Revised Structures of the Two C_{50} Carotenoids C.p. 450 and C.p. 473 from *Corynebacterium poinsettiae*

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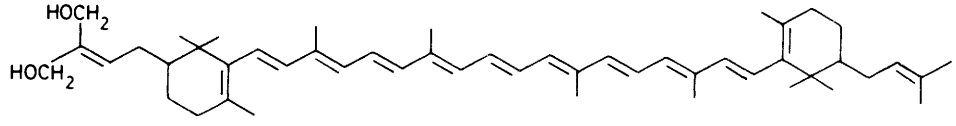
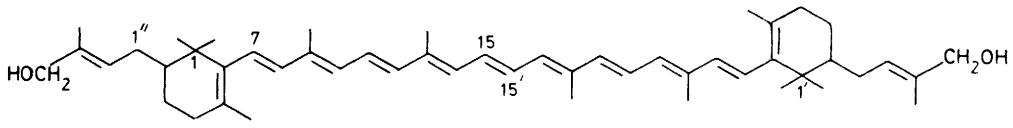
The revised structures 2,2′-bis-(4-hydroxy-3-methylbut-2-enyl)-β,β-carotene and 2-(4-hydroxy-3-methylbut-2-enyl)-2′-(3-methylbut-2-enyl)-3′,4′-didehydro-1′,2′-dihydro-β,ψ-caroten-1′-ol for the two C_{50} carotenoids C.p. 450 and C.p. 473 from *Corynebacterium poinsettiae* were deduced by ^1H and ^{13}C n.m.r. spectroscopy.

The taxonomic position of the plant pathogen *Corynebacterium poinsettiae* is uncertain, but it is described as an aerobic, motile, Gram-positive rod, requiring thiamine for reproduction.[1] The organism is coloured by carotenoids and can be yellow, orange, or pink depending on the thiamine concentration in the growth medium. In the absence of thiamine, or at low thiamine concentrations, the cultures are pink due to the main pigment C.p. 496, which has been identified as bisanhydrobacterioruberin [2,2′-bis-(3-methylbut-2-enyl)-3,4,3′,4′-tetradehydro-1,2,1′,2′-tetrahydro-ψ,ψ-carotene-1,1′-diol]. At higher thiamine concentrations, two shorter chromophore carotenoids, C.p. 450 and C.p. 473, predominate. These were assigned the C_{50} bicyclic and monocyclic diol structures 2-[4-hydroxy-3-(hydroxymethyl)but-2-enyl]-2′-(3-methylbut-2-enyl)-β,β-carotene (**1**), and 2′-(4-hydroxy-3-methylbut-2-enyl)-2-(3-methylbut-2-enyl)-3′,4′-didehydro-1′,2′-dihydro-β,ψ-caroten-1′-ol (**3**), respectively, by Norgard *et al.* on the basis of spectroscopic studies (u.v.–visible, i.r., m.s., and ^1H n.m.r.).[2,3]

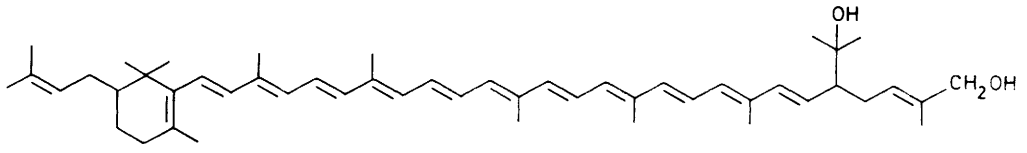
We have recently elucidated the stereochemistry of the cyclization reaction in the biosynthesis of the conventional C_{40} β,β-carotene-3,3′-diol, zeaxanthin, in a *Flavobacterium*.[4] The results obtained indicate the stereochemistry of cyclization in the C_{40} and C_{50} carotenoid series to be different. Thus, in the formation of zeaxanthin the initial step in the cyclization involves proton attack at C-2 of the acyclic precursor, and the hydrogen introduced occupies the β-position at C-2 of the β-ring produced. Cyclization in the C_{50} series is generally considered to be initiated by similar electrophilic attack at C-2 of the acyclic carotenoid precursor, this time by a C_5 species instead of a proton. The C_5 group introduced, however, occupies the α-position at C-2 of the cyclic C_{50} carotenoids produced. It is therefore of interest to determine the behaviour of the C-1 methyl substituents during cyclization in the C_{50} series, as has been done for the C_{40} compound zeaxanthin by a ^{13}C-labelling procedure. A necessary preliminary to this is the full assignment of the ^1H and natural abundance ^{13}C n.m.r. spectra of the C_{50} compounds, so that the position of any



