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MONOHYDROXYCAROTENOIDS FROM DIPHENYLAMINE-INHIBITED CULTURES OF *RHODOSPIRILLUM RUBRUM*

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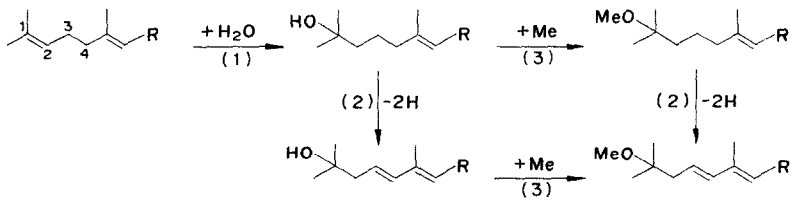
Abstract—The monohydroxycarotenoids formed by diphenylamine-inhibited cultures of *Rhodospirillum rubrum* have been investigated Nine have been isolated and identified as 1-hydroxy-1,2-dihydrophytofluene (**1**), 1-hydroxy-1,2,7′,8′,11′,12′-hexahydrolycopene (**2**), chloroxanthin (**3**), 1-methoxy-1′-hydroxy-1,2,1′,2′-tetrahydrophytofluene (**4a**), 1′-hydroxy-3,4,1′,2′,11′,12′-hexahydrospheroidene (**5**, 1′-hydroxy-3,4,1′,2′-tetrahydrospheroidene (**6**, 1′-hydroxy-1′ 2′-dihydrospheroidene (**7**), rhodovibrin (**8a**) and monodemethylated spirilloxanthin (**9**) **4a**, **5** and **6** are novel carotenoids, and a definite structure has been assigned to **2** for the first time, the structure of **1** has been amended The possible role of these carotenoids in spirilloxanthin biosynthesis is discussed

INTRODUCTION

MATURE illuminated anaerobic cultures of the purple non-sulphur photosynthetic bacterium *Rhodospirillum rubrum* (Rhodospirillaceae,[1] formerly Athiorhodaceae) normally accumulate spirilloxanthin (1,1′-dimethoxy-3,4,3′,4′-tetradehydro-1,2,1′,2′-tetrahydro-ψ,ψ-carotene) as the predominant carotenoid [2–4] When *R rubrum* is cultured anaerobically in the light in the presence of diphenylamine (DPA), however, the formation of spirilloxanthin is inhibited and other carotenoids accumulate [5] A study of the kinetics of the disappearance of these carotenoids on removing the DPA showed that they are precursors of spirilloxanthin[6] and it was concluded that lycopene (ψ,ψ-carotene) is converted into spirilloxanthin by a sequence of three consecutive reactions which operates first at one end of the molecule and then at the other [7] More recent studies of the carotenoids formed under conditions of DPA inhibition have suggested that not only lycopene, but the more saturated precursors of lycopene, namely phytofluene (7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene), 7,8,11,12-tetrahydrolycopene (7,8,11,12-tetrahydro-ψ,ψ-carotene) and neurosporene (7,8-dihydro-ψ,ψ-carotene), are also able to undergo these reactions [8] The resulting mono- and di-methoxycarotenoids[9] could be converted by dehydrogenation into either anhydrorhodovibrin (1-methoxy-3,4-didehydro-1,2-dihydro-ψ,ψ-carotene), a precursor of spirilloxanthin,[7] or spirilloxanthin, as appropriate

[1] PFENNIG, N and TRUPER, H G (1971) *Int J Syst Bacteriol* **21,** 17
[2] VAN NIEL, C B and SMITH, J H C (1935) *Arch Mikrobiol* **6,** 219
[3] POLGAR, A , VAN NIEL, C B and ZECHMEISTER, L (1944) *Arch Biochem.* **5,** 243
[4] GOODWIN, T W and OSMAN, H G (1953) *Biochem J* **53,** 541
[5] GOODWIN, T W and OSMAN, H G (1954) *Biochem J* **56,** 222
[6] LIAAEN JENSEN, S , COHEN-BAZIRE, G , NAKAYAMA, T O M and STANIER, R Y (1958) *Biochim Biophys Acta* **29,** 477
[7] LIAAEN JENSEN, S , COHEN-BAZIRE, G and STANIER, R Y (1961) *Nature* **192,** 1168
[8] DAVIES, B H (1970) *Biochem J* **116,** 101
[9] MALHOTRA, H C BRITTON, G and GOODWIN, T W (1970) *Phytochemistry* **9,** 2369

The first step in the formation of all these methoxycarotenoids from the corresponding carotene is always hydration (1) of the isopropylidene end group to introduce the hydroxyl group at C-1 (Scheme 1) The 3,4-didehydrogenation reaction (2), which normally extends the polyene chromophore by one conjugated double bond, may precede or follow the methylation (3) of the hydroxyl group [8,10]



SCHEME 1 ALTERNATIVE PATHWAYS FOR THE FORMATION OF METHOXYCAROTENOIDS FROM CAROTENES IN *Rhodospirillum rubrum* [8]

The methoxycarotenoids of DPA-inhibited cultures of *R rubrum* have been described in detail by a number of workers[8,9,11] but investigations of the structures of the hydroxycarotenoids, with the exception of studies of the hydroxy-derivatives of phytoene (7,8,11,12,7',8',11',12'-octahydro-ψ,ψ-carotene) and phytofluene[12] and of 'hydroxyspheroidene' (1'-methoxy-3' 4'-didehydro-1,2,7,8,1',2'-hexahydro-ψ,ψ-caroten-1-ol),[8,13] have been less rigorous,[5,6,8] The present paper reports the results of a detailed study of the monohydroxycarotenoids of DPA-inhibited cultures of *R rubrum*

