CAROTENOID BIOSYNTHESIS IN *RHODOCOCCUS MARIS* STRAIN N1020

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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Chapter 1

General Introduction

1.1 Introduction

Compounds are coloured because they absorb light in the visible region of the spectrum in the wavelength range 380-750 nm. The delocalization of π-electrons along the chromophore that is either aromatic or conjugated determines the light absorption properties. The more extended the conjugation of the chromophore, the less energy is required to promote electrons and this corresponds to the absorption of light of longer wavelength. Electronic transition of the molecules, especially π to π* transitions, are responsible for the light absorption.

Five main groups of natural pigments are melanins, quinones, flavonoids tetrapyrroles and carotenoids. Carotenoids may be considered to be the most important group of pigments in many biological processes, for instance, photosynthesis, photoprotection, photooxidation, camouflage, colour and vision, *etc.* They are widely distributed throughout the animal and plant kingdoms and in microbes. In animals, especially mammals, β-carotene is the precursor of vitamin A. In plants, carotenoids are distributed in both photosynthetic and non-photosynthetic tissues (Britton, 1983). They are involved in photosynthesis and colouration. In microbes, they are found in yeasts, algae and bacteria, both photosynthetic and non-photosynthetic. Approximately six hundred carotenoids have been identified and characterised (Straub, 1987).

1.2 Characteristics of carotenoids

Carotenoids are generally derived from hydrocarbon $C_{40}H_{56}$ compounds which may or may not have rings at the ends of the molecule. Modification in the rings is normally in the form of oxygen substitution that may be hydroxy, ketone, epoxy or carboxylic acid groups.

1.2.1 Physical and chemical properties

Carotenoids are unstable compounds in the normal environment and are sensitive to oxygen, light, heat, acids, and in some cases alkali, and especially to combinations of these factors, for example, light and oxygen (Britton, 1985). They are soluble in organic solvents. Binding of carotenoids to protein or lipo(glyco)proteins causes the molecule to be more stable than in the free form.

Owing to the long conjugated double-bond system in the chromophore, *cis-trans* isomerization can take place in many positions of the polyene chain. Normally *cis*-carotenoids are less stable than the *trans*-isomer. Isomerization of the carotenoids may be caused by light with and without catalyst (Weedon, 1971).

1.2.2 Spectral properties

The spectrum of a carotenoid is a property of the conjugated polyene chain chromophore. The λmax is determined by the length of the chromophore. The shape of the spectrum is also characteristic and is determined by the number of double bonds, the presence of cyclic end groups and substituents, *cis-trans* isomerism and the nature of the organic solvent. As the length of the conjugated double-bond chain increases, the introduction of additional double bonds has a diminishing effect on the position of the main absorption band (Vetter *et al.*, 1971).

1.3 Structural features and nomenclature of carotenoids

Carotenoids are polyene compounds basically consisting of eight isoprenoid units (C_5). They differ in their functional groups. The basic carotenoid hydrocarbons are called carotenes and oxygenated carotenes xanthophylls. The carotenoids have not only trivial names which are usually derived from the biological source from which they were first extracted but also semisystematic names which indicate structural information. The carotenoid molecule is considered in two halves. Seven end groups have been classified and designated by Greek letter prefixes and the presence of substituent groups is then indicated by use of conventional prefixes and suffixes. The specific names are based on the stem name carotene combined with the corresponding substituent groups. Hence, β-carotene, canthaxanthin and astaxanthin are called β,β-carotene, β,β-carotene-4,4'-dione and 3,3'-dihydroxy-β,β-carotene-4,4'-dione, respectively, as shown in Figure 1.1-1.3 (Britton, 1983; IUPAC, 1972; IUPAC and IUB, 1975; Weedon and Moss, 1995; Schmidt *et al.*, 1994). In this thesis, well known trivial names are used in preference to semisystematic names except when the latter provide more clarity.

1.4 The role of carotenoids in living organisms

Carotenoids are distributed in a great diversity of organisms. They are important in many biological processes, for instance, light harvesting in photosynthesis, photoprotection, photooxidation, sex-related colouration pattern and vision (Britton, 1995).

1.4.1 Carotenoids in photosynthesis

Photosynthesis in different organisms employs different systems, which contain various carotenoids. In the thylakoids of plants as well as the reaction centre

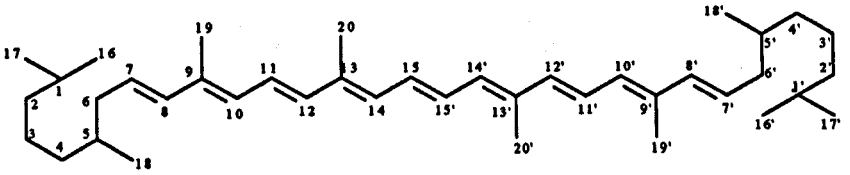


Figure 1.1: Numbering of the carotenoid skeleton

From IUPAC (1972) *J. Biol. Chem.*, 247, 2634

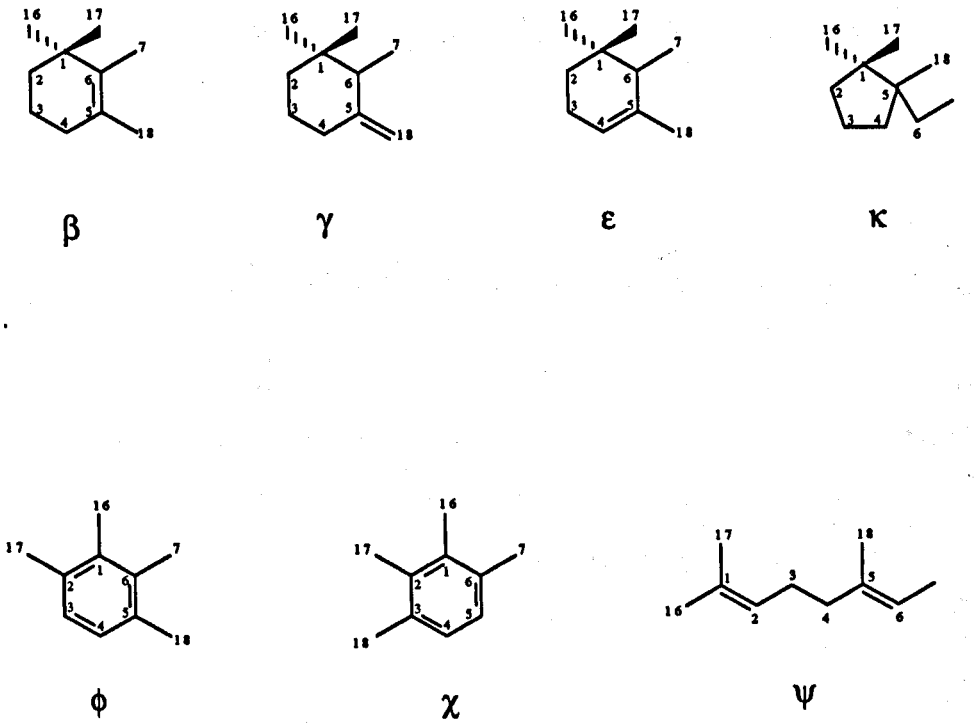


Figure 1.2: The seven carotenoid end groups

From IUPAC (1972) *J. Biol. Chem.*, 247, 2634

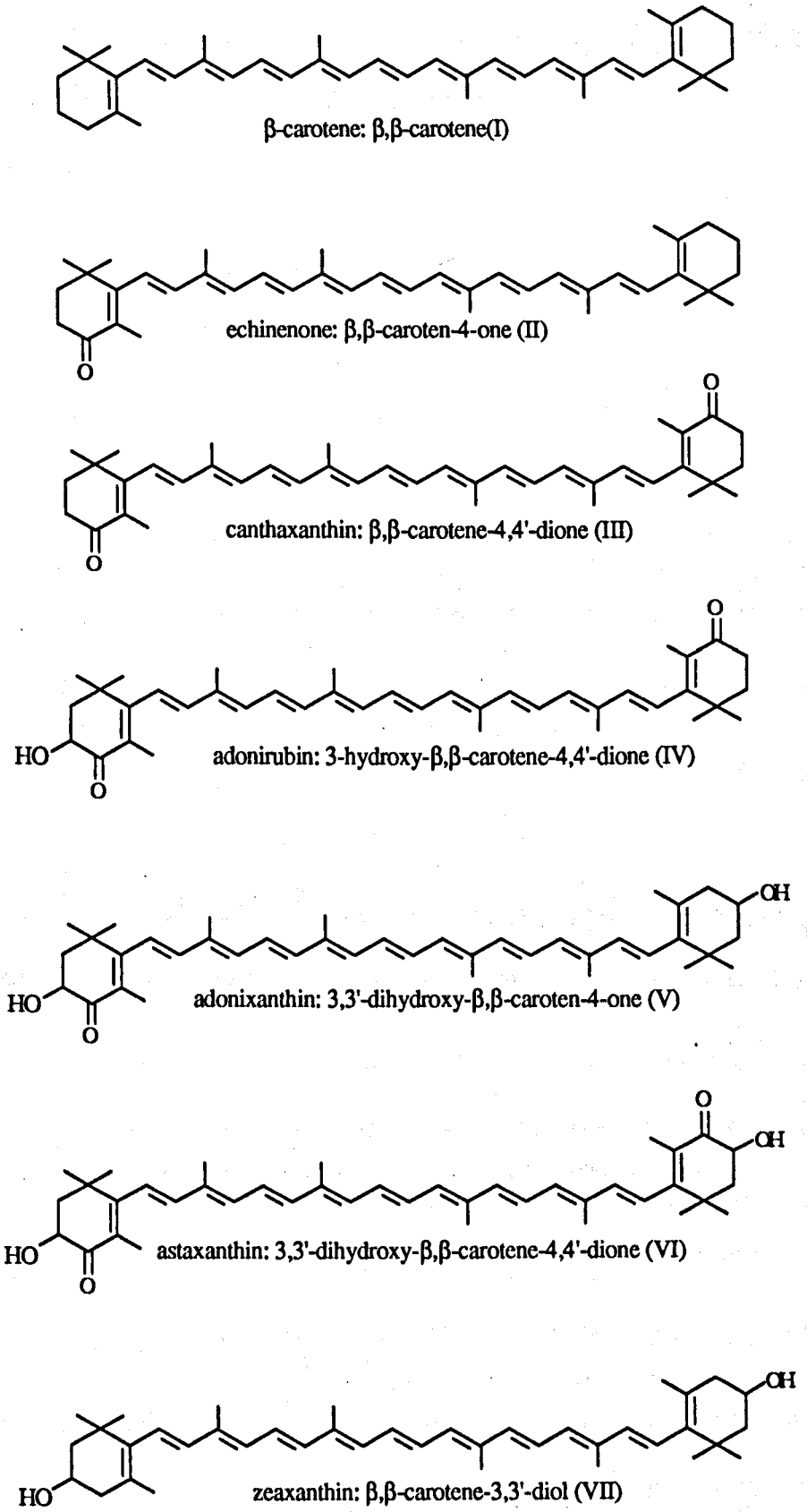
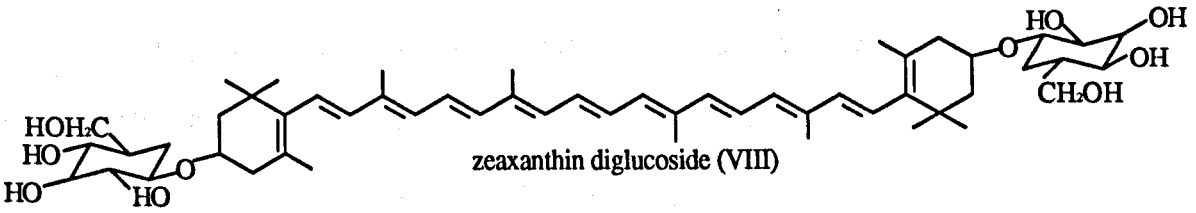
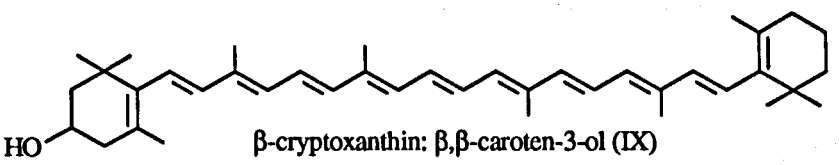


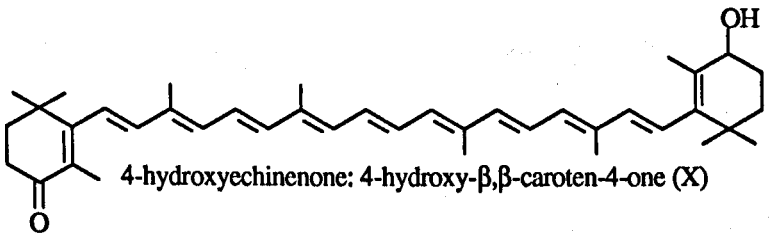
Figure 1.3: Trivial names and semisystematic names of carotenoids



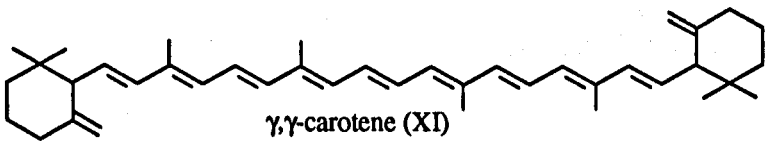
zeaxanthin diglucoside (VIII)



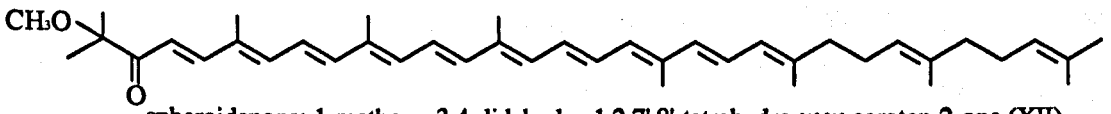
β-cryptoxanthin: β,β-caroten-3-ol (IX)



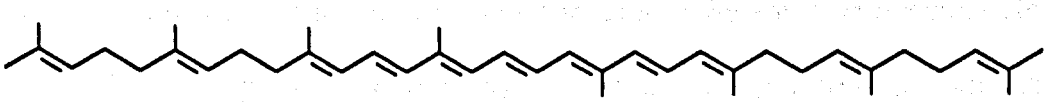
4-hydroxyechinenone: 4-hydroxy-β,β-caroten-4-one (X)



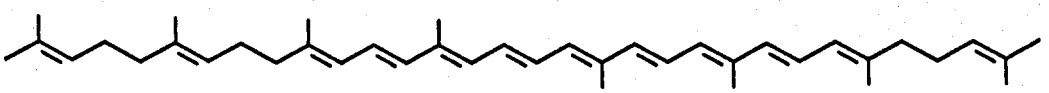
γ,γ-carotene (XI)



spheroidenone: 1-methoxy-3,4-didehydro-1,2,7',8'-tetrahydro-ψ,ψ-caroten-2-one (XII)



ζ-carotene: 7,8,7',8'-tetrahydro-ψ,ψ-carotene (XIII)



neurosporene: 7,8-dihydro-ψ,ψ-carotene (XIV)

Figure 1.3: (continue): Trivial names and semisystematic names of some carotenoids

and antenna system of phototrophic bacteria, carotenoids are found in membrane-bound protein complexes rather than in the free form.

1.4.1.1 Carotenoids in plant photosynthesis

It is universally agreed that carotenoids are found within the thylakoid membrane as integral components of pigment-protein complexes. In general, the reaction centre of the photosynthetic unit contains carotene and the light-harvesting antenna contains xanthophylls that absorb light energy that can be used to drive electron transport from water to $NADP^+$. NADPH is then utilized as an energy source for many biological pathways, for instance providing the ATP that is used for carbon dioxide fixation and carbohydrate production.

The light harvesting function is the result of singlet-singlet energy transfer between carotenoids and chlorophylls.

$$^1Car \xrightarrow{h\nu} {}^1Car^* \quad \text{(Carotenoid excitation)}$$

$$^1Car^* + Chl \longrightarrow Car + {}^1Chl^* \quad \text{(Singlet-singlet energy transfer)}$$

The captured energy ultimately reaches the reaction centre of the photosynthetic unit where it aids in the generation of a transmembrane potential via electron transfer between a series of donors and acceptors. This electrical potential is then converted into a chemical potential that serves as the thermodynamic driving force for the photosynthetic process (Britton, 1983).

1.4.1.2 Carotenoids in photoprotection

There is evidence to show that carotenoids have the ability to protect plants, animals and micro-organisms from the harmful effects of photodynamic action, by quenching energy from a triplet-state photosensitizer, such as chlorophyll, or from

singlet oxygen ($^1O_2$).

Carotenoids in microbial communities dominated by cyanobacteria and diatoms consist mainly of β-carotene and various xanthophylls. Total carotenoid: chlorophyll *a* ratio is high in diatom mats, suggesting that carotenoids serve a photoprotective role in the high light environment (Palmisano, *et al.*, 1989).

In the bacterium *Micrococcus roseus*, the main carotenoid components are canthaxanthin and echinenone. The organism was found to be killed when exposed to light in the presence of air and an exogenous photosensitizer, toluidene blue. This result indicates that a photodynamic process is lethal in this species. The sensitivity to photokilling may be related to the amount of carotenoid, and to the length of the chromophore of the carotenoid. When the carotenoid biosynthesis gene cluster from *Erwinia herbicola* was cloned and transformed into *E. coli*, the presence of carotenoid caused protection of the cell membrane against not only light in the near uv region (320-400 nm) but also in the presence of photosensitising agents activated by near uv light (Tuveson, *et al.*, 1988).

In the alga, *Haematococcus lacustris*, exposed to different photon irradiances for photoinhibition, the photosynthetic activities of green akinetes were less resistant to photodamage than red ones in which the pigment was mainly astaxanthin (Yong and Lee, 1991). The extrachromoplastic secondary carotenoids such as canthaxanthin and astaxanthin may present a photoprotective barrier, preventing the cell and particularly the genome from free radical-mediated damage (Hagen, *et al.*, 1993).

The photoprotective function of carotenoids in phototrophic bacteria occurs in the reaction centre. The reaction is based on triplet energy transfer from T_1 bacteriochlorophyll (^{3}Bchl*) to the ground state S_0 carotenoid ^{1}car, and energy dissipation by the 15-*cis* carotenoid through the non-radiative relaxation of the

resultant T_1 carotenoid ($^3Car^*$).

$$^3Bchl^* + {}^1Car \longrightarrow {}^1Bchl + {}^3Car^*$$

$$^3Car^* \longrightarrow {}^1Car + heat$$

The quenching of $^3Bchl^*$ prevents the generation of singlet oxygen (Koyama, 1991) as shown in Figure 1.4.

In plants, the reaction centre carotenoids have a photoprotective role, participating in triplet-triplet energy transfer from chlorophyll to prevent the chlorophyll-sensitised formation of singlet oxygen; they also may scavenge singlet oxygen directly (Borland *et al.*, 1987).

$$Chl \xrightarrow{h\nu} {}^1Chl^* \text{ (Chlorophyll excitation)}$$

$$^1Chl^* \longrightarrow {}^3Chl^* \text{ (Triplet-state formation)}$$

$$^3Chl^* + Car \longrightarrow Chl + {}^3Car^* \text{ (Triplet-triplet energy transfer)}$$

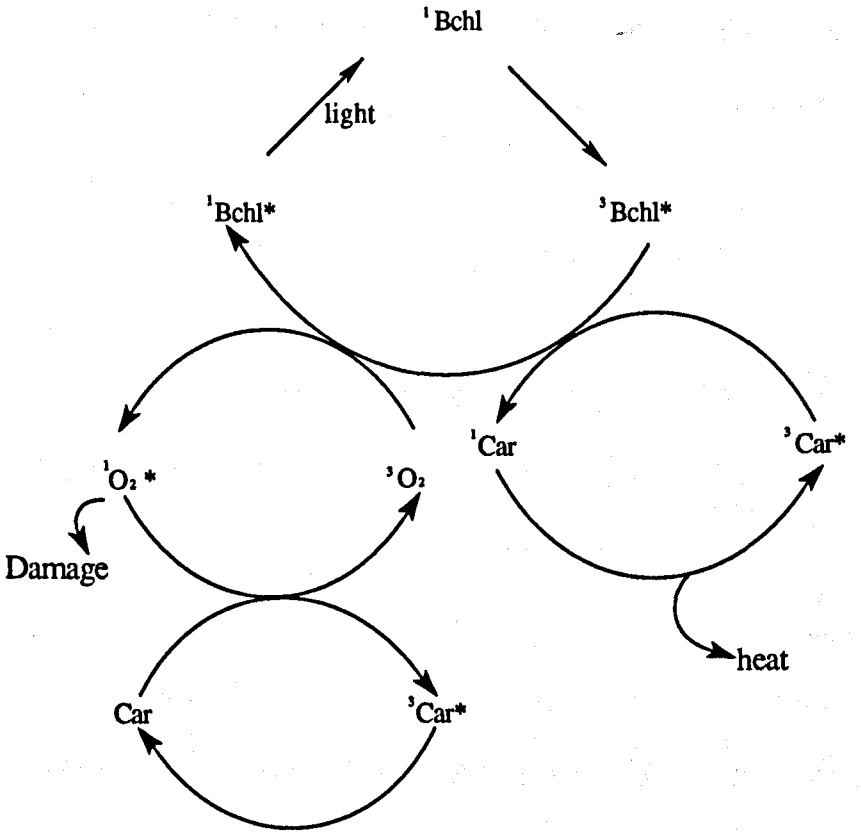
$$^3Car^* \longrightarrow Car + heat \text{ (Carotenoid deactivation)}$$

$$^3Chl^* + O_2 \longrightarrow Chl + {}^1\Delta gO_2^* \text{ (Singlet oxygen formation)}$$

$$^1\Delta gO_2^* + Car \longrightarrow O_2 + {}^3Car^* \text{ (Singlet oxygen scavenging)}$$

1.4.1.3 Carotenoids in phototrophic bacteria

The pigment compositions of phototrophic bacteria have been reviewed extensively in recent years. Many carotenoids have been found in the variety of bacterial strains. In phototrophic bacteria, the photosynthetic unit contains only one photosystem which is related to photosystem II in plants. The photosynthetic unit contains carotenoids, which function in light absorption and the singlet-state energy



Notes

Bchl : Bacteriochlorophyll

Car : Carotenoid

1* : Singlet excited state

3* : Triplet excited state

Figure 1.4: The protective function of carotenoids as triplet state energy quenchers in photosynthesis
Modified from Young, A. J. (1993) In "Carotenoid in Photosynthesis" (Young, A. J. and Britton, G. eds) p170

transfer to bacteriochlorophyll. Carotenoids that are involved in this process may have acyclic, cyclic or aryl endgroups and carotenoid glycosides are sometimes found, *e.g.* in *Rhodobacter acidophila*. The carotenoids also function as energy acceptors from triplet state bacteriochlorophyll in order to protect the cell from damage by singlet oxygen (Goodwin, 1980). The photosynthetic process in phototrophic bacteria is anoxygenic as bacteriochlorophyll is not a powerful enough oxidant to remove an electron from water (Connor, 1991).

In cyanobacteria, the photosystems are similar to those of higher plants. Phycobilisomes may be present to function as light-harvesting antennae that are sensitive to the alteration of light and carbon dioxide (Reuter and Muller, 1993).

1.4.2 Carotenoproteins

In marine invertebrates, especially crustacea, carotenoproteins such as crustacyanin that are present in the carapace may play a role in photoprotection. It has been proposed that the yellow astaxanthin protein in the outermost part of the endocuticle of the lobster (*Homarus gammarus*) may act as a light receptor by passing energy inwards other pigments and transferring energy to the deep blue α-crustacyanin which absorbs light of longer wavelength (Salares, *et al.*, 1979). Possible mechanisms for the perturbed optical properties of astaxanthin in crustacyanin include intermolecular interactions of the π-electron systems and/ or distortion induced by the protein (Buchwald and Jencks, 1968).

Askin (1992) and Weesie *et al* (1995) studied the interaction between carotenoid and protein in the carotenoprotein complexes by means of reconstitution with synthetic and labelled carotenoids, *e.g.* [14,14'-$^{13}C_2$] astaxanthin and detected the chemical shifts by solid state ^{13}C NMR spectroscopy. The results showed that binding between protein and carotenoid causes a reduction of the electronic density

near the C-14 and C-14' positions of the polyene chain. The interaction between carotenoid and protein may involve more than one part of the molecule, that is the rings and along the polyene chain.

1.4.3 Carotenoids as antioxidants

The role of carotenoids in protecting cells against reactive oxygen species has been widely studied in order to understand the mechanism and the potential for using carotenoids as cancer preventing agents. β-Carotene is known to be efficient in protecting cells from oxidative damage which is one of the main factors in the development and progression of cancer.

Canthaxanthin and astaxanthin had the capability to act as antioxidants in the rat liver microsomal membrane, when the rat microsomes were treated with either a generator of oxyradicals (NADPH/ ATP/ Fe_2^+) or a generator of peroxyl radicals, such as 2,2'-azobis(2-amidinopropane, AAPH). At the concentration of only 2-3 nmole/ mg protein, astaxanthin is a potent inhibitor of oxyradical induced lipid peroxidation while β-carotene was needed in a higher concentration of 10 nmole/ mg protein (Palozza and Krinsky, 1992).

In higher plants such as maize, mutants in which the accumulation of carotenoid pigments was inhibited showed retardation of chromoplast development at a very early stage owing to the extensive photooxidative damage within the chloroplasts. Apparently, normal chloroplast development can occur when carotenoid deficient seedings are grown in very dim light but illumination with higher intensity of light causes rapid chlorophyll destruction, showing the role of carotenoid in protecting chlorophylls against photooxidative damage (Mayfield and Taylor, 1987).

1.4.4 Carotenoids and vitamin A

Animals cannot biosynthesise carotenoids from non-carotenoid precursors *de novo* but they can modify carotenoids obtained from dietary sources. In mammals, most vitamin A (retinol) is obtained from dietary β-carotene which is cleaved in the intestine by dioxygenase enzyme to yield vitamin A aldehyde, retinaldehyde, which is then converted into retinol (Stryer, 1988). Vitamin A plays essential roles in vision and in the control of growth and differentiation.

1.4.5 Carotenoids and reproduction

The distribution of carotenoids in the reproductive organs of various marine, fresh water and aquatic species infers a function of these carotenoids in reproduction. Astaxanthin in the eggs of a gastropod mollusc, *Pomacea canaliculata austrilis,* is known to be in the form of a red carotenoglycoprotein, ovorubin (Cheesman, 1958). Ovoverdin, the green protein of lobster eggs is a glycolipoprotein bound with astaxanthin (Renstrom, *et al.*, 1982). Lipovitellins, ovary carotenoprotein complexes are found in a lamellabranch mollusc *P. maximus* (Zagalsky, 1972). Colour in this reproductive organ may probably for attractive purposes in the process of reproduction.

1.5 The importance of ketocarotenoids in aquaculture

In most marine invertebrate animals, for instance shrimps, prawns, lobsters and starfish, the typical carotenoids present are the keto compounds canthaxanthin and astaxanthin. They are found in the free form, or as carotenoprotein and lipo(glyco)protein complexes. Caroteno(lipo)protein complexes are more stable than the free carotenoids and show large changes in the absorption spectrum. The keto groups conjugated with the polyene chain are essential to the spectral shift, and

polarization of the chromophore appears to be important.

Astaxanthin and canthaxanthin are the main carotenoids present in many protozoan zooplankton which are important in marine food webs (Kleppel and Lessard, 1992). In higher species (fish) such as Atlantic salmon (*Salmo salar L.*), the flesh is coloured pink by astaxanthin, but the fish cannot biosynthesise canthaxanthin and astaxanthin from non-carotenoid precursors so that, in salmon farming, these carotenoids have to be supplemented in the feed. There is evidence to demonstrate that there is no significant preferable utilisation of the (3S, 3'S), or (3R, 3'S), (3R, 3'R) optical isomers of astaxanthin and no epimerization at the chiral centres at C-3 and C-3' of astaxanthin (Storebakken, *et al.*, 1985). The natural deposition of astaxanthin in salmon eggs seems to be important in reproduction rather than simply for protection against cell damage by light.

Xanthophylls such as canthaxanthin, astaxanthin, zeaxanthin, lutein and tunaxanthin have been found to be precursors of retinols in freshwater and marine fish fries (Matsuno, 1991). Astaxanthin and canthaxanthin are found to promote the early growth stage of fry. The average cumulative growth rate of fry fed with diets supplemented by these carotenoids was higher than in the ones which were fed a non-carotenoid diet. This positive effect of carotenoid supplementation on growth rate during the early feeding period might be either a metabolic effect with carotenoids acting as biological antioxidants or an effect on behaviour associated with the skin colour (Torrissen, 1984).

The green alga *Haematococcus pluvialis,* containing β-carotene and astaxanthin mainly as monoesters and diesters, was fed to rainbow trout (*Oncorhynchus mykiss)* in comparison with synthetic carotenoids. The mixture of astaxanthin and canthaxanthin fed caused higher pigmentation of trout flesh than did

the algal carotenoids which show the better efficacy of free astaxanthin than of the esterified form, and indicates that the cleavage of the astaxanthin ester may be a limiting step for the pigmentation (Choubert and Heinrich, 1993).

Astaxanthin was first introduced to the fish feed industry at the beginning of the 1970s. The demand for this carotenoid is increasing rapidly because it is required for not only salmon farming but also lobster, prawn, and shrimp farming. Current commercially available astaxanthin is produced by total chemical synthesis via β-ionone or by partial synthesis from canthaxanthin; these processes are extremely expensive (Bernhard, 1990; Johnson and An, 1991).

