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7′,8′,11′,12′-TETRAHYDRO-γ-CAROTENE: A NOVEL CAROTENE FROM *PHYCOMYCES BLAKESLEEANUS*

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Key Word Index—*Phycomyces blakesleeanus*; Mucoraceae; fungi 7′,8′,11′,12′-tetrahydro-γ-carotene; carotene cyclization; carotenoid biosynthesis.

Abstract—The natural occurrence of 7′,8′,11′,12′-tetrahydro-γ-carotene (7′,8′,11′,12′-tetrahydro-β,ψ-carotene, IV) is predicted and its isolation from diphenylamine-inhibited cultures of a mutant of *Phycomyces blakesleeanus* and characterization are described. The possible role of this novel monocyclic carotene in β-carotene biosynthesis is discussed.

INTRODUCTION

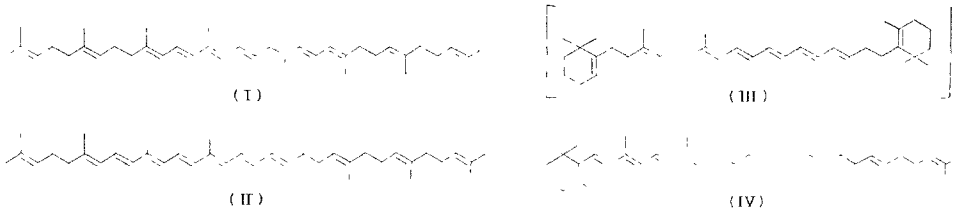
ALTHOUGH a general mechanism for the cyclization of the ψ-end group of an acyclic carotene to a β-ring has been proposed by Goodwin and Williams[1] as a result of their work on β-carotene (β,β-carotene) formation in *Phycomyces blakesleeanus*, the precise nature of the acyclic carotene substrate has not yet been defined (Fig. 1). The isolation of β-zeacarotene (7′,8′-dihydro-β,ψ-carotene) from diphenylamine (DPA)-inhibited cultures of *P. blakesleeanus*[2,3] indicated that neurosporene (7,8-dihydro-ψ,ψ-carotene) can cyclize and thus provided an alternative candidate to lycopene (ψ,ψ-carotene) as the intermediate between neurosporene and γ-carotene (β,ψ-carotene) in β-carotene biosynthesis. Neurosporene and lycopene are the penultimate and final products, respectively, of a dehydrogenation sequence in which phytoene (7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene) is converted first into phytofluene (7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene) and then, in higher plants and some microorganisms, into the conjugated heptaene, ζ-carotene (7,8,7′,8′-tetrahydro-ψ,ψ-carotene, I); subsequent dehydrogenations yield neurosporene and lycopene.[4,5]

The possibility of the cyclization of ζ-carotene (or of phytofluene or phytoene) has often been considered in the past but, apart from a report of the occurrence in berries of *Lonicera japonica* of a conjugated heptaene with a polarity far too low for all-*trans* ζ-carotene,[6] no further observations have been made to support this. Indeed, it could be argued (*vide infra*) that ζ-carotene, phytofluene and phytoene, each lacking an olefinic bond between carbons 7 and 8, might not lend themselves to cyclization at all.

We have recently observed that the conjugated heptaene isolated from DPA-inhibited cultures of the C115 strain of *P. blakesleeanus* [genotype *mad-107*(–)] is a mixture of

[1] GOODWIN, T. W. and WILLIAMS, R. J. H. (1965) *Biochem. J.* **94**, 5C.
[2] DAVIES, B. H., VILLOUTREIX, J., WILLIAMS, R. J. H. and GOODWIN, T. W. (1963) *Biochem. J.* **89**, 96P.
[3] WILLIAMS, R. J. H., DAVIES, B. H. and GOODWIN, T. W. (1965) *Phytochemistry* **4**, 759.
[4] PORTER, J. W. and LINCOLN, R. E. (1950) *Arch. Biochem.* **27**, 390.
[5] DAVIS, J. B., JACKMAN, L. M., SIDDONS, P. T. and WEEDON, B. C. L. (1966) *J. Chem. Soc.* **C**, 2154.
[6] GOODWIN, T. W. (1952) *Biochem. J.* **51**, 458.