RESULTS

Chromatography of the unsaponifiable fraction of DPA-inhibited cultures of *R rubrum* on a column of alumina (grade III), using increasing concentrations of diethyl ether in light petroleum as the developing solvent, led to the separation of a very large number of carotenoids The elution of the carotenes and the mono- and di-methoxycarotenoids which have been described elsewhere,[8,9] was complete by the time 20% diethyl ether in light petroleum (E/P) was being used as the solvent, and further development with higher concentrations of ether led to the elution of three poorly resolved and mixed hydroxycarotenoid fractions, designated 'A', 'B' and 'C'

Fraction 'A' was subjected to a silylation procedure and the resulting mixture of trimethylsilyl (TMS) ethers was resolved on a column of alumina (grade III) into three components (Table 1) The first to be eluted, with petrol was colourless and showed, in UV light, the green fluorescence characteristic of the chromophore of phytofluene,[14] its absorption spectrum was also typical of a conjugated pentaene [15] The second and third TMS ethers, eluted with 0 5 and 1-2% E/P respectively had the absorption spectra of a conjugated heptaene and a conjugated nonaene respectively[15] The fact that all of the carotenoids from fractions 'A', 'B' and 'C' became much less polar after silylation confirmed the presence of hydroxyl groups and the polarities of the natural pigments were consistent with

[10] DAVIES B H (1970) *Pure Appl Chem* **20**, 545
[11] DAVIES, B H, HOLMES, E A, LOEBER D E TOLBI, T P and WILDON B C L (1969) *J Chem Soc* **C**, 1266
[12] MALHOTRA H C, BRITTON, G and GOODWIN T W (1970) *FEBS Letters* **6**, 334
[13] MALHOTRA, H C BRITTON G and GOODWIN, T W (1969) *Phytochemistry* **8**, 1047
[14] DAVIES B H (1965) in *Chemistry and Biochemistry of Plant Pigments* (GOODWIN T W ed) p 489 Academic Press New York
[15] DAVIES B H (1970) *Biochem J* **116**, 93

their all being monohydroxy- (rather than dihydroxy-) carotenoids The differences in chromatographic mobility of the three TMS ethers arising from fraction 'A' were consistent with the differences in their numbers of conjugated double bonds (Table 1) The three carotenoids from fraction 'A' were tentatively formulated as 1-hydroxy-1,2-dihydrophytofluene (**1**, 1,2,7,8,7′,8′,11′,12′-octahydro-ψ,ψ-caroten-1-ol), 1-hydroxy-1,2,7′,8′,11′,12′-hexahydrolycopene (**2**, 1,2,7′,8′,11′,12′-hexahydro-ψ,ψ-caroten-1-ol) and chloroxanthin (**3**, 1,2,7′,8′-tetrahydro-ψ,ψ-caroten-1-ol) The tentative identification of chloroxanthin, which was present in quantities too small to allow an unambiguous identification by MS, was confirmed by a spectroscopic comparison and co-chromatography on thin layers (Table 2) with a fully authenticated sample[16] isolated from a green mutant of *Rhodopseudomonas spheroides* The structure of the other two fraction 'A' carotenoids were subsequently confirmed by MS of their TMS ethers (see below)

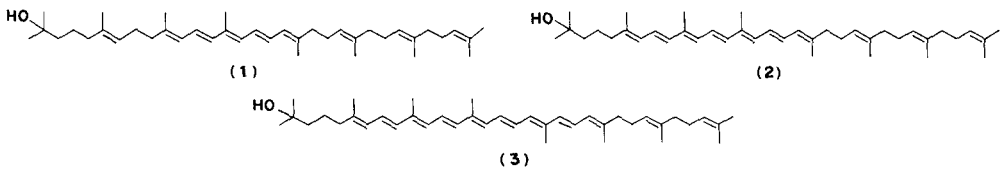


TABLE 1 CHROMATOGRAPHIC AND SPECTROSCOPIC CHARACTERISTICS OF THE MONOHYDROXYCAROTENOIDS (AND THEIR TMS ETHERS) ISOLATED FROM DIPHENYLAMINE-INHIBITED CULTURES OF *Rhodospirillum rubrum*

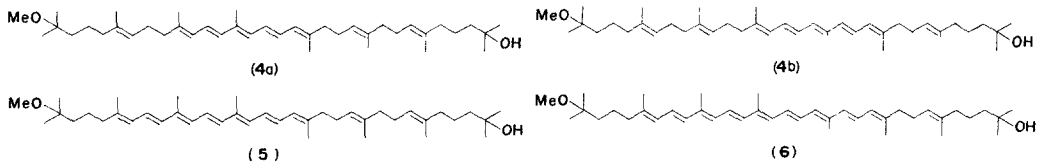
Fraction	%E/P* required for elution from alumina (grade III) by Carotenoid	TMS ether	Absorption maxima (nm) in petrol	Conjugated double bonds	Hydroxyl groups	Methoxyl groups	Structure
A	20–30	0	331 347 366	5	1	0	(1) 1 2 7 8 7 8 11 12-Octahydro ψ ψ caroten 1 ol
A	20–30	0 5	(354) 375 396 420	7	1	0	(2) 1 2 7 8 11 12 Hexahydro ψ ψ-caroten-1-ol
A	20–30	1–2	(394) 412 438 466	9	1	0	(3) 1,2 7 8-Tetrahydro ψ ψ caroten 1 ol
B	30–40	2–3	331 347 367	5	1	1	(4a) 1 Methoxy 1 2 7 8 11 12 1 2 7 8 deca-hydro-ψ ψ caroten 1 ol
B	30–40	3–4	(354) 374 395 419	7	1	1	(5) 1-Methoxy-1 2 7 8 11-12 1 2-octahydro-ψ ψ-caroten-1-ol
B	30–40	4	(395) 414 438 467	9	1	1	(6) 1 Methoxy 1 2 7 8 1 2-hexahydro-ψ ψ-caroten 1 ol
C	40–60	5	(404) 426 451 481	10	1	1	(7) 1 Methoxy 3 4 di dehydro 1 2 7 8 1 2 hexahydro ψ ψ-caroten 1 ol
C	40–60	5–8	(430) 454 480 512	12	1	1	(8a) 1 Methoxy 3 4 di dehydro-1 2 1 2 tetra hydro ψ ψ-caroten 1 ol
C	40–60	5–8	(433) 462 490 524	13	1	1	(9) 1 Methoxy 3 4 3 4-tetradehydro-1 2 1 2 tetrahydro-ψ ψ-caroten 1-ol