Carotenoids from natural extracts are likely to be more acceptable for use as a foodstuff additive than chemically synthesised carotenoids. Natural sources of astaxanthin are widely sought; these may be normal natural sources or genetically engineered organisms.

1.6 Carotenoid biosynthesis: general pathway

Carotenoids are biosynthesised by the common isoprenoid pathway either from mevalonic acid or by an alternative pathway from pyruvate and glyceraldehyde diphosphate. Four C_5 isoprenoid units are united to build the chain of geranylgeranyl diphosphate (GGDP), then two GGDP are combined to give phytoene. Phytoene is desaturated to lycopene, both ends of which can be cyclised to give β-carotene. The final steps are the modification of the ring such as the introduction of oxygen functions (Bramley, 1985; Britton, 1990; Britton, 1991).

1.6.1 The formation of GGDP

According to the classical pathway of isoprenoid biosynthesis, the first general isoprenoid precursor is usually considered to be acetyl CoA. Three molecules

of acetyl CoA form 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) which is then reduced to mevalonic acid (MVA) by the enzyme HMG CoA reductase and NADPH, as cofactor. MVA is then phosphorylated twice to give mevalonic acid 5-phosphate and 5-diphosphate. The next reaction involves the decarboxylation of the molecule to give the isoprene unit as isopentenyl diphosphate, IDP. IDP is converted by an isomerase enzyme into dimethylallyl diphosphate (DMADP) in a reversible reaction. These two molecules are substrates for the prenyl transferase enzymes that build the chain. DMADP acts as a primer for the addition of IDP (C_5) to form geranyl diphosphate (GDP, C_{10}), farnesyl diphosphate (FDP, C_{15}) and geranylgeranyl diphosphate (GGDP, C_{20}) as shown in Figure 1.5.

An alternative pathway of isoprenoid biosynthesis was observed in three non-sulphur bacteria: *Rhodobacter acidophila, Rhodobacter palustris* and *Methylobacterium organophilum*. A study with labelled [1-^{13}C] acetate showed only one ^{13}C molecule per isoprenoid unit, a result was not consistent with the acetate-mevalonate pathway. A new alternative pathway was proposed (Flesch and Rohmer, 1988).

1.6.2 The formation of phytoene

The first C_{40} hydrocarbon phytoene is formed from two molecules of GGDP through prephytoene diphosphate (PPDP). In the final step of this reaction, one of two alternative hydrogen atoms is lost, and this determines whether the product is either (all-*trans*) or (15-*cis*)-phytoene as shown in Figure 1.6. Phytoene synthase is the enzyme that catalyses the reaction.

1.6.3 Desaturation of phytoene

Phytoene undergoes a series of desaturation reaction to give phytofluene, ζ-carotene, neurosporene and lycopene. At each stage, two hydrogen atoms are removed

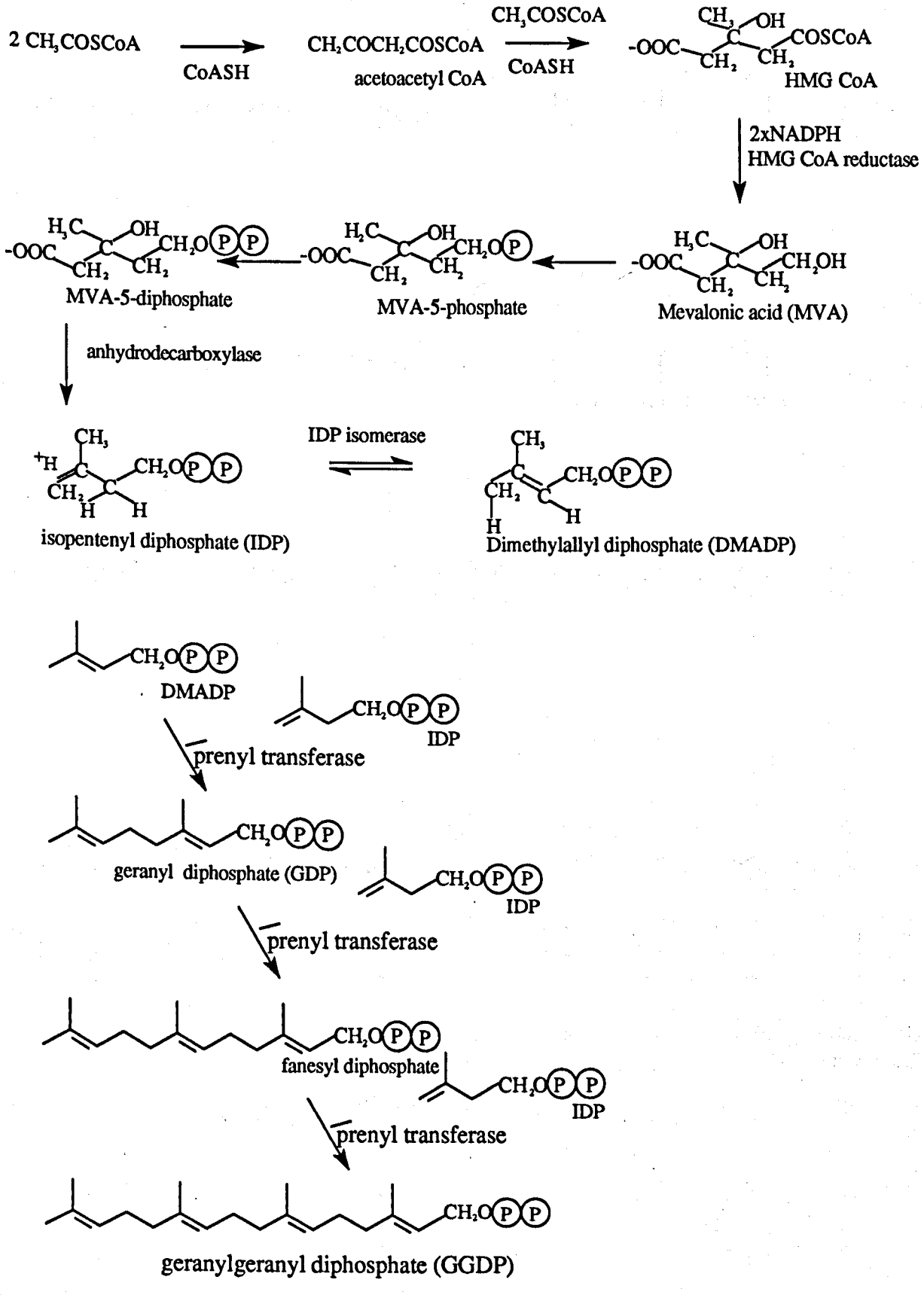


Figure 1.5: GGDP formation

From Britton (1983) In "The Biochemistry of Natural Pigments" p47

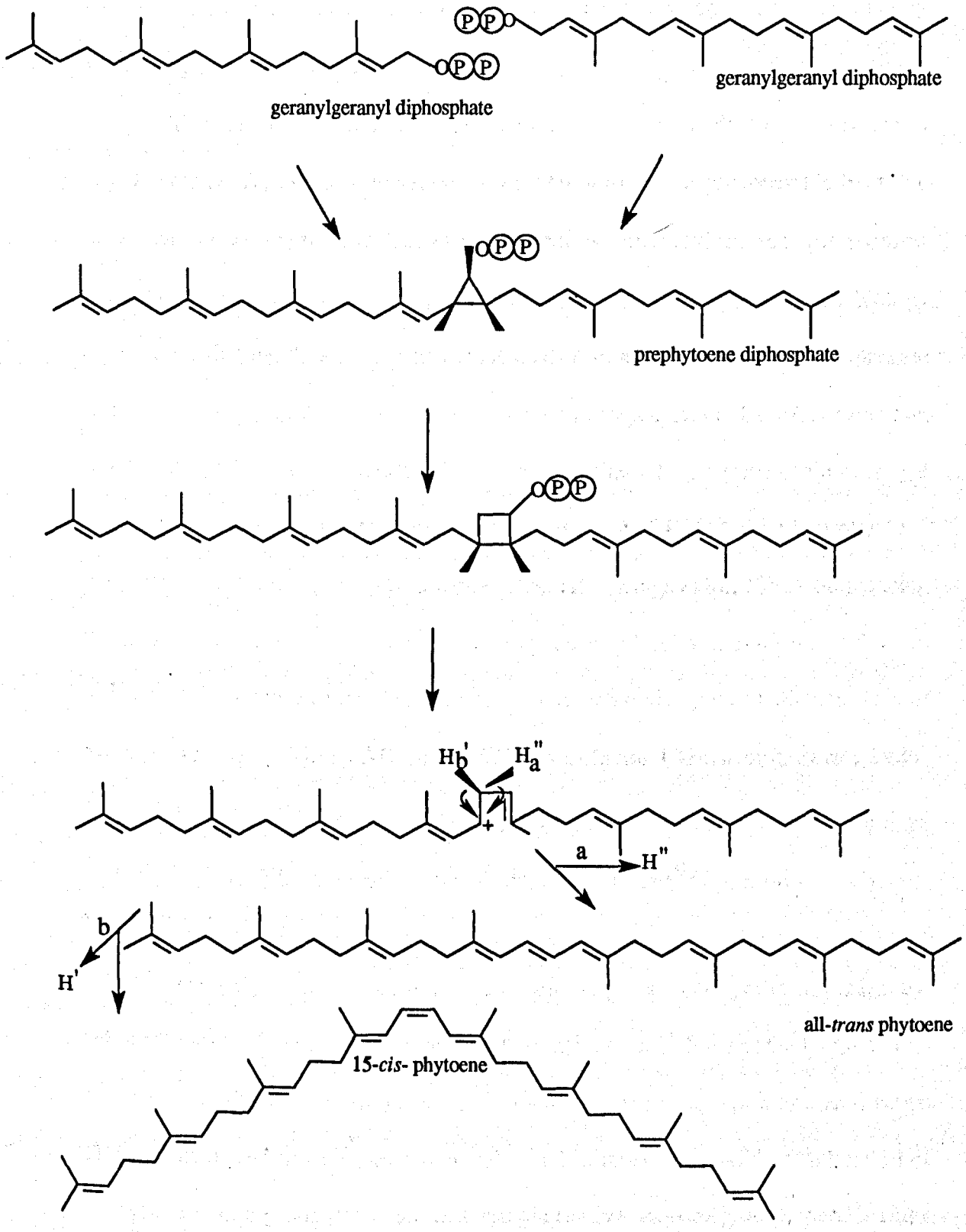


Figure 1.6: The alternative formation of all-*trans* (all-*E*) and 15-*cis* (15*Z*) phytoene
From Britton (1990) In "Carotenoid: Chemistry and Biology" p169

from the molecule, introducing a new double bond and extending the polyene chain by two conjugated double bonds.

The desaturase enzymes are membrane bound in daffodil (*Narcissus pseudonarcissus*), tomato (*Lycopersicon esculentum*) and in phototrophic bacteria, *e.g. Rhodobacter capsulatus*. The enzyme is bifunctional and the reaction requires oxygen (Bartley and Scolnik, 1989; Beyer, *et al.*, 1989). However, molecular biology work shows that the desaturase enzyme isolated from *R. capsulatus* can be expressed in *E. coli* similarly in the presence and absence of oxygen. In the *Capsicum annuum* chromoplast membrane, the desaturase enzyme is a single polypeptide chain of 56 ± 2 kDa and its absorption spectrum suggests the presence of FAD^+, *i.e.* plant phytoene or phytofluene desaturase may be a flavoprotein (Hugueney, *et al.*, 1992). Sequencing of the bacterial gene reveals that the enzyme has a hydrophobic N-terminal domain which contains a putative ADP-binding site and a βαβ fold is present characteristic of enzymes known to bind FAD or NAD(P) as cofactor (Armstrong, *et al.*, 1989; Armstrong, *et al.*, 1990). In cyanobacteria, phytoene desaturation is considered to be the rate limiting step in carotenogenesis (Chamovitz, *et al.*, 1993).

1.6.4 Cyclization

The polyene chain in carotenoids is quite rigid so that cyclization is limited only to the ends of the molecule provided these have a single bond at the C-3,4 and C-3',4' position. Cyclic end groups are found with either five or six membered rings. The six membered rings are common and found in most carotenoids. Cyclization of the lycopene end group can occur in three alternative ways to give β, γ and ε rings through a carbocation intermediate and stabilized by loss of alternative protons as shown in Figure 1.7. Cyclic carotenoids of higher plants, algae, fungi and bacteria normally have β and ε end groups. The mechanism of cyclization for β and ε rings

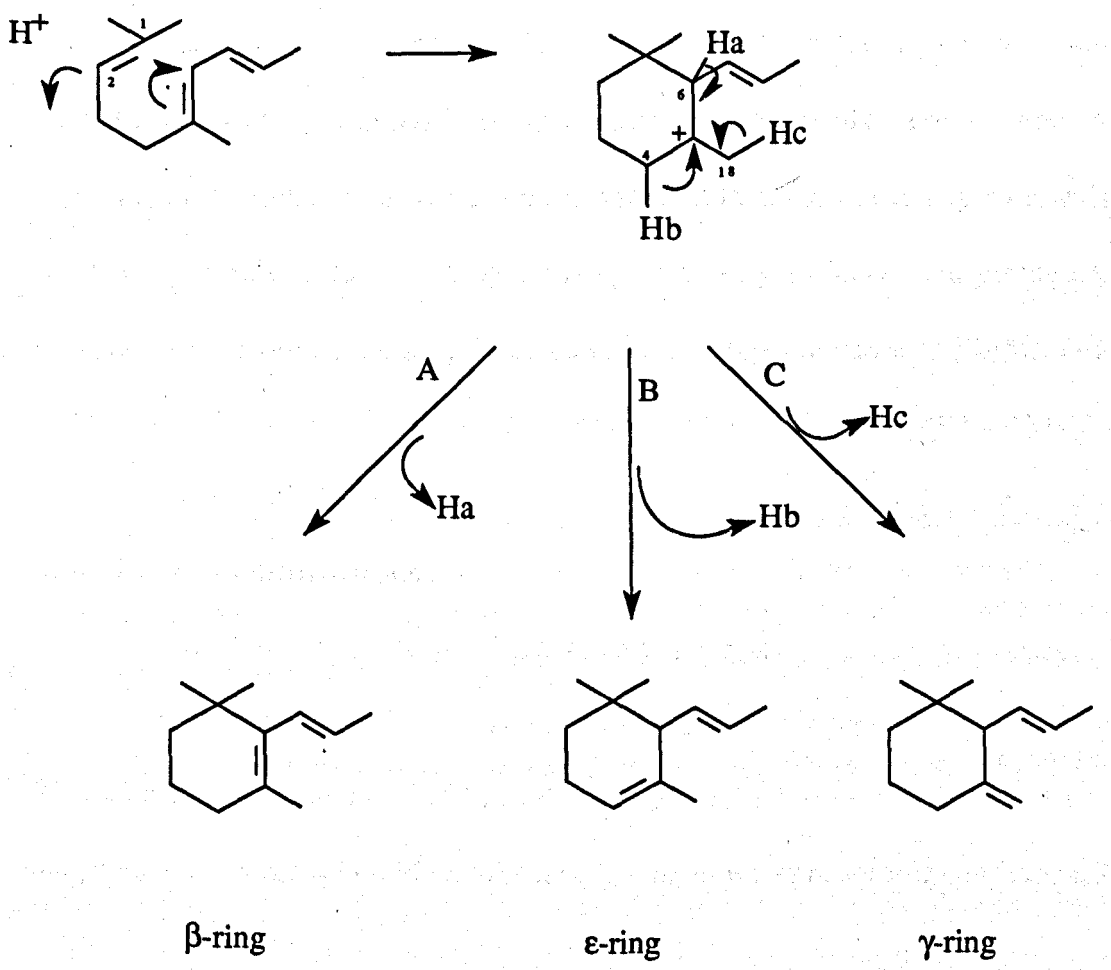


Figure 1.7: Mechanism of cyclization to form the β, γ or ε ring

From Britton (1976) *Pure Appl. Chem.*, 47, 225

involves proton attack at C-2 of the acyclic precursor followed by the ring closure. The different ring types appear to be formed independently, and therefore presumably require different cyclases (Britton, 1976; Britton, 1990). In the alga *Scenedesmus obliquus* strain PG1, cultured in the dark, acyclic carotenoids were accumulated. When cultures grown in the dark were transferred to D_2O, the newly synthesised cyclic carotenoids were shown by mass spectrometry to have incorporated two deuterium atoms, one for each cyclization. Labelling experiments with [2-^{14}C, 2-$^{3}H_2$] mevalonate in tomato confirmed that the β-ring is not formed by isomerization of an ε-ring (Williams, *et al.*, 1967).

1.6.5 Later modifications

1.6.5.1 Introduction of carbonyl groups

In the phototrophic bacterium, *Rhodobacter sphaeroides*, spheroidenone (IX), with an oxo group in the C-2 position, is formed in the presence of oxygen from the parent carotenoid spheroidene which accumulates under anaerobic conditions. The double bond at C-3,4 is necessary for the introduction of the C-2 oxo group.

1.6.5.2 Hydroxylation

Hydroxylation of cyclic carotenoids at the C-3 position occurs by direct replacement of a C-3 hydrogen atom of the hydrocarbon precursor by a hydroxy group (Britton, 1976). The reaction is thought to be catalysed by a mixed-function oxidase enzyme, and may involve cytochrome P450 (Britton, 1990).

1.7 Factors that influence carotenoid biosynthesis

Many environmental factors such as light, temperature, oxygen, carbon and nitrogen sources can effect the accumulation of carotenoids in tissues and organisms. Carotenogenesis in some organisms, particularly fungi and some bacteria, is strictly

photoregulated. In *Neurospora crassa*, the expression of the *al3* gene that encodes for GGDP synthase was shown by analysis of mRNA not to be expressed in the dark grown mycelia but the enzyme is induced by blue light after a short pulse of illumination (Carattoli, *et al.*, 1991; Nelson, *et al.*, 1989). Individual carotenoids responded differently to light conditions in the cyanobacterium *Spirulina platensis*. Under white light, β-carotene and echinenone were most abundant. Under red and blue light, myxoxanthophyll decreased while β-carotene increased (Olaizola and Duerr, 1990). Induction of carotenogenesis by light in the gram negative, non-phototrophic bacterium *Myxococcus xanthus* has been studied extensively. The accumulation of carotenoids in the membrane occurred when the strain was exposed to blue light. The dark grown cultures or colonies of wild-type strains appeared yellow due to the presence of a non-carotenoid pigment. At the molecular genetics level, there are two unlinked structural genes, *carB* and *carC*, which are both activated by light through the regulatory genes *carR* and *carQ*. The *carR* gene product is a negative regulator of *carQ*. The protein product of *carQ* was activated on the expression of *carB* through *carA*. Light activation of *carC* was also mediated through *carQ* and *car*A but the process required cell starvation (Martinez-Laborda, *et al.*, 1990; Ruiz-Vazquez, *et al.*, 1993). The process is illustrated in Figure 1.8. The *carC* gene has been cloned and sequenced. The enzyme phytoene dehydrogenase is the product of this gene. Lack of carbon source and nitrogen source promoted the sensitivity of *carC* to light (Fontes, *et al.*, 1993). The gene *carR* encoded the regulator of a light-inducible promoter. The role of *carQ* in the regulation mechanism has been elucidated by studying a *carQ* mutant that was generated and in which the expression of the light-inducible promoter was examined. The introduction into a plasmid containing promoterless *lacz* fused to the light-inducible promoter was manipulated, then the cells were exposed to light. The

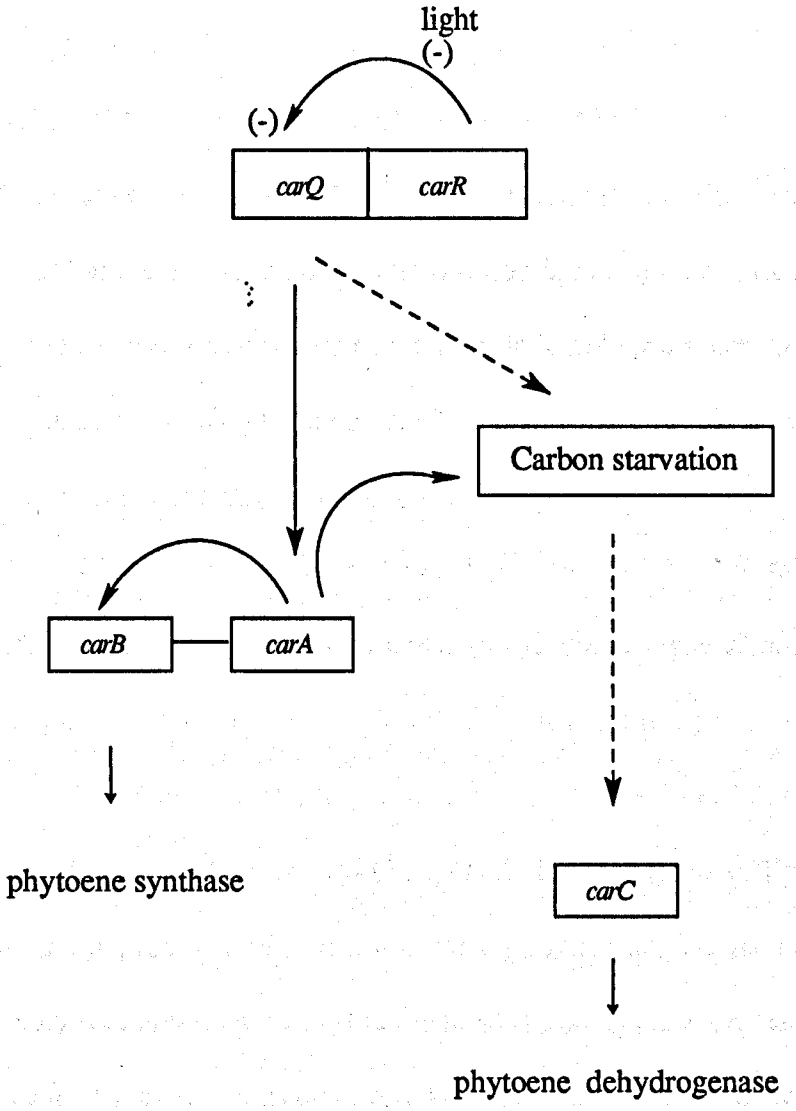


Figure 1.8: The regulation of gene expression in *Myxococcus xanthus*

From Ruiz-Vazquez, *et al.* (1993) *Mol. Microbiol.*, 10, 26

specific activity of β-galactosidase in the cell remained the same as in cells which were kept in the dark. Therefore, *carQ* is required for the expression of the light-inducible promoter. *CarR* has been shown to be activated by *carS* which is involved in stimulating the *car* regulon (M^{c} Gowan, *et al.*, 1993). The *carR* gene has been cloned and identified by using Tn5 transposon mutagenesis. The gene expression studies proved that there was not only a light-inducible promoter in this region, but also two genetic functions downstream of the light-inducible promoter, *i.e.* a negative regulator of the light-inducible promoter (*carR*) and a positive regulator of the structural genes for the carotenogenesis (Hodgson, 1993).

Light and nitrogen have been proved to be factors that regulate carotenogenesis in many organisms. In the green algae *Dunaliella bardawil* and *Trentepohlia odorata*, when high light intensity or nitrogen deficiency was applied, a synergistic effect on carotenogenesis was seen. The effect of light was smaller with *T. odorata* than with *D. bardawil* (Tan, *et al.*, 1993). Effects of light and nitrogen were studied for growth of the fresh-water alga *Haematococcus pluvialis*. When light intensity was greatly increased to the light exposed culture, a massive accumulation of astaxanthin (as ester) was observed. Under conditions of nitrogen deprivation imposed by lowering $NaNO_3$ concentration but with the same light intensity, the accumulation of carotenoid started earlier than when the nitrogen level was normal (Boussiba and Vonshak, 1991). The concentration of nitrate and phosphate can affect the carotenoid accumulation. It has been concluded that the main factor leading to carotenoid accumulation was nitrogen starvation which was enhanced by high phosphate concentrations (Borowitzka, *et al.*, 1991).

Oxygen has been demonstrated to be involved in the regulation of *crt* gene expression in phototrophic bacteria. In *Rhodobacter capsulatus*, under anaerobic

conditions, mRNA levels from *crtA*, *crtC* and *crtE* were increased, the greatest increase being seen in the case of *crtE*. *CrtA* is responsible for conversion of demethylspheroidene into demethylspheroidenone and spheroidenone, *crtC* for converting neurosporene to hydroxyneurosporene and *crtE* for converting GGDP to phytoene. Oxygen starvation caused increased transcription of a gene. That gene may be transcribed under the regulation of an anaerobiosis-inducible promoter (Giuliano, *et al.*, 1988).

1.8 Astaxanthin formation

In the biosynthesis of ketocarotenoids, the introduction of the oxygen function into the cyclic end groups is supposed to be the final stage of the pathway. Some marine animals can produce ketocarotenoids from carotene precursors, *e.g.* the hermit crab *Clibanarius erythropus* can transform dietary β-carotene to oxygenated carotenoids such as astaxanthin, echinenone and canthaxanthin (Castillo, 1980). In goldfish, when pure lutein and zeaxanthin were fed, these pigments were converted into astaxanthin. Canthaxanthin and adonirubin are probably intermediates in the pathway from β-carotene to astaxanthin in crustacea. In other cases, *e.g.* sea bream, ingested canthaxanthin and zeaxanthin cannot be converted into astaxanthin but are deposited in the flesh without modification.

In *Micrococcus roseus* astaxanthin appeared to be formed by the conversion of β-carotene via the intermediates echinenone and canthaxanthin (Schwartzel and Cooney, 1974).

There are two configurations of astaxanthin most commonly present in natural organisms namely (3S, 3'S) and (3R, 3'R). Normally naturally occurring astaxanthin is the (3S, 3'S) form but the yeast *Phaffia rhodozyma* accumulates the

other (3R, 3R')-isomer. The difference of configuration might be a consequence of the order of the introduction of hydroxy group and ketone group into the ring. In algae, the C-3 hydroxy groups are introduced first to give the (3R)-3-hydroxy-β-ring which leads to (3S, 3'S)-astaxanthin, whereas in *Phaffia rhodozyma,* the C-4 keto groups may be introduced first followed by the hydroxy group to give the (3R, 3'R)-configuration (Andrewes and Starr, 1976; Andrewes, *et al.*, 1976; Britton, 1990).

1.9 Carotenoid biosynthesis genes in micro-organism

The biosynthesis of carotenoids has been studied extensively at the molecular genetic level especially by applying the transposon mutagenesis technique. In recent years, many papers describing genes in the carotenoid biosynthesis pathway have been published.

