Isolation and Identification of Canthaxanthin from Micrococcus roseus

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ABSTRACT

COONEY, J. J. (University of Dayton, Dayton, Ohio), H. W. MARKS, JR., AND ANNE M. SMITH. Isolation and identification of canthaxanthin from Micrococcus roseus. J. Bacteriol. 92:342–345. 1966.—The principal colored carotenoid of Micrococcus roseus was purified by solvent partitioning followed by column and thin-layer chromatography. Absorption spectra, partition coefficients, and infrared spectra suggested that the pigment was a diketo derivative of β-carotene. The pigment was subjected to reduction, and the reduced pigment was subsequently dehydrated. Spectral data and partition coefficients of these derivatives indicated that the original pigment was canthaxanthin (4',4'-diketo-β-carotene). The pigment was an all-trans isomer; it does not exist as an ester in M. roseus. Canthaxanthin has not previously been identified as a bacterial pigment.

Carotenoid pigments occur in a wide variety of chemosynthetic and photosynthetic microorganisms. The pigments of gram-positive cocci reported to date are primarily carotenoids, but many of the pigments have not been identified. When Micrococcus roseus was cultured on the surface of complex media or in a defined medium, the cell mass appeared orange-pink. In the presence of diphenylamine, pigment production decreased only 27%, suggesting that xanthophylls rather than carotenes constitute the major class of pigments (1). This paper describes the isolation and identification of canthaxanthin, the principal colored carotenoid of M. roseus.

MATERIALS AND METHODS

M. roseus ATCC 516 was cultured on the surface of stock culture agar in Koller flasks. Flasks were incubated at 25 C for 5 to 7 days. Cells were rinsed from the surface of the agar with distilled water and washed three times with distilled water. Washed cells were extracted twice with absolute methanol; during each extraction, the suspension was agitated at room temperature for 30 min. The cell pellet which remained after the second extraction was colorless. In early experiments, the pooled methanol extracts were saponified for 30 min at 65 C with 6% (w/v) KOH, but in later experiments this step was omitted without altering the characteristics of the pigment. Throughout the work, pigments were stored in the dark under nitrogen.

Sufficient distilled water was added to the extract to dilute the methanol to 90% (v/v). This solution was partitioned against petroleum ether (boiling point, 30 to 60 C), with the addition of Na2SO4 or a saturated aqueous NaCl solution to facilitate phase separation. The extraction was repeated (usually three times) until the ether phase was colorless. Glassware with Teflon stopcocks was used throughout to avoid contamination of pigments with silcones which interfered with chromatography. The pooled epiphase, which contained the bulk of the colored material, was dried over several portions of anhydrous Na2SO4 and the petroleum ether was evaporated under a stream of nitrogen.

The residual red oil was dissolved in a small quantity of anhydrous diethyl ether and applied to the surface of a column (2.5 by 8 cm) of silica gel G. A broad (1.5 cm) orange band (fraction I) was eluted with diethyl ether, and after this a narrow (0.2 cm) yellow band (fraction II) was eluted with methanol. Subsequent analyses were confined to fraction I.

The ether was evaporated from fraction I under a stream of nitrogen. The residue was dissolved in CS2 and applied to thin-layer chromatography plates coated with CaCO3 (low in alkali; Matheson, Coleman and Bell, East Rutherford, N.J.). Plates were developed in benzene-petroleum ether (3:2; v/v). Pigmented areas were scraped from the plates, and the pigments were eluted from the adsorbent with benzene.

Absorption spectra were determined in spectro-quality solvents. Partition coefficients were determined in a hexane-95% methanol system according to the method of Petracek and Zechmeister (14). Infrared spectra were determined in CS2 and in CHCl3.

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Carbonyl groups were reduced according to Krinsky and Goldsmith (12) by dissolving the pigment in 95% ethyl alcohol and adding a few crystals of NaBH4. The solution was stored under nitrogen at 4°C overnight; it was then chromatographed on CaCO3 plates; the principal spot was eluted as before.

After reduction, some samples were dehydrated by treatment with CHCl3 saturated with HCl gas; the pigment was extracted into petroleum ether and washed three times with distilled water to remove excess NaBH4. The ether extract was dried over Na2SO4, and the solvent was evaporated with nitrogen. The dried pigment was dissolved in 3.5 ml of CHCl3, and two drops of acetic acid CHCl3 was added. After 15 min, the sample was deacetylated by shaking with 5% (w/v) NaHCO3 (16). The CHCl3 solution was washed with water and dried over Na2SO4, and the solvent was evaporated under a stream of nitrogen. The residue was chromatographed on CaCO3 plates and eluted as before.

The method of Curl and Bailey (2) was used to test for 5,6-epoxides; the pigment was dissolved in 3.0 ml of petroleum ether and treated with a drop of ethyl alcoholic 0.05 N HCl. The presence of a 5-6 epoxide group is indicated by a decrease of 16 to 22 mU in the wavelength of maximal absorbance.

Trans isomers were detected by a decrease of 6 to 8 mU in maximal absorbance after treatment with iodine in hexane in the presence of white light (9).