ζ-carotene (I) and its unsymmetrical isomer, 7,8,11,12-tetrahydrolycopene (7,8,11,12-tetrahydro-ψ,ψ-carotene, II).[7] Cyclization of the latter isomer would yield the monocyclic carotene, 7′,8′,11′,12′-tetrahydro-γ-carotene (7′,8′,11′,12′-tetrahydro-β,ψ-carotene, IV). The predictable properties of this carotene would include an absorption barely in the visible region of the spectrum (highest wavelength band at about 400 nm) and a polarity such that it would chromatograph slightly ahead of phytofluene on aluminium oxide. This paper describes a successful attempt to isolate and characterize this novel carotene.



RESULTS

The existence of 7′,8′,11′,12′-tetrahydro-γ-carotene in DPA-inhibited cultures of the C115 strain of *P. blakesleeanus* was first suggested by the pale yellow colour of the leading edge of the normally colourless, but fluorescent, zone of phytofluene revealed by TLC of the unsaponifiable fraction. Repeated larger-scale separations of the carotenes on columns of alumina enabled the isolation of the novel carotene in a chromatographically pure state. It was slightly less polar than all-*trans* phytofluene on alumina and its absorption spectrum in light petrol. had inflexions at 329 and 358 nm, absorption maxima at 378 and 397 nm and a minimum at 389 nm.

TABLE 1. ABSORPTION SPECTRA IN LIGHT PETROL. OF ACYCLIC CAROTENES AND THEIR CORRESPONDING MONO-β-CYCLIC ISOMERS

Acyclic carotene	λ_{max} (nm)			Mono-β-cyclic isomer	λ_{max} (nm)		
3,4-Dehydrolycopene[11]	463	493	527	Torulene[13]	460	484	518
Lycopene[12]	446	473	505	γ-Carotene[13]	437	462	494
Neurosporene[12]	414	439	467	β-Zeacarotene[3]	405	428	455
7,8,11,12-Tetrahydrolycopene[12]	374	394·5	418·5	7′,8′,11′,12′-Tetrahydro-γ-carotene	358	378	397

The MS (using an ionization potential of 70 eV) had *m/e* 69 as its base peak. The molecular ion at *m/e* 540 (78%) corresponds to a molecular formula of $C_{40}H_{60}$ and represents an acyclic carotene with 11 olefinic bonds, a monocyclic carotene with 10 olefinic bonds or a bicyclic carotene with 9 olefinic bonds. The absence of ions at M-135 and M-56, characteristic of γ-rings[8] and ε-rings[9] respectively, showed that these structures were not present. The strength (55%) of an ion at *m/e* 335 (M-205), compared with the weakness (<2%) of ions

[7] DAVIES, B. H. and REES, A. F., unpublished work.
[8] SCHWIETER, U., ENGLERT, G., RIGASSI, N. and VETTER, W. (1969) *Pure Appl. Chem.* **20**, 365. *Phytochemistry* **10**, 1595.
[9] ARPIN, N., FIASSON, J.-L., BOUCHEZ-DANGYE-CAYE, M. P., FRANCIS, G. W. and LIAAEN-JENSEN, S. *Phytochemistry* **10**, 1595. (1971).

for M-69 and M-137, and the presence of a strong metastable ion at *m/e* 208 ($335^2/540 = 207{\cdot}8$) indicates the fully saturated nature of the 11,12- (or 11′,12′-) carbon–carbon bond.[10]

