Isolation of 4'-Hydroxyechinenone from Micrococcus roseus

E. M. SCHWARTZEL AND J. J. COONEY

Department of Biology, University of Dayton, Dayton, Ohio 45409

Received for publication 10 July 1972

The carotenoid 4'-hydroxyechinenone (4'-hydroxy-β,β-carotene-4-one) was isolated from Micrococcus roseus. It is proposed as an intermediate between echinenone and canthaxanthin.

Canthaxanthin (β,β-carotene-4,4'-dione, reference 6; Fig. 1e) is the major carotenoid pigment of Micrococcus roseus (2, 11). The organism also contains the monoketo pigment echinenone (β,β-carotene-4-one) (Fig. 1d; reference 10), which is presumed to be a precursor of canthaxanthin, and a number of other carotenoids (10, 11). Although these compounds are present in a variety of bacteria, algae, plants, and animals, relatively little is known of the manner of insertion of keto functions. The homologous hydroxy derivatives, isocryptoxanthin (Fig. 1b) and isozeaxanthin (Fig. 1c), have been proposed as intermediates between β-carotene (β,β-carotene; Fig. 1a) and these keto carotenoids. The limited evidence available is from crustaceans: in the brine shrimp Artemia salina, the hydroxy compounds do not appear to be intermediates (3), but they can be converted into the keto compounds by the cladoceran branchipod Daphnia magna (4), and they are present in other branchiopods (5). We therefore examined the minor carotenoids of M. roseus for potential hydroxy precursors of echinenone and canthaxanthin.

M. roseus ATCC 516 was cultured as described previously (10). Solvents were reagent grade and were distilled prior to use. Washed, stationary-phase cells from about 30 liters of medium were extracted three times with methanol under nitrogen and in the dark. MgSO₄ in methanol was added to the pooled extracts to a final concentration of 2 × 10⁻⁴ M to enhance lipid precipitation. The extract was held at −10°C for 12 hr and then centrifuged at −10°C. The clear pigmented supernatant fluid was decanted. The pellet of precipitated lipids contained considerable pigment. It was suspended in methanol, and the suspension was shaken at room temperature for 12 hr under nitrogen in a foil-covered flask, after which the MgSO₄ precipitation was repeated. Three such extractions removed most of the carotenoids from the lipid pellet. Preliminary spectral analysis indicated that lipid precipitation did not result in a selective separation of carotenoids; therefore all the methanol extracts were combined.

The extract was diluted to 90% (v/v) methanol with distilled water, and the solution was partitioned against petroleum ether (boiling point 30 to 60°C) until the ether phase was colorless. The petroleum ether epiphase was dried over anhydrous Na₂SO₄, and the solvent was removed under reduced pressure. The oily residue was dissolved in acetone and stored at −10°C for 12 hr. A white flocculent precipitate which formed was removed by centrifugation. The solution was then concentrated and stored at 5°C under nitrogen in the dark until it was used.

The epibasic carotenoids were applied to a Silica Gel G-Celite (3:1, w/w) column. Four colored fractions were eluted: (i) with petroleum ether, (ii) with 3 to 5% acetone in petroleum ether, (iii) with 6 to 12% acetone in petroleum ether, and (iv) with methanol. Thin layer chromatography (TLC) of the four fractions revealed that each was a mixture of carotenoids, many of which were present in quantities too small to be identified with certainty. TLC plates were developed in the dark. TLC of fraction 2 on Silica Gel G, developed with 6 to 12% acetone in petroleum ether, gave three pigmented zones. After elution from the adsorbent, the most mobile pigment was identified as echinenone, based on its absorption spectrum and on co-chromatography with authentic echinenone. A second had a β-carotene chromophore and its greater polarity in chromatographic systems suggested it could be a monohydroxy carotenoid. TLC of fraction 3 on

1 Present address: Life Sciences Division, Syracuse University Research Corporation, Syracuse, N.Y. 13210.
Silica Gel G, developed with 15% acetone in petroleum ether, resolved canthaxanthin as the major and most mobile component, plus a mixture of more polar unidentified minor components. Canthaxanthin was identified by its spectral properties and by co-chromatography with the authentic pigment.

Fraction 4 was applied to Silica Gel G TLC plates which were developed with 20% acetone in petroleum ether. Three pigments were resolved. The least polar pigment was eluted from the adsorbent. Its absorption spectrum had a single peak which was slightly asymmetrical and which had a $\lambda_{\text{max}}$ of 468 nm (Fig. 2), identical with the spectrum of echinenone. Treatment of the pigment with sodium borohydride (7) increased the polarity of the compound, suggesting that a keto group had been reduced to a hydroxy group. The product had a three-peak spectrum typical of a $\beta$-carotene chromophore (Fig. 2). Increased polarity in TLC systems, the change in shape of the spectrum and the decrease in $\lambda_{\text{max}}$ of 8 nm suggested that one conjugated keto group had been reduced. The product co-chromatographed with isozeaxanthin ($\beta, \beta$-carotene-4, 4'-diol; Fig. 1c), the expected product of reduction of 4-hydroxyechinenone (4'-hydroxy-$\beta, \beta$-carotene-4-one). Isozeaxanthin was prepared from authentic canthaxanthin by reduction with sodium borohydride. The pigment is thus identified as 4'-hydroxyechinenone (Fig. 3). The other two pigments in fraction 4 had $\beta$-carotene chromophores. They are sufficiently polar to be dihydroxy compounds, but they have not been collected in sufficient quantities to permit identification.

Pigment content was estimated by using an extinction coefficient ($E_{1\%\text{cm}}$) of 2,200 for total pigment extracted from cells and for canthaxanthin, and 2,158 for echinenone and 4'-hy-
droxyechinenone. Crude extracts contained about 0.58 μg of pigment per mg of cell dry weight. Canthaxanthin comprised about 56%, echinenone 5%, and 4'-hydroxyechinenone 6% of the pigment recovered after fractionation.

4'-Hydroxyechinenone has been isolated from marine isopods (9), from hydra (8), and it has been tentatively identified in a pea crab in free and esterified forms (1). To our knowledge this is the first report of it as a bacterial pigment. It has been postulated as a precursor of isozeaxanthin (9), and as an intermediate between echinenone and canthaxanthin (1, 4, 8, 9), and its presence in M. roseus suggests that it is an intermediate in bacteria.

Pigments suspected as being isozeaxanthin and isocryptoxanthin (β, β-carotene-4-ol; Fig. 1b) were detected only when extracts were treated with MgSO₄ and acetone to remove contaminating lipidoidal materials. Even after extensive purification, their spectra showed high absorbance in the ultraviolet range. Saponification of crude extracts or of fractions obtained by column chromatography did not yield cleaner preparations. If their presence is confirmed it will provide presumptive evidence for formation of keto carotenoids in bacteria from β-carotene via hydroxy compounds.

LITERATURE CITED