RESULTS AND DISCUSSION

When a diethyl ether solution of the epiphatic pigments was layered over concentrated HCl or H2SO4, a blue color was obtained. This reaction is characteristic of 4'-diketo-o-carotenoids (3). Lack of fine structure in spectra suggested the presence of at least one carbonyl at each end of the molecule in conjugation with the polyene chain (12, 15). Carbonyls were also indicated by a peak at 1,745 cm⁻¹ in infrared spectra. The partition coefficient (Table 2) was characteristic of a carotenoid with two carbonyls (14). Tests for epoxides were negative. These data suggested that the pigment was canthaxanthin (4,4'-diketo-β-carotene, Fig. 2). Upon treatment with iodine in hexane, the absorption maximum decreased 5 to 9 mU, indicating the presence of all-trans isomers.

After treatment with NaBH4, the pigment was less mobile on CaCO3 thin-layer chromatography plates (Table 1), indicating reduction of carbonyls to more polar hydroxyl groups. The partition coefficient (Table 2) indicated that the reduction product had two hydroxyl groups (14). After NaBH4 treatment, spots on thin-layer chromatography plates were yellow (Table 1), and absorption spectra of the reduced pigment (Fig. 1, Table 2) were typical of β-carotene (12).

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Before reduction</th>
<th>After reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rf value</td>
<td>Color</td>
</tr>
<tr>
<td>1</td>
<td>0.89</td>
<td>Yellow</td>
</tr>
<tr>
<td>2</td>
<td>0.59</td>
<td>Orange</td>
</tr>
<tr>
<td>3</td>
<td>0.27</td>
<td>Orange</td>
</tr>
</tbody>
</table>

FIG. 1. Absorption spectra of the principal pigment from Micrococcus roseus and derivatives of the pigment. Solid line, pigment in CS2; dashed line, after reduction in CHCl3; dot-dash line, the reduced pigment after dehydrated in CHCl3. Curves have been shifted on the y-axis for clarity.
They were also typical of isoxeazanthen (4,4'-
dihydroxy-β-carotene), the expected reduction
product of canthaxanthin, since addition of
hydroxyl groups to β-carotene has very little
influence on the shape of spectra or the position
of absorption maxima (3). Displacement of the
principal absorption maximum upon reduction
(17 μm in CHCl₃, Table 2) was also consistent
with reduction of two conjugated carbonyl
groups to hydroxyls. Reduction of two noncon-
jugated (3,3') carbonyls or one conjugated and
one nonconjugated (3,4') carbonyl would have
resulted in a smaller decrease in the wavelength
of the principal peak (3). Infrared spectra, how-
ever, showed a small peak at 1,745 cm⁻¹.

When the reduced compound (isoxeazanthen) was
dehydrated, the product had an absorption
spectrum with a single broad maximum, and
the spectrum was slightly asymmetrical. The
absorption maximum of the dehydrated product
was 16 to 24 μm higher than the principal max-
imum of the reduced pigment (Fig. 1, Table 2),
suggesting that a conjugated carbonyl and a
conjugated double bond had been introduced by
HCl treatment. The dehydration product also
had a partition coefficient (Table 2) typical of a
carotenoid with one carbonyl (14). Infrared
spectra showed a prominent peak at 1,745 cm⁻¹.
Upon treatment with HCl in CHCl₃ (3), isoxea-
zanthen yields 3',4'-dehydroechinenone (4-keto-
3',4'-dehydro-β-carotene).

These data indicated that the original pigment
was canthaxanthin (Fig. 2). A sample of cantha-
xanthin was provided by Dennis L. Fox (Scripps
Institution of Oceanography, La Jolla, Calif.).
When pigment from M. roseus was cochromato-
ographed with canthaxanthin on CaCO₃ thin-layer
chromatography plates, a single spot was ob-
served. The two pigments could not be dis-
tinguished on the basis of their absorption spectra.

Canthaxanthin does not exist in M. roseus as
an ester, since saponification could be omitted
without altering the properties of the pigment.

Canthaxanthin was first isolated from the mush-
room Cantharellus cinnabarinus by Haxo

TABLE 2. Absorption maxima and partition coefficients of the principal pigment of
Micrococcus roseus and of derivatives of the pigment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CS₂ (μm)</th>
<th>Hexane (μm)</th>
<th>CHCl₃ (μm)</th>
<th>Ethyl alcohol (μm)</th>
<th>Partition coefficient (% hypobasic)</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>498</td>
<td>479</td>
<td>479</td>
<td>473-478</td>
<td>54-56</td>
</tr>
<tr>
<td>Reduced</td>
<td>498</td>
<td>479</td>
<td>(435),</td>
<td>473-478</td>
<td>88-89</td>
</tr>
<tr>
<td>Reduced and dehydrated</td>
<td>498</td>
<td>479</td>
<td>478-486</td>
<td>473-478</td>
<td>5-11</td>
</tr>
</tbody>
</table>

* Varied from experiment to experiment.
  b Parentheses indicate a shoulder, rather than a distinct peak.

ACKNOWLEDGMENTS

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