# *cis*-Stereoisomers of β-Carotene and its Congeners in the Alga *Dunaliella bardawil*, and their Biogenetic Interrelationships

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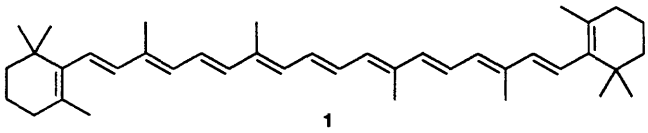
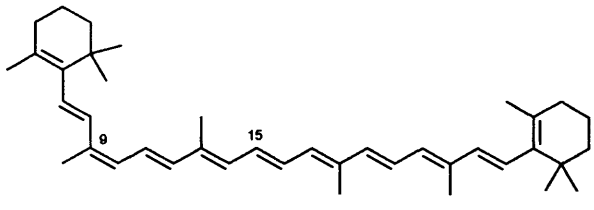
The acyclic carotenoids phytoene and phytofluene, which are precursors of 9-*cis*-β-carotene produced by the alga *Dunaliella bardawil*, have been separated and characterised. Spectroscopic data, and comparison with synthetic model compounds and corresponding carotenoids found in the fruits of the Tangerine tomato *Lycopersicon esculentum* var., establish that the pigments have the stereochemistries 15-*cis*-**6** and 9-*cis*-**11** respectively. The data suggest that phytoene **6** is the clear branch point for the formation of 9-*cis*-β-carotene **2** in *D. bardawil*. In addition, spectroscopic data for β-carotene isolated from *D. bardawil* showed that it was isomerically pure 9-*cis*-β-carotene **2**.

The halotolerant green alga *Dunaliella* is commercially very important, and it is grown on a large scale in parts of Israel, Australia and the USA. The considerable amounts of β-carotene **1** that the alga produces (*ca.* 10% dry weight) is harvested for use as a natural food colourant and as a vitamin supplement.[1,2] Indeed, the two strains of *Dunaliella*, *i.e.* *D. salina* Teod and *D. bardawil*, that produce β-carotene, actually appear red rather than green owing to the significant accumulation of carotenoids. The rate and extent of β-carotene accumulation in *Dunaliella* is critically dependent on the growth conditions. Thus, when the light intensity is greater than the intensity required for normal growth, and when nutrient levels are reduced to limit growth, β-carotene accumulates to the highest levels.[3] This feature has led some researchers to believe that β-carotene is accumulated in *Dunaliella* to protect the alga against the harmful effects of high intensity radiation; indeed strains of *Dunaliella* which do not accumulate β-carotene die on exposure to high light intensity.



