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BIOSYNTHESIS OF 1,2-DIHYDROCAROTENOIDS IN *RHODOPSEUDOMONAS VIRIDIS*: EXPERIMENTS WITH INHIBITORS

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Key Word Index—*Rhodopseudomonas viridis*; Rhodospirillaceae; 1,2-dihydrocarotenoids; biosynthesis; nicotine; diphenylamine; CPTA.

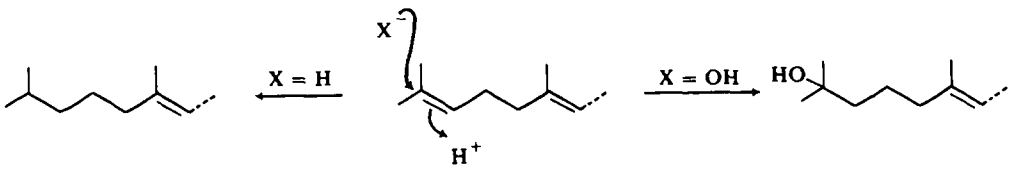
Abstract—The effects of the inhibitors diphenylamine (DPA), 2-(4-chlorophenylthio) triethylammonium chloride (CPTA) and nicotine on the biosynthesis of 1,2-dihydrocarotenoids by *Rhodopseudomonas viridis* (Rhodospirillaceae) have been investigated. Small amounts of 1,2-dihydro derivatives of phytoene, phytofluene and ζ-carotene and its unsymmetrical isomer, and 1,2,1′,2′,-tetrahydro derivatives of neurosporene and lycopene were isolated from *R. viridis* grown in the presence of DPA, although there was virtually no quantitative effect on the levels of the normal main carotenoids, neurosporene and lycopene and their 1,2-dihydro derivatives. Nicotine also had little effect on the overall carotenoid composition, but the formation of 1,2-dihydrocarotenoids was inhibited to some extent by CPTA. The 1,2-dihydro end group may thus be introduced by a hydrogenation reaction similar to the more familiar C-1,2 hydration reaction characteristic of carotenoid biosynthesis in other photosynthetic bacteria.

INTRODUCTION

The carotenoid pigments of the purple, non-sulphur photosynthetic bacteria of the Rhodospirillaceae are characteristically acyclic compounds with tertiary hydroxy or methoxy substituents at C-1 and C-1′ [1]. One species however, *Rhodopseudomonas viridis*, produces mainly carotenoid hydrocarbons, the most abundant having the 1,2-dihydro end-group, a structural feature that has not been found in the carotenoids of any other organism. The main pigments of *Rps. viridis* have been identified as neurosporene (7,8-dihydro-ψ,ψ-carotene, **9**) and lycopene (ψ,ψ-carotene, **12**) and their 1,2- dihydro derivatives 1,2,7,8-tetrahydro-ψ,ψ-carotene (**10**) and 1,2-dihydro-ψ,ψ-carotene, (**13**) [2, 3]. The synthesis of these and other 1,2-dihydrocarotenes has recently been reported [4]. Only very small amounts of xanthophylls are present in *Rps. viridis*, and these have not been investigated in detail, but the presence of cross-conjugated caroten-13-als, one of which has the 1,2-dihydro end group, has recently been reported [5]. No work on the biosynthesis of the 1,2-dihydrocarotenoids of *Rps. viridis* has yet been published.

In studies of the biosynthesis of the hydroxy- and methoxycarotenoids commonly found in members of the Rhodospirillaceae, much use has been made of inhibitors [6, 7]. In particular, diphenylamine (DPA) has been very widely used. In many microorganisms, this compound blocks the sequence of desaturation reactions of carotenoid biosynthesis, and phytoene (7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene, **1**) accumulates, often accompanied by a variety of other carotenoids not normally present; the identification of these may give some indication of possible biosynthetic pathways. Recently two substances, nicotine and CPTA [2-(4-chlorophenylthio) triethylammonium chloride] have been found to inhibit formation of β- and ε-rings in the biosynthesis of cyclic carotenoids, and also the hydration of the C-1,2 double bond in the biosynthesis of the characteristic hydroxy- and methoxycarotenoids in organisms of the Rhodospirillaceae. These inhibitors have been particularly useful in studies of the biosynthesis of spheroidene (1-methoxy-1,2,7′,8′-tetrahydro-ψ,ψ-carotene) and hydroxyspheroidene (1′-methoxy-1,2,7,8,1′,2′-hexahydro-ψ,ψ-caroten-1-ol) [8, 9] and of rhodopin (1,2-dihydro-ψ,ψ-caroten-1-ol) [9, 10].