1.9.1 Phototrophic bacteria

Transposon mutagenesis has been applied for understanding carotenoid biosynthesis at the molecular genetic level. A cluster of genes for carotenoid biosynthesis in the purple non-sulphur phototrophic bacterium *Rhodobacter capsulatus* has been isolated and identified. It contained nine carotenoid genes within the cluster of 46 kb. They were identified and named as *crtA*, *crtB*, *crtC*, *crtD*, *crtE*, *crtF*, *crtI*, *crtJ* and *crtK*. The *crt* gene cluster contained a minimum of four district operons which are *crtA*, *crtIBK*, *crtDC* and *crtEF*. These genes are responsible for the pathway from geranylgeranyl diphosphate to hydroxyspheroidene (Armstrong, *et al.*, 1989; Armstrong *et al.*, 1990; Giuliano *et al.*, 1988) as shown in Figure 1.9. *crtI*, which encodes for the enzyme phytoene desaturase in the early stage of the biosynthesis pathway is a membrane protein of approximately 60 kDa. Mutant strains with a 5-fold lower or 10-fold higher level of *crtI* with respect to wild type have only a small

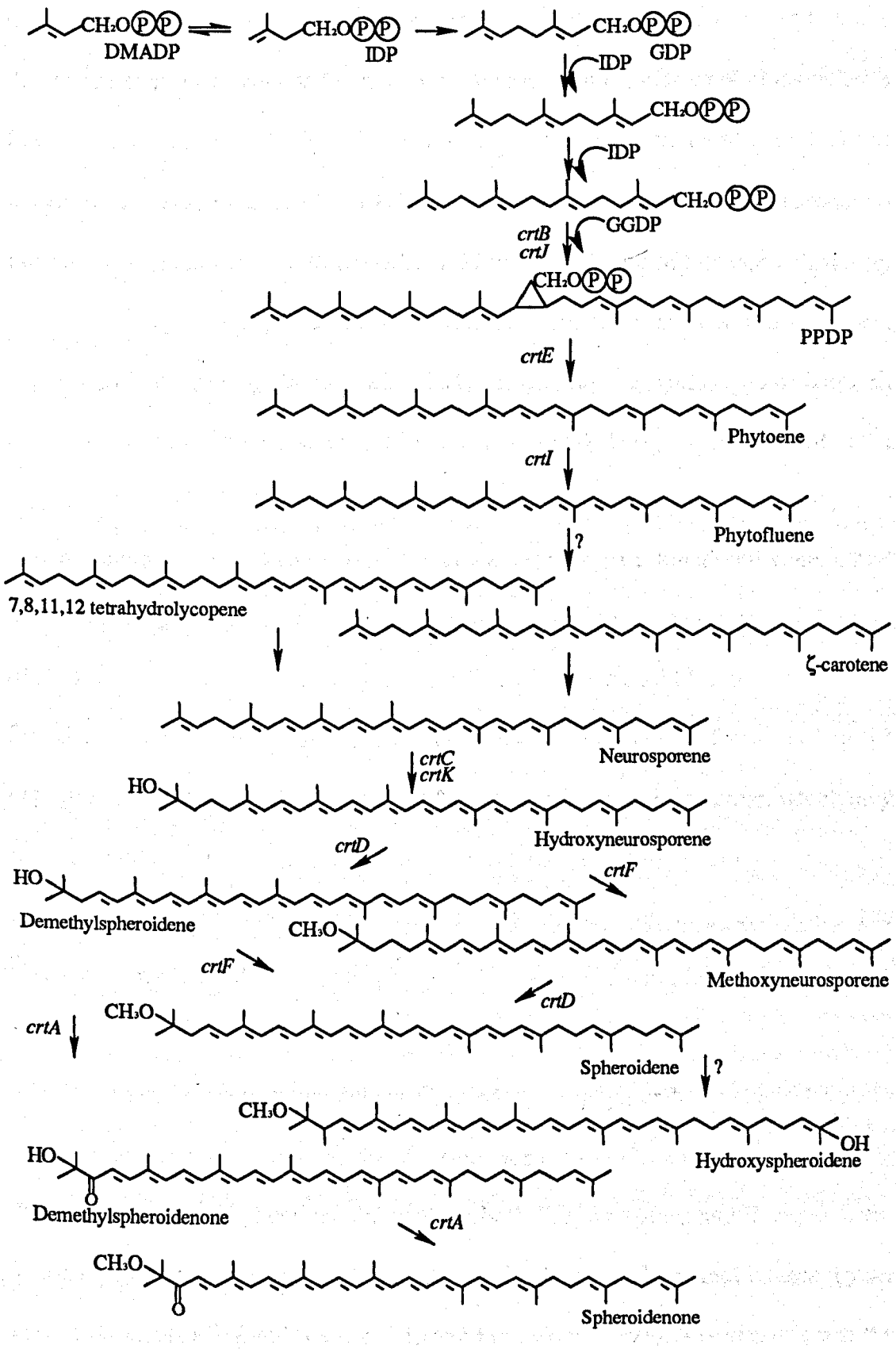


Figure 1.9: Carotenoid biosynthesis genes in *Rhodobacter capsulatus*
From Armstrong, *et al.* (1990) *J. Biol. Chem.*, 256, 8330

difference in their carotenoid content so that the cellular concentration of phytoene desaturase expressed from *crtI* is not a limiting factor in the carotenoid biosynthesis (Bartley and Scolnik 1989). Tn5.7 that contained the transposition ability of Tn5 and the antibiotic resistant factors from Tn7 has been applied to mutagenize the carotenoid biosynthesis gene cluster in *R. capsulatus*. Physical mapping of this gene cluster by restriction enzyme study showed endonuclease recognition sites which are *Xho*I, *Bgl*II, *Kpn*I and *Sst*I (Zsebo, *et al.*, 1984). The lycopene cyclase gene from *E. herbicola* is approximately 43 kDa (Hundle, *et al*, 1993). This gene is different in the fungus *Phycomyces blakesleeanus* as the function was related to *carA* and *carR* genes which are closely linked and designated as a *carRA* single bifunctional gene. *CarR* coded for lycopene cyclase and *carA* coded for substrate transfer (Torres-Martinez, *et al*, 1980). The hydroxylation enzyme from *E. herbicola* is a 22 kDa protein which is found in the supernatant of the cell lysate by overproduction from *crtZ* in T7 expression vector. Membrane seems to reduce the activity of the enzyme, which may not be membrane bound (Hundle *et al*, 1993). *CrtX* coded for glucosyltransferase. The binding of zeaxanthin to the enzyme is the rate limiting step for glycosylation. The glycosylation occurred after xanthophylls had been formed completely (Hundle *et al*, 1992).

There is a correlation between bacteriochlorophyll and carotenoid levels in phototrophic bacteria. It may be that the gene *pps* could induce trans suppression of bacteriochlorophyll and carotenoid levels in both *R. sphaeroides* and *R. capsulatus*. The suppression of *crt* by *pps* is quantitatively different from that caused by an absence of mature *Bch* mRNA (Penfold and Pemberton, 1991). It is possible that the 3' end of *crtA* may overlap a transcription initiation signal downstream of a gene which was responsible for bacteriochlorophyll biosynthesis (Armstrong *et al*, 1989).

The *Bch* operon must be located within the *crtA* structural gene as the promoter of the operon initiated by the *bchI* gene is located within an upstream operon for carotenoid biosynthesis. The stop codon for the *car*A gene overlaps the start codon of the downstream *bchI* gene (Young, *et al.*, 1992). The *bchI* gene product of *R. capsulatus* has 49% sequence identity with the nuclear encoded *Cs* chloroplast protein that is required for normal pigmentation in the higher plants *Arabidopsis thaliana*. The suppression of *R. capsulatus crtI*, *crtA* and *bchCXYZ* genes expression was dependent upon oxygen tension. Pigment accumulation can be triggered by decreasing oxygen tension in the dark grown culture. Anaerobiosis of these strains could be reflected by post-transcriptional regulation (Armstrong, *et al.*, 1993).

1.9.2 Non-phototrophic bacteria

Most of the genes for cyclic carotenoid biosynthesis have been studied and classified in the phytopathogenic bacteria *Erwinia herbicola* and *E. uredovora*.

From *E. uredovora*, a carotenoid biosynthesis gene cluster of 6918 bp was isolated and cloned in *E. coli*. With reference to the *R. capsulatus* carotenoid biosynthesis genes, six open reading frames were proposed and designated as *crtE*, *crtB*, *crtI*, *crtY*, *crtZ* and *crtX*. They code for the enzymes in the carotenoid biosynthesis pathway which catalyse the reactions from geranylgeranyl diphosphate through prephytoene diphosphate, phytoene, lycopene and β-carotene, to zeaxanthin and zeaxanthin β-diglucoside (Misawa, *et al.*, 1990). Conjugation of the genes *crtB*, *crtE*, *crtI* and *crtY* to those bacteria that did not produce any carotenoids, can cause carotenoid biosynthesis, as shown with the ethanol-producing bacterium *Zymomonas mobilis* and a phytopathogenic bacterium *Agrobacterium tumefaciens*. The transconjugants of *Z. mobilis* and *A. tumefaciens* carrying these genes formed yellow colonies and biosynthesised β-carotene (Misawa, *et al.*, 1991).

The cyanobacterium *Synecococcus* pCC7942 carries a gene *pys* responsible for phytoene synthase. The gene *pys* that can co-transcribe with *E. uredovora* gene *crtE* from their endogenous promoter in *E. coli* (Chamovitz, *et al.*, 1992). However, enzymes in the early pathway from different species may need the same precursors but the conversions give different end products. Linden *et al.* (1993) showed that the phytoene desaturases from *Synechococcus, R. capsulatus* and *E. uredovora* are different in the number of desaturations they can perform. The enzyme from *Synechococcus* introduced two double bonds at C-11 and C-11' of phytoene yielding ζ-carotene. The phytoene desaturase enzyme from *R. capsulatus* catalyzes three steps which also introduce a further double bond at C-7 to give neurosporene and the one from *E. uredovora* carried out four desaturation steps at position C-7, C-7', C-11 and C-11' forming lycopene as a major carotenoid. Different phytoene desaturase genes from different species, *e.g. R. capsulatus, E. uredovora* and *Synecococcus* PCC 7942 can be functionally complemented with one another by co-transformation into *E. coli.* Work in the carotenogenic fungus *N. crassa* showed that the *al3* gene, when complemented to the *E. uredovora crt* gene cluster from which, the *crtE* gene was deleted, led to the accumulation of carotenoid. It had been demonstrated that the *al3* gene from this fungus codes for GGDP synthase and can be substituted for *E. uredovora crtE* (Sandmann, *et al.*, 1993).

Perry, *et al.* (1986) isolated a carotenoid biosynthesis chromosomal gene cluster from *E. herbicola.* The gene cluster was inserted in cosmid pHC79 to give pPL376 which was transformed into *E. coli* HB101. The major carotenoids then produced were β-cryptoxanthin glucoside, zeaxanthin monoglucoside and zeaxanthin diglucoside, with trace amounts of β-carotene and zeaxanthin. The characteristic carotenoids from the recombinant strains are identical to those of *E. herbicola.* The

biosynthetic pathway of zeaxanthin glucoside has been proposed as shown in Figure 1.10 (Hundle, *et al.*, 1991). Most of the genes in the pathway are similar to those of *E. uredovora*. Genes for the later steps of the pathway, encoding for lycopene cyclase, β-carotene hydroxylase and glycosyl transferase were isolated and cloned successively (Schnurr, *et al.*, 1991).

1.10 The importance of ketocarotenoid biosynthesis genes

Several biological sources have been considered for the production of astaxanthin (Table 1). Benemann (1992) has reviewed astaxanthin accumulation in *Haematococcus* which reaches about 2-5% of the dry weight. The application of *Haematococcus* as an astaxanthin supplement in feed for salmon has been achieved with some success. Biological sources however usually contain astaxanthin only in very small amounts. Methods for overproduction of these valuable carotenoids need extensive research.

The isolation of genes for the biosynthesis of ketocarotenoids may provide the best chance for production of astaxanthin and for studying gene regulation and control of gene expression for the later stages of the carotenoid biosynthesis pathway. A possible source for the gene responsible for ketone biosynthesis was eukaryote cDNA. β-carotene oxygenase cDNA was isolated from *H. pluvialis*. It can also complement to the zeaxanthin biosynthesis gene cluster from *E. uredovora* for (3S, 3'S) astaxanthin biosynthesis (Kajiwara *et al.*, 1995). These results provide an interesting basis for extended research for astaxanthin biosynthesis.

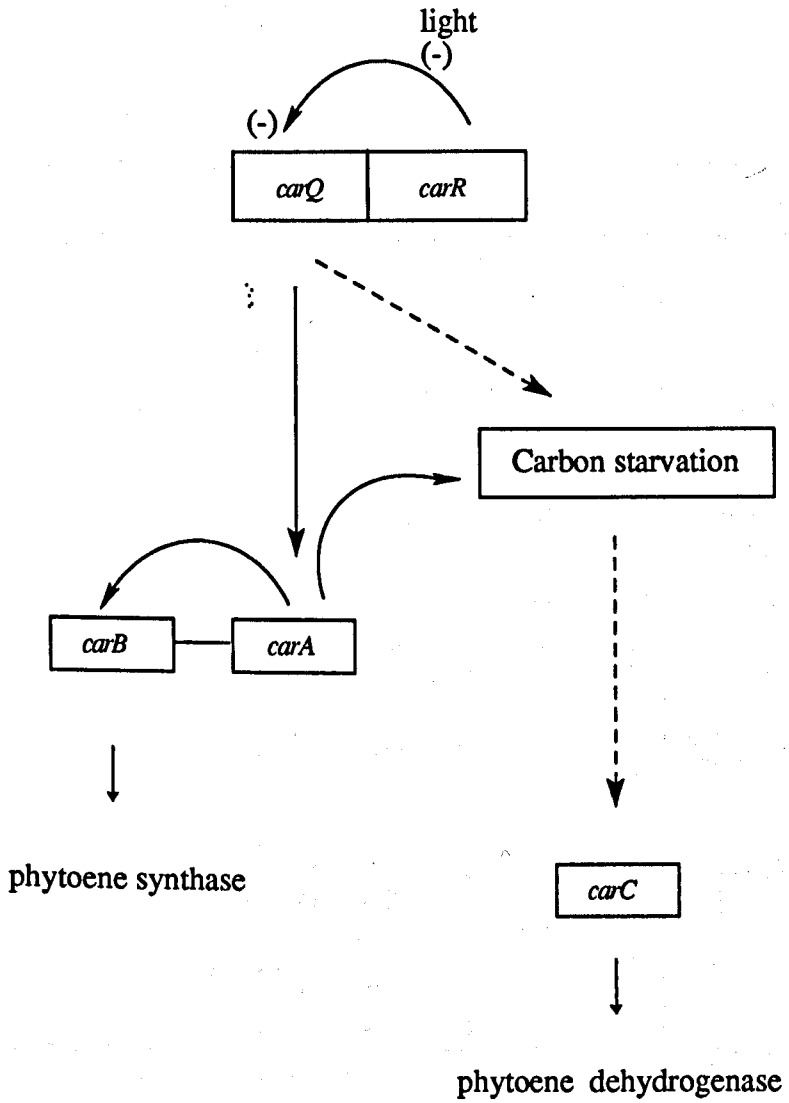


Figure 10: The regulation of gene expression in *Myxococcus xanthus*

From Ruiz-Vazquez, *et al.* (1993) *Mol. Microbiol.*, 10, 26

Table 1: Sources of diketone carotenoids

carotenoids	sources in micro-organisms	references
canthaxanthin	*Dinoflagellate:Gymnodinium* sp. *Microzooplankton*	Kleppel and Lessard, 1992
	Haematococcus pluvialis	Choubert and Heinrich, 1993 Boussiba and Vonshak, 1991
	Haematococcus lacustris	Hagen, *et al.*, 1993 Dokin, 1976
	Brevibacteria strain No103	Iizuka and Nishimura, 1969
	Brevibacteria strain KY4313	Nelis and De Leenheer, 1989
	Micrococcus roseus	Schwartzel and Cooney, 1974
	Scenedesmus quadricuada strain 449 *Scenedesmus obliquus* strain 633 *Chlorella fusca* strain 211-8p,211-15, 211-86	Burczyk, 1987
astaxanthin	*Haematococcus pluvialis*	Choubert and Heinrich, 1993
	Haematococcus lacustris	Hagen, *et al.*, 1993 Yong and Lee, 1991 Dokin, 1976
	Phaffia rhodozyma	Chun, *et al.*, 1992 Schroeder and Johnson, 1993
	Scenedesmus obliquus strain 633	Burczyk, 1987

1.11 Taxonomy of *Rhodococcus*

Rhodococci are classified as true *Actinomycetes* that grow in the form of mycelia. Their natural occurrence is mostly restricted to soil. They are aerobic gram positive bacteria that are related to the Coryneform bacteria and *Mycobacteria* by an almost continuous sequence of intermediate forms (Schlegel, 1988). The main carotenoids in the membrane of some strains of *R. rhodochrous* are monocyclic and contain a tertiary hydroxy group at the C-1 position (Takaichi, *et al.*, 1990). DNA base composition in *Rhodococcus* sp. is within 61-72 mol% G + C (Zakrzewska-Czerwinska, *et al.*, 1988).

Chapter 2

Materials and Methods

Materials

1 Biological materials

1.1 The strains of *Rhodococcus* (*R. aeichiensis* strains N933, strains N934; *R. chlorophenolicus* strains N1053, strains N1131; *R. coprophilus* strains N774, strains N650; *R. equi* strains R71, strains R70; *R. erythropolis* strains N11, strains N63; *R. fascians* strains R260, strains R262; *R. globerulus* strains R58, strains R43; *R. luteus* strains N1008, strains N913; *R. marinonascens* strains N1056; *R. maris* strains N1015, strains N1020; *R. obuensis* strains N935; *R. rhodnii* strains N444, strains N445; *R. rhodochrous* strains N54, strains N5 and *R. ruber* strains N361) and *Brevibacterium* (*B. acetylicum*, *B. casei*, *B. epidermidis*, *B. iodinum* and *B. linens*) used in this work were kindly provided by Professor M. Goodfellow, the University of Newcastle Upon Tyne.

1.2 *E. coli* DH5α containing pAPU211 and mutants were kind gifts from Professor John Hearst, The University of California, Berkeley, USA.

2 Carotenoid standards

Carotenoid standards were kindly donated by F. Hoffmann-La Roche and Co. Ltd., Basel, Switzerland.

3 Chromatographic adsorbents

3.1) Neutral alumina for column chromatography (Woelm, Eschwege, West Germany) was purchased from PS Park Scientific Ltd., Northampton.

3.2) Kieselgel G and Kieselguhr G for thin layer chromatography (E. Merck, Darmstadt, West Germany) were purchased from Anderman and Co. Ltd., London.

3.3) Magnesium oxide for chromatography was purchased from BDH Chemicals Ltd., Poole, Dorset.

4 Culture reagents

4.1 *E. coli* culture reagents

Nutrient broth, nutrient agar and bacteriological agar were purchased from Oxoid Ltd., London, yeast extract was purchased from Beta Lab, Surrey, sodium chloride and sodium hydroxide (Analar grade) were purchased from BDH Supplies, Poole, UK, bactotryptone was purchased from Difco Laboratories, Michigan, USA.

4.2 Plaque culture reagent: NZCYM was purchased from Sigma Chemical Co.

5 Organic solvents

Diethyl ether (ether; Analar grade), chloroform and dichloromethane were purchased from BDH Laboratory Supplies, Poole, UK. The ether was redistilled over reduced iron powder immediately before use.

Petroleum ether (petrol, b.p. 40-60°C) and acetone were purchased from the Department of Chemistry, The University of Liverpool, ethanol and methanol were purchased from Mills Ltd., Liverpool, ethyl acetate and acetonitrile (HPLC grade) were purchased from Fisons, Loughborough, UK.

Diethyl ether and petrol used for carotenoid purification for mass spectrometry and nuclear magnetic resonance analysis were filtered through a column of activated alumina (1 cm diameter, 10 cm long).

6 Materials for electrophoresis

Agarose gel (electrophoresis grade) was purchased from Gibco BRL, Life Technologies, Paisley, Scotland, acrylamide gel was purchased from Severn Biotech Ltd. Kidderminster, UK.

7 Enzymes

The restriction enzymes BamHI, EcoRI, EcoRV, HindIII, SalI and Sau3A were purchased from Promega Limited, Southampton, UK; SmaI was purchased from Stratagene, Cambridge, ligase, DNAse and Taq polymerase were purchased from Promega Limited, UK, T4 polynucleotide kinase was purchased from NBL Gene Sciences, RNAase T1 was purchased from Boehringer Mannheim, lysozyme and proteinase K were purchased from Sigma Chemical Co.

8 Antibiotics: ampicillin, chloramphenicol and tetracycline were purchased from Sigma Chemical Co., Dorset.

9 Radioactive materials: γ-^{32}PdATP, ^{35}SdATP, α^{32}PdCTP were purchased from ICN Biomedicals, Inc.

10 Nucleotides: deoxynucleotide triphosphates and dideoxynucleotide triphosphates were purchased from Promega Limited, UK.

11 X-ray film was purchased from Fuji.

12 DIG-labelled and detection kit was purchased from Boehringer Mannheim.

13 Hybron N$^+$ membrane was purchased from Amersham International plc, UK.

14 Lambda GEM-11 BamHI arms and packagene extract were purchased from Promega Limited, UK.

15 Geneclean kit was purchased from BIO 101 Inc. La Jolla, CA.

Methods

I Analytical techniques

I.1 Carotenoid analysis techniques

I.1.1 General precautions for work with carotenoids

Carotenoids are very sensitive to light, heat, oxygen, acid and in some cases alkali so that carotenoids have to be handled under conditions which minimize degradation and isomerization. All carotenoid manipulations were carried out in subdued light. Developing chromatograms were covered by black cloth or foil. The detection of colourless carotenoids on thin layer chromatography under UV light was done as quickly as possible. Carotenoids eluted from chromatograms were eluted as rapidly as possible and stored under nitrogen at -20°C. Carotenoid solutions were dried under vacuum in a rotary evaporator below 40°C or the samples were placed in sample vials and evaporated under a nitrogen stream at room temperature. Dry samples in vials were placed under nitrogen and capped with airtight stoppers.

I.1.2 Growth of *Rhodococcus* strains

I.1.2.1 Storage

Rhodococccus (*R. maris* strains N1020 and *R. aiechiensis* strains N933) were plated on broth agar (1% NaCl, 1% tryptone, 0.5% yeast extract, 3 m*M* NaOH and 1% bacteriological agar) and incubated at 30°C for 14 days under illumination. Then the culture plates could be kept at 4°C for about 4 months.

1.1.2.2 Screening procedure

All 30 bacterial strains were screened for carotenopid biosynthesis. An initial visual screen was undertaken. Only bacterial colonies which were yellow to orange were selected for further screening. Each strain was scraped off from the original plates and all the carotenoids extracted. The complete carotenoid extract was separated on HPLC. Those in which the main carotenoid showed a single round peak were then used for further examination and identification of each carotenoid by mass spectrometry and ^{1}H-NMR spectroscopy. The strains finally choosen for all future study was *R. maris* strains N1020.

I.1.2.3 Liquid culture

A single colony from a plate culture was inoculated in 100 ml liquid broth and incubated at 30°C, 100 rpm in a 2000 lux illuminated incubator for 4 days. Then the liquid culture was used to inoculate 10 flasks of 1000 ml liquid broth to produce the large amount of carotenoid for extraction. All cultures were grown at 30°C, 100 rpm in a 2000 lux illuminated incubator for a further 10 days.

For the long term storage of bacterial strains, bacterial culture was diluted with 30% glycerol and kept at -70°C.

I.2 Harvesting of the cells

Cultures were harvested by centrifugation at 4,000xg for 30 minutes at 4°C. The supernatant was then decanted, and the pellet was resuspended in 5 ml of the culture media per 1 litre of the original culture under nitrogen for about 30 minutes. Then the cell pellet was transferred to a 250 ml flask for carotenoid extraction.

I.3 Carotenoid extraction

The carotenoids were extracted from the pellets by adding acetone:methanol (3:7) and shaking in a warm water bath (40°C). The extraction was repeated until no more colour were recovered from the pellets; this usually required about 6-7 extractions (total volume of the solvent used was about 100 ml of solvent mixture per 1 litre of culture). The total extract was added to an equal volume of ether followed by two volumes of tap water and the aqueous phase separated from the organic phase. The organic phase, containing the carotenoids, was collected and dried in a vacuum rotary evaporator at about 40°C, 5 ml of absolute ethanol being added in the later stages to ensure removal of water. The whole carotenoid extract was redissolved successively in petrol, ether, acetone and methanol and transferred to a glass vial. The sample was dried under nitrogen and protected against light. The dried sample was subsequently stored at -20°C.

I.4 Purification of carotenoids

I.4.1 Open column chromatography on alumina

A 1.5 x 50 cm^2 column of neutral alumina (deactivated to grade III by addition of 6% water) was used to separate carotenoids into groups of similar chromatographic polarity by stepwise elution. The lipid extract was applied to the column which was equilibrated with 5% ether in petrol. Carotenoid fractions were subsequently eluted by stepwise increase in the polarity of the eluting solvent.

I.4.2 Thin-layer chromatography

Carotenoids from each column fraction were separated and purified by thin-layer chromatography (t.l.c.). Silica gel G plates (five plates, 20 x 20 cm^2, 0.5 mm layer) were prepared from a slurry of silica gel G (40 g) in 80 ml of water. Magnesium oxide:Kieselguhr G 1:1 plates (five plates, 20 x 20 cm^2, 0.5 mm layer) were prepared from a slurry of magnesium oxide (20 g) and Kieselguhr G (20 g) with 80 ml of water. All t.l.c. plates were prepared by mean of a "Quickfit" 8 CR thin layer plate spreader (JA Jobling Ltd. Stone, Staffs). T.l.c. plates were dried at 110°C for at least 1 hour, and allowed to cool at room temperature before use.

Samples were dissolved in a small volume of suitable volatile solvent (normally ether or petrol) and applied to the t.l.c. plates by means of finely drawn pasteur pipettes. Several solvent systems of different polarity were used to develop the chromatograms. Each band of t.l.c. adsorbent which contained carotenoids was quickly scraped off and added into the vial containing eluting solvent. Carotenoid solutions were separated from the adsorbent by filtration through a sintered glass funnel. Solvents were evaporated under a nitrogen stream. Dry carotenoid samples were kept at -20°C.

I.4.3 Silicic acid column chromatography

All samples for mass spectrometry were further purified on small silicic acid columns. Approximately 1 g silicic acid was added into pasteur pipette plugged with cotton wool and equilibrated with 5 ml of hexane. The carotenoid solution was applied and the main fraction was collected with the appropriate solvents and stored under nitrogen. Carotenoid samples were sent for mass spectrometry immediately after purification.

I.5 Quantitative analysis of carotenoids

Carotenoids were estimated quantitatively by UV/ Vis spectrophotometry. The carotenoid samples were redissolved in a known volume of solvent and the net absorbance at λmax was measured. The amount of carotenoid was calculated from the equation.

$$W = \frac{A \times V}{A1\% \times 100}$$

Where W is the weight (g) of carotenoid dissolved in V (ml) volume of solvent and A is the net absorbance. Values of $A1\%/_{cm}$ ($E1\%/_{cm}$) tabulated by Davies (1976) were used.

I.6 Identification of carotenoids

Carotenoids were tentatively identified by their chromatographic properties

and their electronic absorption spectra in different solvent systems. Electronic absorption spectra of carotenoid solutions were determined and recorded on a Cecil CE 590 (Scan speed 5 mm/ sec; sequential mode: 250-700 nm) or a Phillips PU 8750 UV/ vis Scanning Spectrophotometer and colour plotter at the absorption scan speed 250 nm/ sec. The spectra of unknown carotenoids were compared with those of authentic standards.

I.7 $NaBH_4$ reduction

Carotenoids for which the absorption spectra showed little or no fine structure are likely to be ketocarotenoids. This was confirmed by reduction of the keto groups by addition of about 5% of $NaBH_4$ into the sample in ethanol. The spectrum was then recorded after 30 seconds, 5 minutes, 30 minutes and 1 hour.

I.8 Acetylation

Very polar compounds, containing many hydroxy groups (especially glycosides) were acetylated to reduce the polarity and facilitate chromatographic purification. Sample (10-50 μg) was dissolved in two drops of pyridine and one drop of acetic anhydride added. The mixture was left overnight. Pyridine and excess acetic anhydride were removed under nitrogen in the fume hood before the acetylated carotenoid was purified by t.l.c.

I.9 High performance liquid chromatograghy (HPLC)

The total carotenoid extract was separated on a Spherisorb ODS_2 column (5 μm, 25 cm long, 0.46 cm in diameter). The mobile phase was a gradient of ethyl acetate (0-90%) in acetonitrile-water (9:1) over 25-30 minutes at a flow rate of 1.5 ml/ minute. Chromatograms were monitored continuously over the wavelength range between 250-600 nm. The HPLC apparatus consisted of a Waters 990 solvent delivery system with Rheodyne injector (7125), 600E photodiode array detector with a NEC APC IV computer and plotter.

I.10 Mass spectrometry

Electron impact mass spectra of purified carotenoids were obtained with a VG Quattro system. The direct insertion probe was used with ion source temperature

of 200-220°C and the emission current was 200 mA and an ionization potential of 4 kV with OKI printer. Spectra were kindly determined by Mr. Mark Prescott.

I.11 Nuclear Magnetic Resonance Spectroscopy

The ^{1}H-NMR spectrum of canthaxanthin (in $CDCl_3$) was detemined in the University of Leiden, The Netherlands on a Bruker BMX 600 MHz machine. TMS was used as a reference. The ^{1}H-NMR spectra was kindly determined by Mr. Roland Weesie.

II Molecular biology techniques

Plasticware and glassware were sterilized before use. All contaminated glassware was soaked with 2% Stericol for 72 hours before cleaning. Waste materials were autoclaved before disposal. Pure cell lines of bacterial cultures were stored in 20% glycerol in large eppendorf tubes at -70°C (Blumberg, 1987).

II.1 Growth curve

Rhodococcus maris strain N1020 was inoculated in 100 ml Luaria Bertani medium and incubated at 30°C for 14 days under 2,000 lux illumination. 2 ml of culture was collected daily and the absorbance at 600 nm measured. The absorbance was then plotted against time (day).

II.2 Cell density

Cell density of *R. maris* strain N1020 was determined by placing 5 µl of the culture on the grid of a haemocytometer and covering with the cover slip. Cells were counted for 30 squares under the microscope with magnification scale 10 x 40. The mean value of cells/ cm^3 was then related to the absorbance of the culture at 600 nm.

II.3 Extraction and purification of chromosomal DNA from *Rhodococcus*

Liquid medium (100 ml) containing *Rhodococcus* cells (in the stationary phase) was harvested by centrifugation at 6,500xg for 15 minutes at 4°C. The orange sediment was washed once with 10 m*M* Tris EDTA (pH 8). The cell pellet

was resuspended in 10 ml of buffer containing 0.05 *M* tris, 0.01 *M* EDTA, 0.05 *M* NaCl and 20% sucrose plus lysozyme 5 mg/ ml and incubated at 37°C for 2 hours. The weakened cell pellet was collected by centrifugation at 6500xg for 15 minutes. The pellet was added with 10 ml of 3% SDS and then in 0.05 *M* Tris-HCl at 55°C for 2 hours. 1 ml of 5 *M* potassium acetate (pH 4.8) was added to the opaque orange suspension, together with 25 ml of -20°C absolute ethanol and the mixture was left at -20°C for 1 hour. The floating DNA clump was collected with a blunt glass rod and the DNA was washed twice with 0.5 ml 70% ethanol. The DNA was dried in a vacuum oven for 20 minutes (Vogt-Singer and Finnerty, 1988; Sambrook *et al.*, 1989).

II.4 Plasmid extraction

II.4.1 Preparation of large amount of plasmid pAPU211

All processes were carried out at 4°C. 100 ml culture of exponential phase *E. coli* DH5α carrying pAPU211 was chilled on ice for 15 minutes. The ice-cooled culture was then centrifuged at 6,500xg for 15 minutes and the supernatant was discarded. The pellet was resuspended in 2 ml of TEG (25 m*M* Tris-HCl pH 8, 10 m*M* EDTA and 50 m*M* glucose) and incubated with lysozyme (4 mg/ ml) for 15 minutes. Then 4 ml of 0.2 *M* NaOH containing 1% SDS was added to the suspension. Then 3 ml of 3 *M* sodium acetate (pH 5) was added and mixed in, followed by 25 ml of cold ethanol and the mixture was left at -20°C for 1 hour. The suspension was then centrifuged for 10 minutes at 10,000xg to precipitate the plasmids. The crude plasmid pellet was resuspended in 2.4 ml of 7.5 *M* ammonium acetate and distributed into 6 eppendorf tubes, so that each eppendorf contained 0.5 ml of the suspension. 1 ml of -20°C absolute ethanol was added to each tube in order to precipitate DNA. The pellet was collected by centrifuging the sample at 13,000xg for 15 minutes, the pellet was collected and dried in a vacuum oven at 50°C for 10 minutes and stored at -20°C. The plasmid was purified by the procedure described in II.5.