**1**



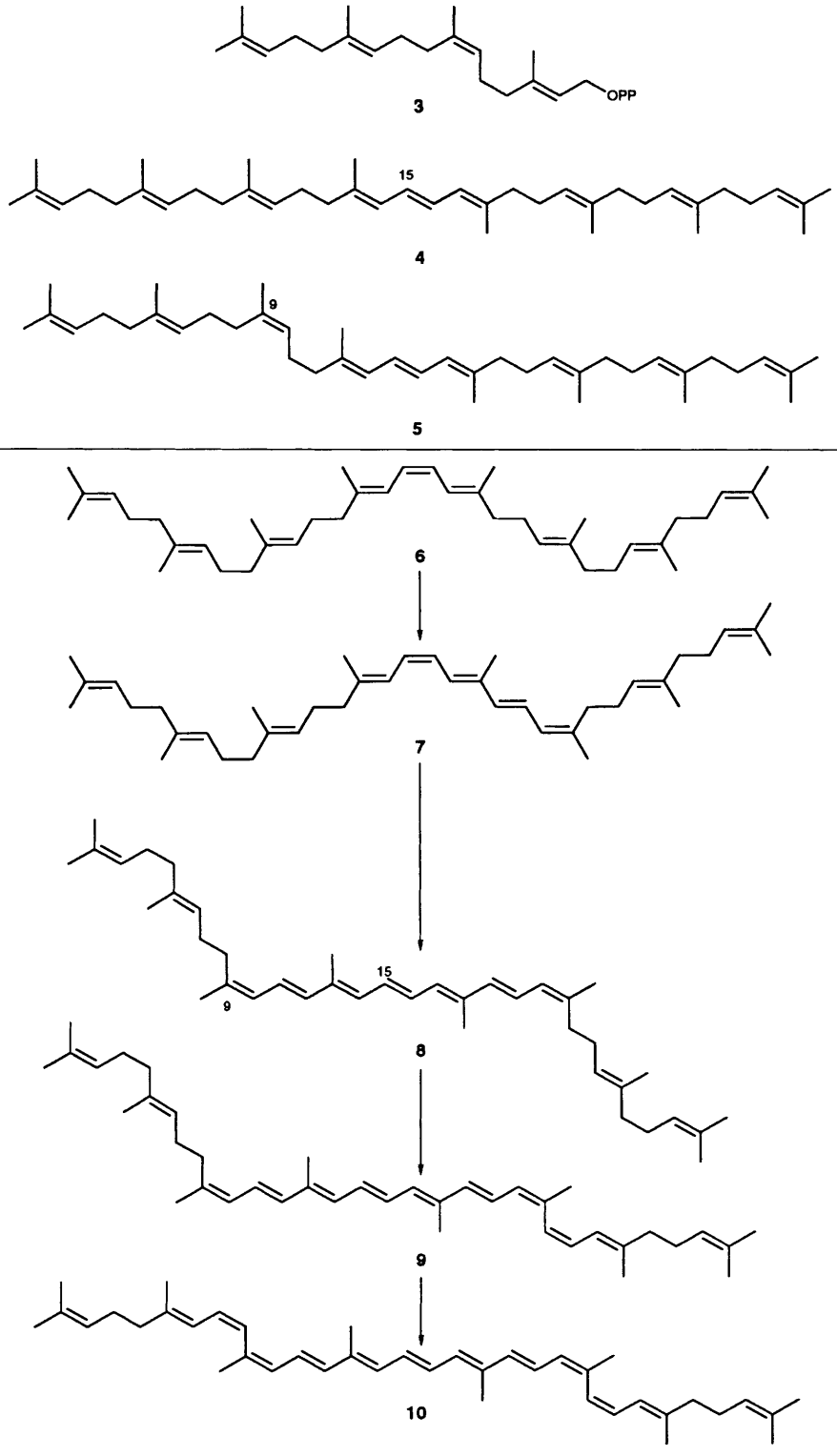

**2**

The most interesting feature about the accumulation of β-carotene by *Dunaliella* is that the carotenoid is not present entirely as the normal all-*trans*-form **1**, but that it is reported to be produced in the alga as a 1:1 mixture of **1** and the 9-*cis*-stereoisomer **2**.[4,5] Although a biological rationale for the accumulation of 9-*cis*-β-carotene **2** in *Dunaliella* is not immediately obvious, it has been proposed that *cis*-carotenoids, with their more open V-shapes, are more efficient radical quenching agents than their all-*trans* isomers, and therefore provide greater protection to the alga against the harmful effects of high light intensity and damage by oxidation processes.

The fact that *D. bardawil* accumulates large amounts of 9-*cis*-β-carotene **2** has contributed greatly to its commercial success. Thus, recent epidemiological and oncological studies have suggested that normal to high levels of β-carotene in humans protect against cancer development, especially lung cancer. In addition, in animal models, carotenoids have been shown to protect against ultraviolet-induced skin cancers and chemically induced tumours. There is now strong evidence that naturally derived carotenoids which contain *cis*-isomers are more efficient in the prevention of cancer formation.[6,7] The exploitation of this feature, in the case of *cis*-β-carotene from *Dunaliella*, for the formulation of various health tablets, skin lotions and body oils available to the public has been, perhaps, predictable.

