Lipids Produced by *Epicoccum nigrum* in Submerged Culture

By F. H. FOPPEN AND OLGA GRIBANOVSKI-SASSU

*Centro Internazionale di Chimica Microbiologica, Laboratori di Chimica Biologica,
 Istituto Superiore di Sanità, Rome, Italy*

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1. The lipids of the red mycelium of the fungus *Epicoccum nigrum* Link were investigated. Four carotenoid pigments were isolated and identified as β -carotene, γ -carotene, rhodoxanthin and torularhodin. 2. For the first time rhodoxanthin was isolated as a fungal metabolite. 3. Linoleic acid was the major fatty acid in the total lipids. 4. Ergosterol was present in the lipids in the non-esterified form.

Several investigators (Naumann, 1912; Moreau & Moreau, 1951) have tried to elucidate the structure of the red pigments of *Epicoccum nigrum* Link. The pigments of this fungus have been reinvestigated by Gribanovski-Sassu & Foppen (1967). From the lipid fraction four carotenoid pigments were isolated and the chemical structure of three of them was determined. In the present paper the whole lipid fraction of the fungus, grown in submerged culture, is described, including the final determination of the structure of the fourth pigment.

MATERIALS AND METHODS

Strain. The strain used was *Epicoccum nigrum* Link received from Professor E. Küster, Department of Industrial Microbiology, University of Dublin, Irish Republic.

Preparation of the culture. Erlenmeyer flasks (500 ml.) containing 100 ml. of culture medium were inoculated from an agar slant (0.5 ml.). The medium used had the following composition: 1% glucose and 0.5% yeast autolysate in distilled water at pH 6.8–7.0. The flasks were put on a rotatory shaker running at 220 rev./min. under continuous illumination for 3 days at a constant temperature of 24°. The illumination was supplied by two lamps (Philips, 60 w, 130 v) installed at a distance of 150 cm. from the shelf of the shaker, providing 100–110 lux.

Lipid extraction and separation. The dark-red mycelium was harvested by filtration on a Buchner filter. From a typical batch of ten flasks about 80 g. of fresh material, corresponding to 5 g. dry wt., was obtained. The mycelium was washed twice with 100 ml. of cold water and extracted three times with 250 ml. of acetone in a Waring Blendor at 12000 rev./min. for 2 min. The acetone extracts were combined, diluted with the same volume of water and re-extracted three times with 100 ml. of hexane. To eliminate traces of acetone the hexane solution was washed five times with 50 ml. portions of water, saturated with NaCl to prevent emulsification. After drying for 30 min. over 10 g. of anhydrous Na_2SO_4 , the ultraviolet and visible-light absorption spectra of the solution were determined (see Figs. 1a and 1b).

The hexane solution was concentrated in the dark in a stream of N_2 at 30° to about 2 ml. and, after it had been kept for 48 hr. at –20°, a white precipitate was obtained. It was filtered off and washed twice with 5 ml. of cold hexane.

The total hexane extract was passed through a cellulose (standard grade; W. and R. Balston Ltd., Maidstone, Kent) column (30 cm. long $\times$ 1.5 cm. diam.). Two fractions were separated; fraction I was eluted rapidly with pure hexane, showing a bright-orange-red band on the column, and fraction II was eluted with hexane–acetone (9:1, v/v).

Fraction I was passed through a $\text{Ca}(\text{OH})_2$ –Celite 503 (3:1, w/w) (Johns–Manville, New York, N.Y., U.S.A.; grade Celite 503) column (15 cm. long $\times$ 1.0 cm. diam.). Three pigments were separated from this column. The first pigment (P_1) was eluted with pure hexane and the second (P_2) and third (P_3) pigments required hexane containing increasing amounts of acetone up to 8% (v/v). Pigment P_1 , and pigment P_2 after washing with water and drying over anhydrous Na_2SO_4 , were rechromatographed on alumina (Brockmann grade I; E. Merck A.-G., Darmstadt, Germany) columns (10 cm. long $\times$ 1.0 cm. diam.) and eluted respectively with pure hexane and hexane containing 1% (v/v) of acetone. Fraction II was rechromatographed on an alumina column (10 cm. long $\times$ 0.5 cm. diam.) with hexane–acetic acid (199:1, v/v) as eluent. Only one pigment, P_4 , was obtained.