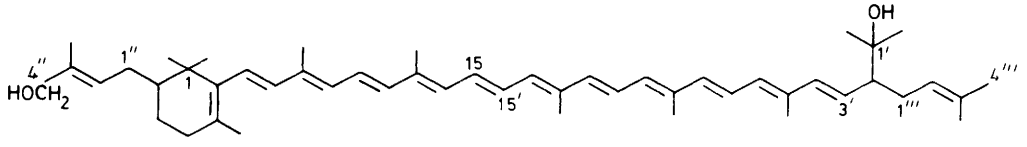
(**1**)



(**2**) C.p. 450



(**3**)



(**4**) C.p. 473

isotopic enrichment in subsequent experiments can be established. The initial spectroscopic data obtained were not fully compatible with the structures previously proposed for C.p. 450 and C.p. 473, so a detailed investigation by ^1H and ^{13}C n.m.r. was undertaken. This has proved conclusively the structures (**2**) and (**4**) instead of (**1**) and (**3**), respectively, for the C.p. 450 and C.p. 473 samples isolated from our strain of *Corynebacterium poinsettiae*.

Spectroscopic Investigation

C.p. 450.—The 400 MHz ^1H and the 100 MHz ^{13}C n.m.r. spectra unequivocally proved the symmetrical structure (**2**) for this compound as revealed by the number of signals in both spectra. The ^1H n.m.r. chemical shifts (2 mg of sample in 0.6 ml $CDCl_3$; 25 °C) are given in Figure 1. The assignments were supported by several decoupling experiments. Irradiation of the signal of the olefinic proton 2″-H (broadened triplet, δ 5.445) helped to locate the two non-equivalent protons 1″-H_a and 1″-H_b at δ 2.214 and 1.798. In the same experiment the signals of 4″-H_2 at δ 4.035 *and* of 3″-Me at δ 1.684 were sharpened, confirming the proposed symmetric structure. Decoupling of 1″-H_a and 1″-H_b in two subsequent experiments gave as an additional information the approximate chemical shift of 2-H near δ 1.30 where, according to the integral, the signals of 4 further protons, probably of one of the 3-H pairs and the two 4″-OHs, are located. Decoupling at δ *ca.* 1.68 with the closely spaced signals of 5-Me, 3″-Me, and one of the two 3-H protons gave decoupling effects at δ *ca.* 1.30 (3-H and 2-H), at δ 5.445 (sharpening of 2″-H), and at δ 6.164 (sharpening of 7-H) in agreement with expectation.

A further confirmation of the proposed structure (**2**) for C.p. 450 was derived by nuclear Overhauser (n.O.) difference measurements. This method was applied recently in a number of cases for the confirmation or elucidation of the structures of carotenoids.[5,6] Thus, pre-irradiation (8 s) of the OCH_2 signal at δ 4.035 resulted in an n.O. enhancement at 2″-H of 15%, thus confirming the *cis* relationship of the two groups of protons. In addition, a small but significant n.O. effect of 3% was observed at the 3″-Me signal in accordance with the proposed structure (**2**).

Saturation of the two geminal methyl groups at δ 0.916 and

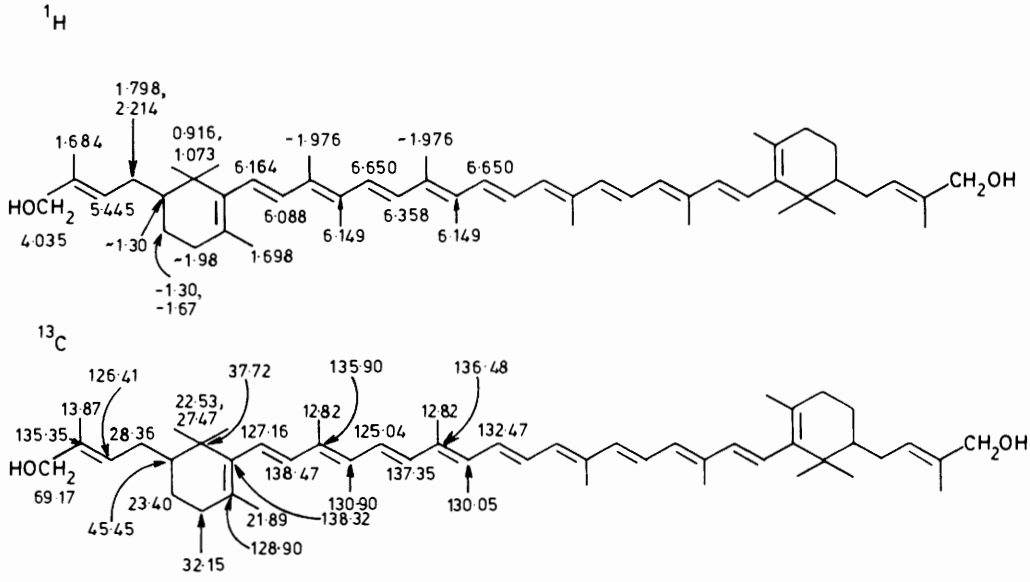


Figure 1. ^1H and ^{13}C n.m.r. data of C.p. 450 (**2**) (δ in p.p.m.)