*cis*-Carotenoids occur only rarely in Nature and their biosynthesis is therefore even the more intriguing. In the case of the biogenesis of β-carotene in *Dunaliella*, Ben-Amotz and his collaborators have clearly demonstrated that the proportion of 9-*cis*-**2** to all-*trans*-**1** carotenoid that accumulates in the alga is dependent on the amount of light absorbed during a division cycle; the higher the light intensity, the higher is the 9-*cis*- to all-*trans*-β-carotene ratio.[1,5] In addition, treatment of *D. bardawil* with the bleaching herbicide norflurazon, has resulted in the accumulation of phytoene at the expense of β-carotene in the alga,[8] and the 9-*cis* and all-*trans* configurations, **5** and **4** respectively, have been assigned to the isolated phytoenes.[1] In early reports, it has also been suggested that the 9-*cis* stereochemistry **2** in β-carotene from *Dunaliella* has its origins in 9-*cis*-geranylgeranyl pyrophosphate **3**.[5]

In studies of the tetra-*cis*-lycopene **10** (also known as 'prolycopene') and its congeners 15-*cis*-phytoene **6**, 9′,15-di-*cis*-phytofluene **7**, 9,9′-di-*cis*-ζ-carotene **8** and proneurosporene **9** in the Tangerine tomato fruit, *Lycopersicon esculentum* we have earlier demonstrated that the 9,9′-di-*cis* stereochemistry in prolycopene is laid down: (*i*) at the phytoene→phytofluene, and (*ii*) at the phytofluene→ζ-carotene stages in its biosynthesis (see Scheme 1).[9] It seemed unlikely to us that the biosynthesis of 9-*cis*-β-carotene **2** in *Dunaliella* would follow a completely different pathway to that followed by *L. esculentum* in the accumulation of the 9- and 9,9′-*cis*-carotenoids **7**→**10**. We decided therefore to examine the stereochemistries of the phytoene and phytofluene carotenoids produced by *Dunaliella* in anticipation that, as observed with *L. esculentum*, it was at this part in the biosynthesis of 9-*cis*-β-carotene in *Dunaliella* that the 9-*cis*-double bond is incorporated, *i.e.* not before as suggested with the involvement of 9-*cis*-geranylgeranyl pyrophosphate **3** and not later by for example enzymic isomerisation of all-*trans*-β-carotene. Fortunately, Shaish *et al.*[10] have already demonstrated that when *D. bardawil* is grown in the presence of the

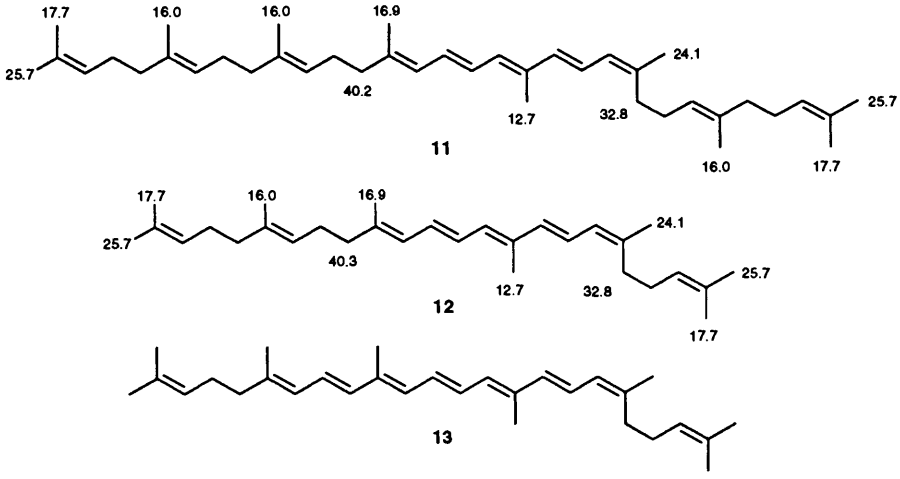



**Scheme 1**

desaturation inhibitor norflurazon, the alga accumulates phytoene and phytofluene, and hence these compounds are readily available for scrutiny of their detailed stereochemistry.