It has been suggested [7, 11] that the hydrogenation of the C-1,2 double bond in the biosynthesis of the *Rps. viridis* carotenoids may be similar mechanistically to the more common hydration reaction (Scheme 1), so that



Scheme 1. Postulated mechanism for formation of the 1,2-dihydro and 1-hydroxy-1,2-dihydro end groups in carotenoid biosynthesis.

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this hydrogenation might also be susceptible to inhibition by nicotine and CPTA. The effect of these inhibitors on carotenoid synthesis by *Rps. viridis* has therefore been investigated. The effect of DPA has also been studied, since this might give useful indications about the early post-phytoene stages in the biosynthetic pathway.

RESULTS

Although the medium used for culturing *Rhodopseudomonas viridis* in much of the present work was not the same as that used in the previous work with this organism [2, 3], the carotenoid composition was similar to that reported previously, with the 1,2-dihydro derivatives of neurosporene and lycopene, 1,2,7,8-tetrahydro-ψ,ψ-carotene (**10**) and 1,2-dihydro-ψ,ψ-carotene (**13**) predominating over the normal neurosporene (**9**) and lycopene (**12**) and 3,4-didehydro-1,2-dihydro-ψ,ψ-carotene (**15**) also present. The addition of DPA had very little overall effect on the carotenoid composition. Even at the highest DPA concentration that the organisms would tolerate (6×10^{-5} M), the amounts of the usual carotenoids present were not significantly altered, but very small amounts of a range of more saturated carotenoids were detected. The amounts present were so small that 40 litres of culture were required to allow sufficient material to be isolated for absorpton spectroscopic and MS analysis. *Rps. viridis* differed from most other microorganisms studied, because the amount of phytoene present in DPA-inhibited cultures was very small, but this phytoene was accompanied by 1,2-dihydrophytoene [1,2,7,8,11,12,7',8',11',12'-decahydro-ψ,ψ-carotene, (**2**) 6% of total phytoenes]. Phytofluene [7,8,11,12,7',8'-hexahydro-ψ,ψ-carotene, (**3**)] was also present, accompanied by an equal amount of its 1,2-dihydro derivative, 1,2,7,8,11,12,7',8'-octahydro-ψ,ψ-carotene (**4**). The main conjugated heptaene present (*ca* 75% of the total) was the symmetrical ζ-carotene (7,8,7',8'-tetrahydro-ψ,ψ-carotene (**5**)), together with a mixture of 1,2-dihydro derivatives, with the unsymmetrical chromophore isomer 1,2,7,8,11,12-hexahydro-ψ,ψ-carotene (**8**) predominating (85%) over 1,2,7,8,7',8'-hexahydro-ψ,ψ-carotene (**7**). The identification of these compounds from their spectroscopic properties has been reported previously [3].

The effects of increasing concentrations of nicotine on the carotenoid composition were then investigated. However the carotenoid composition was not greatly affected (Table 1), even by nicotine concentrations as high as 10 mM. Nicotine thus does not exert a specific inhibitory effect on carotenoid biosynthesis in *Rps. viridis* as it does with many other microorganisms.

Another substance that often has an inhibitory effect similar to that of nicotine is CPTA. The effects of a range of concentrations of CPTA on carotenoid synthesis by *Rps. viridis* are illustrated in Fig. 1. The main effect was that the amounts of 1,2-dihydrocarotenoids present decreased, and this decrease was matched by increases in the amounts of neurosporene and lycopene. Again, however, the inhibition was not so extensive or specific as has been observed with other organisms.

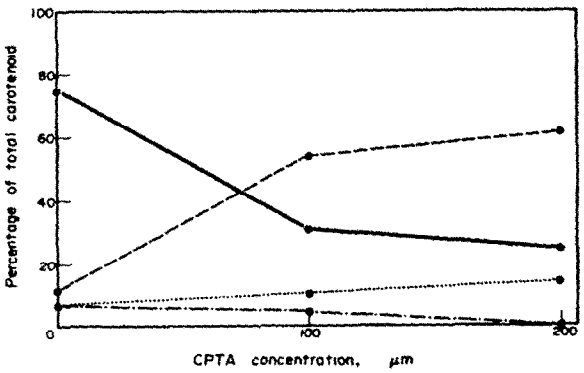

Fig. 1. Effects of increasing concentrations of CPTA on the carotenoid composition of *Rhodopseudomonas viridis*. ——— 1,2-Dihydroneurosporene, – – – – – Neurosporene, – · – · – 1,2-Dihydrolycopene, ····· Lycopene. [Total carotenoid concentrations at 0.100 and 200 μM CPTA were 2050, 1970 and 1850 μg/g dry wt respectively].

