ACTA CHEMICA SCANDINAVICA 23 (1969) 3493–3497

# On the Biosynthesis of Sulochrin

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Questin-O$^{14}CH_3$ has been prepared by enzymic methylation of emodin. Degradation studies demonstrate that questin-O$^{14}CH_3$ when fed to a culture of *Penicillium frequentans* is transformed into sulochrin by the organism.

The isolation of emodin and questin from a sulochrin producing strain of *Aspergillus terreus* suggests an identical pathway of sulochrin formation in this mold.

Two major theories to explain sulochrin biosynthesis have been offered (Fig. 1). One group of investigators favours the formation of sulochrin by a partial oxidative fission of a preformed hydroxyanthraquinone (Raistrick 1950,[1] Birch and Donovan 1953,[2] Gatenbeck 1960,[3] Money 1963,[4] Mahmoodian and Stickings 1964 [5]). Another group suggests sulochrin formation by condensation of two benzeneoid derivatives (Tatum 1944,[6] Aghoramurthy and Seshadri 1954,[7] and Curtis *et al.* 1966, 1968 [8,9]).

Recently, using the mold *Aspergillus terreus*, Curtis *et al.*[8,9] described experiments studying the incorporation into sulochrin of $^{14}$C-labelled acetate

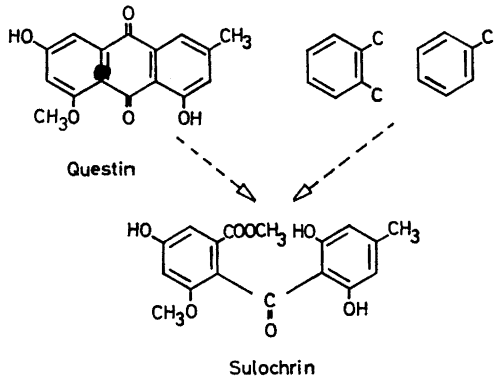


*Fig. 1.* Possible pathways of sulochrin formation.

and malonate, respectively. The data were interpreted as an indication that the biosynthesis of sulochrin proceeds *via* condensation of two benzeneoid metabolites possibly arising from two polyketide chains, each originating from acetate-malonate. These data, however, do not appear to be convincing enough to be conclusive proof for the pathway of sulochrin formation.

In 1964 Mahmoodian and Stickings [5] demonstrated that the mold *Penicillium frequentans* produced, in addition to sulochrin, the anthraquinone questin, that theoretically might be the precursor of sulochrin. This co-occurrence does not indicate *per se* a direct biogenetic relationship between questin and sulochrin. Questin may itself be derived from two benzeneoid precursors, but, if it is formed from a single polyketide chain — a thought supported by $^{14}C$-incorporation experiments in other mold anthraquinones [10] — there is reason to suspect a direct relationship between questin-sulochrin.

Curtis *et al.*[11,12] have reported the occurrence of a large number of phenolic compounds produced by *A. terreus* Thom IMI 16043 (the wild type and mutant strains), but no anthraquinone derivatives. In this publication we will describe the isolation and identification of the anthraquinone emodin and its 5-methylether, questin, from *A. terreus*. Evidently, *P. frequentans* and *A. terreus* have many metabolites in common that might be related to the biosynthesis of sulochrin. Therefore, it seems likely that the pathway for sulochrin formation in the two molds is identical.

Our strain of *P. frequentans* normally produces very small amounts of questin and no detectable emodin. If, however, a shake culture growing in Czapek-Dox medium is treated with aminopterin a few days after inoculation, the broth becomes intense yellow in color after a few hours. The yellow color is caused by the accumulation of emodin in the culture medium. The amount of emodin formed exceeds by far the quantity of questin formed in an untreated culture. Since aminopterin is known to inhibit the synthesis of tetrahydrofolic acid and consequently the synthesis of methyl groups *via* C-1 metabolism, the formation of questin seems to take place by methylation of emodin. If methyl groups in the form of methionine are added in excess to the inhibited culture, emodin disappears in the course of a few hours without the appearance of a corresponding accumulation of questin. The precursor-product relationship between emodin and questin has been verified by the isolation of an O-transmethylase that very actively catalyzes the *in vitro* formation of questin from emodin, using *S*-adenosylmethionine as methyl donor. The observations described show that questin is efficiently transformed into another metabolite by the organism.