* %E/P = Percentage of diethyl ether in petrol (v/v)

The fraction 'B' monohydroxycarotenoids, eluted from the preliminary alumina column with 30–40% E/P, were silylated and the products chromatographed on another column of alumina (grade III) This procedure revealed the presence of three TMS ethers which were eluted in sequence with 2–4% E/P They had absorption spectra characteristic of compounds with 5, 7 and 9 conjugated double bonds (Table 1) and the conjugated pentaene

[16] AUNG THAN and DAVIES, B H unpublished work

had, like phytofluene, a green fluorescence in UV light The least polar was tentatively identified as a monomethoxy-monohydroxyphytofluene, with the two alternative structures, 1′-methoxy-1,2,7,8,11,12,1′,2′,7′,8′-decahydro-ψ,ψ-caroten-1-ol (**4a**) and 1′-methoxy-1,2,7 8 1′,2′,7′ 8′,11′,12′-decahydro-ψ,ψ-caroten-1-ol (**4b**) The former structure (**4a**, i e 1-methoxy-1′-hydroxy-1,2,1′,2′-tetrahydrophytofluene or 1′-hydroxy-3,4,7,8,1′,2′,11′ 12′-octahydrospheroidene) is favoured both by analogy with 1′-methoxy-1,2,7,8,11,12,1′ 2′-octahydro-ψ,ψ-caroten-1-ol (**5**) and 1′-methoxy-1,2,7,8 1′,2′-hexahydro-ψ ψ-caroten-1-ol (**6**) and on the grounds of its possible biosynthetic relationship to 1-methoxy-1,2-dihydrophytofluene (1-methoxy-1,2,7,8,7′,8′,11′,12′-octahydro-ψ,ψ-carotene)[9] The conjugated heptaene from fraction 'B' was identified as 1′-hydroxy-3,4,1′,2′,11′,12′-hexahydrospheroidene (**5**, 1′-methoxy-1 2 7,8,11,12,1′,2′-octahydro-ψ,ψ-caroten-1-ol) while the structure of 1′-hydroxy-3,4,1′,2′-tetrahydrospheroidene(**6**,1′-methoxy-1,2,7 8 1′ 2′-hexahydro-ψ,ψ-caroten-1-ol) was assigned to the conjugated nonaene Both structures were confirmed by MS analysis of the corresponding TMS ethers (see below)



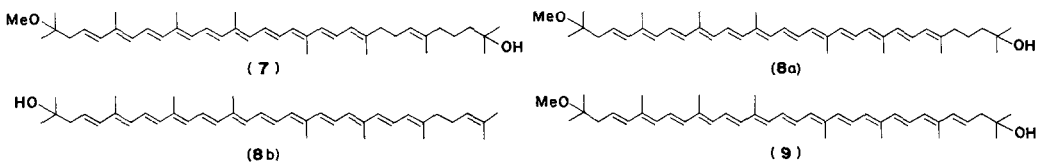
The third monohydroxycarotenoid fraction ('C'), eluted from the preliminary alumina column with 40–60% E/P, had an absorption spectrum in light petroleum with maxima at 426 543 and 481 nm (characteristic of a conjugated decaene[8]) with some absorption at even higher wavelengths Repeated chromatography on columns of alumina (grade IV) with 20–30% E/P enabled the isolation of a chromatographically pure sample of a monohydroxycarotenoid with an inflection in light petroleum at 404 nm and absorption maxima at 427, 452 and 482 nm Previous studies[8 9] have identified this carotenoid as 1′-hydroxy-1′,2′-dihydrospheroidene (**7**, 1′-methoxy-3′,4′-didehydro-1,2,7,8,1′,2′-hexahydro-ψ,ψ-caroten-1-ol) Further confirmation of this structure, based on the MS both of the natural carotenoid and of its TMS ether was obtained (see below)

TABLE 2 BEHAVIOUR OF TMS ETHERS OF MONOHYDROXYCAROTENOIDS FROM DPA-INHIBITED CULTURES OF *Rhodospirillum rubrum* ON TLC

TMS ether of	MgO–Kieselguhr G (1 1) Solvent	R_f	MgO Solvent	R_f	Silica gel G Solvent	R_f
1	15% B/P*	0 68	15% B/P	0 45	3% E P*	0 64
2	30% B P	0 65	25% B/P	0 33		
3	15% A/P*	0 25	25% B/P	0 05	5% F P	0 82
	100% B	0 48				
4a					5% E P	0 34
5	15% B/P	0 21				
	30% B/P	0 40				
6	100% B	0 38				
7	100% B	0 21				
	20% EtOAc/P*	0 37				
8a	100% B	0 06				

