54. Shoji Shibata, Takao Murakami, Osamu Tanaka, Goro Chihara, and Masashi Sumimoto: Metabolic Products of Fungi. IV.* Isolation of the Coloring Matters of Endothia Spp. and the Respective Identities of Endothianin and Redicalisin with Skyrin and Rugulosin.

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In earlier literatures, some descriptions[1~4] have been given concerning the coloring matters produced by *Endothia parasitica* (Murr.) Anderson et Anderson, a plant pathogenic fungus which is known as the organism having caused a serious epidemic disease, chestnut-blight, in the United States at the beginning of this century. However, none elucidated the chemistry of this pigment.

Recently we obtained two crystalline coloring matters from the mycelia of laboratory-cultivated *Endothia parasitica* (Murr.) Anderson et Anderson and *Endothia fluens* Shear et Stevens (syn. *E. radicalis* Fr.).

One of them, which we named endothianin, is an orange pigment with a high melting point and anthraquinone-like properties, while the other, a yellow pigment named radicalisin, is acidic and optically active and does not give any characteristic reaction of the anthraquinone derivative.

Previously we wrote a brief preliminary report on the experimental results on these two pigments.[5] At that time, we proposed a tentative molecular formula, $C_{27}H_{18}O_9$, for endothianin, and showed that the analyses of radicalisin favored $C_{30}H_{22}O_{10}$ (a possibility of $C_{30}H_{24}O_{10}$ is not ruled out).

Shortly after appearance of our preliminary report on the Endothia pigments, one of us (Sh.) visited Professor Raistrick's laboratory in London School of Hygiene & Tropical Medicine and at that time he noted the similarity between endothianin and skyrin, a coloring matter of *Penicillium islandicum* Sopp, and between radicalisin and rugulosin, a pigment isolated from *Penicillium rugulosum* Thom, in all points of their properties. Both pigments of *Penicillium* mentioned above were isolated and studied by Raistrick and his co-workers, but at that time no report had yet been published. We are very much indebted to Professor Raistrick and Dr. Howard for their kindness in having shown the unpublished manuscripts and the records of experiments to one of us (Sh.) in London.

Reëxamination of the Endothia pigments in our laboratory led us to reach the conclusion that endothianin and radicalisin would undoubtedly be respectively identical with skyrin and rugulosin.

The identity of endothianin with skyrin was established by a mixed fusion of its ethoxycarbonyl ether, m.p. 171~173°, which is the only derivative of endothianin ever obtained showing a definite melting point. Comparison of the Rf values of endothianin and skyrin on the paper chromatogram and the infrared absorption spectra of the acetates also agreed with this conclusion.

To establish the identity of radicalisin with rugulosin, mixed fusion of their benzoates, m.p. 224~226°, and methyl ethers, m.p. 279~280°, and the comparison of

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1) Pantanelli: Rend. Acad. Lincei Roma, Sci., **20**, 366(1911).
2) Anderson: Bull. Penn., chestnut tree blight Comm., **7**, 1(1913).
3) A. Hawkins, N. E. Stevens: Am. J. Botany, **4**, 336(1917).
4) C. E. Sando: *Ibid.*, **6**, 242(1919).
5) S. Shibata, O. Tanaka, G. Chihara, H. Mitsuhashi: This Bulletin, **1**, 302(1953).