DISCUSSION

The response of *Rps. viridis* to some of the most useful inhibitors of carotenoid biosynthesis was disappointing. Nicotine, which has proved to be such a valuable tool in recent biosynthetic studies with other members of the Rhodospirillaceae [8–10] had no consistent effect in *Rps. viridis*. CPTA, however, did inhibit formation of the 1,2-dihydro end group, in agreement with the proposal [7, 11] that this occurs by a hydrogenation reaction similar to the hydration reaction involved in the biosynthesis of the 1-hydroxy-1,2-dihydrocarotenoids commonly found in the Rhodospirillaceae (Scheme 1). In particular, neurosporene and lycopene accumulated at the expense of their 1,2-dihydro derivatives, suggesting that the usual main carotenoids of *Rps. viridis*, 1,2-dihydroneurosporene and 1,2-dihydrolycopene are normally produced by hydrogenation of the parent compounds, neurosporene and lycopene. The inhibition by CPTA was not, however, so well-defined as with other organisms.

Even the extensively used DPA had little quantitative effect in *Rps. viridis*, but this inhibitor did allow the

Table 1. Effect of increasing concentrations of nicotine on the carotenoid composition of *Rhodopseudomonas viridis*

Nicotine concentration	0 mM		2.5 mM		5 mM		7.5 mM		10 mM	
Carotenoid content	μg/g dry wt	%	μg/g dry wt	%	μg/g dry wt	%	μg/g dry wt	%	μg/g dry wt	%
1,2-Dihydroneurosporene	1270	52.2	1400	51.4	1390	51.3	1055	51.6	1130	53.2
Neurosporene	265	11.0	675	24.6	645	23.8	595	28.9	580	27.5
1,2-Dihydrolycopene	275	11.3	225	8.3	275	10.2	110	5.3	100	4.9
Lycopene	380	15.6	160	5.9	130	4.8	85	4.1	95	4.6

detection and identification of a number of minor compounds not present in normal cultures. The presence of 1,2-dihydro derivatives of phytoene, phytofluene and the ζ-carotene isomers, even in very small amounts, shows that hydrogenation of the C-1,2 double bond can occur, at least under these abnormal conditions, at early stages in the biosynthetic sequence. The situation with regard to the ζ-carotene group of compounds is interesting in that the 1,2-dihydro derivative of the unsymmetrical 7,8,11,12-tetrahydrolycopene is the predominant 1,2-dihydro 'ζ-carotene' found, but the unsubstituted ζ-carotene present is entirely the symmetrical 7,8,7',8'-tetrahydrolycopene. This may indicate that the desaturation in the normal hydrocarbon series occurs via symmetrical ζ-carotene but desaturation in the 1,2-dihydro series occurs preferentially via the unsymmetrical heptaene. Another unusual feature of the DPA cultures was the presence of small amounts of the tetrahydro derivatives of neurosporene and lycopene, i.e. 1,2,7,8,1',2'-hexahydro-ψ,ψ-carotene (**11**) and 1,2,1',2'-tetrahydro-ψ,ψ-carotene (**14**), not detected in normal, uninhibited cultures.

Although these inhibitors are therefore not likely to be useful for detailed studies with *Rps. viridis*, the experiments reported in this paper do give the first information to be obtained about the biosynthesis of the 1,2-dihydro carotenoids found only in this organism. The overall picture that emerges (summarized in Scheme 2) is that the 1,2-dihydro carotenoids are normally produced by hydrogenation of the parent carotenes, neurosporene and lycopene, but under strained conditions, e.g. in the presence of DPA, hydrogenation of the more saturated carotenes, phytoene, phytofluene and the ζ-carotene isomers and further hydrogenation of dihydroneurosporene and dihydrolycopene can occur. It is unusual that in the normal main carotenoid of *Rps. viridis*, 1,2-dihydroneurosporene, the hydrogenation has occurred in the more saturated half of the molecule. In all other related cases, e.g. spheroidene formation in *Rps. spheroides*, substitution occurs primarily in the less saturated half of the molecule. The significance of these findings is not known.