* Solvents E– Et_2O, P– petrol (40 60°), B C_6H_6 A– Me_2CO

The remainder of fraction 'C' was silylated and the products chromatographed on a column of alumina (grade III) when the TMS ether of 1'-hydroxy-1',2'-dihydrospheroidene (**7**) was eluted with 5% E/P Two further TMS ethers were recovered from the alumina column by elution with 5–8% E/P and were separated by repeated chromatography on columns of the same type The first to be eluted had an absorption spectrum (Table 1) characteristic of a conjugated dodecaene and identical with that reported for anhydrorhodovibrin [8] Two alternative identifications are possible for the monohydroxycarotenoid, namely rhodovibrin (**8a**, 1'-methoxy-3',4'-didehydro-1,2,1',2'-tetrahydro-ψ,ψ-caroten-1-ol) or 3,4-dehydrorhodopin (**8b**, 3,4-didehydro-1,2-dihydro-ψ,ψ-caroten-1-ol) Unfortunately, the amount of material available was not sufficient for mass spectrometric analysis, so it was not possible unambiguously to differentiate between **8a** and **8b** The most polar of the three TMS ethers formed from the fraction 'C' carotenoids had an absorption spectrum (Table 1) of a conjugated tridecaene which was virtually identical with that reported for spirilloxanthin [8] This absorption spectrum and the polarities of the natural carotenoid and its TMS ether lead to the formulation of this pigment as monodemethylated spirilloxanthin (**9**, 1'-methoxy-3,4,3',4'-tetradehydro-1,2,1',2'-tetrahydro-ψ,ψ-caroten-1-ol) Again, the amount of carotenoid isolated did not permit an absolute structural assignment by MS In retrospect, the similarity in chromatographic behaviour, both between monodemethylated spirilloxanthin (**9**) and the carotenoid (**8**) which preceded it on chromatographic elution and between their respective TMS ethers, suggests a structural difference between them of only one olefinic bond rather than also of a methoxyl substituent Thus, the favoured structure for **8** is that of rhodovibrin (**8a**) rather than 3,4-dehydrorhodopin (**8b**)

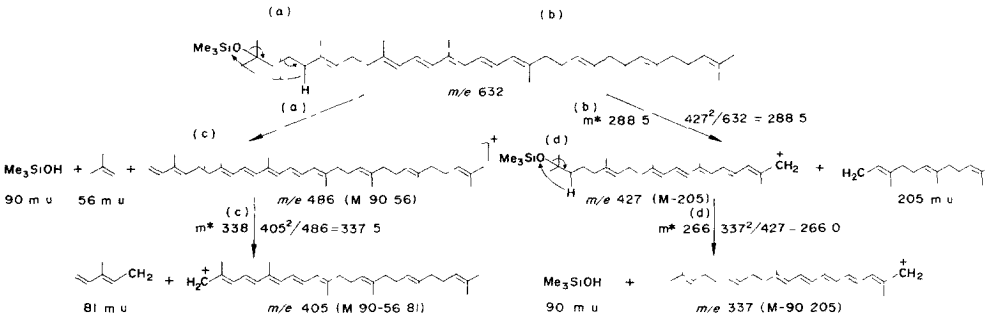


Prior to its examination by MS, each of the TMS ethers derived from the carotenoids of fractions 'A', 'B' and 'C' was further purified by TLC and, finally, by chromatography on a column of alumina (grade III) The behaviour of the ethers on the thin-layer systems (Table 2) lent further support to the above tentative identifications

MS identification of **1** *as* 1-*hydroxy*-1,2-*dihydrophytofluene*

The high resolution measurement of the mass of the molecular ion (16%, 632 5360) corresponded closely to that required for the TMS ether of a carotenoid of the proposed structure (Calc for $C_{43}H_{72}OSi$, 632 5352) The loss of 205 m u from the molecular ion to give *m/e* 427 (4%, M-205), substantiated by a metastable peak (m*) at *m/e* 288 5 ($427^2/632$ = 288 5), indicated the presence of a 3,7,11-trimethyldodeca-2,6,10-trienyl unit (Scheme 2) as in phytoene, phytofluene and 7,8,11,12-tetrahydrolycopene [11 15] There was a loss of trimethylsilanol (Me_3SiOH) both from the molecular ion and from *m/e* 427 to yield, respectively, ions at *m/e* 542 (1%, M-90) and at *m/e* 337 (10%, M-205-90) The latter elimination (Scheme 2), supported by a metastable peak at *m/e* 266 ($337^2/427$ = 266 0) indicated that the hydroxyl group of the natural carotenoid is in that part of the molecule not containing the 3,7,11-trimethyldodeca-2,6,10-trienyl moiety, so that hydration has occurred at the less saturated end of the phytofluene molecule to yield 1-hydroxy-1,2-dihydrophytofluene (**1**, 1,2,7,8,7',8',11',12'-octahydro-ψ,ψ-caroten-1-ol) Further fragmentations yielding ions at

m/e 486 (1 5%, M-90-56) and at *m/e* 405 (3%, M-90-56-81) could be rationalized[17] by a rearrangement involving the simultaneous loss of Me_3SiOH (90 m u) and methylpropene (56 m u) from the molecular ion followed by a 'bis-allylic' fission of the fragment ion *m/e* 486 to yield a 3-methylpenta-2,4-dienyl radical (81 m u) and a fragment ion at *m/e* 405 (Scheme 2), that this fission occurs is indicated by a metastable peak at *m/e* 338 ($405^2/486 = 337\ 5$) The ion at *m/e* 405 can clearly be formulated as M-90-56-81, any interpretation of structure made on the basis of the alternative formulation of this ion as M-90-137 and assuming this exclusively to indicate the loss of Me_3SiOH (90 m u) and a 3 7-dimethylocta-2 6-dienyl radical (137 m u) from the molecular ion would therefore be ambiguous



SCHEME 2 MS FRAGMENTATIONS OF THE TMS ETHER DERIVED FROM 1-HYDROXY-1 2-DIHYDROPHYTOFLUENE (**1**)