Thus, crude extracts of phytoene and of phytofluene were kindly made availabe to us by colleagues in Israel. Each extract was next carefully purified by preparative layer chromatography on Kieselgel plates and the separated isomers were then examined by $^{1}H$ and $^{13}C$ NMR.

Spectroscopic analysis of the phytoene from *Dunaliella* straightaway indicated that the carotenoid was approximately 90% pure isomerically, and that the major isomer was 15-*cis*-phytoene **6**,[11] *i.e.* the same isomer of phytoene produced by both Tangerine and red tomato.[10] Thus, the nine signals in the olefinic region of the $^{13}C$ NMR spectrum of the sample of phytoene demonstrated immediately that the carotene had a symmetrical structure. The ratio (1 : 3) of 'in chain' ($\delta$ 1.68, *E*-1-, *E*-1′-$CH_3$) to 'out of chain' ($\delta$ 1.62, *Z*-1-, *Z*-1′-, 5-, 5′-, 9-, 9′-$CH_3$) methyl resonances in the $^{1}H$ NMR spectrum showed that each of the four non-conjugated double bonds capable of isomerisation in the phytoene had the *E*-configuration;




confirmation of this came from the $^{13}C$ NMR spectrum ($\delta$ 16.02, 5-, 5'-, 9-, 9'-$CH_3$ and $\delta$ 39.73, $CH_2$-4, -4', -8, -8'). The IR absorption at 767 $cm^{-1}$ (and no significant absorption at *ca.* 960 $cm^{-1}$) indicated that the central disubstituted double bond had the *Z*-configuration. Final confirmation was provided by comparison of the $^{13}C$ and $^{1}H$ NMR spectra of this phytoene sample with those of authentic 15-*cis*-phytoene **6** isolated from the Tangerine tomato;[10] the spectra were completely superimposable.

The minor isomer in the phytoene mixture (< 10%), which could only be seen in trace amounts in the $^{13}C$ NMR spectrum, could not be assigned to a particular isomer of phytoene although the lack of signal at $\delta$ 23.5, characteristic of the vinyl methyl of a *cis*-trisubstituted double bond, demonstrated that the second isomer was not 9-*cis*-phytoene **5**.

Analysis of the purified phytofluene obtained from *Dunaliella*, first by IR spectroscopy, showed an absorption at 961 $cm^{-1}$ but no significant absorption at 775 $cm^{-1}$ indicating that the chromophore contained only *E*-disubstituted double bonds. The ratio (2:5) of 'in chain' ($\delta$ 1.68, *E*-1-, *E*-1'-$CH_3$) to 'out of chain' ($\delta$ 1.59, 5-, 5'-, 9-$CH_3$) methyl resonances in the $^{1}H$ NMR spectrum of the compound showed that each of the three non-conjugated double bonds capable of isomerisation had the *E*-configuration; confirmation of this came from the $^{13}C$ NMR spectrum ($\delta$ 16.02, 5-, 5'-, 9-$CH_3$ and $\delta$ 39.73, $CH_2$-4, -4', -8). The carbon chemical shift of 13'-$CH_3$ ($\delta$ 12.73) clearly demonstrated that the double bond at C-13' in the phytofluene was of *E*-configuration (for *Z*-13', predicted 13'-$CH_3$ of approximately, $\delta$ 20). The $^{13}C$ NMR spectrum also showed that a double bond at one end of the pentaene chromophore had an *E*-configuration, while the other, very interestingly, had a *Z*-configuration, *i.e.* carbon chemical shift of the methyl group at the end of the chromophore and of the adjacent methylene group: $\delta$ 16.94, 40.23; $\delta$ 24.13, 32.76.

