

## 3-Hydroxyphthalic Acid, a Metabolite in *Penicillium islandicum* Sopp

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In an investigation of *Penicillium islandicum* Sopp 3-hydroxyphthalic acid was isolated from the culture medium. The structure was proved by paper chromatography and degradation to *m*-hydroxybenzoic acid.

It has been shown previously<sup>1-3</sup> that the mycelium of *Penicillium islandicum* Sopp, when grown on a synthetic medium containing glucose as the sole source of carbon, contains large amounts of red pigments consisting of a mixture of islandicin, iridoskyrin, skyrin, rubroskyrin and erythroskyrine. Of these pigments islandicin is 1,4,5-trihydroxy-2-methylanthraquinone, iridoskyrin a dimeric derivative of islandicin and skyrin a dimeric derivative of *Frangula* emodin (4,5,7-trihydroxy-2-methylanthraquinone). The structures of rubroskyrin and erythroskyrine are unknown.

During the studies of the biological formation of islandicin, the culture medium of *P. islandicum* Sopp has been examined for aromatic substances. The examination has been made by paper chromatographical methods with a technique elaborated at this laboratory by Ehrensvärd and his group<sup>4</sup>.

The ether extract of the acidified culture medium contains very small amounts of aromatic compounds detectable with the reagents used. 3 or 4 different aromatic substances could be detected. One of them has been identified as 3-hydroxyphthalic acid which has not previously been obtained from any natural source.

3-Hydroxyphthalic acid was identified after isolation by fractionation on a silica gel column. The crude fraction was extracted in a countercurrent distribution apparatus. The amount of natural 3-hydroxyphthalic acid was estimated to be 1—2 mg per litre culture medium by comparing the fluorescence in UV light of a solution of the natural product with a standard solution of synthetic 3-hydroxyphthalic acid<sup>5</sup> in ether. Owing to the difficulty in getting the acid in a pure, crystalline form the identification was made by comparing its color reactions and  $R_f$  values in 6 different solvents with those of pure,

synthetic 3-hydroxyphthalic acid. Furthermore, the natural product was decarboxylated to *m*-hydroxybenzoic acid which was identified in the same way by comparison with synthetic *m*-hydroxybenzoic acid.

3-Hydroxyphthalic acid has a very close structural relationship to 3,5-dihydroxyphthalic acid which occurs in *Penicillium brevi-compactum*<sup>6</sup>. According to Oxford and Raistrick<sup>7,8</sup> dihydroxyphthalic acid appears to be an oxidation product of the different propyl derivatives of  $\beta$ -resorcylic acid which accompany 3,5-dihydroxyphthalic acid in *P. brevi-compactum*. The biological formation of 3-hydroxyphthalic acid in *P. islandicum* does not seem to follow the same route because the corresponding methyl- and propyl derivatives of salicylic acid cannot be detected in the culture medium. Nor can the 3-hydroxyphthalic acid in the culture medium be looked upon as a degradation product of islandicin or any other pigment because the acid is formed at an early stage during growth at a time when there is hardly any pigment production. The question whether 3-hydroxyphthalic acid is a metabolite in a reaction series leading to the formation of islandicin or represents a by-product in this chain will be a subject for further investigations.

#### EXPERIMENTAL

*Culture conditions.* Culture medium: Czapek-Dox solution: Glucose, 50.0 g; NaNO<sub>3</sub>, 2.0 g; KH<sub>2</sub>PO<sub>4</sub>, 1.0 g; KCl, 0.5 g; MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.5 g; FeSO<sub>4</sub> · 7H<sub>2</sub>O, 0.01 g; distilled water 1 l.

Ten 1-litre flat-bottomed culture flasks plugged with cotton wool each containing 500 ml of Czapek-Dox solution were sterilized. Small inoculates of *P. islandicum*, strain N.R.R.L. 1036, were placed in the flasks. The cultures were incubated in the dark at 24°C and were harvested after 20 days' incubation.