The visible absorption maxima of the novel carotene are at significantly shorter wavelengths than those of the isomeric carotene, 7,8,11,12-tetrahydrolycopene (Table 1), and there is a relative loss of fine structure in the spectrum. This is characteristic of the situation where the chromophore, in this case a conjugated heptaene, extends into a β-end group; this same phenomenon is shown in the comparison (Table 1) of the absorption maxima of the isomeric pairs, neurosporene and β-zeacarotene, lycopene and γ-carotene, and 3,4-dehydrolycopene (3,4-didehydro-ψ,ψ-carotene) and torulene (3′,4′-didehydro-β,ψ-carotene). This effect is due to steric hindrance (between the ring methyl groups and the acyclic polyene chain) which results in a non-planarity of the structure, limiting the overlap of the π-orbitals associated with the ring (5,6–) double bond and those of the rest of the polyene chain.[14]

TABLE 2. CHROMATOGRAPHIC BEHAVIOUR OF ALL-*trans* CAROTENES ON ALUMINIUM OXIDE (WOELM NEUTRAL), LISTED IN DECREASING ORDER OF POLARITY[7]

Carotene	Conjugated double bonds	Total double bonds	β-Rings
3,4-Dehydrolycopene *	13	14	0
Lycopene	11	13	0
Torulene *	13	13	1
Neurosporene	9	12	0
γ-Carotene *	11	12	1
7,8,11,12-Tetrahydrolycopene	7	11	0
β-Zeacarotene	9	11	1
β-Carotene	11	11	2
Phytofluene	5	10	0
7′,8′,11′,12′-Tetrahydro-γ-carotene *	7	10	1
Phytoene	3	9	0

*A mixture of all 11 carotenes would normally show clear separations only at points marked thus.

Thus the novel carotene must be a mono- or bi-β-cyclic isomer of 7,8,11,12-tetrahydrolycopene (cyclized isomers of the symmetrical ζ-carotene retain the ζ-carotene spectrum[12]). The possibility of the bicyclic structure can be eliminated by considering the chromatographic behaviour of the carotene. It is more polar than all-*trans* phytoene on columns of alumina, but less polar than all-*trans* phytofluene, from which it is separated only with

[10] DAVIES, B. H., HOLMES, E. A., LOEBER, D. E., TOUBE, T. P. and WEEDON, B. C. L. (1969) *J. Chem. Soc.* **C**, 1266.
[11] LIAAEN JENSEN, S. (1965) *Phytochemistry* **4**, 925.
[12] DAVIES, B. H. (1970) *Biochem. J.* **116**, 93.
[13] DAVIES, B. H. (1965) in *Chemistry and Biochemistry of Plant Pigments* (GOODWIN, T. W., ed.), p. 489, Academic Press, New York.
[14] WEEDON, B. C. L. (1969) *Fortschr. Chem. Org. Naturstoffe* **27**, 81.

difficulty. Table 2 shows that the polarities of carotenes on alumina are determined primarily by the total number of olefinic bonds (and not by the number of conjugated double bonds) and then by the number of β-rings A bicyclic isomer of 7,8,11,12-tetrahydrolycopene should be less polar even than all-*trans* phytoene. The novel carotene is clearly the monocyclic isomer, 7′,8′,11′,12′-tetrahydro-γ-carotene (IV).

DISCUSSION

In the absence of any evidence for the existence of a monocyclic phytofluene, which could yield a monocyclic conjugated heptaene by dehydrogenation, the isolation of 7′,8′,11′,12′-tetrahydro-γ-carotene from DPA-inhibited cultures of the C115 mutant of *P. blakesleeanus* must be taken as a clear indication of the ability of 7,8,11,12-tetrahydrolycopene to cyclize. This means that, in fungi, there are four known levels at which the formation of monocyclic carotenes can occur (Fig. 1).