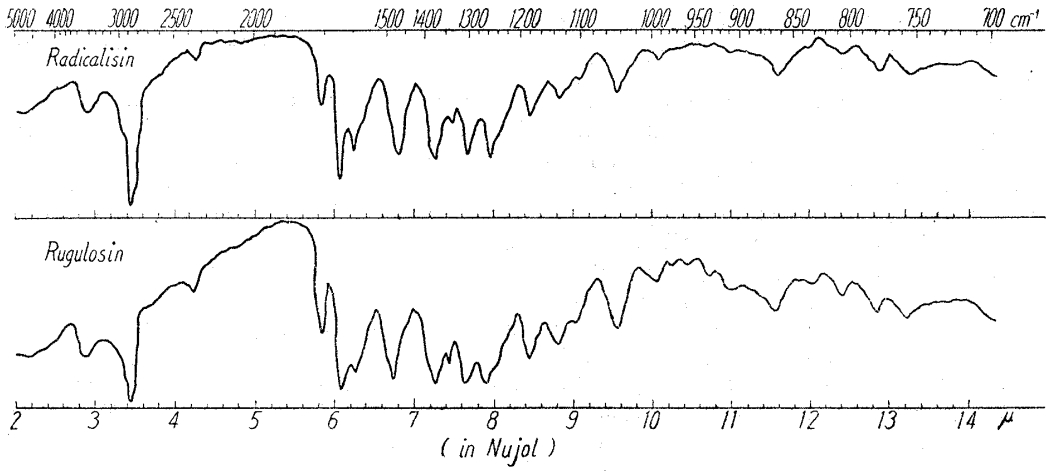

Fig. 1

the infrared spectra were undertaken (Fig. 1).

The works on skyrin[6] and rugulosin[7] have thereafter been published by Raistrick and his co-workers. As for skyrin they proposed a molecular formula, $C_{30}H_{18}O_{10}$, and a bianthraquinone structure consisting of two molecules of emodin, though no satisfactory evidence for the position of linkage of each emodin moiety was given.

By reëxamination of our analytical figures of endothianin (=skyrin), we agree with molecular formula of skyrin proposed by Howard and Raistrick. We therefore withdraw the names, endothianin and radicalisin, to avoid confusion of the literature.

We are greatly indebted to Prof. H. Raistrick, Dr. B. H. Howard and Mr. G. Smith, London School of Hygiene & Tropical Medicine, for their kind help in providing laboratory facilities for one of us (Sh.), giving encouragement and all covenience to pursue this work, and supplying the strain of *P. rugulosum* Thom and the samples of the mold pigments which were used for this part of work. We thank Mr. K. Aoshima, Government Forest Experiment Station, for supplying the strains of *Endothia parasitica* and *E. fluens* and giving advices in mycological problem. The infrared spectral analyses were carried out by Mr. S. Tanaka, Dept. Applied Chemistry of this University and the microanalyses were made by the members of the Microanalytical Laboratories of the Institute for Infectious Diseases, Institute for Health, and this Institute, and by Mr. D. Ohata, Iatrochemical Institute, to all of whom our thanks are due.

Experimental

Strains—The strains of *Endothia parasitica* (Murr.) Anderson et Anderson and *Endothia fluens* Shear et Stevens (Syn. *E. radicalis* Fr.) used in this work were received in April, 1950, from Mr. K. Aoshima, Government Forest Experiment Station, Tokyo. A strain of *Penicillium rugulosum* Thom was received in June, 1954, from Professor H. Raistrick and Mr. G. Smith, London School of Hygiene & Tropical Medicine.

Cultural Conditions—Potato extracts were added with D-glucose in 3% concentration and the pH of the solution was adjusted to 5.0~5.2 with citric acid. The medium (150 cc. in each flask) was sterilized and inoculated with the strain of Endothia sp., then incubated for 3~4 weeks at 25°, when the growth of the mycelium almost completed, producing a highly orange-colored pigments. The yield of dry mycelium was 1.5 g./flask.

The strain of *Penicillium rugulosum* Thom was incubated for 30 days at 25° in Czapek-Dox solution (150 cc./flask). The yield of dry mycelium was 1.5 g./flask.

Isolation of the Pigments—The dried yellow orange mycelium of Endothia sp. was continuously extracted with ether. During the extraction orange crystals separated out which were collected by filtration and recrystallized from pyridine. The red crystals of pyridine-addition compound obtained were boiled in EtOH or acetone when orange crystals of endothianin (=skyrin) separated out. About 1 g. of endothianin was obtained from 100 g. of the dried mycelium. The ethereal extract separated from skyrin crystals was shaken with aq. 5% $NaHCO_3$ solution from which

6) B. H. Howard, H. Raistrick: Biochem. J., **56**, 56(1954).
7) J. Breen, J. C. Dacre, H. Raistrick, G. Smith: Biochem. J., **60**, 618(1955) (by private communication); cf. H. Raistrick: Proc. Roy. Soc., A **199**, 158(1945).

orange yellow precipitate was obtained by acidification. The precipitate was washed with water, dried, and washed again with a small amount of acetone or MeOH to remove accompanying endothianin.

