3-Hydroxyphthalic Acid, a Metabolite in Penicillium islandicum Sopp

STEN GATENBECK

Department of Physiological Chemistry, Wenner-Gren Institute, University of Stockholm, Sweden

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\textsuperscript{1-3} 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,tri-hydroxy-2-methylandraquinone, iridoskyrin a dimeric derivative of islandicin and skyrin a dimeric derivative of Frangula emodin (4,5,7-tri-hydroxy-2-methylandraquinone). The structures of rubroskyrin and erythroskyrine are unknown.

During the studies of the biological formation of islandicin, the culture medium of \textit{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 Ehrensvård and his group\textsuperscript{4}.

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\textsuperscript{5} 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.

\textit{Acta Chem. Scand.} 11 (1957) No. 3
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* 8. According
to Oxford and Raistrick 7, 8 dihydroxyphthalic acid appears to be an oxidation
product of the different propyl derivatives of β-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 pro-
duction. 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; NaNO₃,
2.0 g; KH₂PO₄, 1.0 g; KCl, 0.5 g; MgSO₄ · 7H₂O, 0.5 g; FeSO₄ · 7H₂O, 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. 1038, 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
con. HCl and extracted 6 times with a total amount of 2 l of ether. The ether solution
was dried over anhydrous Na₂SO₄ 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 aceton: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 eva-
porated to dryness. The residue was fractionated in an ordinary manual Craig distribu-
tion 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 illumi-
nation with UV light. The solutions in these tubes were collected and the methyl isob-
utyl ketone was removed by distillation at reduced pressure. The remaining aqueous
solution was extracted several times with ether. The collected ether phases were eva-
porated 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 over-
night. 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

3-HYDROXYPHTHALIC ACID

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 Pencillium 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:

<table>
<thead>
<tr>
<th>Solvent</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R_F ) value</td>
<td>0.60</td>
<td>0.08</td>
<td>0.05</td>
<td>0.00</td>
<td>0.00</td>
<td>0.83</td>
</tr>
</tbody>
</table>

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. \( \text{H}_2\text{SO}_4 \) 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. \( \text{H}_2\text{SO}_4 \)-treated natural product, 2. \( \text{H}_2\text{SO}_4 \)-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.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R_F ) value</td>
<td>0.86</td>
<td>0.45</td>
<td>0.35</td>
<td>0.01</td>
<td>0.09</td>
<td>0.91</td>
</tr>
</tbody>
</table>

No 3-hydroxyphthalic acid could be detected.

Acknowledgment. The author is indebted to Professor G. Ehrensvård for his stimulating interest in this work.

REFERENCES

4. Ehrensvård, G. et al. To be published.

Received January 21, 1957.

Acta Chem. Scand. 11 (1957) No. 3