Thus, the data pointed strongly to the fact that the phytofluene from *Dunaliella bardawil* has the 9-*cis* configuration, *i.e.* **11**. This assignment was confirmed by comparison of the $^{13}C$ NMR spectrum of the natural product with that of the authentic *Z*-$C_{30}$-analogue **12** of phytofluene, synthesised earlier by us;[11] for clarity the most important data are compared in formulae **11** and **12**. The olefinic region of the $^{13}C$ NMR spectrum of the naturally derived phytofluene, also showed a striking similarity to that of the model compound **12**.

Also visible in the $^{13}C$ NMR spectrum of the phytofluene from *Dunaliella* were small peaks due to a second isomer of phytofluene (< 10% of phytofluene present). These peaks bore a very strong resemblance to the peaks expected for *Z*-15,*Z*-9'-phytofluene **7** isolated previously from the Tangerine tomato.

This satisfying result gave very strong support to the suggestion that the 9-*cis* stereochemistry found in β-carotene produced by *Dunaliella bardawil* is introduced during the dehydrogenation step between phytoene and phytofluene; as has previously been observed for prolycopene **10** from the Tangerine tomato. The presence of a trace amount of 15,9'-di-*cis* phytofluene **7** indicated that this is probably the phytofluene isomer first formed, by the dehydrogenation of 15-*cis*-phytoene **6**, as has previously been observed. However, it is known that the 15-*cis* stereochemistry of this molecule is particularly labile[10] and had probably undergone stereomutation, during transit or isolation, to the more stable *E*-15,*Z*-9' phytofluene **11**.

Chromatography of the crude extract from *Dunaliella bardawil* shown to contain phytofluene **11**, also yielded a small sample of ζ-carotene which was free from other carotenoids. A final purification using PLC gave the ζ-carotene as an orange liquid.

$^{13}C$ and $^{1}H$ NMR analysis indicated that the sample was not isomerically pure. However, in the more diagnostic, high field region of the $^{13}C$ NMR spectrum, peaks which corresponded closely with methyl and methylene peaks of the mono-*cis*-model compound **13**, synthesised earlier by us, could easily be discerned. Most significantly, peaks were observed at $\delta$ 24.16 and 32.77 which are indicative of *cis*-stereochemistry at the end of its heptaene chromophore.

Unfortunately the remaining peaks in the $^{13}C$ NMR spectrum could not be assigned to another ζ-carotene isomer. Nevertheless the $^{13}C$ NMR spectrum indicated the presence of *cis*-stereochemistry at the end of the chromophore in this sample of ζ-carotene isolated.

It was interesting to us that in our analysis of the phytofluene fraction from *D. bardawil* we were not able to accumulate any evidence for the co-existence of all-*trans*-phytofluene with the 9-*cis*-isomer **11** in the alga. This was even more interesting since β-carotene found in *D. bardawil* has previously been reported to be present as a 1:1 mixture of 9-*cis* **2** and all-*trans* **1** stereoisomers. We decided therefore to examine the stereochemistry of the β-carotene supplied to us by our Israeli colleagues.

Thus, the crude extract of β-carotene extracted from *D. bardawil* which had been grown under conditions of high light intensity was first purified by PLC and the prominent yellow–orange band was isolated. The $^{13}C$ NMR spectrum of this carotene was next analysed and compared with literature data.[12,13] These data indicated the presence of peaks associated with both 9'-*cis* and 9-*trans* stereochemistry for the β-carotene isolated. This was therefore consistent with a 1:1 mixture of 9'-*cis*-β-carotene **2** and all-*trans*-β-carotene **1**. Two peaks