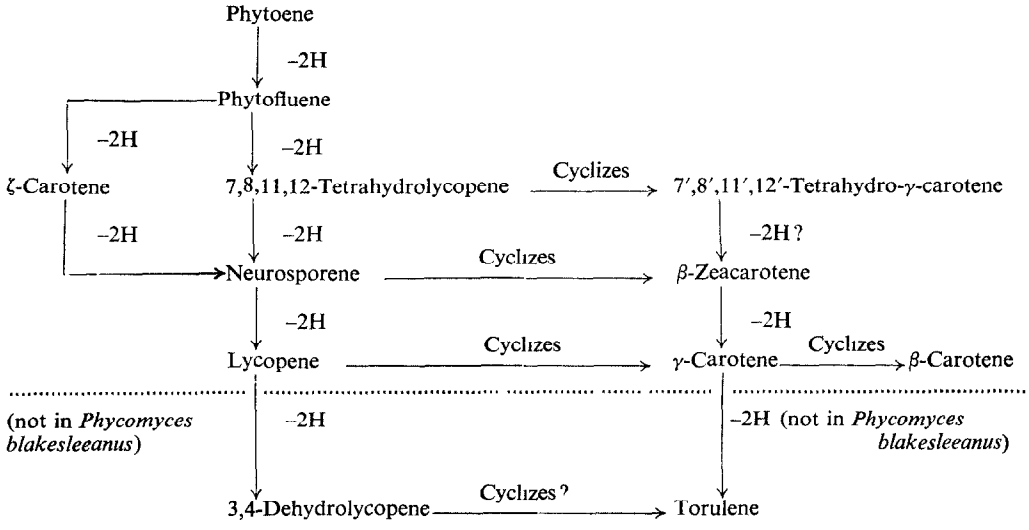


FIG. 1. ALTERNATIVE PATHWAYS OF CAROTENE CYCLIZATION IN FUNGI.

In addition to this novel cyclization at the conjugated heptaene stage of the polyene dehydrogenation sequence, monocyclic isomers are also formed at the conjugated nonaene (neurosporene) and undecaene (lycopene) levels and, in such organisms as *Neurospora crassa*,[15] at the conjugated tridecaene level. Direct evidence of cyclization, obtained by incorporation of the appropriate radioactive substrates, is available only for the conversions of neurosporene into β-zeacarotene and of lycopene into γ-carotene.[15] Torulene is not necessarily formed by the direct cyclization of 3,4-dehydrolycopene; it could be formed, as it presumably is in the red yeasts,[16] by the dehydrogenation of γ-carotene. It is known that, in this mutant of *P. blakesleeanus*, γ-carotene is formed both by lycopene cyclization and by the dehydrogenation of β-zeacarotene;[15] it is also possible that β-zeacarotene could be

[15] DAVIES, B. H. (1973) *Pure Appl Chem.* **35**, 1.
[16] SIMPSON, K. L. (1972) in *The Chemistry of Plant Pigments* (*Adv. Food Res*., Supp 3, CHICHESTER, C O., ed), p 9, Academic Press, New York and London.

formed, not only by the cyclization of neurosporene, but also by the dehydrogenation of 7′,8′,11′,12′-tetrahydro-γ-carotene. The latter step has yet to be demonstrated.

Whether all the primary cyclizations (of an acyclic precursor to a monocyclic carotene) are carried out by separate specific enzymes or by the same, non-specific, enzyme is not clear, although an equivalent sensitivity to nicotine on the part of the cyclizations both of neurosporene to β-zeacarotene and of lycopene to γ-carotene is consistent with, but not necessarily an indication of, the latter alternative.[17]

'η-Carotene' is the name given to a pigment, first isolated from the fruit of a variety of *Lonicera japonica*, which has some of the properties of a cyclized ζ-carotene.[6] It has an absorption spectrum identical with that of ζ-carotene but is much less polar on chromatography; it has been assumed to be the bicyclic isomer of ζ-carotene.[18] Unfortunately, although some of the properties of the synthetic bicyclic conjugated heptaene (7,8,7′,8′-tetrahydro-β,β-carotene, III) have been described,[12,14] a direct comparison of this pigment with natural η-carotene has never been undertaken. In spite of repeated attempts in this laboratory, it has not been possible so far to isolate η-carotene from the fruits of wild *Lonicera periclymenum*; it has never been confirmed that η-carotene is anything other than a stereoisomer of ζ-carotene.