1-Hydroxy-1,2-dihydrophytofluene is not a novel carotenoid as it has been detected on a number of occasions in DPA-inhibited cultures of *R rubrum*[6 8] Its formulation as 1,2,7,8,7',8',11',12'-octahydro-ψ,ψ-caroten-1-ol, however, is at variance with the conclusions of other workers[12] who suggested that the hydroxyl group is at the more saturated end of the molecule (1,2,7 8,11 12 7' 8'-octahydro-ψ,ψ-carotene-1-ol) The latter structure was proposed as a result of a comparison of ion intensities in the MS, the ion at *m/e* 427 (M-205) was recorded as being only weak in comparison with that at *m/e* 495 (M-137) These measurements were made using a probe temperature in excess of 200°[18] Our independent comparisons of ion intensities have shown that at a probe temperature of 180 , the ions at *m/e* 427 (1 06%, M-205) and at *m/e* 337 (2 4%, M-90-205) are more intense, respectively, than those at *m/e* 495 (0 8%, M-137) and at *m/e* 405 (1 33% 'M-90-137') At higher temperatures (e g 200), this is no longer the case (M-137 1 7% M-205 1 7%, M-90-137 3 2%, M-90-205, 3 0%)

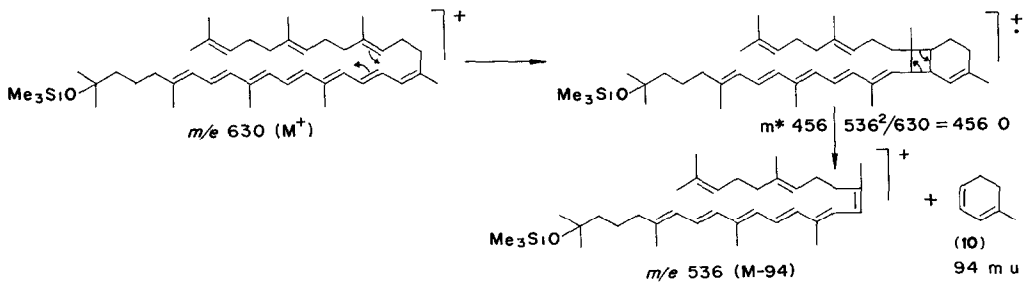
MS identification of **2** *as 1-hydroxy-1,2,7',8',11',12'-hexahydrolycopene*

The MS of the TMS ether of this carotenoid had a molecular ion (84%) with an accurate mass (630 5197) which agreed with that calculated on the basis of the proposed structure ($C_{43}H_{70}OSi$ = 630 5196) 'Bis-allylic' fragmentations led to ions at *m/e* 561 (0 3%, M-69) and at *m/e* 493 (5 3%, M-137 m* 386, $493^2/630$ = 385 8) A similar loss of 205 m u from the molecular ion, giving *m/e* 425 (7 3%, M-205), which was substantiated by a metastable peak at *m/e* 286 5 ($425^2/630$ = 286 7) indicated the presence of an unsubstituted 3,7,11-tri-

[17] LOEBLR D E (1971) Ph D Thesis University of London
[18] BRITTON G personal communication

methyldodeca-2,6,10-trienyl unit at one end of the molecule The presence of a trimethylsilyloxyl function at the other end of the molecule was shown by the loss of Me_3SiOH from the molecule ion to yield *m/e* 540 (2 7%, M-90) This ion underwent further fragmentations to give ions at *m/e* 471 (0 2%, M-90-69), *m/e* 403 (14 4%, M-90-137[?]) and *m/e* 335 (7 8%, M-90-205), the relatively high intensity of the ion at *m/e* 403 is presumably an indication of its also being formed by the alternative route (see Scheme 2) in which it arises from M-90-56 by the loss of a 3-methylpenta-2,4-dienyl radical (81 m u) This was not confirmed by a metastable peak, although an ion was present in the spectrum at *m/e* 484 (0 94%, M-90-56)

A strong metastable peak at *m/e* 456 substantiated the formation of an ion at *m/e* 536 (2 5%, M-94) from the molecular ion ($536^2/630 = 456\ 0$) It is thought that this may represent the loss of 1-methylcyclohexa-1,3-diene (**10**, Scheme 3) from the molecular ion[17] in a manner analogous to that in which toluene (92 m u) and *m*-xylene (106 m u) are commonly lost from the carotenoid polyene chain[19] The loss of 94 m u from the molecular ion was also reported in the case of 3,4,11',12'-tetrahydrospheroidene (1-methoxy-1,2,7',8',11',12'-hexahydro-ψ,ψ-carotene),[11] it is possibly characteristic of carotenoids containing an 11,12- (or 11',12'-) single bond and is probably formed as shown in Scheme 3



SCHEME 3 RATIONALIZATION OF THE LOSS OF 94 m u FROM THE MOLECULAR ION OF THE TMS ETHER DERIVED FROM 1-HYDROXY-1,2,7',8',11 ,12'-HEXAHYDROLYCOPENE[17]

MS identification of **5** *as 1'-hydroxy-3,4,1',2',11',12'-hexahydrospheroidene*

Only a small amount of the TMS ether of this carotenoid was available for analysis and the MS were rather poor Sufficient data were obtained, however, to support the proposed structure (**5**) for the natural carotenoid The TMS ether had a molecular ion (2 3%, 662 544) the accurate mass of which was in fair agreement with that expected (Calc for $C_{44}H_{74}O_2Si$, 662 546) Losses of MeOH to yield an ion at *m/e* 630 (0 5%, M-32), of Me_3SiOH to yield an ion at *m/e* 572 (0 7%, M-90) and of both alcohols to give a weak ion at *m/e* 540 (M-32-90) indicated that the natural carotenoid had methoxyl and hydroxyl groups at the two ends of its molecule Ions at *m/e* 589 (1 0%, M-73) and at *m/e* 73 (**11**, base peak) were also consistent with the presence of a methoxyl group[11] An ion at *m/e* 574 (0 5%, M-32-56) and a weak ion at *m/e* 484 (M-32-90-56) could be rationalized with the simultaneous losses of MeOH and methylpropene (see Scheme 2) from the molecular ion and M-90 respectively, although the complete absence of metastable peaks from the