II.4.2 Minipreparation of plasmids

The method for minipreparation of plasmids was modified from that of Birnboim and Doly (1979), a procedure applied for plasmid DNA extraction from bacterial cells by alkaline denaturation. The procedure started by inoculation of LB medium with a single colony from a fresh plate into 5 ml liquid media. The inoculated culture was incubated overnight at 37°C with shaking (250 rpm). 1.5 ml of the thick overnight culture was transferred into a large eppendorf tube and centrifuged in a bench top centrifuge at room temperature for 2 minutes. The supernatant was discarded, and the pellet was resuspended in 100 µl of ice-cold TEG containing lysozyme (4 mg/ ml) and the mixture left at room temperature for 5 minutes. Then 200 µl of freshly made, ice-cold alkaline SDS solution (0.2 *M* NaOH, 1% SDS) was added, and mixed in by inversion 2-3 times. The mixture was left on ice for 5 minutes. The suspension was added to 150 µl of ice cold 5 *M* potassium acetate and mixed by gentle pipetting. The supernatant, containing crude plasmids, was collected by bench top centrifugation for 5 minutes. 350 µl of supernatant was transferred to a fresh large eppendorf tube, then 700 µl of ethanol at -20°C was added to precipitate the DNA and the reaction was left at -20°C for 30 minutes. The pellet was collected by centrifugation for 5 minutes and the supernatant was discarded. The pellet was washed with 70% ethanol. Plasmids were then dried in the vacuum oven at 50°C for 5 minutes, and resuspended in 20 µl Tris-EDTA (TE; 10 m*M* tris, 1m*M* EDTA) pH 7.5.

II.5 Purification of plasmids

II.5.1 Plasmid purification by NACS PREPAC

Plasmids were purified on the NACS PREPAC column purchased from BRL, Life Technologies Inc. The TE plasmid suspension, containing 0.2 *M* NaCl was loaded to the NACS cartridge pre-equilibrated according to the manufacturer's instructions and eluted with stepwise gradients of NaCl, from 0.7-2.0 *M* NaCl in TE. Purified plasmid fractions were collected in lots of 0.45 ml in eppendorf tubes. Plasmids were precipitated with 1 ml of -20°C ethanol and washed with 200 µl of

70% ethanol, dried under vacuum at room temperature and resuspended in 100 µl of TE.

II.6 Gel electrophoresis

II.6.1 General separation of DNA

DNA was separated on 0.4-1% agarose gel in TAE buffer (0.04 *M* Tris - acetate, 0.001 *M* EDTA) plus 0.5 µg/ ml ethidium bromide to intercalate the DNA. DNA itself is not fluorescent in the visible region but the complex formed between ethidium bromide and DNA causes a fluorescence spectrum with a λmax at 302 nm. The current applied was among 30 and 50 mA, the voltage applied was among 40 and 80 volts for the separation on the gel electrophoresis kit (Pharmacia LKB GNA 100) with Shandon Southern electrophoresis power pack. DNA was detected at 254 nm in the UV illuminator (UVP Inc, San Gabriel) and photographed with a polaroid camera (San Gabriel).

Agarose gel electrophoresis was applied for determining DNA size after separation of the whole DNA sample. The phosphate groups in the phosphodiester bonds along the nucleotide chain cause DNA to be negatively charged so that DNA fragments loaded into a sample well at the cathode (-) end of the gel migrate towards the anode (+). The electrophoretic mobility of the DNA fragments which are larger than oligonucleotides depends on the DNA size, base composition and polynucleotide configuration. The DNA samples are water soluble, so that loading sample onto the agarose well needs the addition of tracking solution for sinking the sample. The addition of 10% v/ v of loading dye (15% Ficoll, 0.25% bromophenol blue and 0.25% xylene cyanole FF) to the DNA sample was generally used for this. The maximum amount of loading sample was about 100 µg/ cm^3. Moreover, 50% glycerol was used to load samples with DNA size between 200 and 600 base pairs to facilitate visualization under uv light.

II.6.2 Estimation of DNA fragment size

DNA fragment size was estimated from its mobility relative to those of known size fragments. The distribution of DNA in the mobility gel was

photographed. The mobility value was measured directly from the photograph. A plot of log L (size) against mobility (m) when low voltage was applied to the running gel was prepared. When high voltage gradients were applied, a plot of L against $1/m$ showed marked curvature. The size estimation was carried out by use of the formula

$$L = k_1/(m - m_0) + k_2$$

$$m_0 = \frac{m_3 - m_1[(L_1 - L_2)/(L_2 - L_3) \times (m_3 - m_2)/(m_2 - m_1)]}{1 - [(L_1 - L_2)/(L_2 - L_3) \times (m_3 - m_2)/(m_2 - m_1)]}$$

$$k_1 = \frac{L_1 - L_2}{1/(m_1 - m_0) - 1/(m_2 - m_0)}$$

$$k_2 = L_1 - k_1/(m_1 - m_0)$$

where m_0 is the correlation factor for three lines joining three points which have the same slope. L_1, L_2 and L_3 are the molecular weight standards with mobility m_1, m_2 and m_3 respectively. The calculation was performed with two different markers, one for the larger size, the other for the smaller size markers (Southern, 1979).

II.6.3 Purification of DNA

II.6.3.1 Gel electrophoresis was used to separate DNA according to size. The gel containing the portion of DNA of interest was then cut off into small pieces for the DNA to be recovered by use of the Geneclean II Kit according to the manufacturer's instructions.

II.6.3.2 DNA produced by PCR was purified by using the low melting point agarose gel to separate the fraction and then cutting the required region for further purification through the Wizard PCR Preps purification system column (Promega).

II.7 Quantitative determination of DNA

II.7.1 Absorbance: double stranded DNA was estimated by its absorbance at

260 nm. The DNA concentration in a solution with A_{260} = 1 is 50 μg/ ml.

II.7.2 Agarose gel electrophoresis: linear double stranded DNA was estimated by separation on agarose gel alongside known quantities of DNA marker such as λHindIII. Circular DNA from plasmids was linearized before application to the gel.

II.7.3 When only a small amount of DNA was present in the sample (a few nanograms), this was detected by use of DNA Dipstick (Invitrogen, UK) according to the manufacturer's instruction.

II.8 Ligation

Genomic DNA of *R. maris* strain N1020 was digested with restriction enzymes and cloned in different sites of plasmids pACYC184, pACYC177 and pUC19 with the various proportions of vector and fragmented DNA. The mixtures were left for the ligation reaction at different temperatures between 14°C and 20°C. The details will be given in Chapter 4.

II.9 Introduction of recombinant DNA to host

II.9.1 Electrotransformation

E. coli DH5α was chosen as host for application of recombinant DNA. A single colony was inoculated in 20 ml LB medium containing the appropriate antibiotic and incubated at 37°C overnight. Then 10 ml of this culture was transferred to 1 litre LB containing the same antibiotic. The culture was then incubated at 37°C with vigorous shaking. The A_{600} was checked every 20 minutes until it reached a value between 0.5 and 1 (this took about 3-4 hours). Then the culture was chilled on ice for 15-30 minutes and centrifuged in a cold rotor at 4,000xg for 15 minutes. As much as possible of the supernatant was discarded. The pellets were resuspended in 1 litre of ice-cold water, centrifuged for 10 minutes and the supernatant was discarded. The pellets were resuspended with 500 ml of ice cold water, recentrifuged for 10 minutes and the supernatant was discarded. The pellets were resuspended with 50 ml of 10% glycerol in ddH_2O. The cell suspension was centrifuged for 10 minutes and the supernatant was discarded. The cell pellet was

resuspended again with 10 ml of glycerol. The cell suspension was then distributed 1 ml per eppendorf tube, and stored at -70°C for up to 6 months.

Stored cells were thawed on ice immediately before transformation. 40 µl of cell suspension was transferred to an ice-cold sterile eppendorf tube and 2 µl of ligated DNA mixture (20 ng) was mixed with the cell suspension, the mixture of cells and DNA was placed on ice for further 2 minutes, and then transferred to an ice-cold 0.2 cm electroporation cuvette. The cuvette was placed in a chilled safety chamber slide which was pushed into the chamber until the cuvette was seated between the contacts in the base of the chamber. One pulse was applied at 25 µF, 2.5 kV 200 Ohm (about 4-5 msec). The transformant cuvette was removed from the chamber and 1 ml of SOC medium used to recover the transformants and the cells were quickly resuspended with a pasteur pipette. The cell suspension was then transferred to a sterile eppendorf tube and incubated at 37°C for 1 hour at 200 rpm to allow the antibiotic gene to be expressed. Then the recovered transformant was plated on a selective medium which contained an appropriate antibiotic. Transformants were collected after 16 hours.

II.9.2 Transformation by $CaCl_2$ treatment

A single colony of bacteria was inoculated, transferred and harvested as for electrotransformation except that the monitored absorbance at 550 nm was between 0.4 and 0.5. The pellet from the 100 ml culture was resuspended in 10 ml of ice-cold 0.1 *M* $CaCl_2$ and stored on ice for 10 minutes. Cells were recovered by centrifugation at 4,000xg for 10 minutes at 4°C. The supernatant was decanted and the pellet was resuspended with 1 ml of ice cold 0.1 *M* $CaCl_2$. 100 µl of each suspension of competent cells was transferred to a cold sterile eppendorf tube by means of a chilled sterile pipette tip. 10 ng of ligation mixture (2 µl) was added to each chilled competent cell tube and the suspension gently swirled. The tubes were stored on ice for 30 minutes then incubated at 42°C for 2 minutes for heat shock to allow the recombinant DNA to enter the competent cell without the need for shaking the tubes. Then the tubes were rapidly transferred to an ice bath for 2 minutes. 900

µl of SOC was added to each tube and the tubes were incubated at 37°C for 1 hour. Each transformant suspension was plated and incubated at 37°C overnight. Transformants on the plates were checked after 16 hours.

II.9.3 Infection

Bacterial strain LE392 was inoculated in 3 ml LB containing 10 m*M* $MgSO_4$ and 0.1% maltose. It was incubated at 37°C at 250 rpm overnight. 1 ml of the overnight culture was transferred to 50 ml LB containing 10 m*M* $MgSO_4$ and 0.1% maltose and further incubated at 37°C until the OD_{600} was between 0.6 and 0.7. The culture was chilled on ice for 10 minutes, transferred to a polypropylene tube and centrifuged at 5,000xg for 10 minutes. The supernatant was discarded and the pellet was resuspended in 3 ml of 10 m*M* $MgSO_4$. 100 µl of the prepared cells were added to 100 µl of diluted phage. The suspension was incubated at 37°C for 30 minutes. The infected cell suspension was then added to 3 ml of 50°C molten LB top agar (1% bactotryptone, 0.5% NaCl, 0.8% agarose gel) which was placed in a 20 ml sterilin tube. The mixture was shaken a few times and immediately poured onto an LB plate prewarmed to 37°C. The LB top agar was allowed to settle at room temperature. Plates were inverted and incubated at 37°C overnight. The following day, plaques were counted and the efficiency was calculated as plaque forming units (pfu).

$$\text{pfu/ ml} = \frac{\text{number of plaques x dilution factor}}{\text{volume of extract plated}}$$

II.10 Southern blotting

In order to screen and identify the genes in the carotenoid biosynthesis pathway, Southern blot hybridization was an important technique to prove the gene homology between *R. maris* and different probe preparations. DNA probes were obtained from *E. herbicola* and *R. maris*.

II.10.1 Membrane preparation

Many enzymes were used to cut total genomic DNA of *R. maris* strain N1020. 10 μg of genomic DNA was digested with single or double restriction enzymes in a total volume of 100 μl in each reaction and loaded on a 16 x 20 x 1 cm^3 gel tank and electrophoresed overnight with a constant voltage of 50 v. Separated DNA gel was then photographed to allow the later identification of significant positive bands after hybridization. The gel was then soaked with 0.25 *M* HCl (300 ml) to purify the DNA at room temperature until the colour at the bromophenol blue position changed to yellow and the gel was left for a further 15 minutes. The gel was then placed up-side down on capillary 3MM paper wetted with 0.4 *M* NaOH on the wick for alkaline transfer of DNA from gel to membrane. Both ends of the 3MM paper were soaked in 0.4 *M* NaOH which filled the grooves of the tank. The Hybron membrane N^+ was prepared slightly bigger than the gel and cut at the right corner to mark the side of membrane facing the probe, and was then placed directly onto the gel, followed by a thick piece of 3MM paper, three sheets of normal 3MM paper and a pile of tissue paper. The system was covered with the lid and pressed with a 1 kg weight overnight. All fragmented DNA was eluted from the agarose gel and the alkaline solvent induced covalent fixation of DNA to the membrane (Reed and Mann, 1985).

The next day, the DNA transferred membrane was dried on tissue paper and wrapped with cling film. In order to fix DNA onto the membrane by crosslinking, the DNA membrane was placed face side down in the UV illuminator at 302 nm for 3 minutes. The fixed membrane was then used for DNA hybridization.

II.10.2 Labelled probe preparation

The method of primer synthesis was used to label the probes. The complementary strand of DNA was synthesized by klenow polymerase using the 3'OH termini of the random oligonucleotide as a primer.

All the procedures were carried out behind a shield. DNA which was used as a probe was frozen at -20°C. The DNA probes used in this procedure ranged

between 230 bp to 1.271 kb. A DNA concentration of 20 ng in 5 µl (in the stock) was used in each preparation and mixed with 1.9 µl of ddH_2O and boiled for 5 minutes to denature the probe, then placed on NaCl-ice for the labelling reaction to take place. The reaction was carried out on ice and usually performed in a total volume of 15 µl as follow

oligo-labelling buffer	3	µl
Bovine serum albumin (1 mg/ ml)	3	µl
α^{32}PdCTP (10 mCi/ ml)	1.5	µl
DNA probe (20 ng)	6.9	µl
DNA polymerase (Klenow fragment)	0.6	µl
total volume	15	µl

The reaction was left at 37°C overnight in a thermal block covered with a lead shield.

In order to determine the specific activity, which should be more than 2×10^9 cpm/ µg for single copy gene detection, 1 µl of the overnight DNA labelling reaction was transferred and mixed with 15 µl ddH_2O, and 4 µl pipetted onto each of 4 pieces of Whatman chromatography paper no 1 and dried. Two pieces of the paper were washed twice with 10 ml ice-cold 5% trichloroacetic acid and the precipitation reaction was left on ice for 5 minutes. These pieces of paper were then each transferred to a scintillation vial which was filled with ddH_2O. All vials were assayed in the scintillation counter (Wallac 1219 Rackbeta Liquid Scintillation counter connected with olivetti computer model M24 and LKB Wallac Plotter). The specific activity was calculated as shown below

$$\text{(a) \% incorporation} = \frac{\text{cpm in washed filter} \times 100}{\text{cpm in unwashed filter}}$$

(b) ng theoretical yield = $\frac{\mu Ci \text{ dNTP added} \times 4 \times 330 \text{ ng/ nmole}}{\text{specific activity dNTP (Ci/ mmole)}}$

(c) ng DNA synthesized = % incorporation x 0.01 x theoretical yield

(d) Specific activity of the product (cpm/ μg) = $\frac{\text{total cpm incorporation}}{\text{(ng of DNA synthesized + ng input DNA) x 0.001}}$

A suitable amount of radioactive probe was then used for hybridization, and the remaining 14 μl of the probe mixture was precipitated by adding 7.5 *M* CH_3COONH_4 (16 μl) and 60 μl of -20°C absolute ethanol. The eppendorf tube was placed at -20°C for 30 minutes. The labelled probe was collected by centrifugation for 10 minutes at 13,000 rpm in a bench top microcentrifuge and the supernatant was discarded. The pellet was then resuspended in 1% SDS (50 μl).

II.10.3 Prehybridization and hybridization

Nylon membrane blots (8" x 10") were prehybridized with 12.5 ml of prehybridization solution (5 x SSPE, 5 x Denhardts solution, 0.5% SDS and 1% milk powder "Marvel") in the drum which was set at 65°C with constant agitation for 3-4 hours in the hybridization oven (Techne). The probe was then added and incubated at 50°C overnight in the oven. The membrane was then washed with 2 x SSPE + 0.1% SDS twice at 50°C for 10 minutes to remove the excess probe, followed by 1 x SSPE + 0.1% SDS which was incubated at 65°C for 15 minutes and stringent washing with 0.2 SSPE + 0.1% SDS twice at 50°C for 10 minutes and drying at room temperature on tissue paper to remove the excess moisture. The membrane was checked for radioactivity in the homology band by means of a Geiger-Muller counter to estimate the duration of exposure to X-ray film needed. The membrane was then wrapped with cling film, placed next to the X-ray film and left at -70°C for 1-5 days. A black bag was used to cover the film cassette for double protection of the film from light.

II.10.4 Removal of the probe from the nylon membrane

As nylon membrane is durable, it can be used repeatedly for hybridization with different probes. Probes were removed from membrane by pouring boiling 0.1% SDS (100 ml) on the membrane and leaving to cool at room temperature. X-ray film was then placed face side up to the membrane and left overnight to check the efficiency of washing.

II.11 Non-radioactive detection

Non-radioactive labelling of probe for DNA hybridization needs fewer precautions than radioactive labelling methods. Different kinds of labelling procedures have been applied for probe preparation, *e.g.* dUTP-biotin. The disadvantage of using non-radioactive labelling is that it is quite difficult to test for the degree of nucleotide analogue incorporation, and there is a possibility of low mobility biotinylated fragments from agarose gel purification (Holmstrφm and Rasmussen, 1990). Digoxigenin (DIG) 11-dUTP is an efficient dUTP analogue for labelling probes (Emanuel, 1991). Its efficiency can be assessed by serial dilution and dot blotting. The intensity of colour that developed from the immunodetection reaction from the control was then used as a reference. The labelled *crtBI* probe which showed lower mobility than the unlabelled ones was then cut from the gel and purified with Geneclean. The purified labelled probe was then used for detection of recombinant DNA on the membrane.

Each membrane of size 66.5 cm^2 was prehybridized with 20 ml of standard hybridization buffer (5 x SSC, 0.1% w/ v N-laurylsarcosine, 0.02% w/ v SDS and 1% blocking reagent) at 65°C for two hours. The prehybridization buffer was added to the denatured probe (20 ng) in a sterilin tube. The probe solution was then added back to the membrane for hybridization. Hybridization was at 55°C for 16 hours overnight. The hybridization membrane was washed twice with 30 ml of washing solution (2 x SSC, 0.1% SDS) for 5 minutes at room temperature. The membrane was then washed with stringency solution (0.1 x SSC, 0.1% SDS) at 68°C for 15 minutes twice under constant agitation in the hybridization oven. The membrane was

then rinsed briefly with 20 ml of maleic acid buffer (0.1 *M* maleic acid, 0.15 *M* NaCl pH 7.5). It was then incubated in 50 ml blocking solution (1% w/ v blocking reagent in maleic acid buffer) for 30 minutes. The blocked membrane was incubated in anti-DIG-alkaline phosphatase conjugate 1:10000 in blocking solution for 30 minutes, then washed twice with 50 ml maleic acid buffer for 15 minutes at room temperature. Conjugated DIG-11-dUTP and anti-DIG membrane were equilibrated in 20 ml detection buffer (0.1 *M* tris.HCl, 0.1 *M* NaCl, 50 m*M* $MgCl_2$, pH 9.5) for 5 minutes at room temperature. Then conjugated membrane was detected with 8 ml freshly prepared colour solution [36 µl of nitroblue tetrazolium (75 mg/ ml in dimethylformamide) and 28 µl of 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (50 mg/ ml in dimethylformamide)] in a sealed plastic bag (10 x 15 cm^2). Air was squeezed from the plastic bag as much as possible. The reaction was placed in a drawer for 10-16 hours then stopped by placing the membrane in 50 ml ddH_2O for 5 minutes. Positive results showed a purple dot on the membrane, which was then colour photocopied.

In order to use other probes for detection, the membrane was washed with 30 ml of dimethylformamide and incubated at 50°C in the closed system with occasional shaking until the blue colour was remove; the membrane was then rinsed with ddH_2O. DIG-labelled probe was removed from the membrane by placing the membrane in probe removal solution (0.2 *M* NaOH, 0.1% SDS). The membrane was rinsed with 2 x SSC and dried at room temperature. The dry membrane was kept at 4°C until the second probe was applied for the hybridization.

II.12 Colony hybridization

Stored recombinant genomic library in bacterial host, which was kept in 20% glycerol, was thawed on ice. 5 µl of the library was serially diluted in liquid media in dilutions between 10^{-3} and 10^{-5}. 3 µl and 6 µl portion of each dilution were plated evenly on the appropriate agar plates. All plates were incubated at 37°C overnight until the colony size was about 0.5-1 mm. The plates which contained about 200-300 colonies per plate were selected to use as master plates for colony hybridization.

Colonies from the plate was transferred to disk Hybron membrane N^+ (a positively charged nylon membrane). The membrane was placed on the plate for 1 minute and marked with a fine drawn pipette to permit the orientation of plating. Each membrane was peeled off and soaked, colony side up, on the 3MM paper wetted with denaturation buffer (1.5 *M* NaCl; 0.5 *M* NaOH) for 10 minutes. Denatured DNA membrane was placed on 3MM paper soaked with neutralising solution (1.5 *M* NaCl; 0.5 *M* Tris.HCl pH 7.2; 0.001 *M* EDTA) for 5 minutes twice. Neutralised membrane was washed by placing membrane on 3MM paper soaked with 2 x SSPE (0.36 *M* NaCl; 0.2 *M* sodium phosphate; 0.02 *M* EDTA pH 7.7). The membrane was baked at 80°C for 2 hours and the dry membrane was kept at 4°C until the hybridization procedure was carried out.

II.13 Polymerase chain reaction

The method of polymerase chain reaction was used for probe synthesis. Template from double stranded genomic DNA or plasmid was denatured by increasing the temperature to 94°C so that the double stranded template was separated. Primers were designed from the known sequence of the carotenoid biosynthesis gene from different species. They were annealed for the complementary strands of the genomic DNA when the temperature was decreased to 50°C in the annealing stage. The 3'-OH end of each primer was provided to polymerise the nucleotide sequence which was complementary to the genomic template at the optimal temperature 72°C by the enzyme Taq DNA polymerase. The time used in each step of the cycle depended on the length of the DNA and the percentage G + C content. Various $MgCl_2$ salt concentrations in the PCR buffer were tried. The reaction mixture used was as follows

ddH_2O	x	µl
10 x PCR buffer	5	µl
Primer 1 (2 µ*M*)	5	µl
Primer 2 (2 µ*M*)	5	µl

DNA template	y	µl
Taq DNA polymerase (5 u/ µl)	1	µl
total	50	µl

The reaction mixture was coated with 50 µl of mineral oil to protect the reaction from evaporation during the high temperature applied in the denaturation step. The reaction was placed in the thermocycler (Techne Ltd. Cambridge, UK) for 30 cycles within 2-3 hours. 5 µl of each reaction product was checked on 1% agarose gel. The PCR product was partitioned on 1% agarose gel and compared with the *E. herbicola* product and marker. The desired bands were cut and eluted from gel by Geneclean DNA Kit. Details of this technique will be described in Chapter 5.

II.14 DNA sequencing

DNA sequencing was undertaken by the chain termination DNA sequencing method which involves the synthesis of DNA with DNA polymerase *in vitro*. The synthesis of DNA was terminated by the incorporation of a nucleotide analogue, 2',3'-dideoxynucleotide-5'-triphosphate (ddNTP), which lacks the necessary 3'-OH group for DNA chain polymerization. Four separate reactions for the four ddNTP provide complete information of sequencing. A radioactive labelled nucleotide was added to allow the product to be visualised by autoradiography after separation by vertical gel electrophoresis.

Recombinant DNA was sequenced by the following steps.

II.14.1 Preparation of covalently closed circular DNA

Recombinant DNA was isolated and purified by the modified QIAGEN preparation. 400 ml of the overnight culture was transferred equally to 2 x 250 ml GSA tubes placed in the GSA rotor and centrifuged at 5,000xg in the Sorvall RC-5B refrigerated superspeed centrifuge (Du Pont Instruments) for 10 minutes; the supernatant was discarded. The pellet, which was about 4 ml, was transferred to a 50 ml polypropylene tube and 8 ml of P1 (50 m*M* tris.HCl; 10 m*M* EDTA pH 8; 100 µg/ ml RNAaseA) and 100 µg lysozyme were added. The mixture was left on the

bench for 10 minutes with occasionally mixing. Then 12 ml of P2 (0.2 *M* NaOH; 1% SDS), was added and the solution was mixed gently and left on the bench for 10 minutes. To the viscous mixture was added 8 ml of 5 *M* potassium acetate pH 5.2, and the tube was inverted a few times. The crude lysate was centrifuged at 4°C, 6,500 rpm for 20 minutes. The supernatant was applied to a column equilibrated with QBT (0.75 *M* NaCl; 50 m*M* MOPS; 15% ethanol and 0.15% Triton-X 100, pH 7). The column was then washed twice with 4 ml of QC (1 *M* NaCl; 50 m*M* MOPS; 15% ethanol pH 7). The covalently closed circular DNA was eluted with 4.5 ml QF (1.25 *M* NaCl; 50 m*M* tris.HCl; 15% ethanol pH 8.5) and precipitated with 3.2 ml isopropanol and placed at -20°C for 30 minutes and centrifuged for 10 minutes. The supernatant was discarded. The pellet was washed with 80% ethanol and dried in a 60°C oven for 5 minutes. The pellet was resuspended with 450 µl of TE buffer and purified by extraction with phenol/ chloroform. The supernatant was treated with 45 µl of 3 *M* sodium acetate at 4°C for 20 minutes followed by centrifugation for 10 minutes. The DNA was precipitated with 990 µl of -20°C ethanol for 30 minutes. The mixture was centrifuged for 10 minutes. The pellet was washed with 80% ethanol and dried at 60°C under vacuum for 5 minutes. The total DNA obtained was about 126 µg.

II.14.2 Denaturation of plasmids

10 µg of DNA was redissolved in 100 µl of denaturing solution (2 *M* NaOH; 0.2 m*M* EDTA) and incubated at 37°C for 30 minutes. The denatured DNA was precipitated with 75 µl of 7.5 *M* ammonium acetate followed by ethanol precipitation. The precipitated DNA was washed with 80% ethanol and dried at 50°C under vacuum for 5 minutes.

II.14.3 DNA sequencing reaction

3 µg of denatured DNA was redissolved in 7 µl of TE. To the DNA suspension, 2 µl sequenase buffer and 1 µl primer (1 pmol) were added. The mixture of template and primer was annealed by placing the mixture at 65°C for 5 minutes and then at 37°C for 20 minutes. The reaction was left on the bench for 10

minutes and placed on ice for 30 minutes.

Annealed template-primer was then used for sequencing. The reaction mixture consisted of

template-primer	10	µl
DTT 0.1 *M*	1	µl
diluted labelling mix	2	µl
(α ^{35}S) dATP(5 µCi)	0.5	µl
diluted sequenase	2	µl
total	15.5	µl

It was mixed thoroughly and incubated at room temperature for 5 minutes.

The reaction mixture was then allocated 3.5 µl to each of four eppendorf tubes labelled A, C, T, G. The tubes were preloaded with 2.5 µl of the corresponding ddXTP prewarmed at 37°C for 1 minute. The reaction was left at 37°C for 5 minutes, and terminated by addition of 4 µl of stop solution.

II.14.4 Sequencing by polymerase chain reaction

By the PCR technique, the template does not need to be denatured as above because the thermocycler temperature in the denaturation stage causes the duplex DNA to be separated. When the temperature of the thermocycler reached the annealing stage, homologous primer was annealed spontaneously to the template, allowing the polymerization to take place at the polymerization stage.

Before the sequencing reaction, each primer was end-labelled with γ^{32}PdATP. The reaction mixture consisted of

ddH_2O	0.8	µl
primer (10 µ*M*)	0.4	µl
kinase buffer	0.4	µl
T4 polynucleotide kinase (5 u/ µl)	0.4	µl

γ^{32}PdATP (20 µCi)	2	µl
total	4	µl

The labelling reaction was then incubated at 37°C for 2 hours. Labelled primer was used for sequencing in the reaction mixture

ddH_2O	y	µl
5 x sequencing buffer	5	µl
DNA template	x	µl
Taq DNA polymerase (5 u/ µl)	1	µl
labelled primer	1.5	µl
total	17.0	µl

The thermocycler was run for 30 cycles with denaturation temperature 94°C, annealing temperature 50°C and polymerization temperature 72°C. The time for each stage was dependent upon the length and G-C content of the DNA to be sequenced. After the themocycler programme was completed, the reaction was inhibited by the addition of 6 µl of stop solution (10 m*M* NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanole).