EXPERIMENTAL

Organism and culture conditions. Cultures of *Rhodopseudomonas viridis* were obtained as kind gifts from Dr. K. Eimhjellen, Trondheim, Norway, Dr. Karin Schmidt, Göttingen, West Germany, and Dr. E. Hilary Evans, Biochemistry Department, University of Liverpool. The bacteria were cultured for 7 days anaerobically in the light at 28 ± 2° as described previously [5]. Inhibitors were added aseptically at the time of inoculation, DPA as a conc ethanolic soln, CPTA as a sterile aq. soln. The final overall concn of DPA was 6×10^{-5} M, and 40 l. of DPA-inhibited culture was used. A series of 1 l. nicotine-inhibited cultures containing 0, 2.5, 5, 10 mM nicotine respectively was analysed, and the carotenoid compositions of these cultures are given in Table 1. The carotenoid compositions of a second series of 1 l. cultures containing CPTA (0, 100, 200 μM) in place of nicotine are illustrated in Fig. 1. The results shown are the mean values obtained from two experiments. Variations between the two experiments were generally not greater than ±5%.

Extraction, purification and identification of carotenoids. Carotenoids were extracted from the harvested cells, purified and identified by their light absorption spectra in petrol (bp 40–60°) and MS as described previously [2, 3]. Phytoene (**1**); λ_{max} nm 275, 285, 296, MS *m/e*: 544 (12%, M^+, $C_{40}H_{64}$), 339 (15%, M-205, *m** 211; $339^2/544 = 211.2$). 1,2-Dihydrophytoene (**2**); λ_{max} nm: 275, 285, 296, MS *m/e*: 546 (10%, M^+, $C_{40}H_{66}$), 341 (8%, M-205, *m** 213; $341^2/546 = 213.0$), 339 (7%, M-207, *m** 210, $339^2/546 = 210.5$). Phytofluene (**3**); λ_{max} nm: 331, 348, 367, MS *m/e*: 542 (15%, M^+, $C_{40}H_{62}$), 337 (8%, M-205, *m** 209; $337^2/542 = 209.5$). 1,2-Dihydrophytofluene (**4**); λ_{max} nm: 331, 348, 367, MS *m/e*: 544 (20%, M^+, $C_{40}H_{64}$), 407 (7%, M-137, *m** 305; $407^2/544 = 304.5$), 337 (10%, M-207, *m** 209; $337^2/544 = 208.7$). ζ-Carotene (**5**). λ_{max} nm 379, 400, 425, MS *m/e*: 540 (30%, M^+, $C_{40}H_{60}$), 403 (12%, M-137, *m** 301; $403^2/540 = 300.8$), no major fragment ion at *m/e* 335 (M-205), showing that 7,8,11,12-tetrahydro-ψ,ψ-carotene (**6**) was not present. '1,2-Dihydro-ζ-carotene'; λ_{max} nm: 375, 395, 419, MS *m/e*: 542 (35%, M^+, $C_{40}H_{62}$), 405 (4%, M-137, *m** 303; $405^2/542 = 302.6$) and 403 (4%, M-139, *m** 300; $403^2/542 = 299.6$) due to 1,2-dihydro-ζ-carotene (**7**), *m/e* 473 (16%, M-69, *m** 413; $473^2/542 = 412.8$) and 335 (25%, M-207, *m** 207; $335^2/542 = 207.1$) due to 1,2,7,8,11,12-hexahydro-ψ,ψ-carotene (**8**). Neurosporene (**9**); λ_{max} nm: 414, 439, 468, MS *m/e*: 538 (25%, M^+, $C_{40}H_{58}$), 469 (7%, M-69, *m** 409; $469^2/538 = 408.8$), 401 (10%, M-137, *m** 299; $401^2/538 = 298.9$). 1,2-Dihydroneurosporene (**10**); λ_{max} nm. 414, 439, 468, MS *m/e*: 540 (32%, M^+, $C_{40}H_{60}$), 471 (8%, M-69, *m** 411; $471^2/540 = 410.8$), 401 (16%, M-139, *m** 298; $401^2/540 = 297.8$). 1,2,1',2'-Tetrahydroneurosporene (**11**); λ_{max} nm 414, 439, 468, MS *m/e*: 542 (42%, M^+, $C_{40}H_{62}$), 403 (17%, M-139, *m** 300; $403^2/542 = 299.6$), 471 (2%, M-71). Lycopene (**12**); λ_{max} nm: 444, 469, 501, MS *m/e* 536 (100%, M^+, $C_{40}H_{56}$), 467 (15%, M-69, *m** 407; $467^2/536 = 406.9$). 1,2-Dihydrolycopene (**13**); λ_{max} nm: 443, 469, 501, MS *m/e*: 538 (100%, M^+, $C_{40}H_{58}$), 469 (10%, M-69, *m** 409; $469^2/538 = 408.8$), 467 (2%, M-71). 1,2,1',2'-Tetrahydrolycopene (**14**); λ_{max} nm: 443, 469, 501, MS *m/e*: 540 (100%, M^+, $C_{40}H_{60}$), 469 (3%, M-71). 3,4-Didehydro-1,2-dihydrolycopene (**15**); λ_{max} nm: 457, 483, 518, MS *m/e*: 536 (100%, M^+, $C_{40}H_{56}$), 467 (6%, M-69, *m** 416; $467^2/536 = 415.6$).