The β-cyclization of a ψ-end group is presumably initiated by a protonic attack at C-2 and occurs with the loss of a proton from C-6 of the acyclic carotene.[1] There seems to be no mechanistic reason, in purely electronic terms, why 7,8,11,12-tetrahydrolycopene should be any more prone to cyclization than ζ-carotene yet, although both acyclic conjugated heptaenes occur in DPA-inhibited cultures of the C115 mutant of *P. blakesleeanus*,[7] only the unsymmetrical isomer appears to cyclize. One possible explanation is that in 7,8,11,12-tetrahydrolycopene the 5′,6′-double bond, which becomes part of the β-ring on cyclization, is the terminal olefinic bond of the rigid conjugated heptaene chromophore. If one binding site of the cyclization enzyme binds with the chromophore, the rigidity of the latter would ensure the correct positioning of the 5′,6′-double bond and C-6′ at the cyclization site of the enzyme. The symmetrical ζ-carotene, on the other hand, lacks the rigidity between its centrally placed chromophore and the 5,6- (or 5′,6′-) double bond so that the combination of this acyclic carotene with the first binding site of the enzyme would not bring the ψ-end group into the correct position for cyclization at the second site. Such a mechanism of primary cyclization would mean that any secondary cyclization (e.g. of γ-carotene to β-carotene) would probably require a second enzyme; this would be consistent with the observations of different sensitivities of the primary and secondary cyclization reactions to inhibition both with 2-(4-chlorophenylthio) triethylamine hydrochloride (CPTA)[19] and with nicotine.[17]

EXPERIMENTAL

Solvents. All solvents used were of AR grade. Light petrol. (b.p. 40–60°) and Et_2O were dried over Na and redistilled from reduced iron powder prior to use.

Growth of organism. The C115 strain of *Phycomyces blakesleeanus* [genotype *mad-107*(–)], which is a high β-carotene *N*-methyl-*N*-nitroso-*N*′-nitroguanidine mutant, was supplied by Prof M. Delbruck (California Institute of Technology, Pasadena, Calif., U.S.A.). Liquid cultures were inoculated from spore suspensions (obtained from Sabouraud dextrose agar slopes) and contained, per l : glucose, 25 g; yeast extract (Difco), 500 mg; $MgSO_4.7H_2O$, 500 mg; thiamin hydrochloride, 0·25 mg, L-asparagine, 1·25 g; L-leucine, 1·25 g and KH_2PO_4, 1·5 g. Starter cultures (100 ml) were grown for a total of 24 hr at 25° under illumination (3200 lx) in a Psychrotherm Incubator Shaker, diphenylamine (DPA) being added in EtOH 12 hr after inoculation in

[17] Davies, B. H., Rainsley, S. and Rees, A. F. unpublished work.
[18] Straub, O. (1971) in *Carotenoids* (Isler, O., ed.), p. 773, Birkhäuser, Basel.
[19] Hsu, W.-J., Yokoyama, H. and Coggins, Jr., C. W. (1972) *Phytochemistry* **11**, 2985.

order to give a concentration of 35 μM. Larger scale cultures (1 l.), also containing 35 μM DPA, were each inoculated by aseptic transfer of a 100 ml starter culture and incubated under the above conditions for 36 hr.