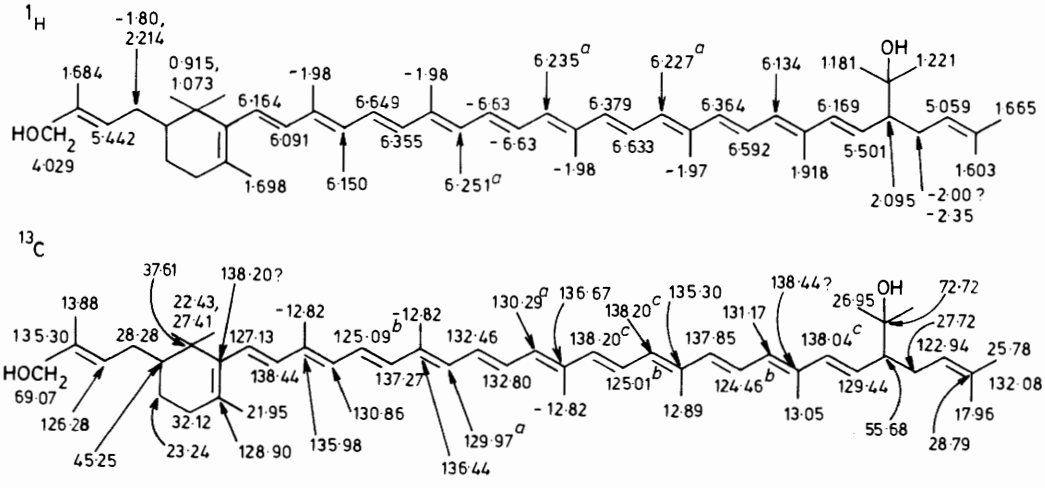


Figure 2. ^1H and ^{13}C n.m.r. data of C.p. 473 (**4**) (δ in p.p.m.). [a,b,c] Corresponding assignments may be interchanged

1.073 in two subsequent experiments gave, besides the expected n.O. effects[5] on 7-H (*ca.* 5% in both experiments) and 8-H (*ca.* 11%), relevant effects at 1″-H_a (3 and 4%) meaning that this proton is close to both methyl groups whereas 1″-H_b is pointing away. However, the n.O. effect is clearly in agreement with the assumed substitution of the β ring at C-2.

The symmetric structure of C.p. 450 is also clearly demonstrated by the ^{13}C n.m.r. studies (10 mg in 0.6 ml $CDCl_3$; 36 °C). The assignments, which were supported by a CW-offset decoupled spectrum and 4 experiments with increasing concentrations of the shift reagent Yb(dpm)$_3$, are also given in Figure 1. The latter measurements were partly of relatively low quality due to the fact that most signals near the complexing site HO–CH_2 were increasingly broadened upon addition of the shift reagent. However, this observation simplified the assignment of the carbon signals of the side-chain.

The c.d. spectrum of C.p. 450 was found to be virtually identical with that published by Andrewes *et al.*,[3] therefore the (2*R*,2′*R*) structure is confirmed.

C.p. 473.—Part of the 400 MHz ^1H n.m.r. spectrum of this C_{50} carotenoid (1.1 mg in 0.4 ml $CDCl_3$; 26 °C) is very crowded even at this high frequency; however, the presence of the same 2-substituted β-end-group with the attached 4-hydroxy-3-methylbut-2-enyl side-chain as in (**2**) immediately follows from the presence in the ^1H n.m.r. spectrum of all the characteristic signals found also for C.p. 450. The assignments, some of which are only tentative, are compiled in Figure 2.

Additional proof for structure (**4**) was obtained by a decoupling experiment: irradiation of the olefinic signal of 2‴-H at δ 5.059 (broadened triplet) resulted in a significant sharpening of the *two* methyl signals at δ 1.665 and 1.603. Furthermore, a stronger decoupling effect was observed at the diffuse, broad multiplet near 2.35 p.p.m., assigned to one of the two protons at C-2‴.