To investigate whether questin gives rise to sulochrin, questin-O$^{14}CH_3$ was prepared. Since our strain of *P. frequentans* produces questin in very low yield, a biological synthesis using methionine-$^{14}CH_3$ as labelled substrate was deemed to be uneconomical. For that reason a cell-free synthesis was carried out using a protein fraction in which O-transmethylase had been enriched by ammonium sulfate precipitation. Emodin and *S*-adenosylmethionine-$^{14}CH_3$ were used as substrates in the reaction. In this way 2 mg (723 000 dpm) of questin were obtained after addition of nonlabelled carrier.

The radioactive questin was added to a four day old shake culture of *P. frequentans* in Czapek-Dox medium and the culture was harvested one day

later. At this time, the yellow color caused by the addition of questin had disappeared. A total of 33.9 mg of labelled sulochrin was isolated from the dry mold mycelium. Its specific radioactivity as measured in a liquid scintillation spectrometer was found to be 10 200 dpm/mg, representing the incorporation of 345 000 dpm or 48 % of the radioactivity from the added questin into the sulochrin formed. The surprisingly high incorporation for this type of experiment indicates, *per se*, a direct relationship between questin and sulochrin.

In order to locate the radioactivity in the labelled sulochrin it was degraded with concentrated sulfuric acid after dilution with nonlabelled carrier. The methyl 3-hydroxy-5-methoxybenzoate formed in the reaction was isolated as described by Curtis *et al.*[8] After hydrolyzing the methyl ester in alkali, the methoxy methyl group was split off with hydroiodic acid and trapped in dimethylaniline. The specific radioactivity of trimethylphenylammonium iodide formed, as well as the specific activities of the other degradation products obtained, are listed in Table 1. The data demonstrate that the radioactivity in the labelled sulochrin is entirely localized in the methyl ether group, which unambiguously indicates a formation of sulochrin from questin. Removal by the organism of the labelled methoxyl group in the added questin and a recycling to sulochrin of the labelled methyl group *via* C-1 metabolism is less likely both because of the high incorporation efficiency and the lack of radioactivity in the methyl ester group of the sulochrin formed.

## EXPERIMENTAL

*Isolation of emodin and questin. Aspergillus terreus* Thom IMI 16043 was grown on a shake table at 28°C in 500 ml conical flasks containing 150 ml of Czapek-Dox medium (glucose 40 g, $NaNO_3$ 2 g, $KH_2PO_4$ 1 g, KCl 0.5 g, $MgSO_4,7H_2O$ 0.5 g, $FeSO_4,7H_2O$ 0.01 g, yeast extract (Difco) 1 g, distilled water 1000 ml). 13 flasks were harvested after 4 days of growth and the cell mass was separated from the culture fluid by filtration. After acidification with concentrated HCl, the filtrate was extracted with ethyl acetate. Reextraction of the ethyl acetate phase with fresh bicarbonate solution followed by 1 M NaOH removed the anthraquinones from the organic solution. The anthraquinones were taken up into ether after acidification of the NaOH solution. After evaporation of the ether, the residue was dissolved in a minimal volume of chloroform-methanol (1:1) and the mixture developed in a column of $Al_2O_3$ with chloroform-methanol (1:1). The pigmented bands close to the top of the column were removed, and the adsorbed anthraquinones were extracted with ether after acidification. The anthraquinone components of the extract were separated by thin-layer chromatography (Kieselgel G, benzene-ethyl formate-formic acid, 100:24:1). Two bands of anthraquinones were obtained. The $R_F$ values corresponded to those of emodin (0.55) and questin (0.25). The bands were separately scraped from the chromatogram, and the anthraquinones were eluted with ether. The pigment obtained from the band corresponding to emodin was purified by sublimation in vacuum at 150°C, followed by recrystallization from toluene. UV and visible spectra were identical with those of authentic emodin and the melting point (260°C) was not depressed on admixture with authentic emodin.

The extract obtained from the pigmented band corresponding to questin was run in a countercurrent distribution apparatus as described by Mahmoodian and Stickings.[5] After 50 transfers the colored fractions were pooled, and the pigment was extracted into the ethyl acetate phase after acidification of the aqueous phase. The material recovered from the evaporated ethyl acetate extract was recrystallized from toluene yielding 3 mg of pigment, m.p. 296—298°C, which was not depressed on admixture with questin isolated

from *P. frequentans*. UV and visible spectra were also identical with those of authentic questin.