[19] SCHWIETER, U , ENGLERT, G , RIGASSI, N and VETTER W (1970) *Pure Appl Chem* **20**, 365

spectra meant that the possibility of an alternative route to M-32-90-56, namely by the loss of Me_3SiOH and methylpropene from M-32, could not be excluded Ions at *m/e* 570 and *m/e* 556 (M-92 and M-106) were so weak as to be insignificant The only evidence of any 'bis-allylic' fission took the form of ions at *m/e* 367 (1 2%, M-90-205) and at *m/e* 335 (0 8%, M-32-90-205) and there was a strong rearrangement ion at *m/e* 368 (24%, M-90-204) There was no M-32-205 ion, so the hydroxyl group of the original molecule is clearly at the more saturated end, the natural carotenoid can therefore be formulated as 1'-methoxy-1 2,7,8 11,12,1',2'-octahydro-ψ,ψ-caroten-1-ol (**5**)

MS identification of **6** *as 1'-hydroxy-3,4 1',2'-tetrahydrospheroidene*

The accurate measurement of the mass of the molecular ion of the TMS ether (30%, 660 529) had a close correspondence to the value calculated ($C_{44}H_{72}O_2Si$ 660 530) on the basis of the proposed structure The presence of a methoxyl group at one end of the molecule was shown by the loss of MeOH from the molecular ion to yield an ion at *m/e* 628 (3 7%, M-32), in some spectra this loss of MeOH was substantiated by a strong metastable peak at *m/e* 597 5 ($628^2/660 = 597\ 55$) The appearance of a weak ion at *m e* 587 (M-73) and of a prominent ion at *m/e* 73 (**11**, base peak) is also indicative of a methoxyl substituent [11] The presence of a trimethylsiloxyl substituent at the other end of the molecule was confirmed by ions at *m/e* 570 (6 4% M-90), representing the loss of Me_3SiOH from the molecular ion and at *m/e* 538 (1 5%, M-32-90) There was no loss of 69 m u either from M-32 or from M-90 and the only possible evidence of 'bis-allylic' fragmentation was the appearance of an ion at *m/e* 433 (3 8%, M-90-137) which then loses MeOH (32 m u) to yield a further ion at *m/e* 401 (3 0%, M-32-90-137, m* 371 5, 401^2 433 = 371 4) The absence of an ion at *m/e* 491 (M-32-137) is significant, it shows that the trimethylsiloxyl group of the TMS ether, and therefore the hydroxyl substituent of the natural carotenoid, is at the more saturated end of the molecule In contrast to the situation in the MS of the more highly saturated carotenoids, there were prominent ions which indicated the losses of toluene (*m/e* 568 3 0%, M-92, m* 489 $568^2/660 = 488\ 8$) and of *m*-xylene (*m/e* 554, 2 5%, M-106) from the molecular ion There were other significant ions in the spectrum namely at *m/e* 478 (0 6% M-90-92), *m/e* 464 (0 4%, M-90-106), *m/e* 341 (4 9%, M-90-92-137), *m/e* 309 (2 3%, M-32-90-92-137) and *m/e* 295 (1 5%, M-32-90-106-137) All the features of the MS of the TMS ether are consistent with the formulation of the parent carotenoid (**6**) as 1'-methoxy-1,2,7 8,1' 2'-hexahydro-ψ,ψ-caroten-1-ol

MS identification of **7** *as 1'-hydroxy-1',2'-dihydrospheroidene*

This was the only pigment in this series which was obtained in sufficient quantity to permit MS analysis of both the natural carotenoid and its TMS ether Many features of these MS have been described previously,[8 13] but the present study has resulted in the acquisition of additional data The high resolution measurement of the mass of the molecular ion of the natural carotenoid (3 3%, 586 474) corresponded to that required for the proposed structure (Calc for $C_{41}H_{62}O_2$, 586 475) A loss of H_2O from the molecular ion to give *m/e* 568 (1 2%, M-18), substantiated by a metastable peak at *m/e* 550 5 ($568^2/586 = 550\ 5$) indicated the presence of a hydroxyl group, while a methoxyl group was lost from the other end of the molecule as MeOH to yield an ion at *m/e* 554 (0 2%, M-32) The presence of the methoxyl group was confirmed by a loss of 73 m u (*m/e* 513, 0 13%, M-73 m* 449, $513^2/586 = 449\ 1$) and by the appearance of an ion at *m/e* 73 (**11**) which was the base peak The loss of both H_2O and MeOH from the molecular ion gave rise to an ion

at *m/e* 536 (0 2%, M-18-32) Losses of toluene (92 m u), *m*-xylene (106 m u) and 1,6-dimethylcyclodecapentaene ($C_{12}H_{14}$, 158 m u), all rearrangement losses from the carotenoid polyene chain,[19] were indicated by ions at *m/e* 494 (2%, M-92), *m/e* 480 (5 7%, M-106), *m/e* 428 (0 13%, M-158), *m/e* 476 (0 65%, M-18-92), *m/e* 462 (4 5%, M-18-106 or M-32-92), *m/e* 448 (0 26%, M-32-106), *m/e* 444 (weak, M-18-32-92) and *m/e* 389 (0 68%, M-18-73-106) 'Bis-allylic' fission followed the loss of H_2O from the molecular ion to give an ion at *m/e* 431 (0 4%, M-18-137) and there was also an ion at *m/e* 325 (0 58%, M-18-106-137) The loss of 137 m u in this way indicates that the hydroxyl group of the carotenoid is at the more saturated end of the molecule and that the carotenoid may be formulated as 1'-methoxy-3',4'-didehydro-1,2,7,8,1',2'-hexahydro-ψ,ψ-caroten-1-ol (**7**),