II.14.5 Analysis of product of the sequencing reaction

The sequencing product was denatured by warming at 80°C for 10 minutes before loading. 3 µl of each reaction was loaded in the well of 7% polyacrylamide gel (1 x TBE; 7% acrylamide/bis; 7% urea; 0.1% ammonium persulphate and 0.05% TEMED) which was prewarmed to 50°C. The gel was run at a constant 25 W for at least 2 hours. When ^{35}SdATP was used, the gel was fixed with 10% acetic acid and 10% methanol for 15 minutes, then transferred to 3MM paper wrapped with cling film and dried at 80°C in vacuum for 90 minutes. The dried gel was monitored with Griger-Muller counter to approximate the required duration of exposure gel to film. The gel was placed cassette face up to the X-ray film. The autoradiography was

carried out at room temperature for 1-7 days. The developed film of the DNA sequence was read through a light box.

Chapter 3

Analysis of Ketocarotenoids from Bacteria

3.1 Introduction

Microbial sources of valuable carotenoids are widely sought as they have advantages over other sources such as plants and animals because of their simple manipulation, collection and controlled production. Carotenoid biosynthesis has been identified in a range of algae, yeasts and bacteria. The ketocarotenoids canthaxanthin and astaxanthin would be particularly valuable products.

3.2 Carotenoids from *Brevibacterium* and *Rhodococcus* species

Numerous carotenoids have been found in *Brevibacterium* and *Rhodococcus* species.

Hsieh *et al.* (1974) found 4-keto and 4,4'-diketo-carotenoids including echinenone and canthaxanthin in *Brevibacterium* strain KY4313.

In *Rhodococcus rhodochrous* strain RNMS1, a ketocarotenoid glycoside, 1'-[(6-*O*-acyl-β-D-glucopyranosyl)oxy]-1',2'-dihydro-β,ψ-caroten-4-one, was found (Takaichi, 1990; Takaichi and Ishidsu, 1992).

Other species of *Brevibacterium* and *Rhodococcus* may be possible producers of ketocarotenoids. *Brevibacterium* and *Rhodococcus* strains which were pink to orange were selected for study and screened for the presence of canthaxanthin and other ketocarotenoids.

3.3 Results

The total carotenoid extracts from *Rhodococcus* and *Brevibacterium* strain were screened by HPLC for the presence of ketocarotenoids. The HPLC profiles were monitored in the wavelength range 250-600 nm. At the wavelength of 450 nm, although many peaks were detected, only two of these were clearly carotenoids one.

The HPLC profile of the whole carotenoid extract from *R. aiechiensis* strain N933 monitored at 480 nm showed two main discrete carotenoid peaks at retention times of 10.55 and 12.1 min (Figure 3.1). For the peak with t_R of 12.1 min, the uv/ vis spectrum had λmax at 478 nm and no fine structure as shown in Figure 3.2. This strain was grown in liquid medium on a larger scale for two weeks, and the main carotenoid was extracted and examined by TLC and HPLC. Its HPLC retention time was confirmed at 12.1 min. This carotenoid was yellowish orange and gave λmax at 470 nm in acetone. When it was reduced with $NaBH_4$, the spectrum changed to give increased fine structure and λmax at 452, 479. The reaction was complete in 30 minutes. This ketocarotenoid represented about 19% of the total carotenoid extract.

For the peak with t_R 10.55 min, the spectrum showed λmax at 445, 475, 505 nm (Figure 3.3) with an additional *cis* peak, at 364 nm. This compound was more polar than canthaxanthin but it was not identified.

Figure 3.4 shows the HPLC profile of the carotenoid extract of *R. maris* strain N1020 monitored at 450 and 480 nm. There were a few small peaks with short retention times less than 10 min in the chromatogram monitored at 450 nm but not at 480 nm. These components showed spectra characteristic of carotenoids. Many minor carotenoid peaks were detected at both monitoring wavelengths.

The most polar peak with tR 11.85 min (Figure 3.5) λmax 457 nm was not identified.

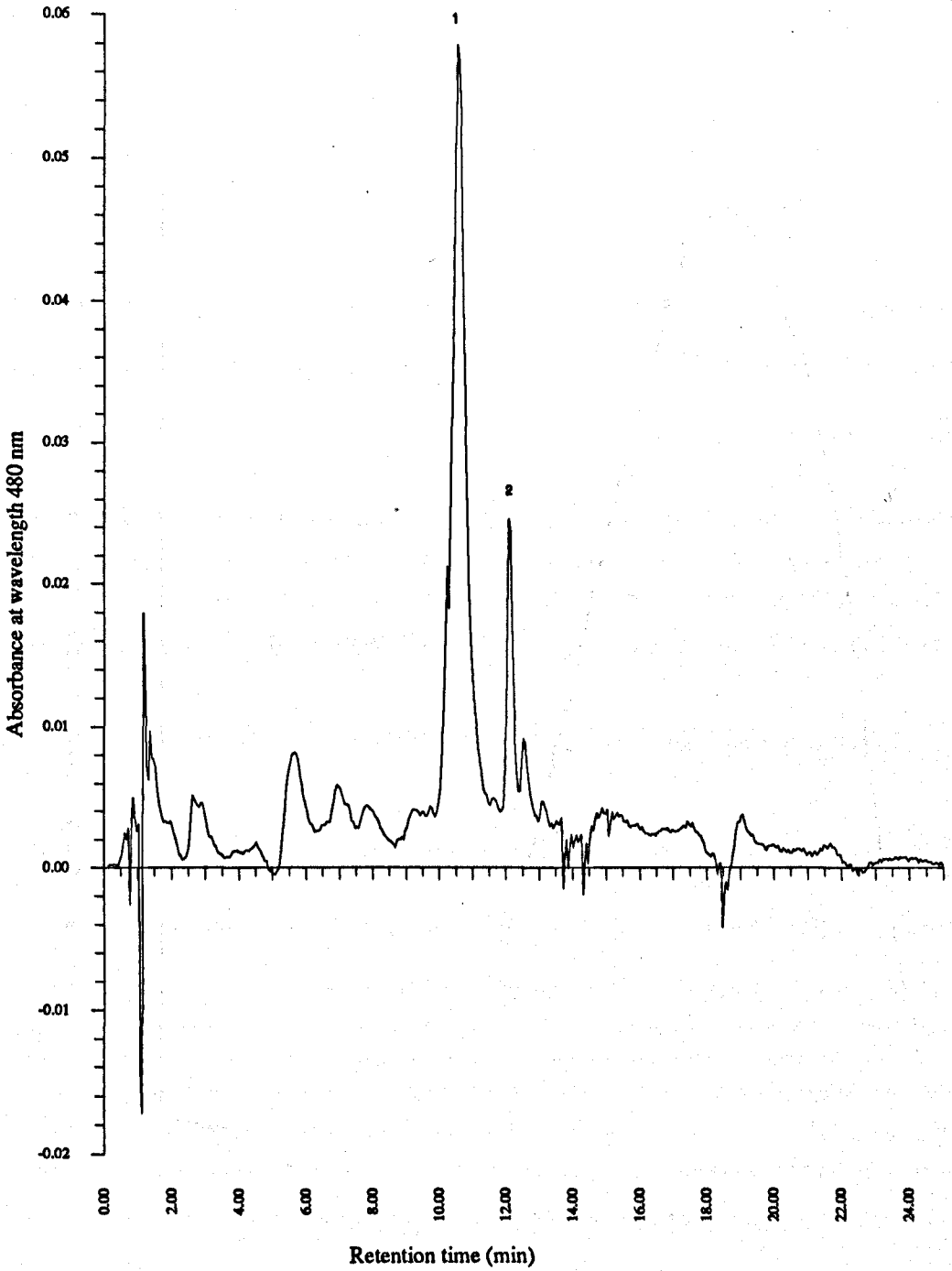


Figure 3.1: HPLC profile of carotenoid extract from *R. aiechiensis* strains N933

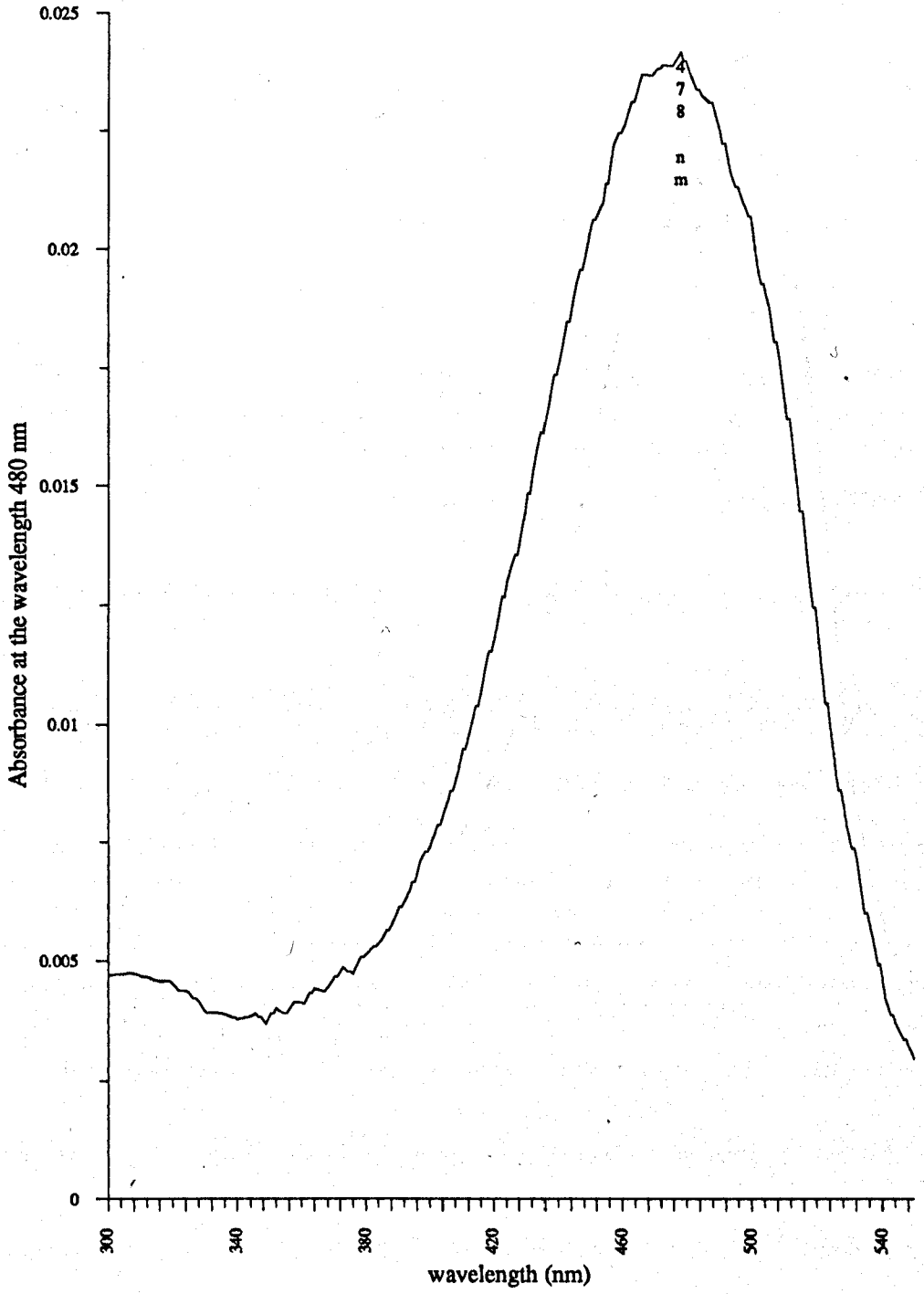


Figure 3.2: UV/ vis absorption spectrum of the carotenoid with t_R 12.1 min from *R. aiechiensis* strains N933

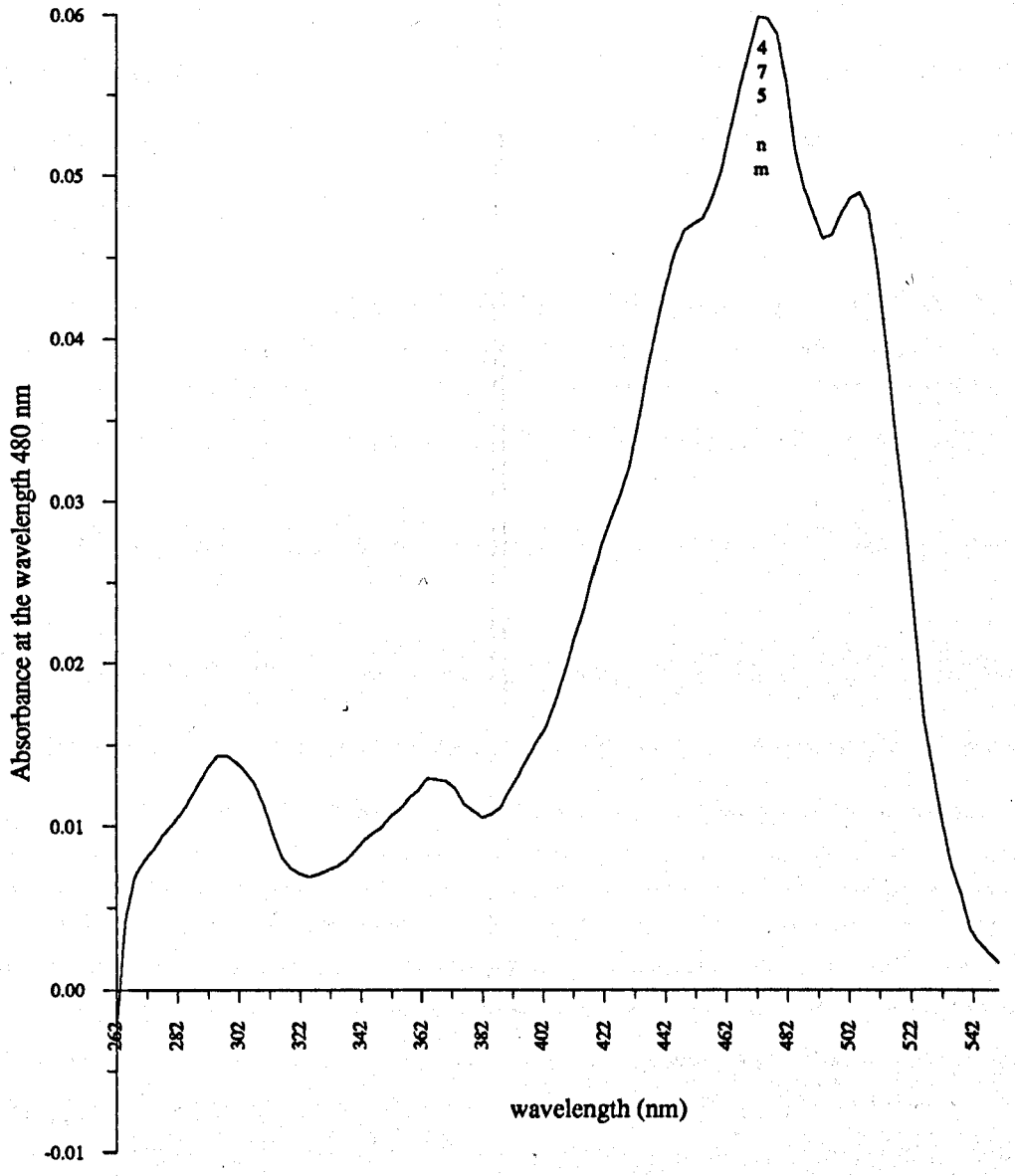


Figure 3.3: UV/ vis absorption spectrum of the carotenoid with t_R 10.55 min from *R. aiechiensis* strains N933

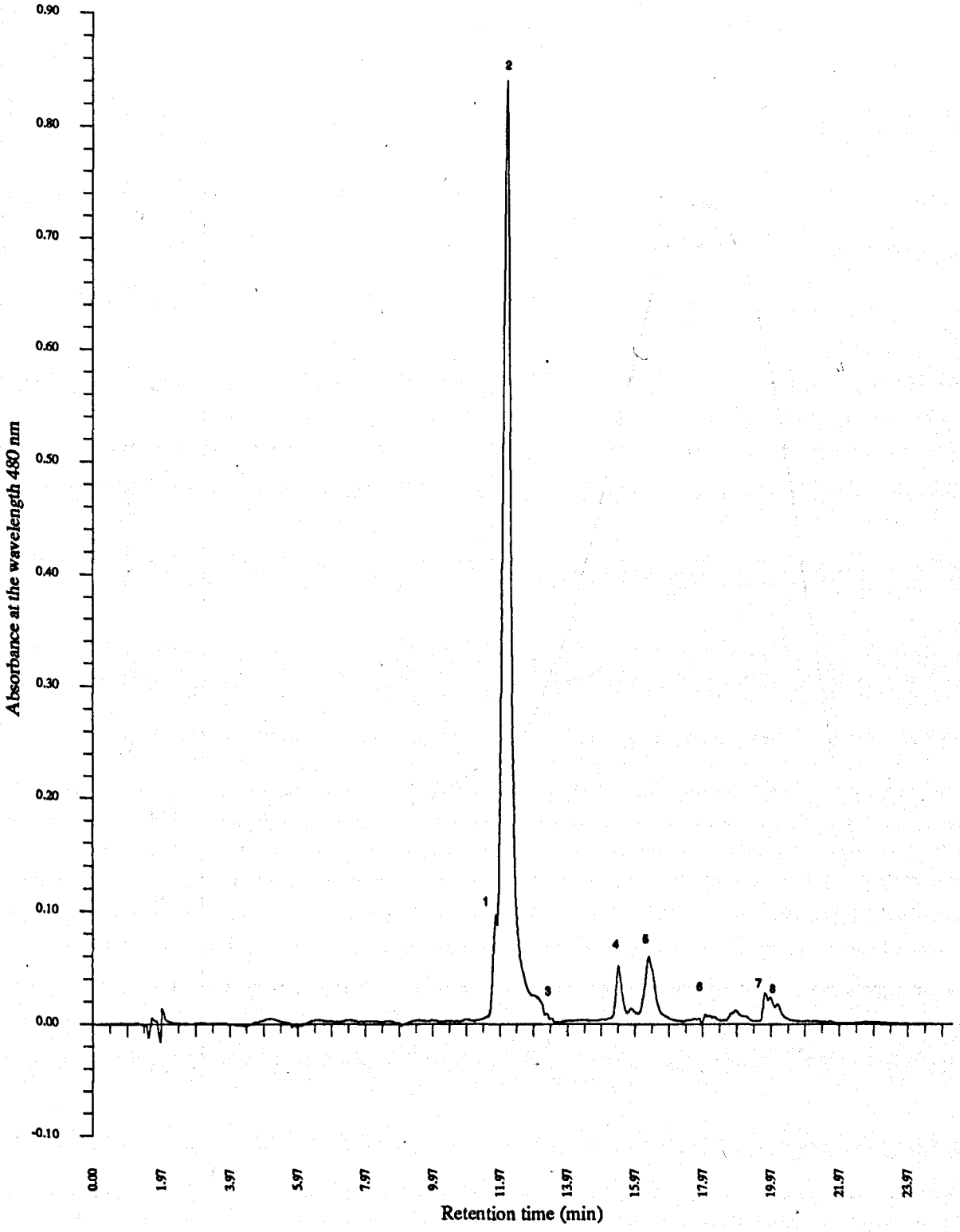


Figure 3.4: HPLC profile of carotenoid extract from *R. maris* strains N1020

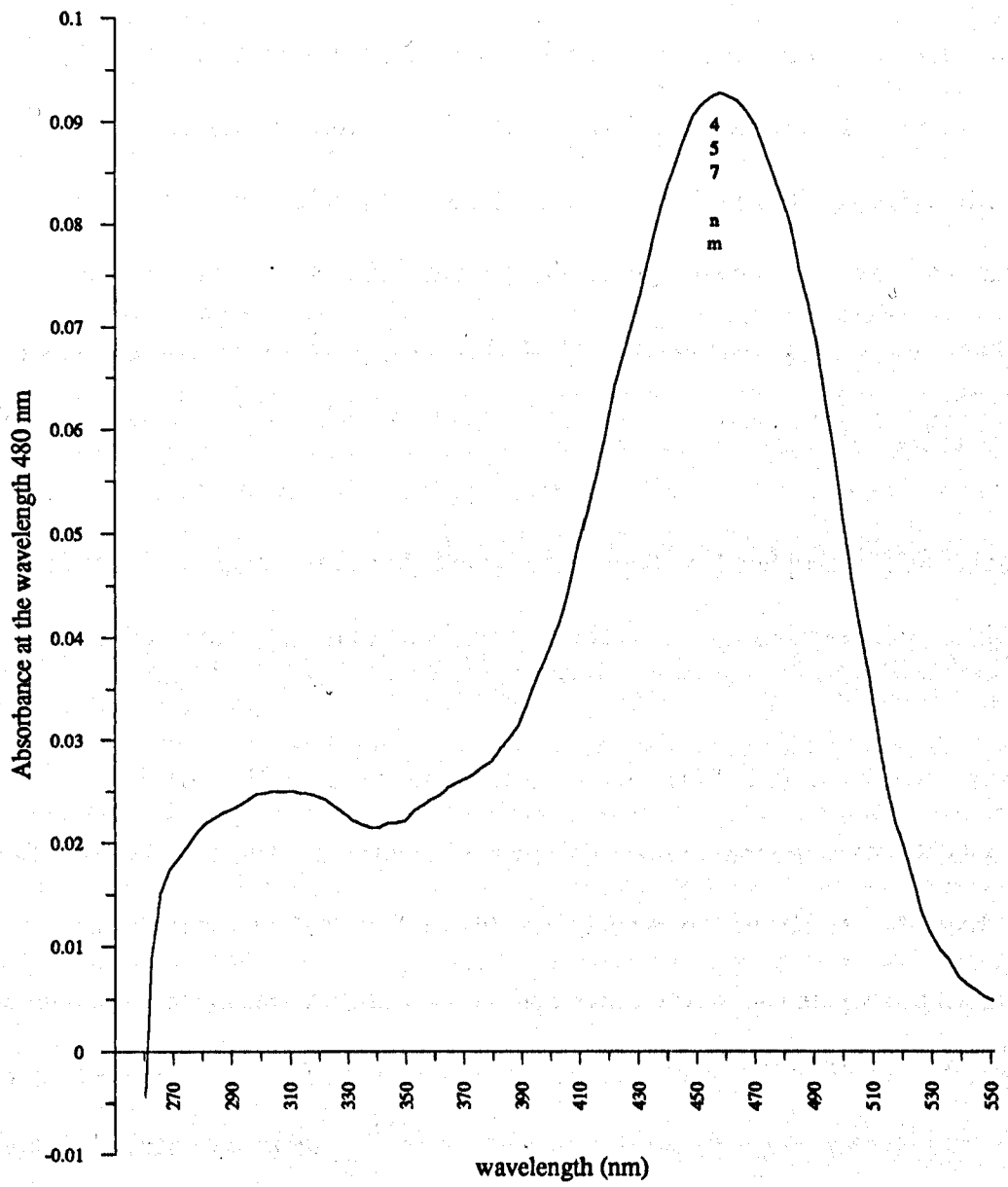


Figure 3.5: UV/ vis absorption spectrum of the carotenoid with t_R 11.85 min from *R. maris* strains N1020

The main carotenoid peak at the retention time of 12.17 min had a spectrum with λmax at 472 nm and no fine structure as shown in Figure 3.6. This carotenoid was purified by t.l.c, and analysed by mass spectrometry. This give a parent ion M^+ at *m/z* 564 corresponding to $C_{40}H_{52}O_2$, with fragment ions at *m/z* 69, 83, 91, 105, 119, 133, 472 (M-92), 458 (M-106) and 360 (M-204) as shown in Figure 3.7. The purified carotenoid was reduced with $NaBH_4$. The reduction product had an absorption spectrum with λmax at 452, 479 nm and increased fine structure. Its mass spectrum had the molecular ion at *m/z* 568, corresponding to $C_{40}H_{56}O_2$ and major fragment ions at *m/z* 550 and 532 due to facile loss of one and two water molecules, respectively as shown in Figure 3.8. Data are consistent with the tentative identification of the isolated carotenoid as canthaxanthin. The absorption spectrum of the compound was shown to be similar to that of authentic synthetic canthaxanthin in several solvents, acetone, ethanol, diethyl ether, methanol and petrol. The reduction product of the ketocarotenoid had chromatographic behaviour and spectrum identical to those of isozeaxanthin.

The ^1H- NMR spectrum of the ketocarotenoid (175 μg) was then determined in $CDCl_3$, which had been deacidified by using sodium carbonate ($NaHCO_3$). The spectrum is shown in Figure 3.9. The assignment of signals is indicated. The assignments were made by comparison of the spectrum with the data for canthaxanthin given in Englert (1995). Therefore, the carotenoid with HPLC t_R 12.17 is definitely identified as canthaxanthin.

The spectrum of a carotenoid with retention time 15.47 min showed some fine structure and λmax at 451 nm (Figure 3.11). It was more polar than echinenone but less polar than canthaxanthin and was not identified.

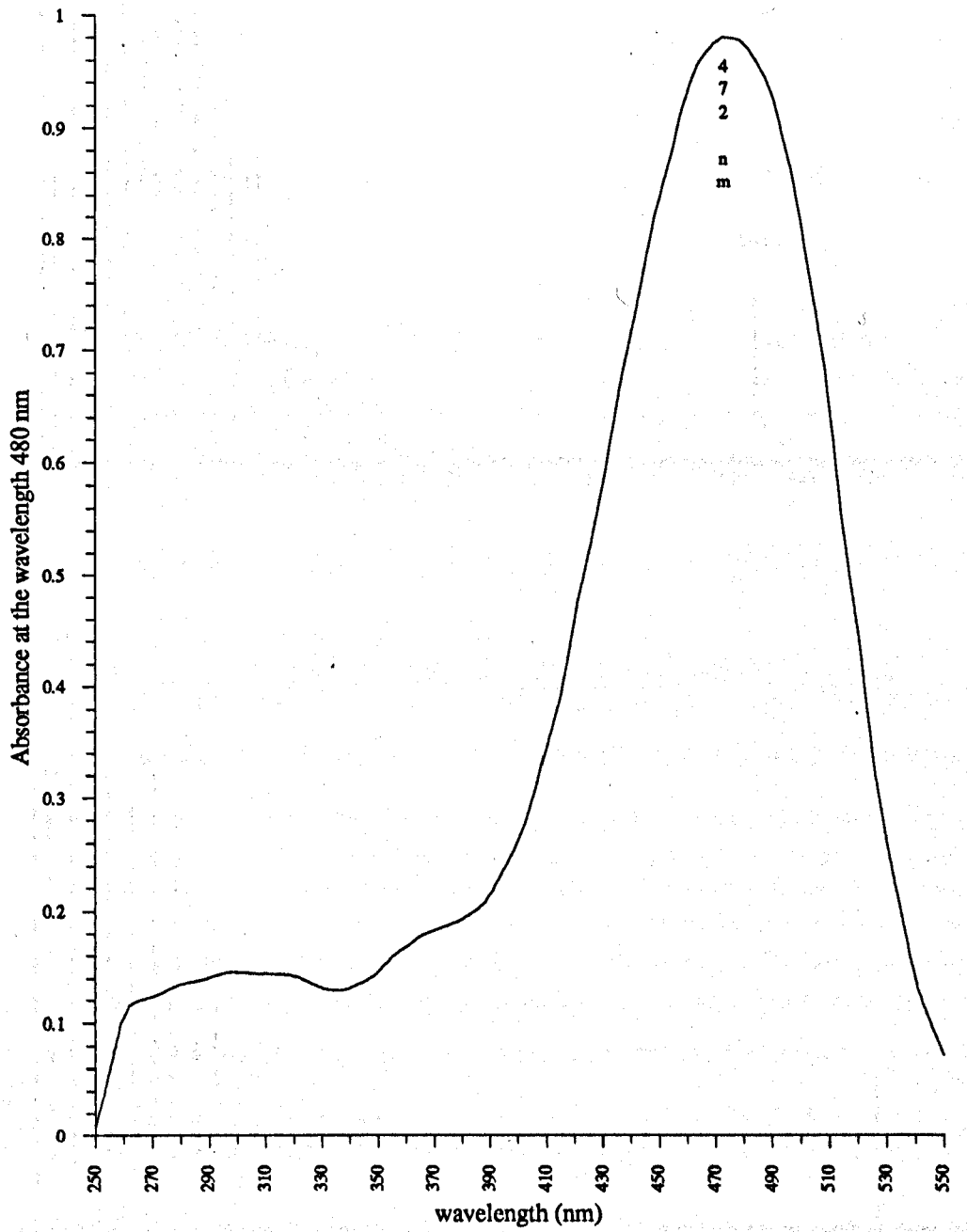


Figure 3.6: UV/ vis absorption spectrum of the carotenoid with t_R 12.17 min from *R. maris* strains N1020

