution pH 6.9 (60 ml.) using $CHCl_3$ as a developing solvent. From the yellowish second band N-(2,4-dinitrophenyl)-N-methylvaline (m.p. 192～194°) was obtained, which was recrystallized from a mixture of $CHCl_3$ and *n*-hexane (yield 5.4%).

Ozonolysis of Erythroskyrine——Erythroskyrine (280 mg.) which was obtained by feeding DL-valine-1-^{14}C to the mold was dissolved in HOAc (10 ml.), and ozonized to furnish N-(2,4-dinitrophenyl)-N-methylvaline (38 mg.).

Photochemical Degradation of N-(2,4-Dinitrophenyl)-N-methylvaline——N-(2,4-Dinitrophenyl)-N-methylvaline (87 mg.) obtained from erythroskyrine was illuminated with ultraviolet light in a solution of Na_2HPO_4 (1.5 g.) in H_2O (100 ml.) under N_2-stream for 19.5 hr. Isobutyraldehyde was introduced into 2,4-dinitrophenylhydrazone which was recrystallized from EtOH to give m.p. 184° (yield 10.7 mg.).

Results and Consideration

The incorporation of radioactivity of ^{14}C into erythroskyrine of *Penicillium islandicum* Sopp strain S fed with the ^{14}C-labeled precursors are shown in the Tables Ⅱ～Ⅵ.

Table Ⅱ. Incorporation of Radioactivity from DL-Valine-1-^{14}C into Erythroskyrine

	d.p.m./mmole	%	Theoretical value (%)
Erythroskyrine	3.3×10^5	100	100
N-(2,4-Dinitrophenyl)-N-methylvaline	2.6×10^5	79	100
Isobutyraldehyde 2,4-dinitrophenylhydrazone	0	0	0

Table Ⅲ. Incorporation of Radioactivity from NaAcetate-1-^{14}C into Erythroskyrine

	d.p.m./mmole	%	Theoretical value (%)
Erythroskyrine	3.8×10^5	100	100
N-(2,4-Dinitrophenyl)-N-methylvaline	4.3×10^3	1.1	0
$BaCO_3$	9.0×10^3	2.4[a)]	5
N-Methyl-2,4-dinitroaniline	4×10^2	0	0
Dodecanedioic acid dihydrazide	2.2×10^5	58.0	60

a) Experimental error.

Table IV. Incorporation of Radioactivity from NaAcetate-2-^{14}C into Erythroskyrine

	d.p.m./mmole	%	Theoretical value (%)
Erythroskyrine	1.1×10^{6}	100	100
$BaCO_3$	0	0	0
N-Methyl-2,4-dinitroaniline	4.9×10^{4}	4.45	5

Table V. Incorporation of Radioactivity from Diethyl Malonate-1-^{14}C into Erythroskyrine

	d.p.m./mmole	%	Theoretical value (%)
Erythroskyrine	4.9×10^{5}	100	100
N-(2,4-Dinitrophenyl)-N-methylvaline	8.4×10^{3}	1.7	0
$BaCO_3$	9.0×10^{3}	1.9	0
N-Methyl-2,4-dinitroaniline	3×10^{2}	0	0
Dodecanedioic acid dihydrazide	3.3×10^{5}	71[a)]	66.7

Table VI. Incorporation of Radioactivity from Diethyl Malonate-2-^{14}C in Erythroskyrine

	d.p.m./mmole	%	Theoretical value (%)
Erythroskyrine	2.2×10^{5}	100	100
N-(2,4-Dinitrophenyl)-N-methylvaline	5.9×10^{3}	2.7	0
$BaCO_3$	8.8×10^{2}	0	0
N-Methyl-2,4-dinitroaniline	4.3×10^{3}	2.0	0
Dodecanedioic acid dihydrazide	1.3×10^{5}	63.5[a)]	66.7

Note to Table V and VI.
a) The ratio of radioactivity of dodecanedioic acid hydrazide and the value, erythroskyrine-(valine +$BaCO_3$+2,4-DNP-methylaniline).

N-(2,4-Dinitrophenyl)-N-methylvaline which was obtained by the degradation of erythroskyrine derived from DL-valine-1-^{14}C showed 79% of total activity, while isobutyraldehyde 2,4-dinitrophenylhydrazone afforded by the photolysis of N-methylvaline gave no radioactivity. Therefore, the ^{14}C-radioactivity was located in $C_{(4)}$ of erythroskyrine. Thus valine was introduced as a whole in the lactam ring portion of the compound.