Fig. 1. Ultraviolet absorption spectrum (a) and visible-light absorption spectrum (b) in hexane of total lipid extract from *Epicoccum nigrum* Link grown in submerged culture.

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The fractions, P₂, P₃ and P₄, were exhaustively washed with water to remove acetone and the resulting hexane solutions were dried over anhydrous Na₂SO₄.

Extraction, chromatography and separation were carried out at 5° in the dark.

Saponification. The three pigments, P₁, P₂ and P₃, after separation, were saponified in 6% (w/v) KOH solution in methanol in the dark for 5 min. at 40° under continuous stirring (Krinsky & Levine, 1964). The mixture was then rapidly cooled in an ice bath. After addition of an equal volume of cold water the pigments were extracted with diethyl ether until no more colour could be observed in the ethereal layer. By washing with water traces of alkali were eliminated. The solution was dried over anhydrous Na₂SO₄ for 30 min., then concentrated in a stream of N₂ and redissolved in hexane.

The fourth pigment, P₄, was not saponified because acidic characteristics had been observed on thin-layer chromatograms.

Determination of the fatty acid content. The fatty acids of the total lipids were determined as their methyl esters by gas-liquid chromatography. Saponification was carried out directly on the filtered mycelium with 2N-KOH solution in 50% (v/v) methanol by refluxing for 3 hr. The solution was extracted twice with diethyl ether, and the aqueous phase was acidified with conc. HCl and extracted twice with diethyl ether. The acids were methylated in methanol containing 3% (v/v) of conc. H₂SO₄ by refluxing for 3 hr. The fatty acid methyl esters were recovered and analysed on a Perkin-Elmer model 800 gas-liquid chromatograph with diethylene glycol succinate on Chromosorb W as stationary phase. Authentic methyl esters of fatty acids were used for the calibration of the column.

Thin-layer chromatography. Thin-layer chromatography was carried out on glass plates covered with a 25 μ layer of silica gel G or silica gel HF₂₅₄ (E. Merck A.-G.). The silica-gel layer was reactivated for 30 min. in an oven at 110°. Solvent systems used were hexane-acetone (22:3, v/v), hexane-propan-1-ol (47:3, v/v), hexane-propan-1-ol (22:3, v/v) and methylene chloride-ethyl acetate (4:1, v/v).

Spectra. Ultraviolet and visible-light absorption spectra were measured with a Cary model 11 MS-50 spectrophotometer; infrared absorption spectra were obtained with a Perkin-Elmer Infracord spectrophotometer in CCl₄ solutions; nuclear-magnetic-resonance spectra were measured with a Varian A-60 instrument; mass spectra were determined with an MSE double-focusing spectrometer;

extinctions were measured on a Bausch and Lomb Spectronic 20 colorimeter.

Identification of the pigments. The pigments were identified by absorption spectra, in both visible light and ultraviolet in various solvents (see Table 1), partition coefficient between hexane and aqueous methanol (Petraček & Zechmeister, 1956), reduction by NaBH₄ in ethanol (95%) at room temperature for 30 min. in the dark in an atmosphere of N₂ (Krinsky, 1963), methylation with diazomethane (Karrer & Rutschmann, 1945) and preparation of the oxime (Kuhn & Brockmann, 1933). The physical and chemical properties of the isolated pigments were also compared with those of authentic samples.

Chemicals. The solvents used were obtained from Carlo Erba S.p.A., Milan, Italy, and were all of chromatographic grade. The diethyl ether was previously freed from peroxides. NaBH₄ was obtained from the Fisher Scientific Co., Fair Lawn, N.J., U.S.A.; digitonin (grade RP) was from Carlo Erba S.p.A.; ergosterol was from E. Merck A.-G.; synthetic β -carotene, rhodoxanthin and torularhodin methyl ester were gifts from F. Hoffmann-La Roche Co. Ltd., Basle, Switzerland. γ -Carotene was extracted from carrots and torularhodin from *Rhodotorula rubra* (Lederer, 1933).

RESULTS

The total pigment extract showed an absorption spectrum with peaks at 272, 282, 293, 460 and 488 m μ and inflexions at 261, 425 and 525 m μ in hexane.