The 100 MHz ^{13}C n.m.r. spectrum (1.1 mg in 0.13 ml $CDCl_3$; 17 °C) is also clearly in agreement with structure (**4**) since all the signals of the left-side part of the molecule are found at practically the same shifts as in (**2**). All the assignments are compiled in Figure 2. For further spectroscopic data see below.

Experimental

Bacterial Cultures.—Corynebacterium poinsettiae strain 854 (from ATCC 9682 ex ICPB CP2, 1960) was obtained from the National Collection of Plant Pathogenic Bacteria, Hatching Green, Harpenden, England. It was cultured first on a small scale (100 ml medium in 250-ml conical flasks modified with three or four indentations to increase aeration) in an illuminated orbital incubator at 18–21 °C in medium[1] containing thiamine (100 μg l^{-1}). After 2 days growth, these cultures were used to inoculate large-scale cultures (ten 10-l flasks, each containing 6 l of medium) which were grown under continuous aeration for 7 d at room temperature and in a normal day/night light régime. The bacteria were then harvested with an Alfa-Laval continuous centrifuge (model LAB-102-B25) operating at a flow rate of *ca.* 30 l h^{-1}.

Pigment Extraction and Purification.—The harvested cells (*ca.* 200 ml packed cell volume) were suspended in 0.5M potassium phosphate buffer (pH 7.7) (300 ml) and incubated for 16 h at room temperature with lysozyme (1 g) before extraction with warm acetone (2 × 1 l) and warm acetone–methanol (1:1; 2 × 1 l). The extracted carotenoids were transferred to diethyl ether and the ethereal solution was washed with water, evaporated, and dried under a stream of nitrogen in the normal way. The total extract was dissolved in the minimal volume of dichloromethane and the solution was diluted 5-fold with light petroleum (b.p. 40—60 °C) before chromatography on a column (150 g) of neutral alumina (activity grade III). After collection of a range of minor carotenoids in fractions eluted with light petroleum and 40% diethyl ether in light petroleum, the major carotenoids were then eluted with diethyl ether. After evaporation of the eluate, the main carotenoids were separated by t.l.c. on silica gel G, with 60% diethyl ether in light petroleum as developing solvent. This gave the three main carotenoids, C.p. 450, C.p. 473, and C.p. 496, R_F value 0.25, 0.4, and 0.5, respectively. C.p. 496 was not examined further, but C.p. 450 and C.p. 473 were purified by t.l.c. on MgO–kieselguhr G (1:1) with 30% acetone in light petroleum (R_F value 0.5 and 0.2 respectively), filtered (in ethereal solution) through a small column of neutral alumina (activity grade III—IV), and finally purified by h.p.l.c. on Partisil-5 before determination of their n.m.r. and mass spectra.

C.p. 450 had $\lambda_{max.}$ 425, 450, and 478 nm in ethanol and 426, 453, and 480 nm in acetone. The mass spectrum showed a molecular ion peak at *m/z* 704 (100%) consistent with $C_{50}H_{72}O_2$. In the high-mass region only one further peak with intensity above 4% was seen, at *m/z* 612 (*M* − 92, 26%).

C.p. 450 readily formed a diacetate (Ac_2O–pyridine), the mass spectrum of which had M^+ at *m/z* 788.

C.p. 473 had $\lambda_{max.}$ 450, 476, and 508 nm in acetone and 447, 473, and 505 nm in ethanol. The mass spectrum had M^+ at *m/z* 704 (20%; consistent with $C_{50}H_{72}O_2$) and major fragment ions at *m/z* 686 (4; *M* − H_2O), 668 (1; *M* − $2H_2O$), 646 (3.5; *M* − 58, Me_2CHOH), 628 (2; *M* − 18 − 58), 612 (6; *M* −92), 598 (19; *M* − 106), 540 (3; *M* − 106 − 58), and 69 (100).

Instrumentation.—Electron impact mass spectra were obtained by Dr. M. E. Rose and Mr. M. Prescott with a VG-Micromass 7070 F mass spectrometer, coupled to a Finnigan Incos Data System. The direct-insertion probe was used, at ion-source temperature 200—220 °C and ion-beam energy 20 or 70 eV. N.m.r. spectra were recorded in $CDCl_3$ on a Bruker WM-400 FT spectrometer with ASPECT 2000 (80 K Memory) and disk unit CDC 9448 (80 + 16 MByte).

Note added in proof. This work is taken from the Ph.D. Thesis of A. P. Mundy, University of Liverpool, 1981. Since it was submitted, other authors have published work leading to the same conclusion (A. G. Andrewes and S. Liaaen-Jensen, *Tetrahedron Lett.*, 1984, **25**, 1191).

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