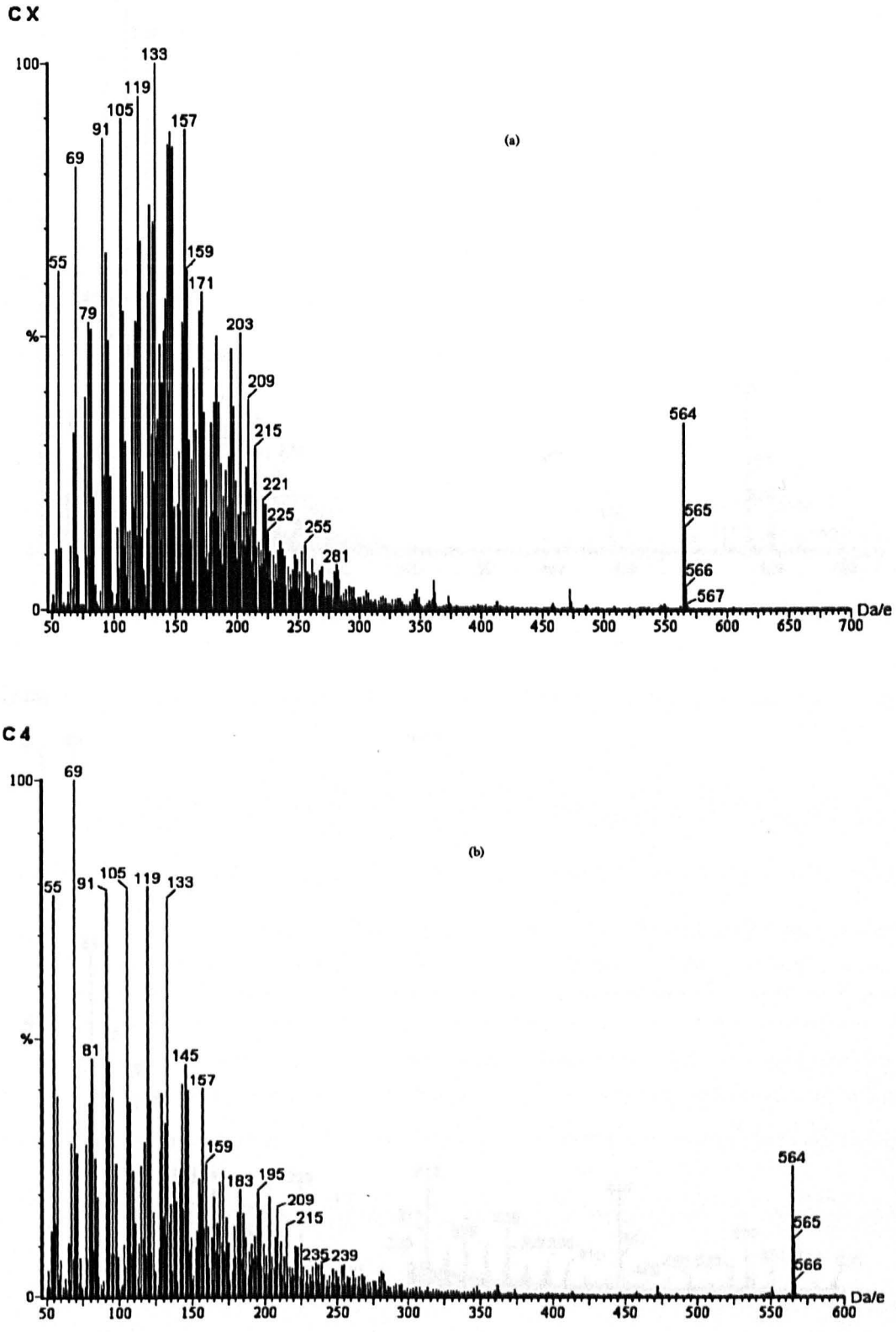


Figure 3.7: Mass spectra of (a) canthaxanthin and (b) the main ketocarotenoid from *R. maris* strain N1020

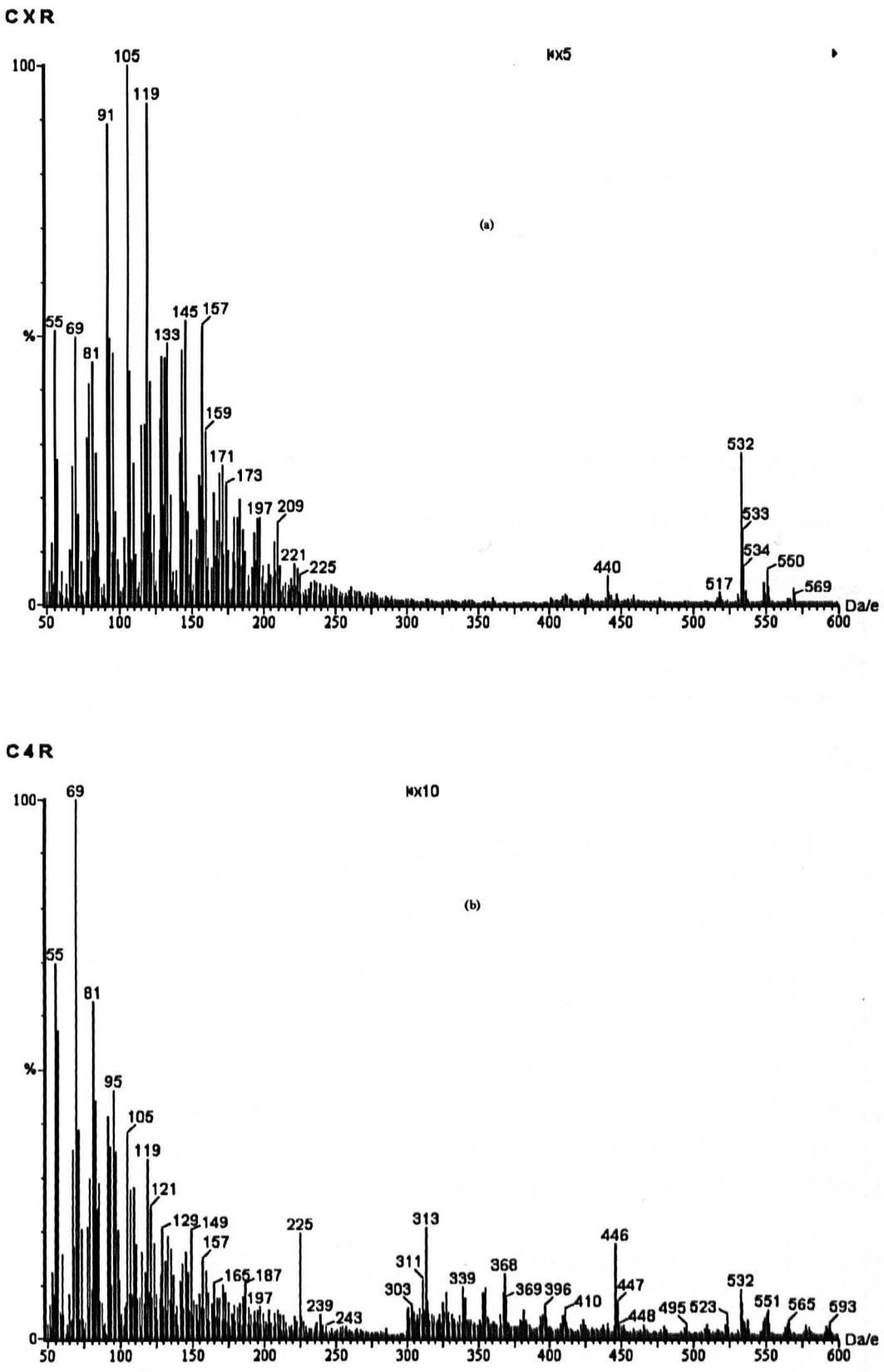
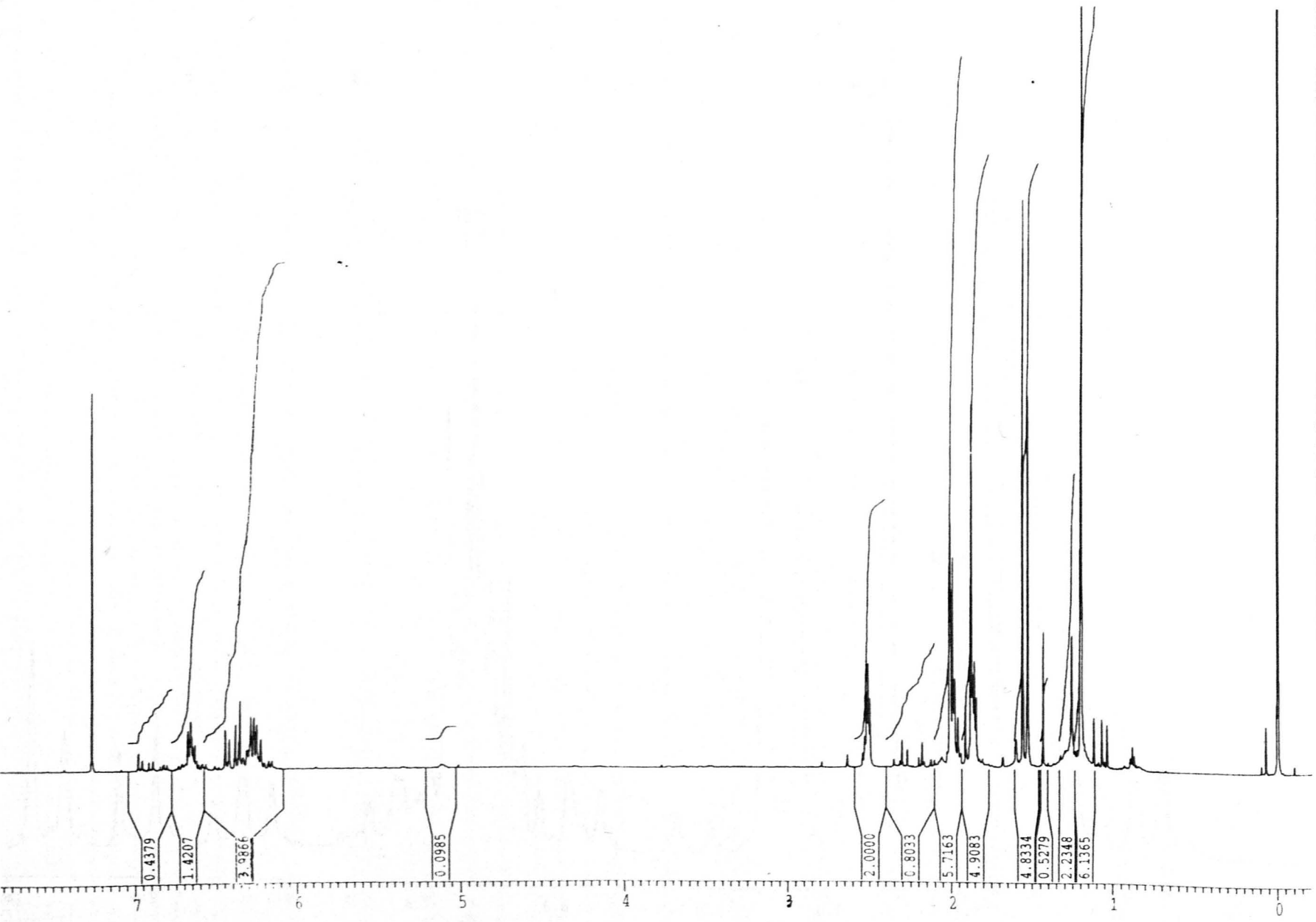


Figure 3.8: Mass spectra of $NaBH_4$ reduction products of (a) canthaxanthin and (b) the main carotenoid from *R. maris* strain N1020

Figure 3.9: NMR spectrum of canthaxanthin from R. maris strains N 1020



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PROCNO 1

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DW 64.2 usec
RG 256
NUCLEUS 1H
D1 1.0000000 sec
P1 6.0 usec
DE 4.5 usec
SFO1 600.1333007 MHz
SWH 7788.16 Hz
TD 65536
NS 139
DS 2

F2 - Processing parameters
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WDW EM
SSB 0
LB 0.50 Hz
GB 0
PC 1.00

1D NMR plot parameters
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F1 4801.04 Hz
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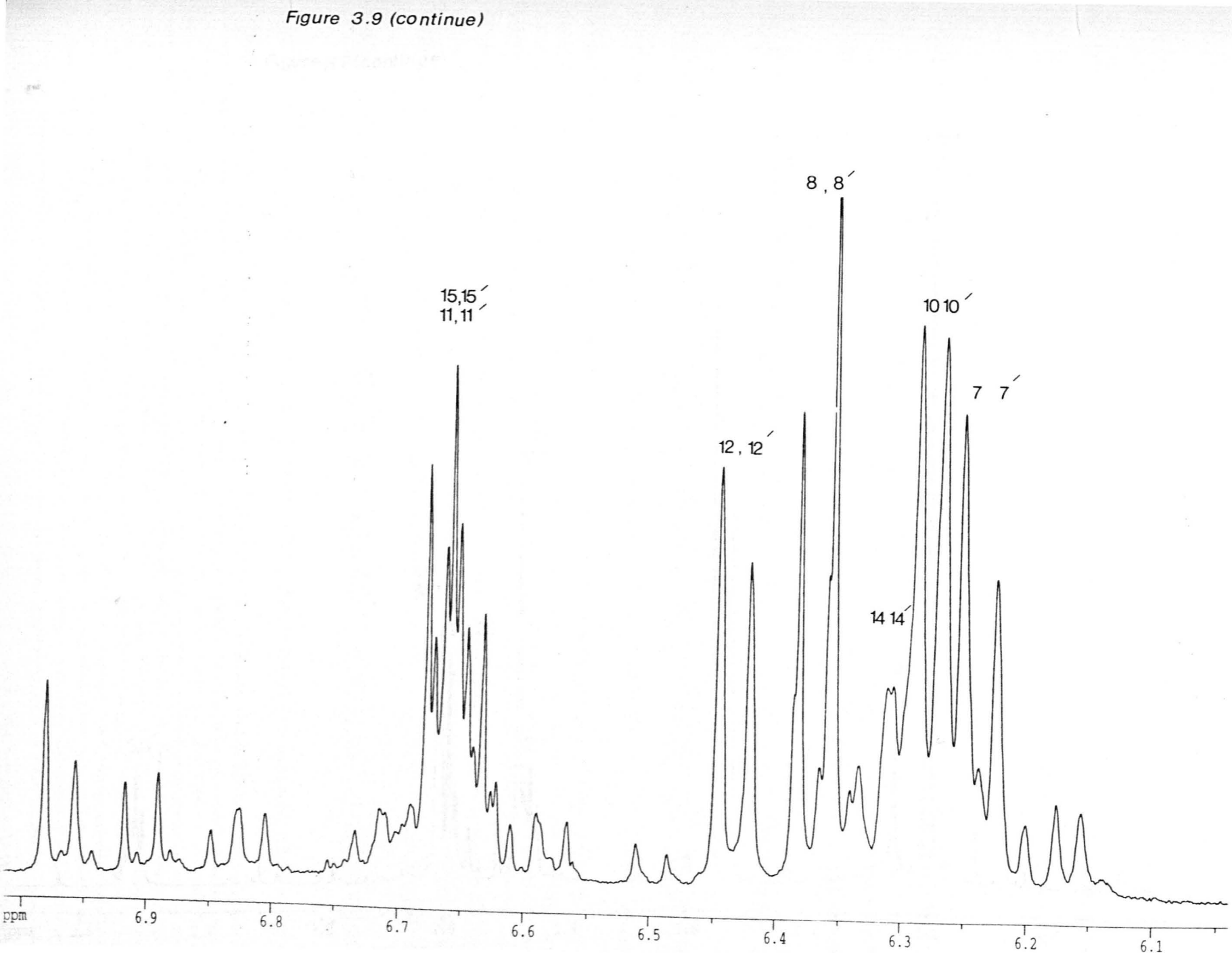
HZCM 205.04442 Hz/cm

(I)

Figure 3.9 (continue)



Current Data Parameters

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PROCNO	1

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DW	64.2 usec
RG	256
NUCLEUS	1H
D1	1.0000000 sec
P1	6.0 usec
DE	4.5 usec
SFO1	600.1333007 MHz
SWH	7788.16 Hz
TD	65536
NS	139
DS	2

F2 - Processing parameters

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SSB	0
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1D NMR plot parameters

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F1P	7.015 ppm
F1	4210.08 Hz
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F2	3625.04 Hz
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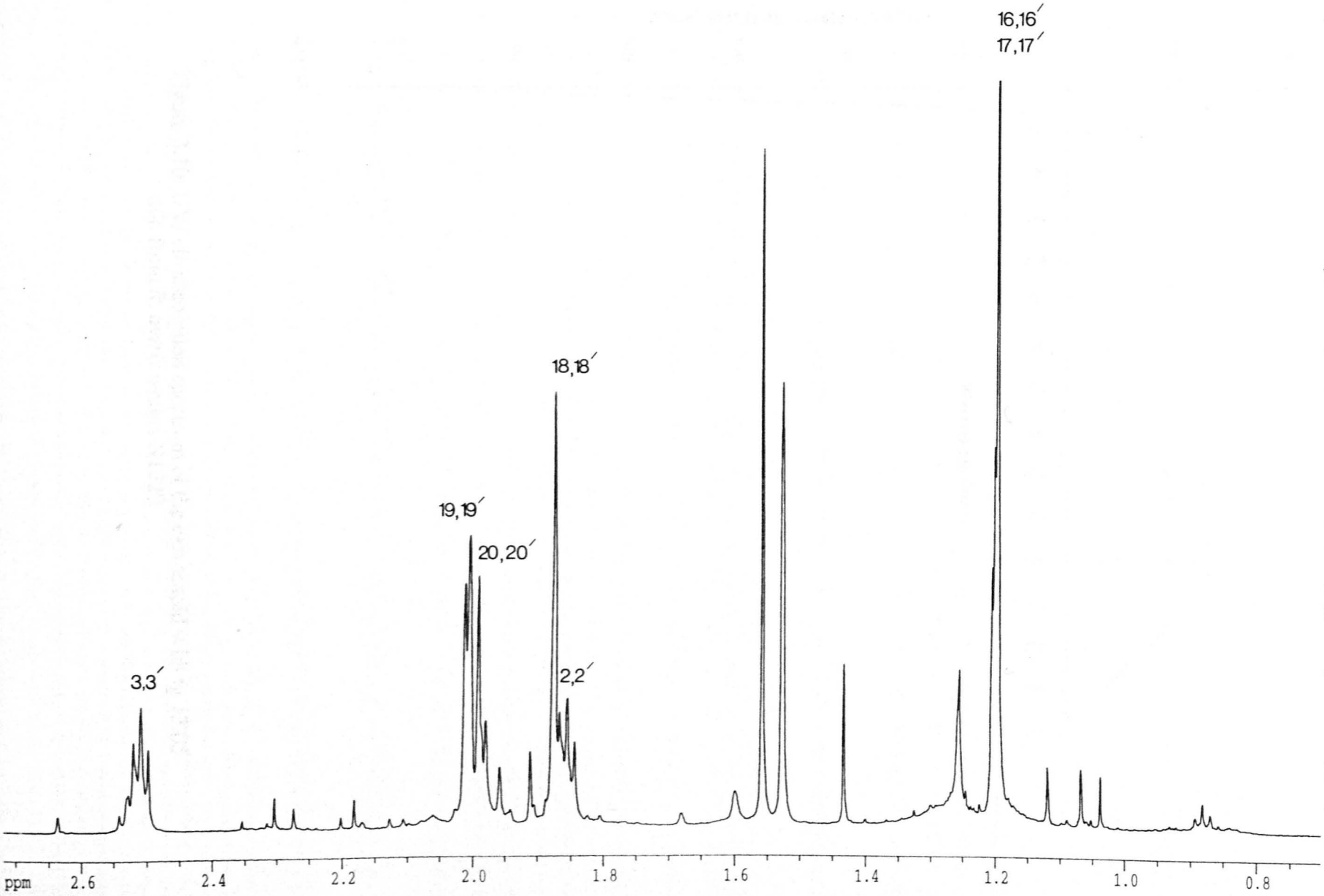
HZCM	24.37634 Hz/cm

(11)

Figure 3.9(continue)



Current Data Parameters

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EXPNO	1
PROCNO	1

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DE	4.5 usec
SFO1	600.1333007 MHz
SWH	7788.16 Hz
TD	65536
NS	139
DS	2

F2 - Processing parameters

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GB	0
PC	1.00

1D NMR plot parameters

CX	24.00 cm
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HZCM	50.35142 Hz/cm

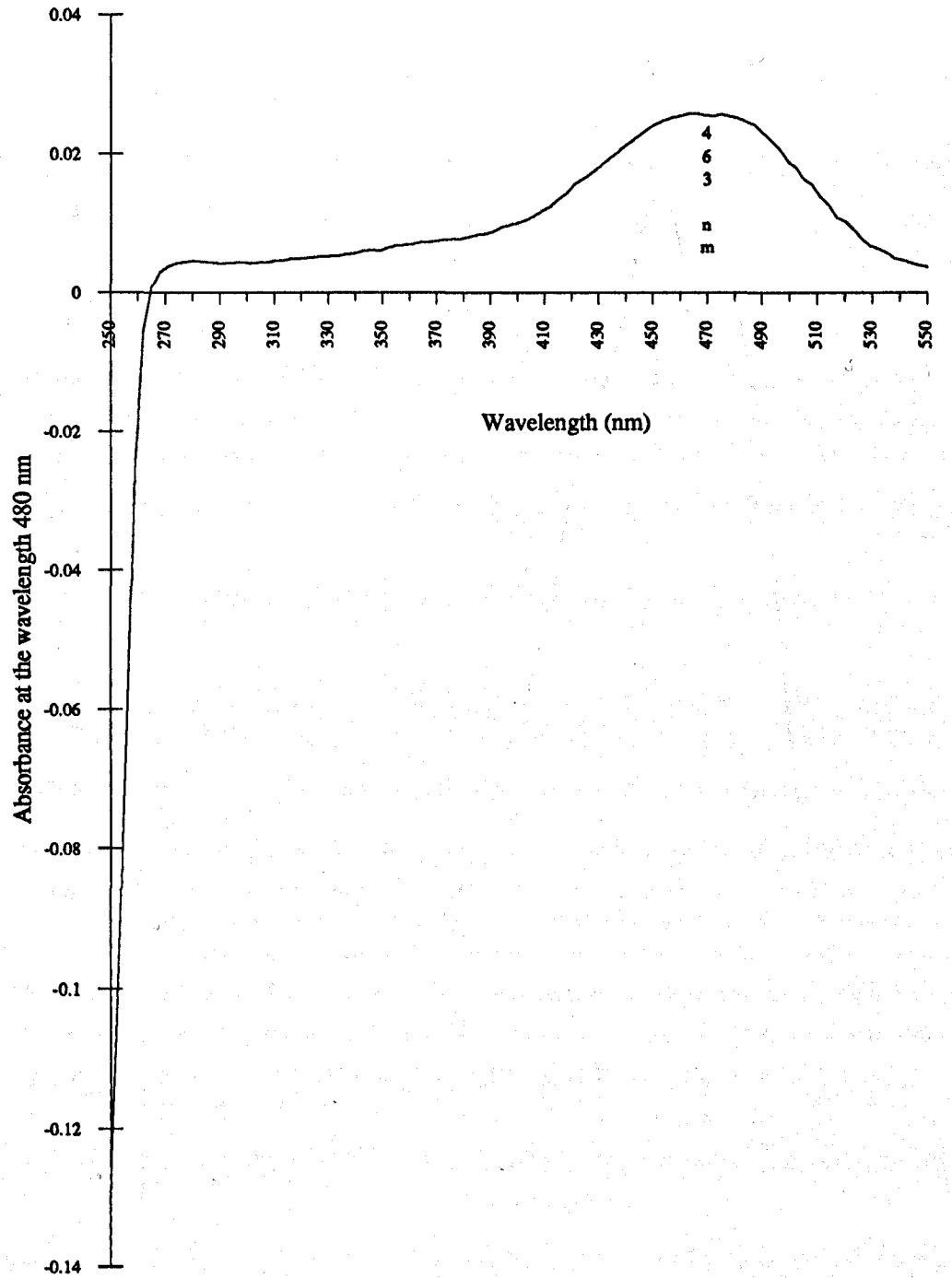


Figure 3.10: UV/ vis absorption spectrum of the carotenoid with t_R 13.05 min from *R. maris* strains N1020

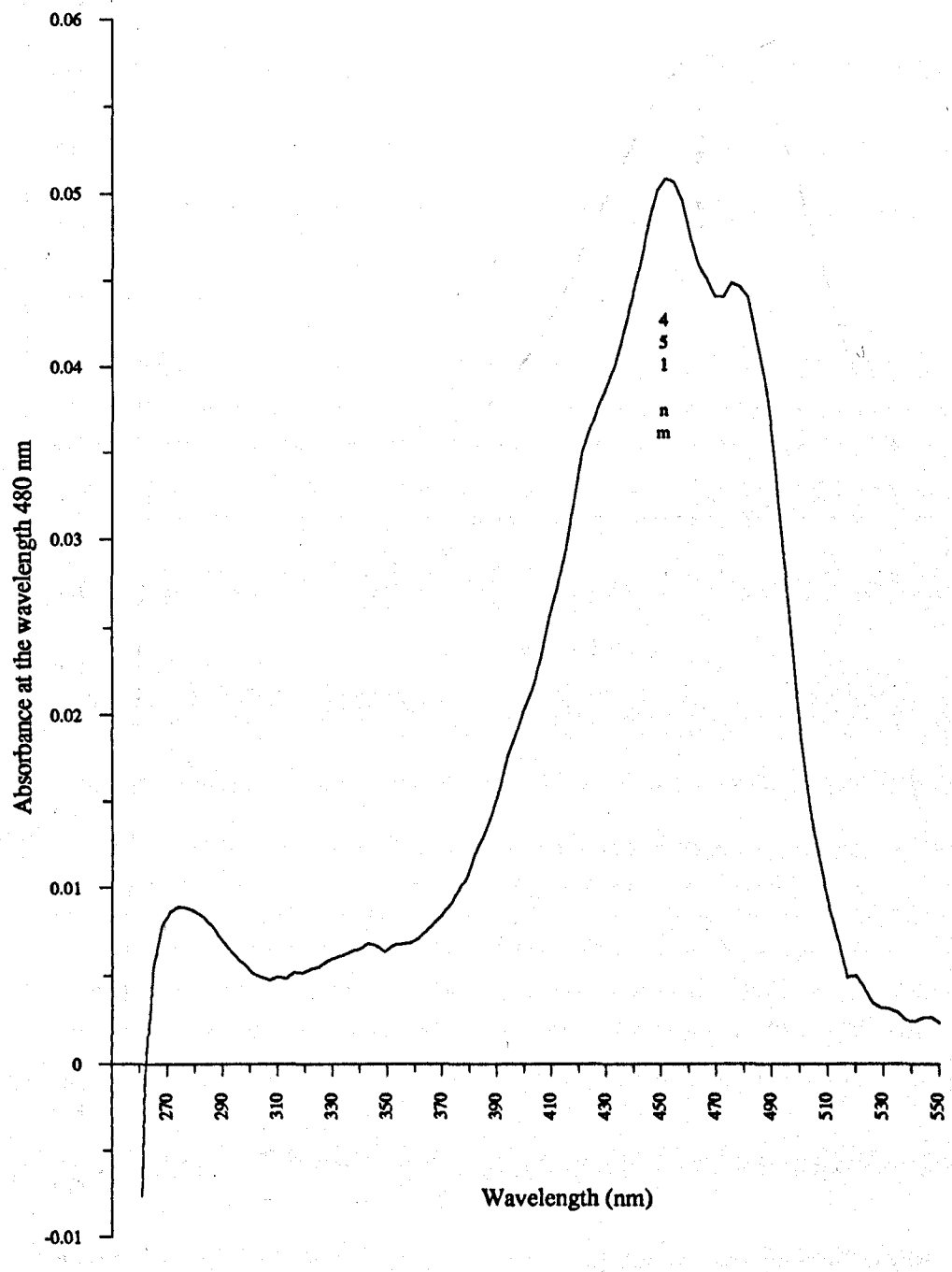


Figure 3.11: UV/ vis absorption spectrum of the carotenoid with t_R 15.47 min from *R. maris* strains N1020

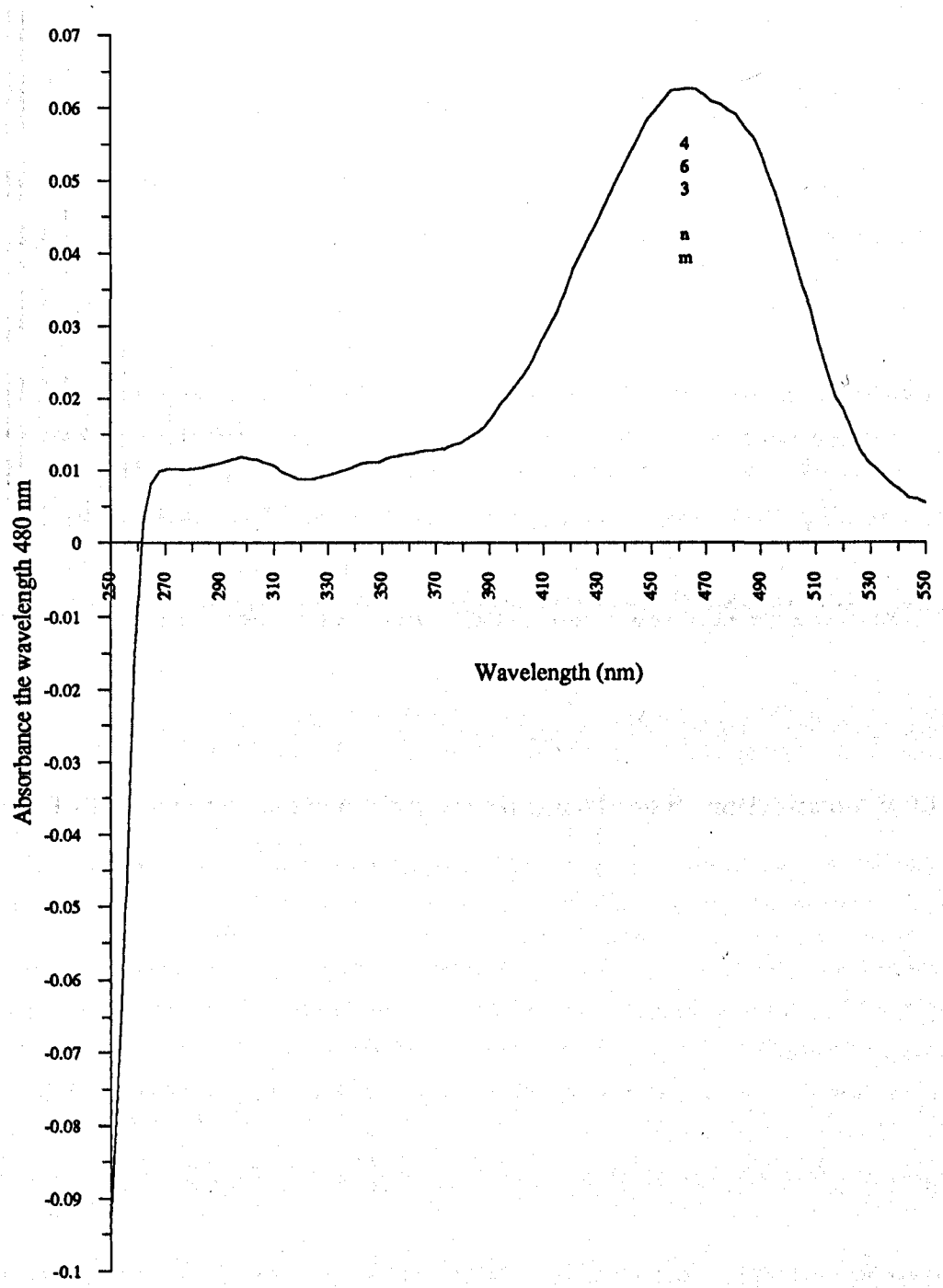


Figure 3.12: UV/ vis absorption spectrum of the carotenoid with t_R 16.37 min from *R. maris* strains N1020