The pigment that remained (radicalisin=rugulosin) was recrystallized from acetone or EtOH, or a mixture of acetone and MeOH. Yellow prisms were obtained from acetone solution and cubes from EtOH solution. One g. of radicalisin was obtained from 100 g. of the dried mycelia.

The dried mycelium of *Penicillium rugulosum* Thom (600 g.) was extracted with ether. During the extraction, orange crystals that separated out and were collected by filtration, washed with a small amount of acetone or MeOH, and recrystallized from acetone, EtOH or acetone-MeOH mixture. Rugulosin forms yellow prisms from acetone and cubes from alcoholic solution. Yield: 30 g. (15% of the weight of the dry mycelium).

Properties of Endothianin (Skyrin)—Endothianin darkens from about 200° and does not melt below 360°. It is insoluble or sparingly soluble in all organic solvents except hot pyridine. It forms red pyridine-addition product from which free endothianin is regenerated by boiling with acetone or EtOH. It is insoluble in $NaHCO_3$ solution but soluble in aq. Na_2CO_3, NH_4OH, and caustic alkali giving a deep purple solution. It gives a red coloration with conc. H_2SO_4 which changes to an emerald green color within a few seconds. With $Mg(OAc)_2$ in EtOH solution endothianin gives a red coloration similar to emodin.

On heating in 40% caustic alkali for 1 hr., endothianin is recovered unchanged, whereas on treatment with strong mineral acids, it is readily converted into a resinous substance.

Endothianin shows an Rf value (0.42) on the paper chromatogram developed by a mixture of acetone: petroleum benzine (b.p. 40~60°): water (5 : 5 : 3.5) at 18~20°. It gives no optical rotation in a saturated solution in acetone. *Anal.* Calcd. for $C_{30}H_{18}O_{10}$: C, 66.91; H, 3.34. Found: C, 66.86, 66.58; H, 3.49, 3.62; OCH_3, nil.

Hexaacetylendothianin (Hexaacetylskyrin)—The acetate prepared from endothianin with Ac_2O and pyridine under ice-cooling was recrystallized from glacial HOAc or acetone-EtOH mixture to yellow needles, m.p. 295~296°(decomp.).* It is sparingly soluble in EtOH and readily in $CHCl_3$. It does not dissolve in cold caustic alkali, whereas on standing it forms gradually a deep purple solution from which endothianin is given on heating. In conc. H_2SO_4 it gives a red coloration which changes to emerald green within a few mins. The infrared spectrum of hexaacetylendothianin coincides with that of hexaacetylskyrin. *Anal.* Calcd. for $C_{42}H_{30}O_{16}$: C, 63.79; H, 3.79; $6\,CH_3CO$, 32.7. $C_{42}H_{30}O_{16}\cdot\frac{1}{2}\,H_2O$: C, 63.01; H, 3.88; $6\,CH_3CO$, 32.2. Found: C, 63.37, 63.16; H, 4.32, 4.01; CH_3CO, 32.4.

Hexamethylendothianin (Hexamethylskyrin)—A mixture of endothianin, dried acetone, Me_2SO_4, and anhyd. K_2CO_3 was boiled under reflux for 20 hrs. Water was added to the reaction mixture and acetone was distilled off. The yellow precipitate was dissolved in $CHCl_3$ and chromatographed on an alumina column using a mixture of acetone and $CHCl_3$ (1 : 1) as a developing solvent. The partially methylated compound was adsorbed on the upper part of the column and hexamethyl ether of endothianin was isolated from the orange yellow eluate. It was crystallized from aq. HOAc to orange yellow needles, m.p. ca. 350°(decomp.).