Spectra. Light absorption spectra and MS were obtained as described previously [3].

Quantitative analysis. Carotenoids were analysed quantitatively by the spectrophotometric method described in ref. [12]. For the 1,2-dihydrocarotenes the $E^{1\%}_{1cm}$ values [12] of the corresponding parent carotenoids were used.

Dry weight measurements. After extraction of the pigments, lipid-free cell debris was dried at 110°, cooled in a desiccator, and weighed.

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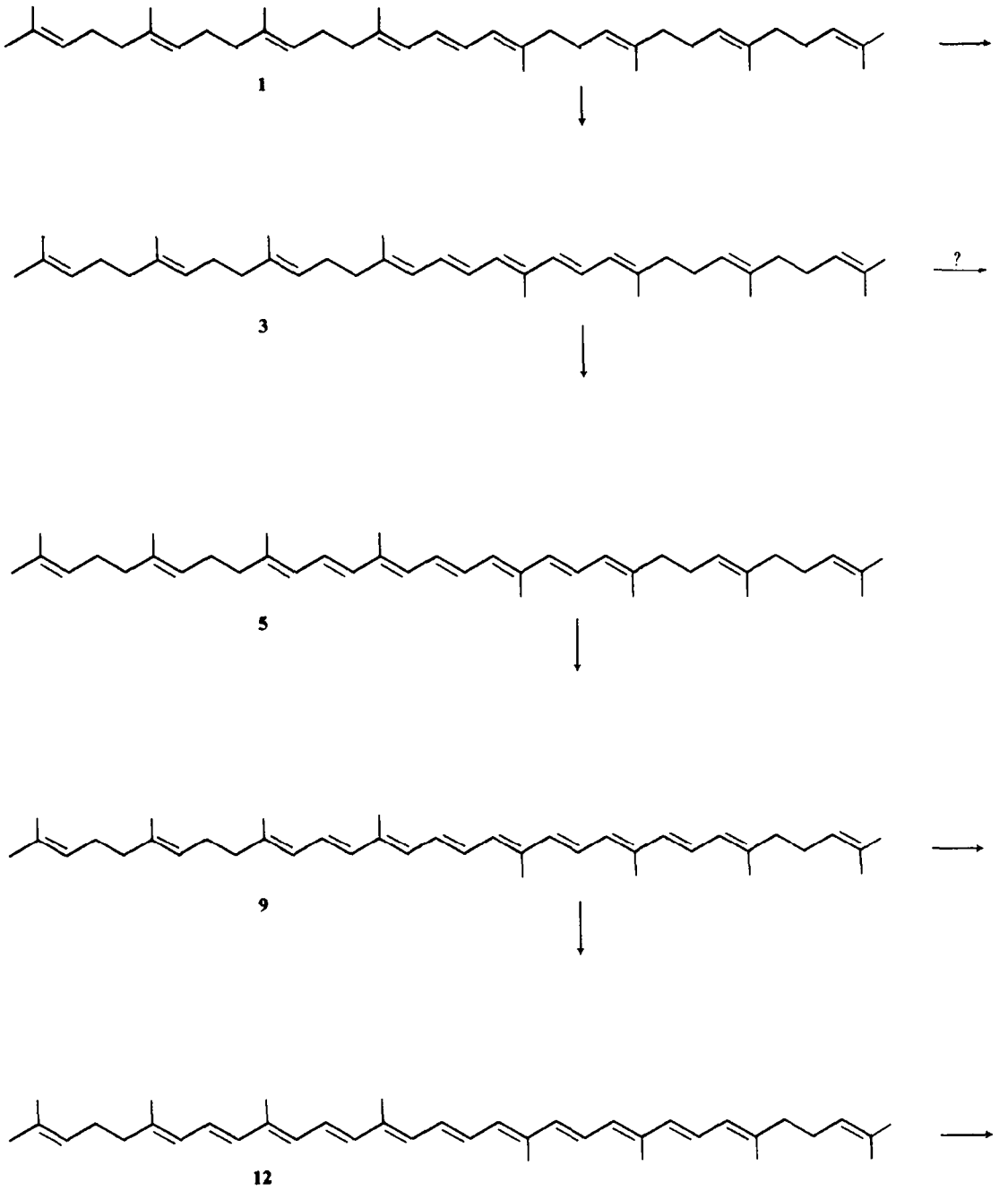
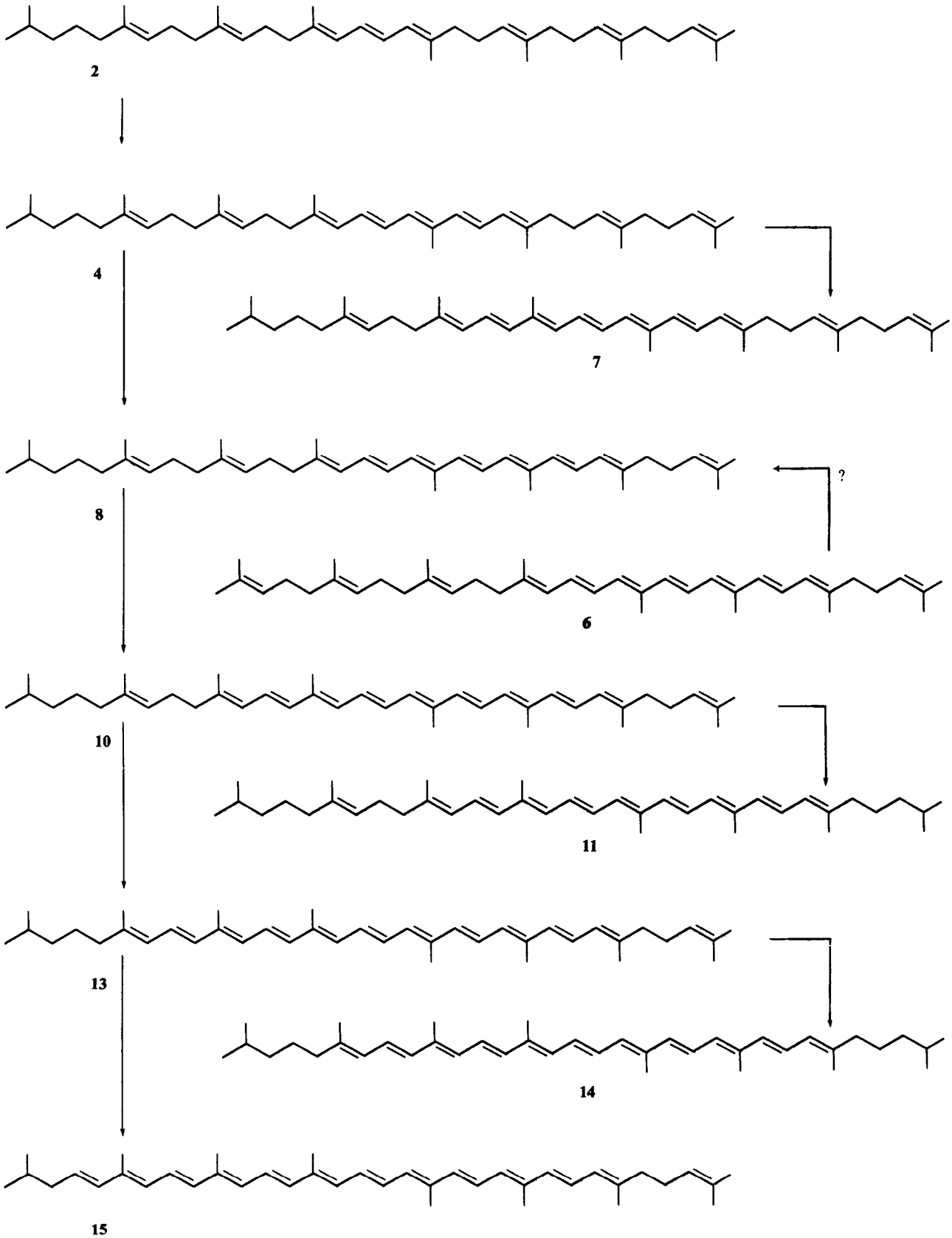


Scheme 2. Possible alternative pathways of carotenoid



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