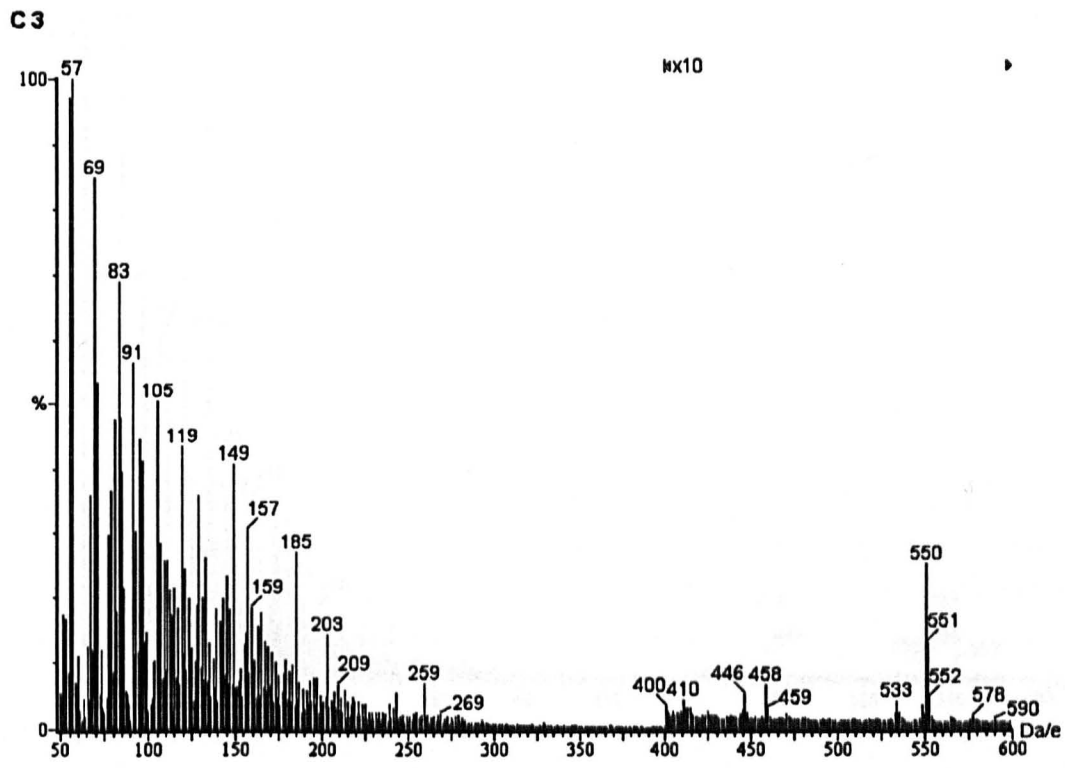


Figure 3.13: Mass spectrum of echinenone extracted from *R. maris* strain N1020

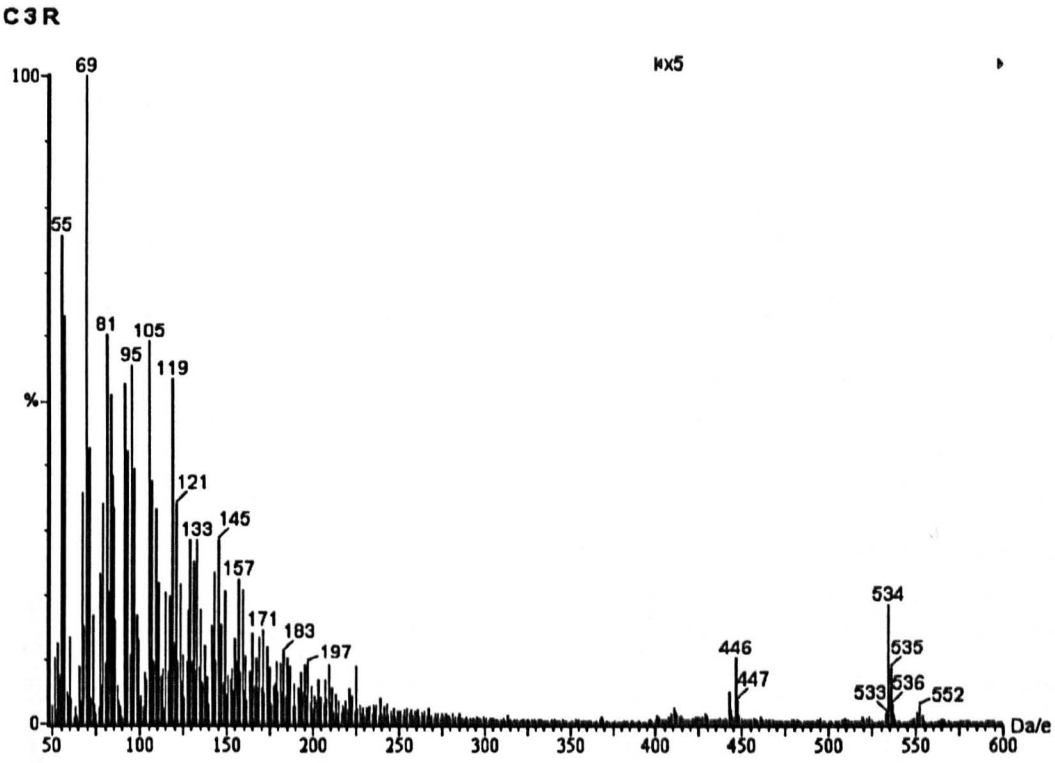


Figure 3.14: Mass spectrum of the $NaBH_4$ reduction product of echinenone from *R. maris* strain N1020

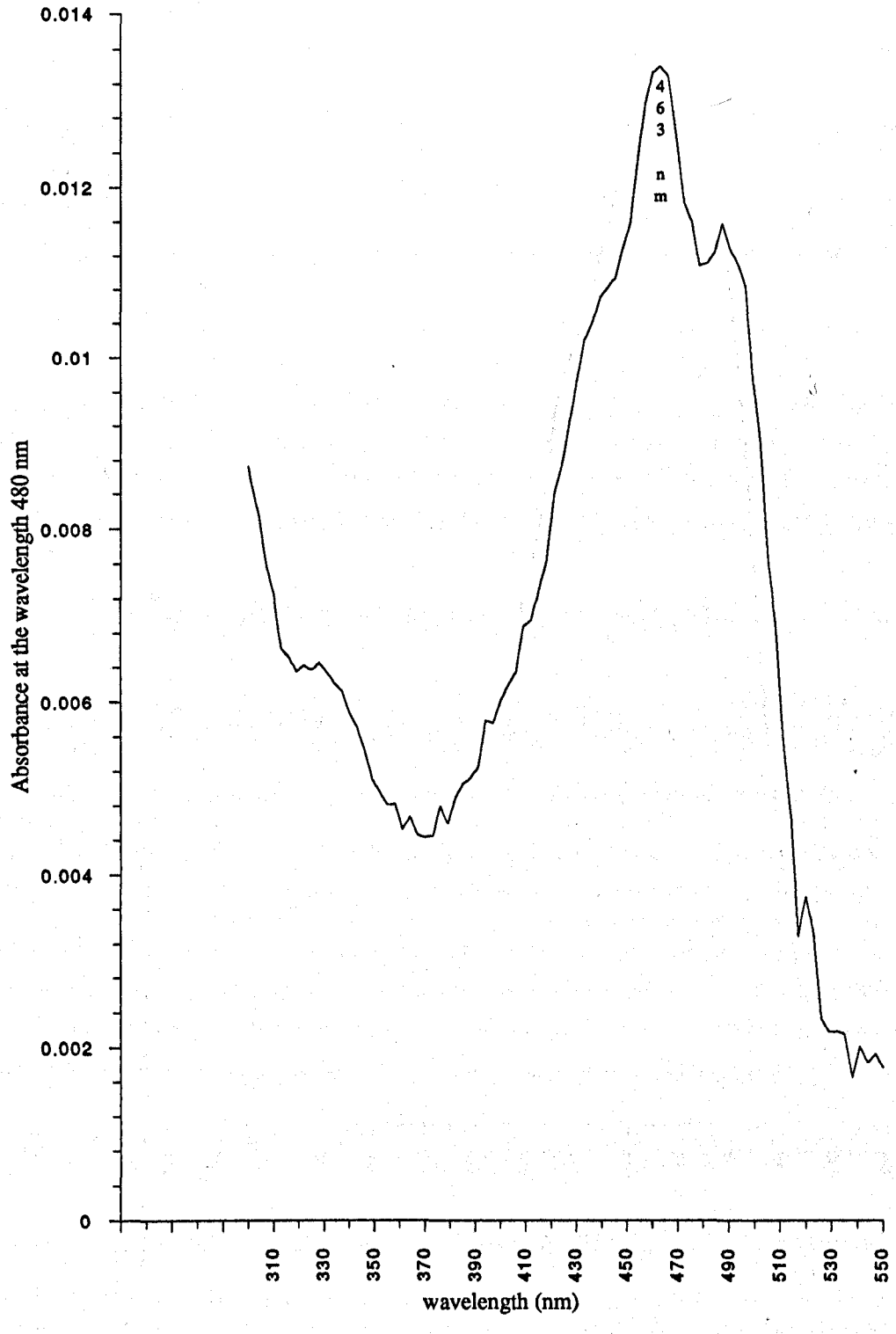


Figure 3.15: UV/ vis absorption spectrum of the carotenoid with t_R 18.9 min from *R. maris* strains N1020

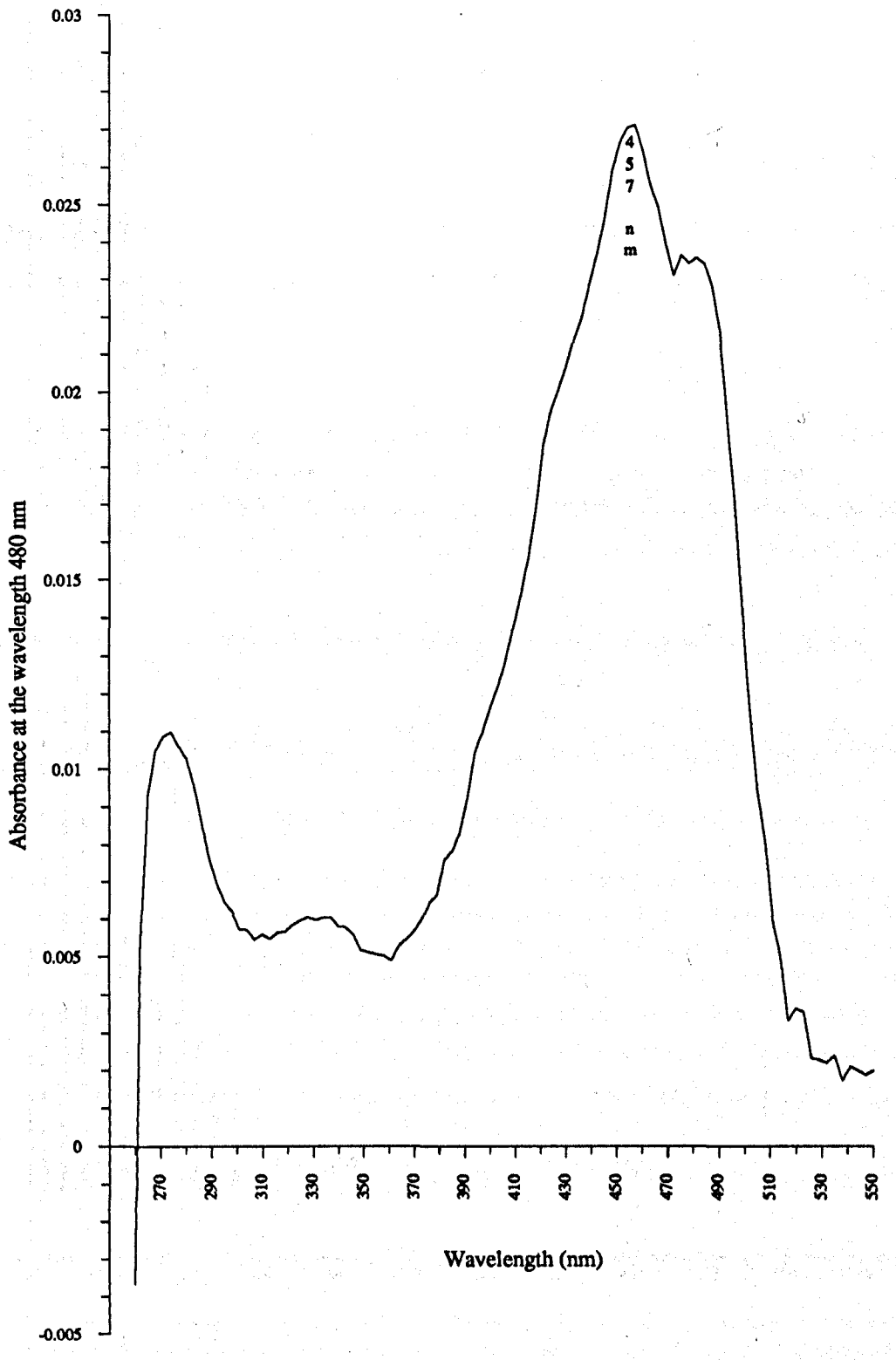


Figure 3.16: UV/ vis absorption spectrum of the carotenoid with t_R 19.82 min from *R. maris* strains N1020

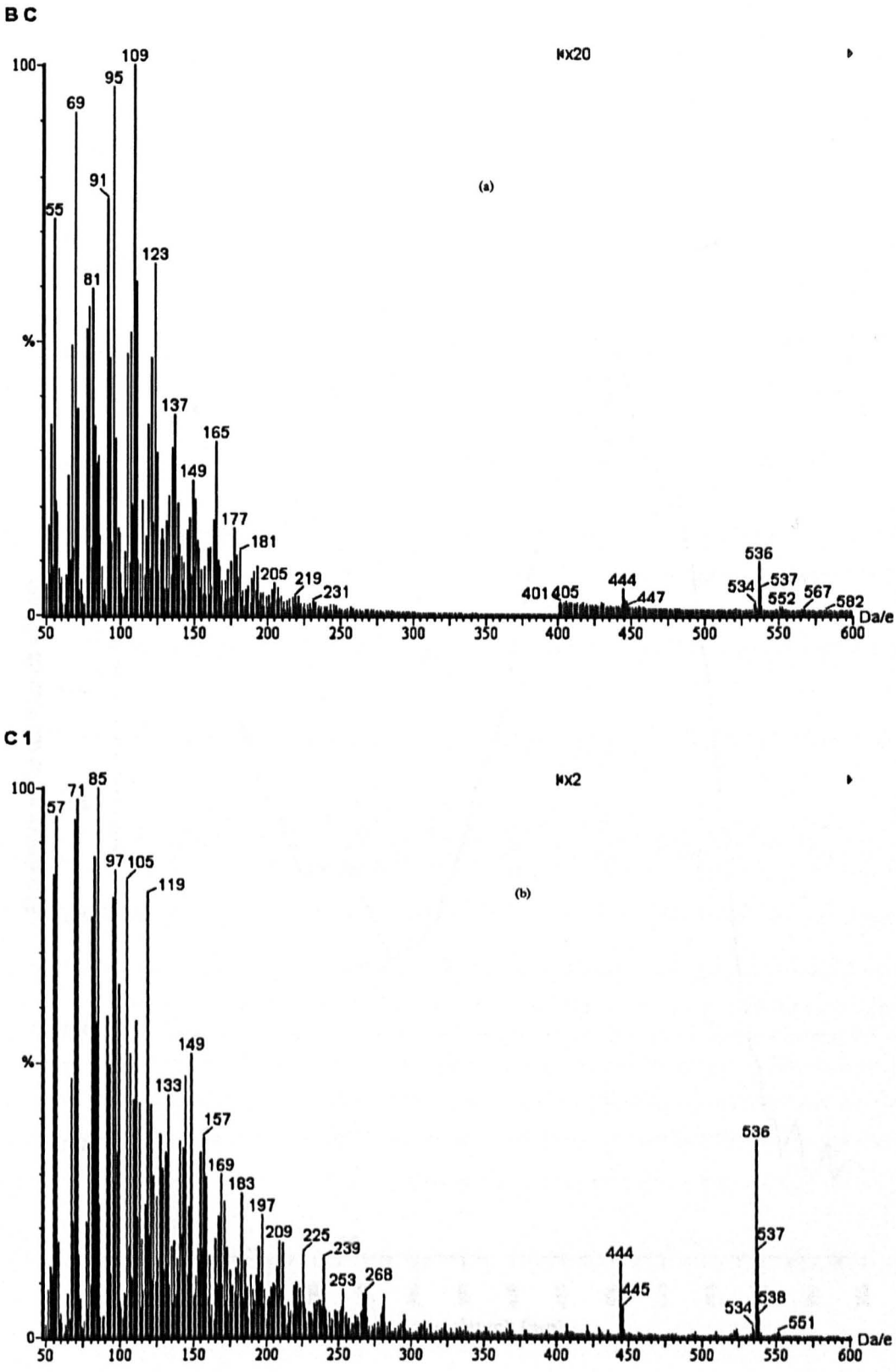


Figure 3.17: Mass spectra of (a) β-carotene standard and (b) β-carotene extracted from *R. maris* strain N1020

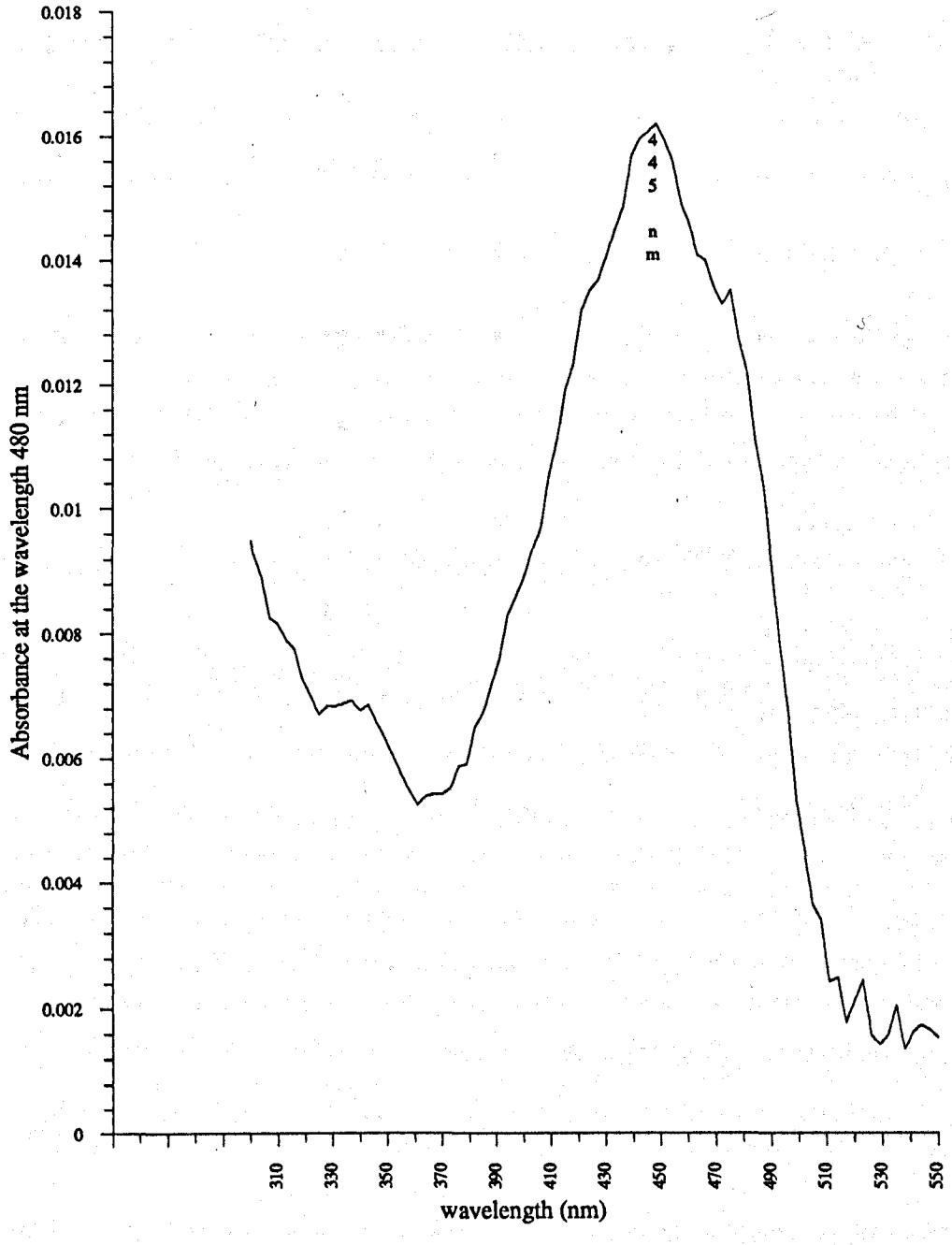


Figure 3.18: UV/ vis absorption spectrum of the carotenoid with t_R 20.1 min from *R. maris* strains N1020

Table 3.1: Carotenoid products from *R. maris* strains N1020

Peak	Retention time (minute)	Molecular mass	Tentative carotenoid
1	11.85	*	*
2	12.17	564	canthaxanthin
3	13.05	*	*
4	15.47	*	*
5	16.37	550	echinenone
6	18.9	*	γ-carotene
7	19.82	536	*trans*-β-carotene
8	20.1	*	*cis*-β-carotene

Note: * represents the unidentified carotenoids

The carotenoid with t_R 16.37 min also had a rounded spectrum with λmax at 469 nm (figure 3.12). Its mass spectrum showed the molecular ion at 550 consistent with $C_{40}H_{54}O$. There were fragment ions at *m/z* 69, 83, 91, 105, 133, 458(M-92), 494(M-56) and 444(M-106) as previously reported for echinenone (Vetter, *et al.*, 1971; Fiksdahl *et al.*, 1983; Bjornland, 1990) as shown in Figure 3.13. Reduction of the ketone group $NaBH_4$ caused a spectral shift and increased fine structure. The reduction product was purified by t.l.c and its mass spectrum determined. The molecular ion was at *m/z* 552. The increase of the molecular weight by two units is consistent with the reduction of one ketone group to the corresponding alcohol. The ion at *m/z* 534 due to the loss of one molecule of water was of high intensity, showing that the hydroxy group was in an allylic position, *i. e.* C-4 (Figure 3.14). The data are consistent with the identification of the isolated compound as echinenone.

The peak with t_R 18.90 min (Figure 3.15), had a spectrum with λmax at 463 nm and more marked fine structure than that of β-carotene. From its retention time and absorption spectrum, this compound is tentatively identified as γ-carotene.

The peak with t_R19.82 min had a spectrum with fine structure and λmax at 451 nm (Figure 3.16). Its mass spectrum had the molecular ion M^+ at *m/z* 536 and fragment ions at *m/z* 69, 83, 91, 105, 430 (M-106), and 444 (M-92) (Figure 3.17). The data are consistent with the identification of this carotenoid as β-carotene.

The peak with t_R 20.1 min (Figure 3.18) had λmax at 446 nm and a *cis*-peak of 334 nm. This compound was not identified but may be a *cis*-isomer of β-carotene.

3.4 Conclusion

The main carotenoids extracted from *R. maris* strain N1020 were identified as canthaxanthin (87%) a monohydroxy carotenoid (possibly β-cryptoxanthin) (4%),

echinenone (5%), γ–carotene (0.1%) and β-carotene (2%) of the total carotenoid extract. *R. maris* strain N1020 appears to be a good source of canthaxanthin.

Chapter 4

Molecular Cloning of Genomic DNA from *Rhodococcus maris* Strain N1020

4.1 Introduction

The 12.7 kb carotenoid gene cluster for the biosynthesis of zeaxanthin diglucoside was isolated from *E. herbicola,* and a genomic DNA library of *E. herbicola* was constructed. By partial Sau3A digestion of genomic DNA, fragmented DNA was purified and cloned in the BamH1 site of cosmid pHC79. Recombinant DNA was transformed to *E. coli* HB101. A coloured colony which contained this gene cluster was then isolated. The recombinant was called pPL376, and it consisted of 12 open reading frames. Assignment was made for *crtB*, *crtI* and *crtE* based on amino acid homology with the *Rhodobacter capsulatus* gene products (Hundle, *et al.*, 1991). Genes which are responsible for the common pathway of carotenoid biosynthesis, especially the early stages, have been given different names from species to species, as shown in Table 4.1. The zeaxanthin glucoside biosynthesis gene cluster provided an important model for studying the molecular genetics of the carotenoid biosynthesis pathway. Moreover, mutants which were derived from this wild type have provided an insight into gene expression in the pathway and have made it possible to prove the carotenoid biosynthesis steps at the genetic level. All the gene base sequences have been elucidated (Armstrong, *et al.*, 1992).

4.2 Mutation

There are many mechanisms for mutation in eukaryotes which might be point mutations or gene mutations. Mutation may be due to transitions, transversions, deletions and insertions. Mutations in different genes in the zeaxanthin diglucoside biosynthesis gene cluster cause the colour of colonies to be changed, ranging from orange to buff. Mutations in the early stages of the pathway, *i.e.*, *crtE, crtB* and *crtI* cause the colonies to be buff instead of orange, but a *crtY* deletion mutant yielded a

Enzyme	Genes (source)	Reference
Geranylgeranyl diphosphate synthase	*crtE* (*E. herbicola*)	*
Phytoene synthase	*crtB*(*E. herbicola*)	*
	pys (Cyanobacteria)	Chamovitz (1992)
	pys 1(Tomato)	Bartley (1992)
Phytoene dehydrogenase	*crtI* (*E. Herbicola*)	*
	pds (Cyanobacteria)	Chamovitz (1993)
	pds (Tomato)	Pecker (1992)
	*al*1 (*N. crassa*)	Schmidhauser (1990)
Lycopene cyclase	*crtY* (*E. herbicola*)	*
	icy (Cyanobacteria)	Cunningham (1993)
β-Carotene hydroxylase	*crtZ* (*E. herbicola*)	*

* Hundle (1991)

Table 4.1: Carotenoid biosynthesis genes from different organisms

pink colony. Therefore, the products of these genes, which are prephytoene diphosphate synthase, phytoene synthase and phytoene desaturase, respectively, are the enzymes which function in the early stages of the pathway and are responsible for the formation of the polyene chain; phytoene desaturase which functions in the desaturation process causes the extension of the polyene chain in the molecule to give the end product. The colour of colonies can be used as the phenotypic marker for the detection of mutants.

4.2.1 Gene mutation

4.2.1.1 Deletion

Deletion is defined as the removal of one or more nucleotides from a gene. It may cause partially or totally deficient gene expression, so that either the protein product does not function properly and is unable to catalyse the reaction or no enzyme is produced. In the bacterial carotenoid biosynthesis gene cluster, if deletion occurs in different parts of the gene, this will cause the colour of colonies to be changed. The obvious example was in Eho13; a recombinant DNA which contains an *E. herbicola* DNA fragment of 9.7 kb was cloned in p8L525 at a SalI site. Eho13 has been transformed into *E. coli* HB101, and causes the bacterial phenotypic change from buff to yellow. Deletion in the recombinant was applied in different part of the gene cluster. The products were then transformed into the bacterial host again. The transformants had different colours which ranged from pink-yellow to buff (Lee and Liu, 1991).

4.2.1.2 Insertion

Insertional mutagenesis, *i.e.* the insertion of a nucleotide or stretch of nucleotides into the gene, has been a valuable tool for studies of the mechanism of transposon mutagenesis. Tn5 was applied for mycobacterial chromosomes in order to isolate several random auxotrophic mutants of *M. smegmatis* (Kalpana, *et al.*, 1991).

4.2.1.3 Transition

Transition refers to a single base pair change in the DNA sequence which results in an alteration from one purine-pyrimidine base pair to one another, for

instance, A-T to G-C.

4.2.1.4 Transversion

Transversion refers to a single base pair change in the DNA sequence results in a purine-pyrimidine base pair, *e.g.* A-T, being replaced by a pyrimidine-purine base pair, for instance A-T to T-A or C-G.