It is soluble in $CHCl_3$, nitrobenzene, pyridine and hot glacial HOAc. It gives a red solution with conc. H_2SO_4, the color of which does not change on standing. Optical activity was not observed in $CHCl_3$ solution. *Anal.* Calcd. for $C_{36}H_{30}O_{10}$: C, 69.45; H, 4.82; $6\,CH_3O$, 29.9; mol wt., 622. Found: C, 69.24; H, 5.00; CH_3O,** 23.19, 23.40; mol. wt. (Akiya-Berger method), 625.

Endothianin Hexaethoxycarbonyl Ether (Skyrin Hexaethoxycarbonyl Ether)—Endothianin was reacted with $ClCO_2Et$ and caustic alkali in acetone under cooling with a freezing mixture. The product was crystallized from MeOH or BuOH to yellow needles, m.p. 171~173°. *Anal.* Calcd. for $C_{48}H_{42}O_{22}$: C, 59.38; H, 4.33. Found: C, 59.66; H, 4.27.

Skyrin hexaethoxycarbonyl ether prepared under the same conditions melts at 171~173° and a mixed fusion with hexaethoxycarbonyl ether of endothianin showed no melting point depression.

Properties of Radicalisin(=Rugulosin)—Radicalisin as prepared above melts at 290°(decomp.). It is soluble in acetone, EtOH, MeOH, EtOAc, glacial HOAc, and pyridine, and very slightly soluble in hot benzene, $CHCl_3$, and petroleum benzine.

It dissolves in aq. $NaHCO_3$ solution to give a yellow solution, while the sodium salt is rather sparingly soluble in water. It gives a yellow solution with cold conc. H_2SO_4, the color of which changes to red by warming in a boiling bath. It exhibits a dark greenish brown coloration with $FeCl_3$ solution. *Anal.* Calcd. for $C_{30}H_{22}O_{10}$: C, 66.42; H, 4.06; mol. wt., 542. $C_{30}H_{24}O_{10}$: C, 66.17;

* m.p. 240°(decomp.) was recorded in the preliminary report. It seemed that the depression of the melting point is caused by the high sensibility of this compound against alkali contamination in the capillary glass.

** Methoxyl value found is lower than that calculated, since β-methoxyl of endothianin(=skyrin) strongly resists demethylation.

H, 4.44; mol. wt., 544. Found: C, 66.13, 66.13, 66.09, 66.29; H, 3.95; 4.18, 4.12, 3.99; mol. wt., 562 (determined by X-ray analysis of the crystal: cell const.: a=13.83, b=15.46, c=12.4, 4 molecules per unit cell; space group: D_2^8: $-P_{2_12_12}$, sp. gr.: 1.4). $[\alpha]_D^{19}$: +492°(0.5% in dioxane), $[\alpha]_D^{19}$: +432°(0.5% in acetone). Rugulosin isolated from *Penicillium rugulosum* Thom shows the same melting point and properties as those observed in radicalisin.

Hexaacetylradicalisin (Hexaacetylrugulosin)—A mixture of radicalisin (100 mg.), pyridine (15 cc.), and Ac_2O (8 cc.) was allowed to stand overnight. The reaction mixture was poured on ice. The precipitate formed was crystallized from a mixture of acetone-MeOH to yellowish rhombic crystals, which show no definite melting point but soften at 171°, begin to decompose at about 176°, and completely melt at 182°. $[\alpha]_D^{20}$: +224°(1% in $CHCl_3$). *Anal.* Calcd. for $C_{30}H_{16}O_4(OCOCH_3)_6$: C, 63.47; H, 4.27. calcd. for $C_{30}H_{18}O_4(OCOCH_3)_6$: C, 63.31: H, 4.55. Found: C, 63.18; H, 4.47.