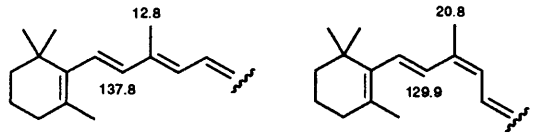



Fig. 1

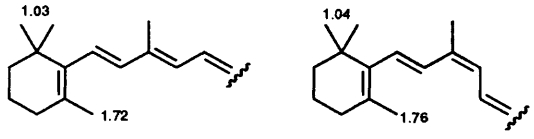



Fig. 2

diagnostic of 9'-*cis* stereochemistry in the $^{13}C$ NMR spectrum were: ($\delta$ 20.79, 9'-$CH_3$) which is deshielded relative to the 9-*trans* situation ($\delta$ 12.82, 9-$CH_3$) and ($\delta$ 129.88, CH-8') which is shielded relative to the 9-*trans* situation ($\delta$ 137.78, CH-8). Both of these shifts are the result of the γ-effect observed earlier by other workers (Fig. 1).

A recent study of the isomers formed by the photoisomerisation of all-*trans* β-carotene **1**, reported the isolation of pure 9'-*cis*-β-carotene **2** using an alumina HPLC system.[14] This enabled us to analyse the high field $^1H$ NMR spectrum of this β-carotene isomer in considerable detail. It was found that there were several small but definite changes in the proton chemical shifts of some signals, when comparing 9'-*cis*- and all-*trans*-β-carotene. In the more diagnostic high field region of the spectrum these changes in proton chemical shifts were most noticeable for the 1- and 5-methyl groups (Fig. 2). Analysis of the $^1H$ NMR spectrum of the β-carotene isolated from *Dunaliella* showed peaks for both 9'-*cis* and all-*trans* stereochemistries; however, the ratio of the integrals for corresponding peaks with 9'-*cis* and all-*trans* stereochemistry was 1 : 1. For an equimolar mixture of 9'-*cis*-β-carotene and all-*trans*-β-carotene, this ratio would be expected to be 3 : 1, as there were three 9-*trans* double bonds for each 9'-*cis* double bond. A 1 : 1 ratio of these integrals corresponds to pure 9'-*cis*-β-carotene **2**, and this assignment was further verified by analysis of the olefinic region of the $^1H$ NMR spectrum and comparison with literature data.[14]

Thus, although the $^{13}C$ NMR data obtained for the sample of β-carotene supplied to us by our Israeli colleagues were consistent with a 1 : 1 mixture of 9-*cis* and all-*trans* isomers, the $^1H$ NMR data and comparison with authentic spectra demonstrated that the material was in fact isomerically pure 9-*cis*-β-carotene **2**. This observation is of course consistent with the fact that we isolated only 9-*cis*-phytofluene **11** from the alga and no accompanying all-*trans*-phytofluene. The isolation of only the 9-*cis*-isomer of β-carotene from *D. bardawil* in our studies is interesting, and contrasts with earlier reports that the alga produces a 1 : 1 mixture of **2** and all-*trans*-β-carotene **1**.[4,5] Whether or not these differences are associated with the fact that the alga which produced the β-carotene supplied to us was grown in the presence of a bleaching herbicide, or that the all-*trans*-β-carotene detected by chromatography in the earlier studies was an artefact, is not clear at this time.

## Experimental

*General Details.*—Owing to the very sensitive nature of carotenoids to isomerisation in light or on heating, and to oxidation, strict methods of handling were adhered to throughout. Carotenoids were left out of solution and in contact with air for the minimum possible time. Solutions containing carotenoids were never left standing for prolonged periods, and they were never heated above room temperature. Solutions of carotenoids were handled in subdued light or darkness, and all columns used in chromatography were wrapped in aluminium foil. Applications to and removals of samples from PLC plates were performed quickly with the plates under a blanket of nitrogen gas, and carotenoids were left adsorbed on active surfaces for the minimum time. For periods of short term storage, samples of carotenoids were kept at −20 °C in the dark under nitrogen.

