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On the Biosynthesis of the Pigments of *Penicillium islandicum*. II

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The acetate origin of islandicin from *P. islandicum* is shown by degradation after feeding the mould with ^{14}C-carboxyl labelled acetate, resulting in a strict alternate labelling of the anthracen structure.

By feeding the mould with ^{18}O-labelled acetate, three hydroxyl groups in islandicin and four in emodin are shown to be derived from the carbonyl group of acetic acid. Furthermore, the incorporation of ^{18}O in the secondary alcoholic groups of rubroskyrin indicates their formation from keto groups, originating from acetate, by hydrogenation.

The acetate origin of skyrin from *Penicillium islandicum* has been demonstrated by the incorporation of ^{14}C-labelled acetate in the molecule[1]. The ^{14}C-distribution in skyrin was experimentally established by reductive cleavage of the dianthraquinone to emodin. Oxidation of emodin to acetic acid according to Kuhn and Roth and hypobromite degradation of tetranitroemodin to bromopicrin left the radioactivities of six C-atoms out of fifteen to be measured directly. The isotope distribution of the remaining C-atoms was calculated by means of the acetate theory and the experimentally determined localizations of ^{14}C-atoms. In spite of the agreement with the theory it was desirable to complete the experiment with an investigation of a genuine anthraquinone and to make a more extensive degradation of the molecule.

Islandicin, which occurs in dominant amounts in the pigments of *P. islandicum*, was chosen as starting material. The degradation of islandicin, isolated from the mould after growing on a Czapek-Dox medium containing ^{14}C-carboxyl labelled acetate, was made according to the reaction series outlined in Fig. 1.

In the reaction series I, ring A of islandicin was obtained as 3-hydroxyphthalic acid by oxidation with hydrogen peroxide in alkali. Decarboxylation of 3-hydroxyphthalic acid yielded *m*-hydroxybenzoic acid, and the carboxyl group in position 2 as carbon dioxide. The radioactivity of the carbon dioxide, measured as barium carbonate, was 1/4 of the total radioactivity of 3-hydroxyphthalic acid. The carboxyl group in position 1 of 3-hydroxyphthalic acid

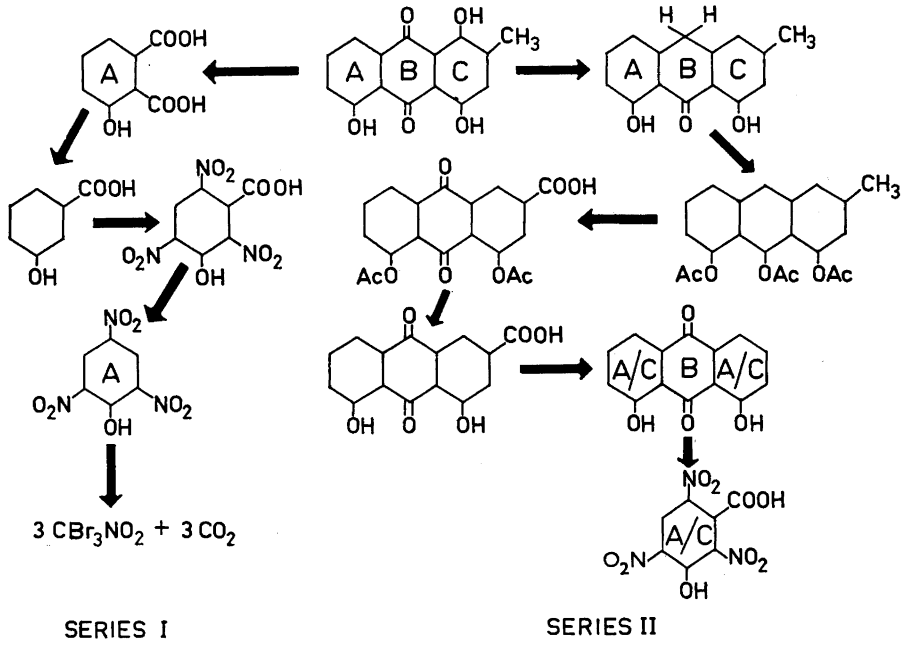

Fig. 1. Scheme of degradation of islandicin.

obtained as carbon dioxide by decarboxylation of the trinitro derivative of *m*-hydroxybenzoic acid, was shown to be nonradioactive. By treating the remaining picric acid with a barium hypobromite solution, the carbon atoms in the 2-, 4-, and 6-positions of the 3-hydroxyphthalic acid were obtained as bromopicrin which was nonradioactive. However, the barium carbonate formed during the reaction, and representing the carbon atoms in the 1-, 3-, and 5-positions, contained the other 3/4 of the total radioactivity of 3-hydroxyphthalic acid.