$MeO^{+} \Rightarrow$ (**11**) $\qquad$ $Me_3SiO^{+} \Rightarrow$ (**12**)

The TMS ether had a molecular ion at *m/e* 658 (88%, 658 5146, Calc for $C_{44}H_{70}O_2Si$, 658 5145) Losses of MeOH (32 m u) and Me_3SiOH (90 m u) from opposite ends of the molecular ion led to ions at *m/e* 626 (4 4%, M-32, m* 595 5, $626^2/658 = 595\ 5$), *m/e* 568 (9 3% M-90) and *m/e* 536 (1%, M-32-90) The presence of the methoxyl group was confirmed by ions at *m/e* 585 (6 2%, M-73) and at *m/e* 73 (**11**, base peak)[11] An ion analogous to the latter, at *m/e* 131 (90%) and formulated as **12**, arises from the trimethylsiloxy-substituted end of the molecule Losses of toluene (92 m u) and *m*-xylene (106 m u) were indicated by ions at *m/e* 566 (5 9%, M-92, m* 487, $566^2/658 = 486\ 9$), *m/e* 552 (19%, M-106), *m/e* 493 (2 3%, M-73-92), *m/e* 403 (0 8%, M-73-90-92) and *m/e* 389 (weak, M-73-90-106) The hydroxyl group of the parent carotenoid must be at the more saturated end of the molecule, for any losses of 137 m u , which would correspond to the loss of a 3,7-dimethylocta-2,6-dienyl radical, were always subsequent to the loss of Me_3SiOH from the TMS ether This was concluded from the presence of ions at *m/e* 431 (5 9%, M-90-137), *m/e* 399 (4%, M-32-90-137), *m/e* 358 (1 8%, M-73-90-137), *m/e* 339 (3%, M-90-92-137), *m/e* 325 (2%, M-90-106-137), *m/e* 307 (1 6%, M-32-90-92-137) and *m/e* 293 (2%, M-32-90-106-137) All the features of the MS of the TMS ether are consistent with the parent carotenoid having the structure 1'-methoxy-3',4'-didehydro-1,2,7,8,1',2'-hexahydro-ψ,ψ-caroten-1-ol (**7**)

DISCUSSION

When normal carotenoid biosynthesis in *R rubrum* is inhibited by DPA, the cultures are capable of forming 1-hydroxy-1,2-dihydro-derivatives of all the carotenes of the phytoene dehydrogenation sequence[15] Both the first and the last of the series, 1-hydroxy-1,2-dihydrophytoene (1,2,7,8,11,12,7',8',11',12'-decahydro-ψ,ψ-caroten-1-ol)[12] and rhodopin (1,2-dihydro-ψ,ψ-caroten-1-ol),[20,21] have been described by other workers but, unaccountably, were not detected in the present study The other three carotenoids of the series, 1-hydroxy-1,2-dihydrophytofluene (**1**), 1-hydroxy-1,2,7',8',11',12'-hexahydrolycopene (**2**) and chloroxanthin (**3**), have been observed in this organism on previous occasions[6,8] but have not been examined in such detail

Now that the structure assigned to the 'hydroxyphytofluene' has been amended to **1** from that reported earlier,[12,22] it is clear that in each instance a carotene with a non-central

[20] LIAAEN JENSEN, S (1959) *Acta Chem Scand* **13**, 842
[21] LIAAEN JENSEN, S (1959) *Acta Chem Scand* **13**, 2142
[22] STRAUB, O (1971) in *Carotenoids* (ISLER, O , ed), p 771, Birkhauser, Basel

chromophore undergoes its primary 1,2-hydration at the less saturated end of the molecule The apparent absence of 3 4-didehydro-derivatives of the monohydroxycarotenoids not only proves that the 1,2-hydration (reaction 1, see Scheme 1) must be the initial step in xanthophyll formation in *R rubrum* but also implies that, under the culture conditions employed, the *O*-methylation (3) precedes the 3,4-didehydrogenation (2) This is true only for methoxycarotenoid formation from carotenes with 3, 5 7 or 9 conjugated double bonds, the identification in DPA-inhibited cultures of *R rubrum* of both 3 4-dehydrorhodopin (**8b**)[6,23] and 3,4-dihydroanhydrorhodovibrin (1-methoxy-1 2-dihydro-ψ,ψ-carotene)[8,9] by different workers shows that alternative pathways can operate (Scheme 1), at least at the lycopene level, under the appropriate conditions

Once the less saturated end of the molecule has undergone at least a hydration reaction, the other end becomes amenable to attack The dihydroxy-derivatives of some of the carotenes have been observed in DPA-inhibited cultures of *R rubrum*[6] but have not yet been examined in detail The present study has revealed the presence of a number of monomethoxy-monohydroxycarotenoids In all cases where an unambiguous structural determination has been possible, it is clear that the methoxyl group is at the less saturated end and hydration has introduced a hydroxyl group at the more saturated end of the molecule Whether these carotenoids are derived biosynthetically from dihydroxycarotenoids or from monomethoxycarotenoids cannot be deduced at present Again, it is clear that at the lycopene (or anhydrorhodovibrin) level the 3,4-dehydrogenation reaction (the conversion of **8a** into **9**) can precede the final *O*-methylation which yields spirilloxanthin although the isolation of 3,4-dihydrospirilloxanthin (1,1'-dimethoxy-3,4-didehydro-1 2 1 2-tetrahydro-ψ,ψ-carotene) and of 3 4 3 ,4'-tetrahydrospirilloxanthin (1,1'-dimethoxy-1 2 1' 2'-tetrahydro-ψ ψ-carotene)[9] indicate that this cannot be the only route available in *R rubrum* for the final steps of spirilloxanthin biosynthesis