*Carotenoid Accumulation and Extraction.* (*A. Shaish, M. Avron and A. Ben-Amotz*).—*Dunaliella bardawil* was cultivated in Israel, under defined conditions, which resulted in the accumulation of high concentrations of β-carotene. The alga was also grown in the presence of inhibitors (*i.e.* norflurazon for phytoene and phytofluene;[15] J-334 for ζ-carotene)[10] to allow the accumulation of carotenoid intermediates towards β-carotene.

Algal pellets were extracted with ethanol–hexane and the pigments transferred to hexane. Extracts of partially purified phytoene, phytofluene and β-carotene in hexane were transported to Nottingham in a cooled container, and were then analysed as quickly as possible.

15-cis-*Phytoene* **6**.—The phytoene rich algal extract in hexane, from *D. bardawil* was evaporated under reduced pressure, and the residue was then purified by chromatography on 40 × 40 cm PLC plates (Fluka Keiselgel $HF_{254}$), developed twice with 99 : 1 hexane–diethyl ether. The zone which was more weakly adsorbed than the highly fluorescent phytofluene band was removed from the plate to give almost pure 15-*cis*-phytoene as a pale-orange oil; $\lambda_{max}$/nm (hexane) 265inf, 276inf, 286, 298 ($\varepsilon$/dm$^3$ mol$^{-1}$ cm$^{-1}$ 16 200, 25 600, 31 000, 23 200); $\nu_{max}$/cm$^{-1}$ (liquid film) 1638, 833, 767; $\delta_H$ 1.59, 1.60, 1.62 (18H, *Z*-1-, *Z*-1'-, 5-, 5'-, 9-, 9'-$CH_3$), 1.68 (6 H, s, *E*-1-, *E*-1'-$CH_3$), 1.77 (6 H, s, 13- 13'-$CH_3$), 1.99, 2.07, 2.13 (24 H, m, $CH_2CH_2$), 5.11 (6 H, br s, olefinic protons on isolated double bonds), 6.09, 6.11, 6.29, 6.31 (4 H, 2 × m, olefinic protons on chromophore); $\delta_C$ 16.02 (5-, 5'-, 9-, 9'-$CH_3$), 16.53 (13-, 13'-$CH_3$), 17.69 (*Z*-1-, *Z*-1'-$CH_3$), 25.71 (*E*-1-, *E*-1'-$CH_3$), 26.69, 26.78 ($CH_2$-3, -3', -7, -7', -11, -11'), 39.73 ($CH_2$-4, -4', -8, -8'), 40.51 ($CH_2$-12, -12'), 120.24 (CH-14, -14'), 123.36 (CH-15, -15'), 123.98, 124.24, 124.43 (CH-2, -2', -6, -6', -10, -10'), 131.24 (C-1, -1'), 134.94, 135.33 (C-5, -5', -9, -9'), 139.50 (C-13, -13'); (Found: $M^+$, 544.5000. $C_{40}H_{64}$ requires *M*, 544.5008).