*Isolation of 3-hydroxyphthalic acid.* The orange-yellow culture medium was separated by filtration from the mold mycelium which was washed with distilled water. The acid medium, pH 4, was neutralized with 33 % aqueous NaOH and evaporated at reduced pressure on a water bath to about 500 ml. The concentrated solution was acidified with conc. HCl and extracted 6 times with a total amount of 2 l of ether. The ether solution was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and then evaporated to about 100 ml. The evaporation was continued in an air stream under a heating lamp to almost dryness. The last trace of solvent was removed in a vacuum-desiccator over silica gel. The residue was dissolved in 4–5 ml of acetone and the solution added to a silica gel column (45 × 2.5 cm). The 3-hydroxyphthalic acid was eluted with a mixture of acetone:chloroform (1:3). The column was illuminated with UV light and the band with the light blue fluorescence of 3-hydroxyphthalic acid was collected. This fraction, intensively red colored, was evaporated to dryness. The residue was fractionated in an ordinary manual Craig distribution apparatus consisting of 50 transfer tubes, each tube holding 20 ml. An aqueous solution of 3 % formic acid was used as stationary phase and the mobile phase consisted of methyl isobutyl ketone equilibrated with the stationary phase. The residue was dissolved in 10 ml of the mobile phase and placed in the first transfer tube together with 10 ml of the stationary phase. The colored substances run in the front and after 40 transfers the 3-hydroxyphthalic acid could be localized to the tubes 25–34 by illumination with UV light. The solutions in these tubes were collected and the methyl isobutyl ketone was removed by distillation at reduced pressure. The remaining aqueous solution was extracted several times with ether. The collected ether phases were evaporated in an air stream under a heating lamp. The residue that had a faint yellow color was dissolved in about 1.5 ml of boiling water. The solution was treated with a little active carbon and filtered hot, leaving a colorless filtrate.

*Identification of 3-hydroxyphthalic acid.* The filtrate was cooled in a refrigerator overnight. A very small precipitate was formed. The mixture was shaken with an equal volume of ether. Paper chromatograms were run from the ether solution on Whatman

filter paper No. 1 in 6 different solvents at 22°C. The composition of the solvents has been elaborated at this laboratory and they have been extensively used for identification of organic substances in the *Penicillium* series. The solvents are:

- A. methyl isobutyl ketone/4 % aqueous formic acid
- B. chloroform/methanol, 2 % aqueous formic acid
- C. benzene, methyl ethyl ketone/2 % aqueous formic acid
- D. benzene/2 % aqueous formic acid
- E. water, methyl ethyl ketone, diethylamine
- F. methyl ethyl ketone, acetone, aqueous formic acid

On each paper chromatogram were placed: I, natural product; II, pure, synthetic 3-hydroxyphthalic acid; III, a mixture of I and II. The chromatograms were analyzed with a) UV light, b) *p*-nitrobenzenediazoniumfluoborate in 50 % aqueous ethanol + ammonia, c) aqueous ferric chloride. I, II and III had the same  $R_F$  values and gave spots indistinguishable from each other with all the reagents used; a) gave a light blue fluorescence, b) an orange color, c) a violet color.

The following  $R_F$  values were obtained:

Solvent	A	B	C	D	E	F
$R_F$ value	0.60	0.08	0.05	0.00	0.00	0.83

The water-ether solution of natural 3-hydroxyphthalic acid was heated on a water bath until the ether had evaporated. An equal volume of conc.  $H_2SO_4$  was added to the remaining aqueous solution and the mixture was refluxed for 2 h at 125°C. A corresponding amount of synthetic 3-hydroxyphthalic acid was treated in the same way. The solutions were cooled to room temperature and extracted with ether. The ether solutions were tested paper-chromatographically in the same solvents as above. On each chromatogram were placed: 1.  $H_2SO_4$ -treated natural product, 2.  $H_2SO_4$ -treated synthetic 3-hydroxyphthalic acid, 3. synthetic *m*-hydroxybenzoic acid. The chromatograms were analyzed with d) *p*-nitrobenzenediazoniumfluoborate in 50 % aqueous ethanol + ammonia, e) 2,6-dibromoquinonechlorimide in dioxan + ammonia. 1, 2 and 3 gave identical spots with the same  $R_F$  values and the same color reactions, *i. e.* with d) a reddish brown color, e) a blue color.

Solvent	A	B	C	D	E	F
$R_F$ value	0.86	0.45	0.35	0.01	0.09	0.91

No 3-hydroxyphthalic acid could be detected.

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