Thin-layer chromatography on silica gel HF₂₅₄ of the total hexane extract showed no fluorescence under ultraviolet radiation above the spot of β -carotene; therefore it was concluded that the colourless polyenes were absent.

Pigment P₁. The absorption spectra in different solvents of this yellowish-green fraction indicated the presence of β -carotene. The pigment crystallized in orange plates from hexane during 2 days at -20°, m.p. 178°. The identity of the compound with β -carotene was confirmed by comparison with an authentic sample on thin-layer and alumina and cellulose column chromatography.

Pigment P₂. The second fraction, orange-red,

Table 1. *Spectral characteristics of the carotenoids produced by Epicoccum nigrum Link grown in submerged culture*

Compound	$\lambda_{\max}$ (m μ)				
	Hexane	Ethanol (95%)	Chloroform	CS ₂	Benzene
Pigment P ₁	(426), 453, 482	(420), 452, 478	(435), 467, 496	(450), 484, 518	(435), 464, 494
Pigment P ₂	438, 463, 495		(447), 477, 509	464, 497, 531	448, 478, 510
Pigment P ₃	458, 489, 524	(462), 496, 530		491, 524, 562	474, 504, 540
Dioxime	451, 478, 513				
Pigment P ₄	468, 502, 538		480, 516, 556	502, 542, 585	486, 518, 553
Methyl ester	468, 502, 537				

showed absorption spectra in different solvents identical with those of γ -carotene. It occupied the same positions as pure γ -carotene on alumina and cellulose columns; on thin-layer co-chromatography one single spot was obtained.

Pigment P_3 . The third fraction, which changed its rose-violet colour in hexane on an alumina column to orange after addition of acetone, showed absorption spectra in different solvents that were consistent for a carotenoid pigment with 14 conjugated double bonds/mol. ($\lambda_{\max}$ in hexane 489m μ).

The compound gave negative reactions for carboxyl, allylic hydroxyl, *O*-methyl, aliphatic hydroxyl or epoxy groups. The presence of oxo groups was shown by the reaction of the pigment with hydroxylamine. The infrared spectrum of the pigment in carbon tetrachloride solution did not show absorption at about 770cm.⁻¹ characteristic for the 15,15'-*cis*-double bond, nor the peak at about 1000cm.⁻¹ characteristic for the central double bond in the *trans*-position. Also, the hydroxyl absorption peak was absent. In the region between 1660 and 1750cm.⁻¹ a series of absorption peaks of weak intensity were observed, with a major peak at 1670cm.⁻¹ consistent with carbonyl absorption.

These properties of the pigment were indicative of the possible identity with rhodoxanthin (3,3'-dioxo-*retro*- β -carotene) (Kuhn & Brockmann, 1933). This was substantiated by a direct comparison of our compound with synthetic rhodoxanthin by co-chromatography on thin-layer plates and on alumina columns. The pigment required hexane-acetone (93:7, v/v) for the elution from the calcium hydroxide-Celite (3:1, w/w) column. On this column it was retained slightly more strongly than echinenone (4-oxo- β -carotene) and almost the same as canthaxanthin (4,4'-dioxo- β -carotene).

The absorption spectra in different solvents of our pigment showed maxima and minima identical with those of synthetic rhodoxanthin, a compound for which Bodea, Nicoara, Tâmas & Mantsch (1963) observed an infrared carbonyl absorption peak in carbon tetrachloride solution at 1669cm.⁻¹. The unique *retro*-configuration was confirmed by the total absence of the *cis* peak after refluxing the pigment in ethanol or after illuminating a pigment solution.

Pigment P_4 . The fourth pigment was strongly absorbed on alumina. It could be eluted only with a solvent containing a small quantity of acetic acid. In the system hexane and methanol-water (9:1, v/v) the two layers were coloured, and after addition of a little more water and a few drops of 2N-potassium hydroxide the pigment was observed as an oil drop in the interphase between the two layers, showing its acidic character (Karrer

& Jucker, 1950). Its absorption spectra in different solvents agreed with that of torularhodin (Karrer & Rutschmann, 1943, 1945, 1946). The characteristic shift of the absorption spectrum in carbon disulphide was observed. The methyl ester, prepared with diazomethane in hexane at 0°, showed also an absorption spectrum in hexane identical with that of authentic torularhodin methyl ester. It was epiphasic in the system hexane and methanol-water (19:1, v/v). Co-chromatography of this pigment and of its methyl ester with torularhodin and its methyl ester respectively on alumina columns and on thin-layer plates confirmed the identification.