*Preparation of the O-transmethylase solution. P. frequentans* was grown as a surface culture on Czapek-Dox medium in 3 Fernbach flasks, each containing 400 ml of medium. After 9 days of growth, the mycelia were filtered off, washed with buffer solution (0.005 M Na-phosphate, 0.001 M EDTA, pH 7.5), and then disintegrated in an X-press. The supernatant obtained after centrifugation of the homogenate for 40 min at 40 000 *g* and +4°C was treated with ammonium sulfate up to 35 % of saturation. The precipitate was removed by centrifugation at 10 000 *g* for 15 min at +4°C. Addition of ammonium sulfate to the clear solution to 65 % of saturation yielded after centrifugation a protein fraction containing the O-transmethylase activity. The precipitate was dissolved in phosphate buffer (pH 7.5) and dialyzed against this buffer overnight. The dialyzed protein solution was used without further purification for the synthesis of questin. The incubation mixture contained 0.02 μmoles (0.95 μC) of *S*-adenosylmethionine$^{14}CH_3$, 2 mg of emodin (dissolved in 1 ml of ethanol), 10 ml of enzyme solution, 40 ml of phosphate buffer, pH 7.5. After 45 min at 28°C the reaction was stopped by acidification with concentrated HCl. The mixture was extracted with ether (2 × 50 ml). The questin formed was separated by thin-layer chromatography (kieselgel G, benzene-dioxane-glacial acetic acid, 90:25:4). The band on the chromatogram containing questin was collected, and the questin was eluted with ether. After addition of 2 mg of nonlabelled questin the eluted questin was further purified by adsorption on an $Al_2O_3$ column (chloroform-methanol, 1:1). The red zone containing questin was removed from the column and after acidification and extraction with ether, the questin obtained was free of radioactive contaminants or other impurities. Yield 2 mg (723 071 dpm).

*Questin-$^{14}C$ feeding.* The labelled questin, dissolved in 1 ml of ethanol, was added to a 4 day old shake culture of *P. frequetans* (150 ml of medium in a 500 ml conical flask). After an additional 20 h of growth, the mycelia were removed by filtration and dried in a stream of hot air. During ether extraction of the dried mycelia in a Soxhlet apparatus, a precipitate formed in the ether solution. Recrystallization of the precipitate from a mixture of methanol and water yielded 12.3 mg of chromatographically pure sulochrin. The specific radioactivity (10 172 dpm/mg) was determined in a liquid scintillation spectrometer by dissolving 0.85 mg of sulochrin in 1 ml of methanol in a scintillation vial with 7 ml of the phosphor solution (0.4 g of BBOT in toluene-methanol, 1:1). The efficiency of the measurement was determined by using toluene-$^{14}C$ as an internal standard.

To the evaporated mother liquor from the ether extract 100 mg of nonlabelled sulochrin were added. After repeated recrystallizations and determination of the specific radioactivity of the diluted sulochrin, another 21.6 mg of sulochrin were found by calculation to have been formed by the organism.

*Degradation of sulochrin-$^{14}C$.* 11.5 mg of the undiluted sulochrin were recrystallized with 247 mg of carrier sulochrin from methanol-water. The specific radioactivity of the sulochrin obtained was measured as above (Table 1). 224 mg of the labelled sulochrin

*Table 1.* Specific radioactivities of degradation products.

| Compound | Spec. radioact. dpm/mmole |
|---|---|
| Sulochrin | $110 \times 10^3$ |
| Methyl 3-hydroxy-5-methoxy-benzoate | $111 \times 10^3$ |
| 3-Hydroxy-5-methoxybenzoic acid | $106 \times 10^3$ |
| Trimethylphenylammonium iodide | $117 \times 10^3$ |

were treated with 200 μl concentrated $H_2SO_4$ as described by Curtis *et al.*[8] The yield of methyl 3-hydroxy-5-methoxybenzoate was 55 mg after recrystallization from cyclohexane. The specific radioactivity of the ester was measured as above. The ester (49.6 mg) was hydrolyzed by refluxing in 20 ml of 2 M NaOH in a nitrogen atmosphere, giving 20 mg of the acid, m.p. 202°C, after extraction of the acidified solution followed by recrystallization from water. The specific radioactivity of the 3-hydroxy-5-methoxybenzoic acid is listed in Table 1.

18 mg of the benzoic acid derivative were demethylated according to Zeisel and the methyl iodide formed was trapped in undiluted dimethylaniline. The precipitate consisting of trimethylphenylammonium iodide was collected and washed with ethanol and ether prior to the determination of its specific radioactivity (see Table 1).

This work has been supported by a grant from the *Swedish Natural Science Research Council.*

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Received April 22, 1969.