Pentaacetylradicalisin (Pentaacetylrugulosin)—A mixture of radicalisin (100 mg.), Ac_2O (20 cc.), and 2 drops of conc. H_2SO_4 was allowed to stand overnight and then poured on ice. The precipitate formed was recrystallized from a mixture of $CHCl_3$ and petroleum benzine (b.p. 40~60°) to give rhombic crystals. It gives no definite melting point, but softens at 144°, and completely melts at about 180°with decomposition. $[\alpha]_D^{20}$: +210°(0.5% in $CHCl_3$). *Anal.* Calcd. for $C_{30}H_{17}O_5(OCOCH_3)_5$: C, 63.83; H, 4.25. $C_{30}H_{19}O_5(OCOCH_3)_5$: C, 63.66; H, 4.51. Found: C, 63.39; H, 4.78.

Hexabenzoylradicalisin (Hexabenzoylrugulosin)—Radicalisin (100 mg.) dissolved in pyridine (10 cc.) was added with BzCl (3 cc.) under ice-cooling. After standing overnight, ether was added to the reaction mixture and the solution was shaken successively with water and dil. HCl, and finally washed again with water. The solvent was removed and the residue was washed with MeOH, and recrystallized from a mixture of acetone and petroleum benzine (b.p. 60°) to give pale yellow rhombic crystals, m.p. 224~226°. $[\alpha]_D^{18}$: +246°(0.5% $CHCl_3$). It gives no melting point depression by mixed fusion with the benzoate prepared as above from rugulosin obtained from *Penicillium rugulosum* Thom. It is soluble in cold acetone, $CHCl_3$, and benzene, and sparingly soluble in cold MeOH and EtOH. *Anal.* Calcd. for $C_{30}H_{16}O_4(OCOC_6H_5)_6$: C, 74.00; H, 3.94. Calcd. for $C_{30}H_{18}O_4$-$(OCOCH_3)_6$: C, 23.97; H, 4,11. Found (Hexabenzoylradicalisin): C, 73.51; H, 3.63. Found (hexabenzoylrugulosin): C, 73.86, 74.50; H, 4.06, 4.08.

Hexamethylradicalisin (Hexamethylrugulosin)—A mixture of radicalisin (1 g.), CH_3I (50 cc.), and Ag_2O (0.5 g.) was refluxed for 50 hrs. (at every 10 hrs., Ag_2O (0.5 g.) and CH_3I (10 cc.) were added), shielded from light. Radicalisin, which was at first insoluble in CH_3I, dissolved gradually to form a solution as reaction progressed. The reaction mixture, which was separated from Ag_2O, was concentrated to remove CH_3I. Pale yellow crystals separarted out were recrystallized from EtOH to yellow needles which were dissolved again in a mixture of EtOAc and benzene (1 : 5) and chromatographed on an alumina column, developing with the same solvent mixture. The methylated product was separated into three yellow bands on the column and from the first eluate pale yellow needles, m.p. 279~280°, $[\alpha]_D^{18}$: +724°(0.5% in $CHCl_3$), were obtained by recrystallization from EtOH. It gives no melting point depression by admixture with methyl ether of rugulosin, m.p. 279~280°, prepared by the above procedure. *Anal.* Calcd. for $C_{30}H_{16}O_4(OCH_3)_6$: C, 69.01; H, 5.43. Calcd. for $C_{30}H_{18}O_4(OCH_3)_6$: C, 68.78; H, 5.77. Found (Hexamethylradicalisin): C, 69.37; H, 5.92. Found (Hexamethylrugulosin): C, 69.10; H, 5.95.

Summary

Endothianin isolated from *Endothia parasitica* (Murr.) Anderson et Anderson and *E. fluens* Shear et Stevens (syn. *E. radicalis* Fr.) was proved to be identical with skyrin, $C_{30}H_{18}O_{10}$, which was obtained from some strains of *Penicillium islandicum* Sopp. The identity was established by admixture of tetraethoxycarbonyl ethers (m.p. and mixed m.p. 171~173°) of both pigments.

Radicalisin, $C_{30}H_{22}O_{19}$ or $C_{30}H_{24}O_{10}$, isolated from the above Endothia sp. along with endothianin was identified with rugulosin produced by *Penicillium rugulosum* Thom by the comparison of the infrared spectra and by a mixed fusion of their hexabenzoates (m.p. and mixed m.p. 224~226°) and hexamethyl ethers (m.p. and mixed m.p. 279~280°).

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