The ^{14}C-isotope distribution in 3-hydroxyphthalic acid obtained by oxidative cleavage of islandicin is apparently the same as that in the 3-hydroxyphthalic acid formed *in vivo* by *P. islandicum* [2].

On the presumption that the methyl group of islandicin is nonlabelled, which is later confirmed by the experiment, the specific radioactivities of the 3-hydroxyphthalic acid and the islandicin (2.37×10^4 cpm/mg C and 2.23×10^4 cpm/mg C, resp.) indicate that the radioactivity is equally distributed between the A and C rings of islandicin.

In the reaction series II, islandicin was reduced with hydriodic acid to chrysophanol anthron according to Howard and Raistrick [3]. Oxidation of the triacetyl derivative of chrysophanol anthron with chromic anhydride left diacetylrhein. After hydrolysis of the acetyl derivative, the rhein formed

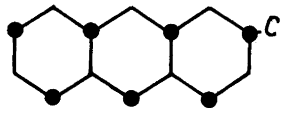

Fig. 2. ^{14}C-Distribution in anthraquinones derived from ^{14}C-carboxyl labelled acetate.

was decarboxylated and the evolved carbon dioxide shown to be nonradioactive. The symmetrical anthraquinone, chrysazin, obtained during the decarboxylation was oxidized with fuming nitric acid to trinitro-*m*-hydroxybenzoic acid. The benzene ring of this substance represents a statistical mixture of the A and C rings of islandicin. The further degradation of trinitro-*m*-hydroxybenzoic acid was performed with the same procedures as described for the degradation of 3-hydroxyphthalic acid. The ^{14}C-distribution in the benzene ring, *i.e.* the rings A + C of islandicin, was identical with that of ring A.

Combination of the ^{14}C-distributions obtained from the two reaction series gives a strict alternate labelling of the carbon atoms all over the molecule of islandicin, as shown in Fig. 2.

As shown in an earlier paper [4] ^{18}O-labelled acetic acid is an available tool for confirmation of the acetate origin of phenolic substances produced by moulds. In some cases it should also be possible to distinguish in the phenolic compound between the oxygen atoms derived from the carbonyl groups of the acetyl units and the oxygen atoms secondarily introduced.

To obtain some information of the character of the oxygen atoms in the polyhydroxyanthraquinone derivatives produced by *P. islandicum*, the mould was grown as above with acetic acid labelled with ^{14}C and ^{18}O in the carboxyl group as the tracer substrate.

Islandicin, skyrin and rubroskyrin were isolated from the mycelium. The radioactivity and the ^{18}O-content of islandicin and emodin, obtained by cleavage of skyrin, were determined.

As seen from Table 3, islandicin and emodin have approximately the same radioactivity, indicating a steady state of the ^{14}C metabolism. Under this condition the ^{18}O-content of the oxygen containing groups originating from the carboxyl group of acetic acid should be the same in islandicin and emodin.

Guided by the acetate theory, the positions of the ^{18}O-labelled groups in phenolic substances could be deduced. Islandicin will theoretically contain three ^{18}O-labelled positions (4, 5 and 10); emodin four labelled positions (4, 5, 7 and 10).

The average amounts of ^{18}O in emodin and islandicin were 0.82 atom% and 0.64 atom%, resp. By calculating the ^{18}O-content in the expected labelled positions, 1.02 atom% (emodin) and 1.07 atom% (islandicin) are obtained. The coincidence of these two values is a support of the validity of the theoretically calculated number of labelled positions in the two substances.

The localization of the labelled oxygen in emodin and islandicin is not established by the ^{18}O-analysis. Emodin anthron and chrysophanol anthron were prepared from the anthraquinones by refluxing with hydriodic acid. The two anthrons should theoretically contain only the labelled oxygen groups but ^{18}O-analysis of the two compounds, however, showed that the ^{18}O groups were interchanged with the nonlabelled aqueous medium during the reaction.

The isolated rubroskyrin was thermally decomposed at 270°C whereby the secondary alcoholic groups of rubroskyrin are split off as water. The ^{18}O-analysis of the water (0.54 atom%) indicated that the alcoholic groups originate from acetic acid and are formed from carbonyl groups by hydrogenation at some stage during the biosynthesis of rubroskyrin. The rather low value of ^{18}O compared with the expected value (1.05 atom%) taken from the ^{18}O-analysis of the anthraquinones, could be explained with a faster exchange of a secondary alcoholic group than of a phenolic hydroxyl group.

In column 7 of Table 3 are given the dilution ratios of ^{18}O and ^{14}C in the substances studied. The ratios 1 obtained for emodin and islandicin show that the activation of exogenous acetate and the formation of these anthraquinones are extremely rapid processes in *P. islandicum*.