Identification of the white precipitate. The white compound, precipitated after concentration of the hexane extract, was crystallized from ethanol (95%) and had m.p. 160–162°, not depressed after admixture with authentic ergosterol (m.p. 163–164°). It was present in the mycelium in the non-esterified form, because after saponification it did not change its physical properties. An identical white precipitate was obtained after treatment with the sterol precipitant digitonin. A chloroform solution of the white compound gave a green-blue colour on addition of a few drops of acetic anhydride and concentrated sulphuric acid (Liebermann-Burchard test).

The ultraviolet absorption spectrum in ethanol (95%) showed a shoulder at 262m μ and three maxima at 271, 281.5 and 293m μ . This is the characteristic absorption spectrum of a homoannular conjugated diene system. The mass spectrograph gave a parent peak for this compound of *m/e* 396, consistent for C₂₈H₄₄O. Infrared and nuclear-magnetic-resonance spectra were identical with those of authentic ergosterol monohydrate, C₂₈H₄₄O.H₂O. This identity was confirmed by determination of the physicochemical data (ultraviolet, infrared and nuclear-magnetic-resonance spectra) of the acetyl derivative.

The hexane extracts of the mycelial material contained about 40 μ g. of total carotenoid pigments and about 10mg. of ergosterol/g. of dried mycelium.

The quantity of the separated pigments was determined by using the $E_{1\text{cm}}^{1\%}$ values given by Goodwin (1965) (Table 2).

Table 2. *Quantity of the carotenoid pigments produced by Epicoccum nigrum Link after 3 days of growth in submerged culture*

Carotenoid	Quantity (μ g./g. of dried mycelium)
β -Carotene	12
γ -Carotene	8
Rhodoxanthin	15
Torularhodin	2

Table 3. *Fatty acids of the lipids of Epicoccum nigrum Link grown in submerged culture*

Fatty acids are designated $C_{x,y}$, where x is the no. of C atoms/mol. and y is the no. of double bonds/mol.

Fatty acid	% recovered	Fatty acid	% recovered
$C_{14:0}$	3	$C_{18:0}$	7
$C_{15:0}$	2	$C_{18:1}$	18
$C_{16:0}$	15	$C_{18:2}$	34
$C_{16:1}$	3	$C_{18:3}$	4
$C_{17:0}$	4		

The determination by gas-liquid chromatography of the fatty acid methyl esters showed that palmitic acid, oleic acid and linoleic acid were present in major quantities with minor amounts of stearic acid and linolenic acid (Table 3).

DISCUSSION

Although several investigators (Naumann, 1912; Moreau & Moreau, 1951; Schol-Schwarz, 1959; Bamford, Norris & Ward, 1961) have worked on the pigments of *Epicoccum nigrum* Link, only Bamford *et al.* (1961) succeeded in the isolation of a yellow compound. It was identified as flavipin (3,4,5 - trihydroxy - 6 - methylphthalaldehyde), a known antibiotic metabolite of *Aspergillus niger* and *Aspergillus terreus* (Raistrick & Rudman, 1956). Under our growth conditions this antibiotic was not detected in the culture fluid.

Of the four carotenoid pigments isolated and identified, rhodoxanthin is described for the first time as a fungal metabolite. Kuhn & Brockmann (1933), studying the carotenoids present in *Taxus baccata*, regarded zeaxanthin (3,3'-dihydroxy- β -carotene) as a possible precursor of rhodoxanthin (3,3'-dioxo-retro- β -carotene), but little is known so far about the biosynthetic pathway of oxocarotenoid synthesis.

In general, the main fatty acids in the lipids of fungi are palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid, e.g. in *Aspergillus nidulans* (Singh & Walker, 1956), *Phycomyces blakesleeianus* (Bernhard & Albrecht, 1948), *Neurospora crassa* (Todd, Stone, Hechter & Nussbaum, 1957) and *Phytomyces chartarum* (Hartman, Hawke,

Morice & Shorland, 1960). The present results show that the lipids from *Epicoccum nigrum* Link contain a similar fatty acid pattern.

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