Erythroskyrine isolated from the mold fed with acetate-1-^{14}C, malonate-1-^{14}C, and malonate-2-^{14}C, respectively, afforded dodecanedioic acid giving respective activity 58.0% (6/10), 71% (6/9) and 63.5% (6/9) of the total radioactivity of erythroskyrine.

The activity of $C_{(26)}$ of erythroskyrine isolated from the mold fed with acetate-2-^{14}C was shown to be 4.45% of the total activity by the degradation product, N-methyl-2,4-dinitroaniline, which was yielded by the Kuhn-Roth oxidation followed by the Schmidt reaction.

Thus it has been proved that erythroskyrine is biosynthesized by the condensation of valine and a polyketide moiety. According to the result of present experiment, it would be probable that $C_{(2)}$ and $C_{(3)}$ are originated from malonate, though there has been considered another possibility of the acetate incorporation.

Consequently, it has been concluded that erythroskyrine is biosynthesized from 1 acetate (for $C_{(25)}$ $C_{(26)}$), 9 malonate (for $C_{(2)(3)(9\sim24)}$) and valine (for $N_{(1)}$ $C_{(4\sim8)}$). However,

there remained still two possibilities of biosynthesis condensing (A) C_{20}-polyketide and valine or (B) C_{18}-polyketide and a 5 membered ring which is formed from valine and 1 malonate as shown in the case of tetronic acid biosynthesis.[5]

A small incorporation of ^{14}C in $C_{(25)}$ and $C_{(26)}$ (ca. 2%) in the experiment using diethyl malonate-1-^{14}C and -2-^{14}C would be resulted by the decarboxylation of malonate into acetate.

The relatively small incorporation of ^{14}C into $C_{(25)}$ in the experiment using acetate-1^{14}-C would be caused by some error in experimental technique.

Stickings and Townsend[6] reported on the biosynthesis of tenuazonic acid which is closely related with erythroskyrine in structure to prove the incorporation of acetate.

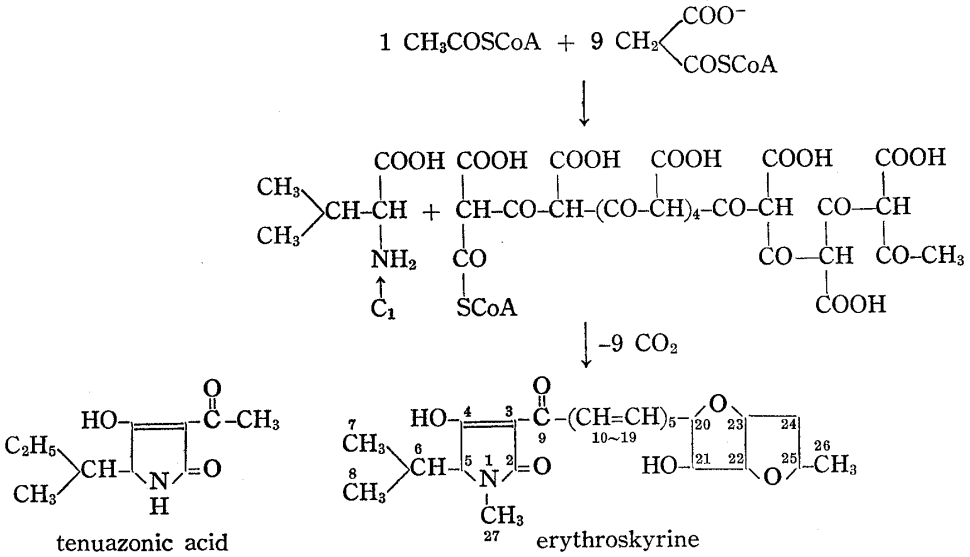

Chart 2.

We are grateful to Dr. J. Shoji who was in this laboratory at that time of this investigation, now in Showa University, for his kind co-operation. Thanks are also due to Dr. H. Tsunoda, Food Research Institute, Tokyo, for supply of mold culture.

Summary

Erythroskyrine, a nitrogen containing coloring matter of *Penicillium islandicum* Sopp is biosynthesized from 1 acetate (for $C_{(25)(26)}$) 9 malonate (for $C_{(2)(3)(9\sim24)}$) and valine (for $N_{(1)}$ $C_{(4\sim8)}$).

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5) R. Bentley, *et al.*: J. Biol. Chem., **237**, 859 (1962).
6) C. E. Stickings, R. S. Townsend: Biochem. J., **78**, 412 (1961).