Of the six monomethoxy-monohydroxycarotenoids described here, three have olefinic bonds between carbons 3 and 4 and have been described previously[8,13,24,25] Another possible member of this series the 1'-hydroxy-1 ,2'-dihydro-derivative of 11 12'-dihydrospheroidene (1-methoxy-3,4-didehydro-1,2,7',8' 11' 12'-hexahydro-ψ ψ-carotene)[8] was not detected The other three compounds described are novel carotenoids and can be considered as the 1' 2'-hydration products of 1-methoxy-1,2-dihydrophytofluene[9] 3,4,11 ,12'-tetrahydrospheroidene (1-methoxy-1,2,7 ,8',11',12'-hexahydro-ψ,ψ-carotene)[11] and 3,4-dihydrospheroidene (1-methoxy-1 2,7' 8'-tetrahydro-ψ ψ-carotene)[8]

It must be emphasized that it is not known whether the carotenoids described here all of which are present in only small quantities, are true intermediates or biosynthetic artifacts Any proof of their role in spirilloxanthin biosynthesis must await the development of techniques capable of following the quantitative changes of nearly 40 carotenoids on liberating cultures of *R rubrum* from conditions of DPA inhibition Many of the carotenoids may result, however, from a lack of specificity on the part of the enzymes which catalyze the three basic reactions of methoxycarotenoid formation so that they are formed only when inhibition by DPA blocks the phytoene dehydrogenation sequence and provides the enzymes not with lycopene, their normal substrate but with its more saturated precursors

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EXPERIMENTAL

Organism and culture conditions Cultures of *Rhodospirillum rubrum* (NCIB 8255) were obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland, and were maintained as agar stabs (1 5% [w/v] agar, 0 2% [w/v] Difco bacteriological yeast extract) The bacteria were grown anaerobically in 15 l batches in completely filled Roux bottles, fitted with Al caps, in the light (tungsten, 4000 lx) at 29° for 7 days on a standard medium [26 27] DPA was added at inoculation (2 5 mg/ml EtOH) to give a final concentration of 65 μM

Solvents All the solvents used were of AR grade Petrol (40–60°) was dried over Na wire, redistilled from reduced Fe powder, passed through a column of silica gel and redistilled again, the fraction distilling between 40 and 55° was used for chromatography Both C_6H_6 and Et_2O were dried over Na wire and redistilled (the latter from reduced Fe powder) prior to use, while pyridine was refluxed for 1 5 hr over KOH pellets before being redistilled with the exclusion of moisture ($CaCl_2$ trap).

Extraction of carotenoids The bacterial cells were collected by centrifugation (Sharples continuous flow centrifuge), washed with 0 1 M K-phosphate buffer (pH 6 8), centrifuged again (8000 *g* for 15 min, Sorvall RC-2B centrifuge, GSA head) and extracted by homogenization in MeOH The methanolic mixture was centrifuged (8000 *g*, 15 min) to sediment the cell residue and the supernatant was decanted Three such extractions with MeOH were sufficient to remove all the bacteriochlorophyll, the methanolic extracts were bulked and the bacterial residue was extracted 3 × with acetone The bulked acetone extracts were concentrated almost to dryness by rotary evaporation at 30° and the methanolic soln was added The entire extract was saponified for 15 hr at 3° under N_2 with aq 60% (w/v) KOH (1 ml/15 ml extract) and the unsaponifiable fraction, isolated by our standard procedure,[14] was dissolved in the minimum vol of petrol prior to column chromatography

Column chromatography Preparative chromatography (to yield fractions 'A', 'B' and 'C') was carried out on a column (25 × 2 2 cm) of alumina (Woelm neutral) which had been deactivated (to Brockmann activity grade III) by the addition of H_2O (6%, v/w) The unsaponifiable fraction was added in petrol and was washed on to the column with the same solvent, development was by increasing concentrations of Et_2O in petrol (Table 1) and fractions were collected on elution from the bottom of the column Further chromatography (Table 1) on smaller columns was also on alumina, deactivated to grade III or IV (10% H_2O, v/w)

TLC Three types of layer were used, namely (a) Silica Gel G (Merck), (b) MgO (B D H Ltd, for chromatographic adsorption analysis), and (c) a mixture (1 1, w/w) of MgO (B D H) and Kieselguhr G (Merck) All the layers were prepared as aqueous slurries and spread to a thickness of 250 μm on glass plates (20 × 20 cm), the layers were dried for 2 hr at 110° and stored in a desiccator Details of the developing solvents are recorded in Table 2

Silylation of hydroxyl groups TMS ethers were prepared from the monohydrocarotenoids by an adaptation of a method described previously [8 28] The carotenoid (*ca* 0 5 mg) was dissolved in 0 5 ml dry pyridine and 0 2 ml hexamethyldisilazane and 0 1 ml trimethylchlorosilane were added The reaction was allowed to proceed for 1 hr at room temp and the reaction mixture was evaporated to dryness under N_2, extracted with petrol and the TMS ethers chromatographed (Table 1) The overall efficiency, of silylation and extraction, was measured spectrophotometrically and was 76–80%

Absorption spectra All electronic spectra were recorded in petrol on a recording spectrophotometer, the wavelength scale of which was calibrated with the appropriate absorption bands of a holmium oxide filter Quantitative measurements (of silylation efficiency) were made on solutions of known vol in one of a matched pair of 1 cm silica cuvettes

MS Some of the MS were determined on an A E I MS 12 instrument (probe temp 220°, ionization potential 70 eV) at the Department of Biochemistry, University of Liverpool, through the courtesy of Dr G Britton Other spectra (probe temps 180–200°) and accurate ion masses (relative to heptacosafluorotributylamine) were determined on an A E I MS 902 instrument at the Department of Chemistry, Queen Mary College, London, with the kind collaboration of Professor B C L Weedon and Dr T P Toube

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