9-cis-*Phytofluene* **11**.—The phytofluene rich algal extract in hexane, from *D. bardawil* was evaporated under reduced pressure, and the residue was then purified by column chromatography on magnesium oxide using increasing proportions of acetone in hexane as eluent. The fractions were analysed by UV–VIS absorption spectroscopy, and by TLC, and those containing phytofluene were combined and concentrated. The residue was purified by chromatography on a 40 × 40 cm PLC plate (Fluka Kieselgel $HF_{254}$), developed twice with 99 : 1 hexane–acetone. The highly fluorescent zone [when irradiated with UV light ($\lambda$ 366 nm)] was removed from the plate to give almost pure 9-*cis*-phytofluene as a pale-yellow liquid; $\lambda_{max}$/nm (hexane) 319inf, 332, 348, 367; $\nu_{max}$/cm$^{-1}$ (liquid film) 2922, 1631, 961, 830, 772; $\delta_H$ 1.59 (15 H, br s, *Z*-1-, *Z*-1'-, 5-, 5'-, 9-$CH_3$) 1.68 (6 H, s, *E*-1-, *E*-1'-$CH_3$), 1.79, 1.82 (6 H, 2 × s, 9'-, 13-$CH_3$), 1.90 (3 H, s, 13'-$CH_3$), 1.99, 2.05, 2.07, 2.11, 2.32 (20 H, m, $CH_2CH_2$), 5.11 (5 H, br s, olefinic protons on isolated double bonds), 5.94, 6.18, 6.21, 6.45 (7 H, m, olefinic protons on chromophore); $\delta_C$ 12.73 (13'-$CH_3$), 16.02 (5-, 5'-, 9-$CH_3$), 16.94 (13-$CH_3$), 17.70 (*Z*-1-, *Z*-1'-$CH_3$), 24.13 (9'-$CH_3$), 25.71 (*E*-1-, *E*-1'-$CH_3$), 26.69, 26.79 ($CH_2$-3, -3', -7, -7', -11), 32.76 ($CH_2$-8'), 39.73 ($CH_2$-4, -4', -8), 40.23 ($CH_2$-12), 123.81, 123.86, 124.22, 124.34, 124.42, 125.74, 126.48, 127.36, 129.54, 131.27, 131.36, 134.96, 135.22, 135.42, 135.59, 139.24, 139.68.

9-cis-β-*Carotene* **2**.—The β-carotene rich algal extract in hexane, from *D. bardawil* was evaporated under reduced pressure, and the residue was purified by chromatography on 40 × 40 cm PLC plates (Fluka Kieselgel $HF_{254}$), developed with 99:1 hexane–diethyl ether. The high running yellow–orange band was removed from the plate to give almost pure 9-*cis*-β-carotene as an orange oil; $\lambda_{max}$/nm ($CH_2Cl_2$), 276, 350, 432inf, 455, 482inf, ($\varepsilon$/dm$^3$ mol$^{-1}$ cm$^{-1}$ 31 200, 30 100, 146 100, 197 200, 168 300); $\nu_{max}$/cm$^{-1}$ (liquid film) 3030, 2926, 1566, 1026, 1007, 964; $\delta_H$ 1.03 (6 H, s, 2 × 1-$CH_3$), 1.04 (6 H, s, 2 × 1′-$CH_3$), 1.48 (4 H, m, $CH_2$-2, -2′), 1.60 (4 H, m, $CH_2$-3, -3′), 1.72 (3 H, s, 5-$CH_3$), 1.76 (3 H, s, 5′-$CH_3$), 1.97 (12 H, br s, 9-, 9′-, 13-, 13′-$CH_3$), 2.02 (4 H, m, $CH_2$-4, -4′), 6.03, 6.14, 6.16, 6.33, 6.37, 6.61, 6.65, 6.69, 6.79 (14 H, 2 × m, olefinic protons); $\delta_C$ 12.82 (9-, 13-, 13′-$CH_3$), 19.27 ($CH_2$-3, -3′), 20.79 (9′-$CH_3$), 21.78 (5 $CH_3$), 21.90 (5′-$CH_3$), 28.98 (2 × 1-, 1′-$CH_3$), 33.11 ($CH_2$-4, -4′), 34.27 (C-1, -1′), 39.55, ($CH_2$-2), 39.64 ($CH_2$-2′), 123.84, 125.00, 126.64, 128.41, 129.36, 129.51, 129.86, 129.99, 130.85, 132.29, 132.42, 134.59, 136.00, 136.36, 136.40, 136.51, 137.24, 137.78, 137.92, 138.22; (Found: $M^+$ 536.4377. $C_{40}H_{56}$ requires *M*, 536.4382).

## Acknowledgements

We thank Professors M. Avron and A. Ben-Amotz, and Mr. A. Shaish for kindly supplying samples of carotenoid extracts from *Dunaliella bardawil*, and for exchange of information. We also thank the SERC for a research studentship (to W. J. E.).

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*Paper* 3/01923C
*Received* 5*th April* 1993
*Accepted* 27*th April* 1993