Degradation of islandicin

EXPERIMENTAL

The culture conditions and the isolation of the islandicin used are described elsewhere [2].

Reaction series I. 104.5 mg of labelled islandicin were dissolved in 25 ml of 1 N sodium hydroxide. The solution was heated on a steam bath and an aqueous solution of 30 % hydrogen peroxide added dropwise until the islandicin solution was colourless. After cooling, the solution was acidified with hydrochloric acid and extracted exhaustively with ether. The residue, after evaporation of the ether solution, was developed on a paper chromatogram with chloroform-methanol-formic acid (4 %) (10:1:1) as the solvent. The

Table 1. ^{14}C-Distribution in 3-hydroxyphtalic acid obtained by oxidation of islandicin.

Material	Numbers of C-atoms from 3-hydroxy-phthalic acid	Radio-activity cpm	Total radio-activity of the material cpm	Number of ^{14}C-labelled positions	
				Found	Calculated
1	2	3	4	5	6
3-Hydroxyphthalic acid (total comb.)	8	483	3 864	3.99	4
COOH-Group (2-position)	1	960	960	1	1
m-Hydroxybenzoic acid (total comb.)	7	405	2 835	3.06	3
COOH-Group (1-position)	1	61	61	0.06	0
Bromopicrin (C-atoms from 2,4,6-positions)	3	0	0	0	0
CO_2 from the hypobromite degradation (C-atoms from 1,3,5-positions)	3	920	2 760	2.89	3

The numbers of ^{14}C-labelled positions in column 5 are obtained by taking the radio-activity of the COOH-group from 2-position as the basis for the calculations. This COOH-group is supposed to be derived directly from the COOH-group in acetic acid.

region of the paper containing 3-hydroxyphthalic acid (R_F *ca.* 0.15) was cut out and the phthalic acid extracted with ethanol. The yield of 3-hydroxyphthalic acid, determined spectrophotometrically (ε_{333} 1 665) was 60 %. The ethanolic solution was evaporated to dryness at room temperature and the specific radioactivity of the 3-hydroxyphthalic acid determined (2.37×10^4 cpm/mg C). To the labelled compound was added 1.5 g of non-labelled 3-hydroxyphthalic acid and the mixture recrystallized to constant radioactivity from water.

The degradation of the 3-hydroxyphthalic acid and the radioactivity measurements (Table 1) were performed as described in a previous paper [2].

Reaction series II. A mixture of 150 mg of islandicin, 3 ml of glacial acetic acid, 150 mg of red phosphorus and 0.6 ml of hydriodic acid (*d*, 1.67) was refluxed, with stirring, for 5 h. After filtration of the hot solution, chrysophanol anthron crystallized on cooling. Recrystallization from glacial acetic acid yielded 53 mg (40 %) of chrysophanol anthron, m. p. 206°C.

A mixture of the labelled anthron and 705 mg of nonlabelled chrysophanol anthron was recrystallized to constant radioactivity. After combustion of the anthron according to van Slyke and Folch [5], the radioactivity of the carbon dioxide was measured as barium carbonate (the same amount of barium carbonate (15 mg) was used throughout this work for the radioactivity measurements).

Acetylation of the anthron was made with the method described by Naylor and Gardner [6]. A mixture of 500 mg of the chrysophanol anthron, 11 ml of acetic anhydride, and 560 mg of anhydrous sodium acetate was boiled for 5 h and then poured onto crushed ice, and the precipitate formed filtered off and recrystallized from glacial acetic acid. Yield of triacetylchrysophanol anthron: 360 mg (47 %), m. p. 237°C.

The triacetylchrysophanol anthron was oxidized to diacetylrhein according to Hesse [7] by dissolving in a solution of 4 ml of glacial acetic acid and 4 ml of acetic anhydride, and heating with a solution of 750 mg of chromic anhydride in 5 ml of glacial acetic acid at 70°C for 4 h. After addition of water and boiling for a while in order to decompose the acetic anhydride, the precipitate formed was filtered off and redissolved in 1 N sodium carbonate without further purification and the unreacted triacetylchrysophanol anthron separated by filtration. The sodium carbonate solution was acidified with hydrochloric acid and the diacetylrhein sucked off and hydrolyzed with 1 N sodium hydroxide by

Table 2. ^{14}C-Distribution in islandicin.

Material	Number of C-atoms	Radio-activity cpm	Total radio-activity of the material cpm	Number of ^{14}C-labelled positions	
				Found	Calculated
1	2	3	4	5	6
Islandicin (total comb.)	15	97	1 455	7	7
Chrysazin (total comb.)	14	104	1 456	7	7
COOH-Group (rhein)	1	1	1	0	0
COOH-Group (trinitro-*m*-hydroxy-benzoic acid)	1	13	13	0.06	0
Picric acid (total comb.)	6	104	624	3.00	3
Bromopicrin	3	0	0	0	0
CO_2 from the hypobromite degradation	3	190	570	2.74	3

The numbers of ^{14}C-labelled positions in column 5 are obtained by placing seven theoretically labelled positions in chrysazin as basis for the calculations.