Extraction of unsaponifiable lipid. The mycelium was harvested by filtering the cultures through muslin and was then washed with dist. H_2O and squeezed dry by hand. Extraction of the lipids, 2× with acetone and 2× with Et_2O, was accomplished using an Ultra Turrax homogenizer. Sufficient H_2O was added to the bulked organic extract to transfer the lipid to the Et_2O phase. The lipid was washed 3× with H_2O and, after the solvent had been distilled off, the lipid was saponified and the unsaponifiable fraction isolated by standard methods.[13]

TLC. A small sample of the unsaponifiable lipid was chromatographed by TLC (250 μm) on a 1·1 (w/w) mixture of MgO (B.D.H., for chromatographic adsorption analysis) and Silica Gel G (Merck), which had been activated for 2 hr at 110°. Development with 17% C_6H_6 in light petrol. (v/v) gave a good separation of the coloured carotenes, the least polar of which was β-carotene (R_f 0·80). Comparison with authentic phytoene[20] showed that this colourless carotene, revealed by treatment with iodine vapour,[21] ran with the solvent front while phytofluene, detected by its green fluorescence in 360 nm UV light,[13] had an R_f of 0·90. Phytofluene normally appears colourless on an unstained thin-layer plate in daylight but, on this occasion, the leading edge of its zone had a greenish-yellow colour so pale that it indicated the presence of a pigment absorbing barely in the visible region of the spectrum.

Column chromatography. The rest of the total unsaponifiable lipid extracted from a 2 l. culture was chromatographed initially on a 30 g column of aluminium oxide (Woelm neutral, Brockmann activity grade III). The sample was applied in light petrol. and the chromatogram was developed with light petrol. followed by 0·5% (v/v) diethyl ether in light petrol. Early fractions, which had no UV absorption, were discarded and the phytoene (λ_{max} 276 [infl.], 286 and 297 [infl.] nm) and the fluorescent phytofluene (λ_{max} 331, 346 and 368 nm) were collected. Phytoene was eluted with light petrol. alone and 0·5% ether was used to elute the phytofluene band. The collection of the phytofluene was terminated as soon as β-carotene (λ_{max} 425 [infl.], 448 and 475 nm) appeared in the eluate. Concentration of the phytoene and phytofluene fractions resulted in very pale yellow solutions, spectrophotometric examination of these revealed additional absorption bands at 358, 378 and 397 nm in the phytoene fraction and at 397 nm in the phytofluene. The phytoene and phytofluene fractions were recombined and chromatographed on a column of aluminium oxide (10 g, Woelm neutral) deactivated with 2% H_2O (between Brockmann activity grades I and II). The developing solvent was light petrol. containing steadily increasing concentrations of Et_2O; 10 ml fractions were collected. The first 200 ml solvent (zero to 3% Et_2O) eluted, in turn, 15-*cis* phytoene[20] (λ_{max} 276 [infl.], 286 and 297 [infl.] nm) and small amounts of all-*trans* phytoene[20] (λ_{max} 276 [infl.], 286 and 297 nm) and of *cis*-phytofluene (λ_{max} 328, 342 and 364 nm). The yellow carotene was eluted with the next 100 ml solvent (4–6% Et_2O) and was followed by all-*trans* phytofluene (λ_{max} 332, 346 and 368 nm) which left the column in 7% ether in light petrol. Those fractions which contained the coloured carotene in a chromatographically pure form were bulked to yield about 90 μg of the pigment.

Absorption spectra. All electronic spectra were recorded in light petrol. on a Unicam S.P. 800 recording spectrophotometer, the wavelength scale of which was calibrated with the appropriate absorption bands of a holmium oxide filter. Quantitative measurements were made on solutions of known vol. in one of a matched pair of 1 cm silica cuvettes; carotenoid concentrations were calculated using standard values[13] for the specific extinction coefficients ($E^{1\%}_{1\,cm}$). A nominal value of 1800 was assigned to 7′,8′,11′,12′-tetrahydro-γ-carotene.

MS. MS were determined on an A.E.I. MS 12 mass spectrometer at the Department of Biochemistry of the University of Liverpool by Mr. J. Ireland and through the courtesy of Dr. G. Britton. The probe temp. was 220° and the ionization potential was 70 eV.

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[21] MERCER, E. I., DAVIES, B. H. and GOODWIN, T. W. (1963) *Biochem. J.* **87**, 317.