Table 3. ^{18}O and ^{14}C analysis.

Material	Radio-activity (total) cpm	Radio-activity of labelled C cpm	Average ^{18}O-content atom %	Theoretical number of ^{18}O-labelled positions	Calculated ^{18}O-content of labelled positions	Dilution ^{18}O *versus* ^{14}C
Sodium acetate	47 600	95 200	21.6			1
Islandicin	2 375	5 089	0.64	3	1.07	1.08
Emodin	2 300	4 928	0.82	4	1.02	1.09
Rubroskyrin			0.54 (the alcoholic group)			

boiling for 15 min. The rhein was isolated after acidification of the solution and recrystallized from glacial acetic acid. Yield 160 mg (57 %), m. p. 320°C.

The rhein was decarboxylated by heating with 5 ml of pure quinoline and 100 mg of copper chromite (prepared according to the method of Adkins and Connor [8]) at 220°C for 2 h. The radioactivity of the carbon dioxide evolved was measured as barium carbonate. After cooling, the reaction mixture was treated with excess of dilute hydrochloric acid, boiled for a few minutes and extracted with ether. The residue, after evaporation of the ether, consisting of chrysazin, was recrystallized from dilute acetic acid. Yield 128 mg (95 %), m. p. 196°C.

The labelled chrysazin was recrystallized together with 2.0 g of nonlabelled chrysazin from glacial acetic acid and the radioactivity determined after combustion as above.

The chrysazin was refluxed with nitric acid (*d*, 1.52) for 7 h and then evaporated to dryness on the steam bath. A filtered water solution of the residue was exhaustively extracted with ether after acidification with hydrochloric acid. Evaporation of the ether phase, containing trinitro-*m*-hydroxybenzoic acid and very small amounts of picric acid, and recrystallization of the residue from dilute sulfuric acid yielded 700 mg of trinitro-*m*-hydroxybenzoic acid, m. p. 186°C.

The ^{14}C-distribution in the trinitro-*m*-hydroxybenzoic acid was experimentally established with the same methods used for the degradation of 3-hydroxyphthalic acid.

The values of the radioactivities in Table 2 are transformed to the same level of dilution.

Analysis of ^{18}O-incorporation

^{18}O-Labelled sodium acetate was prepared by the reaction of 294 mg of water (containing 90 atom% ^{18}O) with an equivalent amount of acetyl chloride at 0°C. The acetic acid and the remaining hydrochloric acid formed were neutralized with 1 N sodium hydroxide. 16.8 mg (0.1 mC) of ^{14}C-carboxyl labelled sodium acetate were added and a small volume withdrawn for isotope analyses. The radioactivity of the dried substance was made after combustion as above, and the ^{14}O content measured after cracking as described earlier [4].

The culture conditions were the same as previously mentioned but with the doubly labelled acetate as the tracer substrate.

After drying at room temperature, the mycelium was extracted in a Soxhlet apparatus with petroleum ether (b. p. 40—60°C) for 3 h, and then with acetone until the extraction solvent was almost uncoloured. The acetone solution was evaporated to dryness, and the pigments separated in a column of silica gel with chloroform as solvent. Islandicin and iridoskyrin together with small amounts of lipids followed the front and a mixture of skyrin and rubroskyrin was eluted by the addition of 10 % acetone to the chloroform.

The islandicin was isolated from its evaporated fraction by sublimation at 150—160°C in vacuum and recrystallization from chloroform. Yield 115 mg, m. p. 218°C.

Skyrin and rubroskyrin were separated in a second silica gel column with ether as the solvent. Emodin, obtained by cleavage of skyrin with sodium dithionite according to Howard and Raistrick [9], was purified by sublimation at 180—190°C in vacuum and recrystallization from toluene. Yield 55 mg, m. p. 257°C.

The radioactivities and ^{18}O contents of islandicin and emodin were determined by the methods described above.

The fraction containing rubroskyrin was developed on a paper chromatogram (Whatman 3 MM) with *iso*propylether, saturated with water, as the solvent. The region containing rubroskyrin (R_F 0.40) was cut out and extracted with acetone. Evaporation of the acetone yielded 30 mg of chromatographically pure rubroskyrin, m. p. 275—280°C (decomp.).

The rubroskyrin was thermally decomposed at 270°C in the apparatus used for the preparation of carbon dioxide for ^{18}O-analysis. Special precautions were taken and test samples run to secure that no decomposition product other than water entered the cracking oven during the process.

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