4.2.2 Causes of mutation

4.2.2.1 Radiation

The rearrangement of genomic DNA can be caused by DNA damaging agents such as ultraviolet irradiation. This can induce the formation of a thymine dimer from adjacent nucleotides. Exponentially growing cells of *Rhizobiaceae* were treated by ultraviolet irradiation which caused genetic rearrangement of the pigmented strains (Heumann *et al.*, 1984). In *E. coli,* ultraviolet mutagenesis was related to survival rate. The survival rate was greater in a low irradiation group than with an acute irradiation group due to higher capacity of DNA repair (Dzidic, *et al.*, 1986).

4.2.2.2 Chemical mutagenesis

In *Mycobacterium aurum*, N-methyl-N-nitro-N-nitrosoguanidine (MNNG) has been applied to mutagenize the carotenoid biosynthesis pathway. The minimal inhibitory concentration of MNNG was 50-100 μg/ ml in nutrient broth and the mutation frequency increased with the concentration (Levy-Frebault and David, 1979).

4.3 Control of gene expression

E. coli has been most widely used and its promoter has been studied intensively. The *E. coli* promoter contains two main important regions, -35 and -10. Position -35, with nucleotide sequence TTG, is known to be a recognition sequence for RNA polymerase. TA...T in the position -10 is a well known conserved sequence. Both positions are essential for initiation of gene transcription downstream starting from position +1.

4.3.1 Transcription of genes

The recognition of promoters by DNA polymerases is mediated by protein subunits known as σ factors. σ^{70} is a 70 kDa factor which recognises a broad spectrum of *E. coli* promoters (Cowing, *et al.*, 1985). As promoters are recognised by different RNA polymerase holoenzymes it is reasonable to consider that the σ subunit recognises only the -10 region directly and indirectly affects recognition at the -35 region by altering the geometry of the holoenzyme RNA complex. σ factors are the factors responsible for the recognition and specificity of promoters. *In vitro*, an active hybrid holoenzyme of *E. coli* RNA polymerase consists of subunits β, β', α, and the σ subunit of *Micrococcus luteus* RNA polymerase possesses less efficient promoter specificity for φχ174 RFI fragment DNA containing gene E. In this case *M. luteus* RNA polymerase used a promoter in the gene E region of φχ174 which is usually not recognised well by hybrid *E. coli* RNA polymerases containing the *M. luteus* σ factor. Therefore, the function of the sigma subunit for initiation of transcription is concerned with species specificity (Ernst, *et al.*, 1982).

4.4 Compatibility of plasmids

Plasmids carrying the p15A replicon rather than the ColE1 replicon are gaining increased interest in gene cloning. There is high DNA sequence homology with the ColE1 plasmid within the replication region of p15A and it shares other replication properties with the ColE1. It is therefore compatible with ColE1 type plasmids as they require the same DNA polymerase for replication, and they continue to replicate in the presence of protein synthesis inhibitors, for example chloramphenicol and spectinomycin. They are compatible with ColE1 plasmids such as pUC19. The prototype vectors of the plasmids are pACYC177 and pACYC184 (Chang and Cohen, 1978). Complement mutation has been applied to isolate isoleucine biosynthesis genes (*ilv*) from *Corynebacterium glutamicum*. DNA which was cut with Sau3A was cloned in the BamHI site of cosmid pHC79 and transformed into *E. coli*. Threonine dehydrogenase (TD), acetohydroxy acid synthase (AHAS)

and isomeroreductase (IR) catalyse three subsequent reactions in Ile synthesis. The gene *ilvC* (IR) is located directly downstream from *ilvB* (AHAS) (Cordes, *et al.*, 1992).

4.4.1 pUC19

One of the most commonly used pUC derivatives for general cloning purposes is pUC19. It is a small high copy number plasmid of 2686 base pairs in length. Nucleotide numbering starts at the first T in the sequence ...TCGCGCGTTT... It carries a 54 base pair multicloning site polylinker. The ColE1 replicon site is at position 867 (Miller, 1992).

4.4.2 pACYC177

The low copy number plasmid pACYC177 was constructed by ligating restriction fragments from three different sources: Tn903, Tn3 and p15A. The nucleotide sequence of pACYC177 which is 3940 base pairs in length has been published. Nucleotide number 1 of pACYC177 is the first G of the unique HincII site GTTGAC. Tn903 contains a Kanamycin resistance determinant which extends from base 1922 (ATG) to 2734 flanked by 226 base pairs inverted repeats which are derived from plasmid R6-5. The p15A origin of replication extends from base 766 to 1595 (Rose, 1988).

4.4.3 pACYC184

pACYC184 contains the same p15A replicon as pACYC177 but was constructed by ligating restriction fragments from pSC101, Tn9 and p15A. The length of this plasmid is 4244 base pairs. The chloramphenicol resistance segment was from Tn9. Bases 1494 to 3275 are derived from pSC101 which contains a tetracycline resistance gene. The whole nucleotide sequence has been published (Rose, 1988). Nucleotide number 1 of pACYC184 is the first G of the unique EcoRI site GAATTC.

Because pUC19 and plasmid pACYC share common replication factors, there is the possibility to have two types of plasmids contained in the same host for the co-expression of different genes which originate from different sources.

4.5 Carotenoid biosynthesis genes

crtB which encodes for phytoene synthase in *E. herbicola* is homologous to the *pys* gene of *Synecococcus* PCC 7942 and its product is a single polypeptide enzyme which catalyses the formation of phytoene from GGDP. *Pds* codes for phytoene desaturase which catalyses the four step desaturation from phytoene to lycopene. The arrangement of the gene *pds* followed by *pys* in the same operon is similar to that of *crtI* and *crtB* in *R. capsulatus* and *E. uredovora.* In higher plants such as tomato, *pys* was found to be highly conserved with that of pTom5: a tomato cDNA recombinant. This gene was expressed differentially during fruit ripening (Chamovitz *et al.*, 1992). mRNA was at a low level when the tomato was immature. The level of mRNA was high during fruit ripening (Gray *et al.*, 1992). The *psy*1 encodes phytoene synthase which is a peripheral plastid membrane protein (Bartley *et al.*, 1992).

The lycopene cyclase gene (*lcy*) in *Synecococcus* PCC7942 was proved to be responsible for both cyclizations of lycopene to β-carotene via γ-carotene and it also conveyed resistance to the herbicide 2-(4-methylphenoxy)triethylamine hydrochloride MPTA (Cunningham, *et al*, 1993).

The zeaxanthin diglucoside gene cluster was first isolated from *E. herbicola* in the recombinant pPL376, which contained the zeaxanthin diglucoside biosynthesis gene cluster in pHC79 (Perry *et al.*, 1986). pAPU211 was derived from pPL376. pAPU211 contains the 12.7 kb zeaxanthin diglucoside biosynthesis gene cluster which was inserted in pUC19 at Sph and EcoRI sites which lack open reading frames 2 and 3 of the original pPL376. The frameshift mutants from this plasmid at the position 9468 had the pathway blocked at the early stage of phytoene synthase so that the transformant was buff. The other early stage of mutation at the position 8427 affected the function of phytoene dehydrogenase and also caused the transformant to be buff.

4.6 Complementation of carotenoid biosynthesis gene

The zeaxanthin diglucoside biosynthesis gene cluster recombinant and other mutants are available as are mutants of individual genes in this carotenoid biosynthesis pathway. The colour of the mutant transformant depends on the step that is blocked. If the blockage is in the early stage, the mutants are buff. Mutants in which gene expression is blocked in the later stages than phytoene dehydrogenase are coloured ranging from pink, yellow to orange. Complement mutation is one possibility to isolate the complement gene from the other species to study in detail. The complement gene for a mutant blocked in an early stage of carotenoid biosynthesis would reverse the mutation and produce a wild type phenotype. Therefore, carotenoid biosynthesis genes could be identified from other species. An interesting example of gene combination for carotenoid biosynthesis from different species has been produced. Known *crt* genes from *E. herbicola* were transferred to and expressed in *Rhodobacter sphaeroides*. β-Carotene was the expression product from *E. herbicola crt* gene cluster complemented to *R. sphaeroides* and was found to function in light harvesting (Hunter, *et al.*, 1994).

In order to screen carotenoid biosynthesis genes responsible for the early pathway in *R. maris* strain N1020, a complete genomic DNA library was constructed. The cloned DNA fragments in the library should cover the whole genomic DNA. Under the conditions used the desired probability (P) that a unique DNA sequence is represented in the necessary number of recombinant (N) plaques is given by

$$N = \ln (1 - P)/\ln (1 - f)$$

where f is the fractional proportion of the genome in a single recombinant and is derived from the average size of the DNA fragment inserted into the vector divided by the size of total genome.

In order to construct the genomic DNA library, Sau3A is the common enzyme preferred for the digestion of genomic DNA to give fragmented inserts. It

produces sticky ends which can be ligated in the cloning site of BamH1 available vectors.

4.6.1 Transformation

The introduction of recombinant DNA into bacteria by transformation is an essential step in the generation of recombinant clones. Electrotransformation by means of a pulse of high voltage electric discharge to render cells permeable to DNA has revolutionized the transformation of bacteria (Chassy *et al.*, 1988). Electrotransformation is a highly efficient method. In the M13 cloning system electrotransformation has proved to be 1000 times more efficient than the conventional $CaCl_2$ method (Heery and Dunican, 1989).

Electrotransformation of *Rhodococcus facians* by non-replicating plasmids containing a suitable marker resulted in stable transformants by integration of these constructs at various sites in the genome, thereby generating different mutations. Tagged genes could be isolated in *E. coli* because of the presence of a ColEI replicon and an ampicillin resistance gene in the inserted sequences (Desomer, *et al.*, 1991). In *Corynebacteria*, transformation efficiency depends on many factors such as electric field strength and pulse length, DNA concentration, physiological stage and concentration of competent cells (Bonamy, *et al.*, 1990). In a gram positive bacterium, *Corynebacterium glutamicum*, transformation efficiency could be increased by freezing and thawing the cells twice, and the yield was approximately seven times better than with the conventional electroporation when the cells were pulsed at the cell density of 1-3 x 10^8 cells/ ml (Wolf *et al.*, 1989). Transformation through protoplasts was achieved with plasmid DNA and the transformation efficiency was 10^7 cells/ µg DNA (Dunican and Shivnan, 1989). The phase of the cell cycle is another factor that influences transformation efficiency. In *E. coli*, transformation was normally carried out at mid-log phase. In eukaryotes, such as mouse cells, the G_2/ M phase is the most efficient period for stable gene transfer by electrotransformation (Yolifuji, *et al.*, 1989).

Conventional transformation can be achieved by simple chemical treatment. The most efficient chemicals for competent cell preparation are DMSO and $CaCl_2$. Transformation of ligation mixture by DMSO treated cells has been proved to improve transformation efficiency in the absence of cations (Hill, *et al.*, 1991). In the work reported in this thesis, $CaCl_2$ treatment technique was chosen to prepare competent cells for transformation because of the simple manipulation. Transformation efficiency was calculated as follows

$$\text{Transformation efficiency [TE]} = \frac{\text{number of transformants/ ml}}{\text{1 µg DNA/ ml}}$$

4.7 Results

4.7.1 The growth of *Rhodococcus maris* strain N1020

Rhodococcus maris strain N1020 was grown at 30°C which is the optimum temperature. It is an aerobic strain and cannot grow under anaerobic conditions. Illumination of 2000 lux was used to stimulate carotenoid production in the culture. The growth curve is shown in Figure 4.1. Lag phase was 1-8 days. Log phase was in days 10-11. The stationary phase was days 12-14. Culture colour developed from yellow in the lag phase to orange in the stationary phase.

4.7.2 Total genomic DNA library of *Rhodococcus maris* strain N1020 construction

Total genomic DNA was cut with the restriction enzyme Sau3A at a concentration of 0.03 u/ µg DNA. The Sau3A fragmented DNA was ligated in the BamHI site of pACYC184 in molarity ratios of 1:1, 4:1 and 8:1. The electrophoresis pattern of the DNA is shown in Figure 4.2. Lane 1 represents the uncut genomic DNA of *R. maris* strain N1020. When the total genomic DNA was cut with the restriction enzyme Sau3A at 0.03 u/ µg, the main fragmented DNA migrated to a position corresponding to 9-12 kb (lane 2) compared with the 1 kb marker in lane 4. After phenol/ chloroform purification of the fragmented DNA, the whole purified

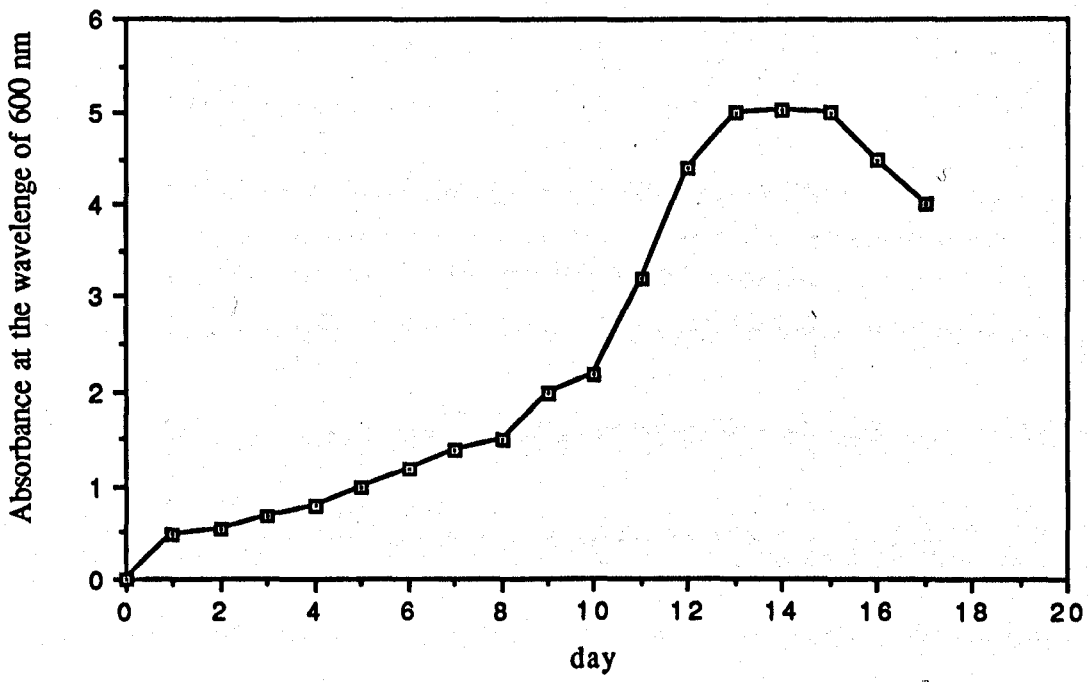


Figure 4.1: Growth curve of *Rhodococcus maris* strains N1020

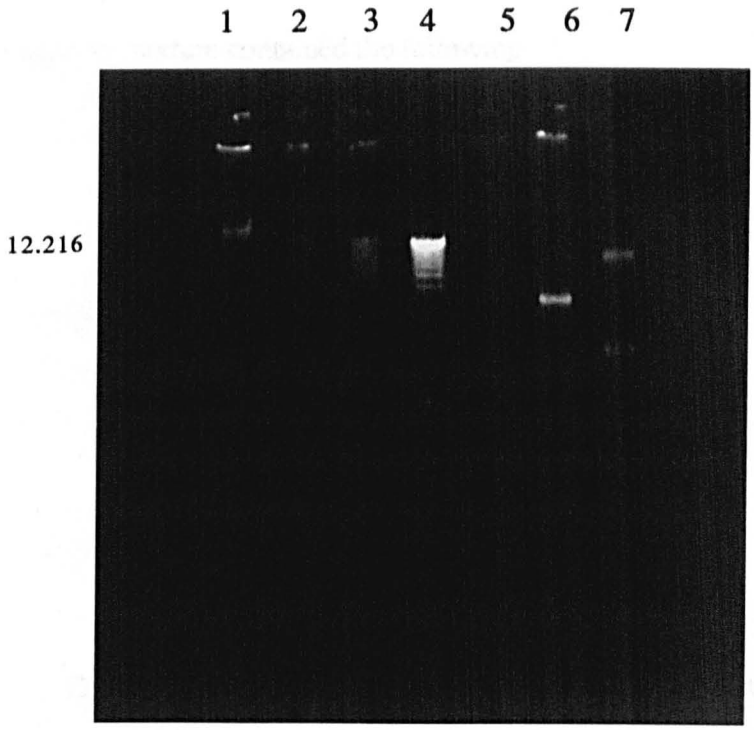


Figure 4.2: Agarose gel electrophoresis illustrating partial genomic digestion of *R. maris* strain N1020 and pACYC184 cloning vector

(1) Total genomic DNA of *R. maris* strain N1020
(2) Genomic DNA of *R. maris* strain N1020 cut with Sau3A 0.03 u/ µg before purification
(3) Genomic DNA of *R. maris* strain N1020 cut with Sau3A 0.03u/ µg after purification
(4) 1kb marker ladder
(5) pACYC184 cut with BamHI after CIP
(6) pACYC184 cut with BamHI before CIP
(7) pACYC184 uncut

Sau3A fragmented DNA migrated a little more rapidly than the unpurified one (lane 3). The purified fragmented DNA was cloned in pACYC184 which was cut with BamHI and dephosphorylated with calf intestinal phosphatase (lane 5). Dephosphorylated pACYC184 did not migrate significantly differently to the non-dephosphorylated one (lane 6).

Each ligation mixture contained the following

ddH2O	x	µl
vector (100 ng)	1	µl
fragmented DNA	y	µl
100 m*M* DTT	3	µl
10 m*M* ATP	3	µl
ligase (10 u/ µl)	1	µl
total	30	µl

The reaction was incubated at 14°C overnight. Portions of the overnight ligation mixture (1 µl, 3 µl or 5 µl) were then transformed by $CaCl_2$ treatment into *E. coli* DH5α. The transformation procedure was described in Chapter 2 (II.9.2). 1,250 transformants were kept each in 5 ml of LB medium as a *R. maris* strain N1020 genomic library which was maintained in 20% glycerol at -70°C.

4.7.3 Partial genomic DNA library construction of *Rhodococcus maris* strain N1020

Total DNA of *R. maris* strain N1020 was completely digested with BamHI, EcoRI or HindIII. The purified fragmented DNA of interest was cloned. Figure 4.3 illustrates the electrophoretic behaviour of the fragmented genomic DNA of *R. maris* strain N1020 after digestion with those enzymes. Lanes 2, 4 and 6 represent *R. maris* strain N1020 restricted with BamHI, EcoRI and HindIII, respectively, at a concentration of 10 u/ µg DNA at 37°C overnight. The fragmented DNA was compared with a 1 kb marker. The highly positive regions which resulted from

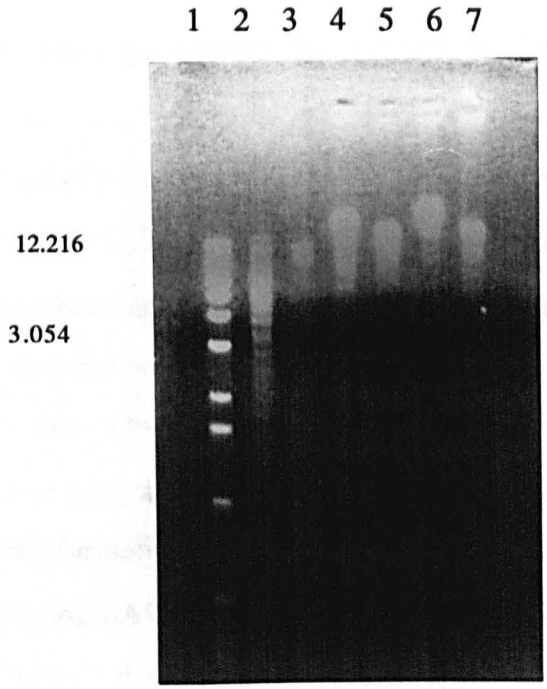


Figure 4.3: Agarose gel electrophoresis showing recovery of genomic DNA of *R. maris* strain N1020 at the highly conserved region for *crtBI* and *crtE*

(1) 1 kb ladder
(2), (4), (6) DNA cut with BamHI, EcoRI and HindIII before purification
(1), (3), (7) DNA cut with BamHI, EcoRI and HindIII after purification

southern blot hybridization will be mentioned in section 4.7.6. The DNA bands of interest which corresponded to the positive region of the *crtE, crtB* and *crtI* DNA probes were cut from the agarose gel and purified on the Geneclean system according to the manufacturer's instructions. Lanes 3, 5 and 7 show the purified *R. maris* DNA which was fragmented from BamHI, EcoRI and HindIII respectively. The purified fragmented DNA was then ligated to the low copy number plasmids pACYC184 and pACYC177 and the high copy number plasmid pUC19. The BamHI fragmented DNA was cloned in the BamHI site of pACYC184. The EcoRI fragmented DNA was cloned in the EcoRI site of pACYC184 but the HindIII fragmented DNA was cloned in the HindIII site of pACYC177 because the pACYC184 HindIII cloning site is not in the antibiotic marker region which is used for insertional inactivation. Transformants were screened with suitable antibiotics.

The ligation was carried out as mentioned in section 4.7.2. Portions of the ligation mixture (1, 3 or 5 µl) were transformed into 100 µl $CaCl_2$ treated competent cells DH5α. The transformation procedure was described in Chapter 2 (II.9.2). Transformants containing pACYC184 recombinants were selected on LB plates containing chloramphenicol (25 µg/ ml) plates and transformants containing pACYC177 were selected on LB containing ampicillin (50 µg/ ml). The transformation procedure was repeated until no more ligation mixture was left. The transformation efficiency was 2.1 x 10^5. Unfortunately, no colour transformants were seen. Transformation efficiency was 1.2 x 10^4/ µg and 2 x 10^5/ µg respectively.

In order to prove the success of the cloning technique, ten fresh pACYC177 recombinant transformants were randomly chosen and inoculated individually in 5 ml LB containing ampicillin (50 µg/ ml) and incubated at 37°C overnight at 250 rpm. The overnight culture was then used for plasmid minipreparation. Each recombinant plasmid was cut with restriction enzyme to check the presence of plasmids and insert as shown in Figure 4.4. Lanes 4, 6, 8 and 10 show randomly chosen recombinants between pACYC177 and fragmented DNA from *R. maris* strain N1020 in the HindIII site. All recombinants migrated significantly slower than pACYC177 alone (lane 2).

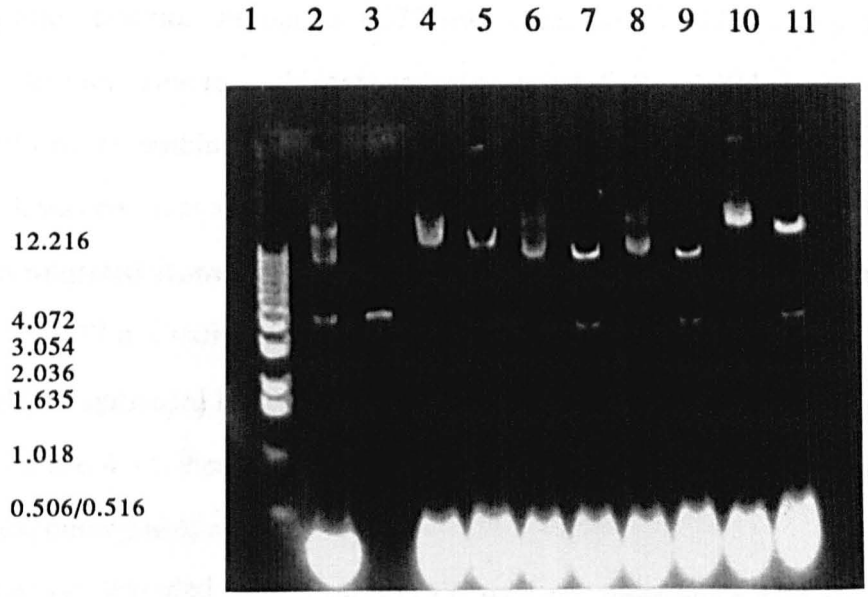


Figure 4.4: Agarose gel electrophoresis illustrating the presence of inserted *R. maris* strain N1020 DNA at the cloning site of HindIII of pACYC177

(1) 1kb ladder
(2) pACYC177 uncut
(3) pACYC177 cut with HindIII
(4), (6), (8), (10) recombinants between *R. maris* and pACYC177
(5), (7), (9), (11) recombinants from (4), (6), (8), (10) cut with HindIII respectively

In lane 2, there were four bands present at the positions similar to the 2.036, 4.072, 11.198 and 12.216 kb of the marker (lane 1). The first two bands should be pACYC177 monomers and the other two multimers because their linear form at the HindIII site showed only a single band at the migration position similar to that of the 4.072 marker band (lane 3). Therefore, the DNA band with migration position similar to the 2.036 marker should be a covalently closed circle. The DNA band at the migration position similar to 4.072 was linear pACYC177. Lanes 5, 7, 9 and 11 represent the same recombinants as from lanes 4, 6, 8 and 10 but cut with HindIII (5 u/ 100 ng recombinant DNA). Each lane shows two discrete DNA bands any of which was constant at the position similar to linear pACYC177 in lane 3 and the other band migrated slower than linear pACYC177. The constant band was from vector pACYC177 cut with HindIII and the other should be from *R. maris* strain N1020 HindIII fragmented DNA which was cloned into the HindIII site of pACYC177. In lanes 2 and 4-11, there was a major component which migrated very fast to a position corresponding to lower than 506 bp. This was a RNA band which was absent in lane 3 as it was degraded by RNAse A (10 u/ μg). The other lanes were from the plasmid minipreparation that had not been treated with RNAse.

The *R. maris* strain N1020 fragmented DNA was cloned in pUC19 at the BamHI, EcoRI and HindIII multicloning sites. Each ligation mixture was composed of vector and the purified corresponding restriction enzyme fragmented DNA in the proportion 1:1, 1:4 or 1:8. The ligation reaction is described in detail in section 4.7.2. 1, 3 or 5 μl of each ligation mixture was transformed to $CaCl_2$ treated competent DH5α (100 μl). Details of the transformation were described in Chapter 2 (II.9.2). The transformation efficiency was 1.93×10^4. Transformation was carried out until no more ligation mixture was left. 2,013 colonies were collected for the *R. maris* strain N1020 genomic library in a total volume of 3 ml LB. The library was kept in 20% glycerol at -20°C.

4.7.4 Complement mutation of the carotenoid biosynthesis genes

Ligation mixture with vector from pACYC was transformed to pAPU211 mutant transformants containing pAPU211B. pAPU211B is the mutant which is deficient in phytoene synthase. In order to obtain colour transformants for reverse gene mutation, complement mutation was applied. The transformants were selected on LB containing ampicillin (50 μg/ ml) and chloramphenicol 25 μg/ ml. The former antibiotic was the marker in pAPU211B recombinants and the later antibiotic was in pACYC184. Two orange colonies were found. Minipreparation of these two colonies showed two different types of plasmids, one similar in migration to pAPU211B the other migrating a little slower than the pACYC184. However, after purification of recombinant pACYC184 from these two colonies and retransfomation to pAPU211B, there were no coloured colonies to be seen. However, only co-transformant colonies can grow in the ampicillin and tetracycline resistance plates.

4.7.5 Probe preparation

4.7.5.1 Preparation of *crtBI* probe

The *crtBI* probe was derived from pAPU211 which contains the zeaxanthin diglucoside biosynthesis gene cluster which is 12.753 kb in length. It contains the six genes *crtB*, *crtE*, *crtI*, *crtX*, *crtY* and *crtZ*. All the nucleotide sequences have been elucidated. SmaI for which the recognition site is CCCGGG, was used to cut pAPU211. Three product bands were obtained which were 1.271, 1.725 and 9.757 kb as shown in Figure 4.5 (lane 3). The smallest band (1.271 kb) was purified and used as a *crtBI* probe. It contains downstream *crtI* gene 732 bp and upstream *crtB* gene 523 bp in length with 16 bp between these two genes. This smallest DNA band was then purified by Geneclean. Purified *crtBI* was then used for *crtB* and *crtI* probe preparation. In order to remove the intervening sequence, purified *crtBI* was double digested with NcoI and PvuII. The product fragments are 16 bp, 230 bp, 279 bp, 278 bp and 468 bp. The product corresponding to 230 bp was part of *crtI* and the product at the position corresponding to 468 bp was part of *crtB*. These were then further purified by Geneclean as shown in Figure 4.6. Lane 5 was the 468 bp fragment of

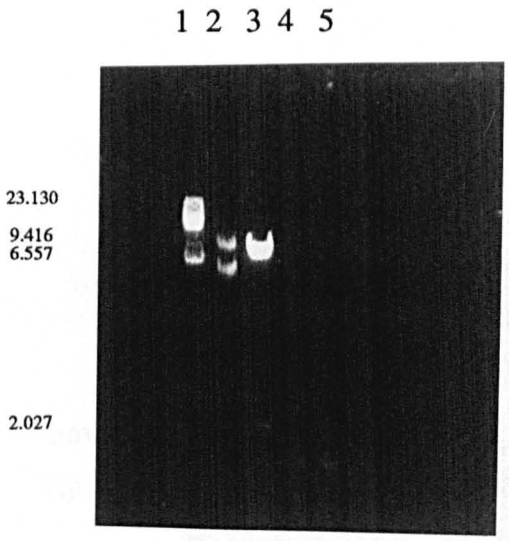


Figure 4.5: Agarose gel electrophoresis showing *crtBI* probe preparation

(1) λ HindIII marker
(2) uncut pAPU211
(3) pAPU211 cut with SmaI 5 u/ μg DNA
(4) SmaI *crtBI* which is 1.271 kb after purification with Geneclean (10 ng)
(5) SmaI *crtBI* the same as in lane 4 but 20 ng

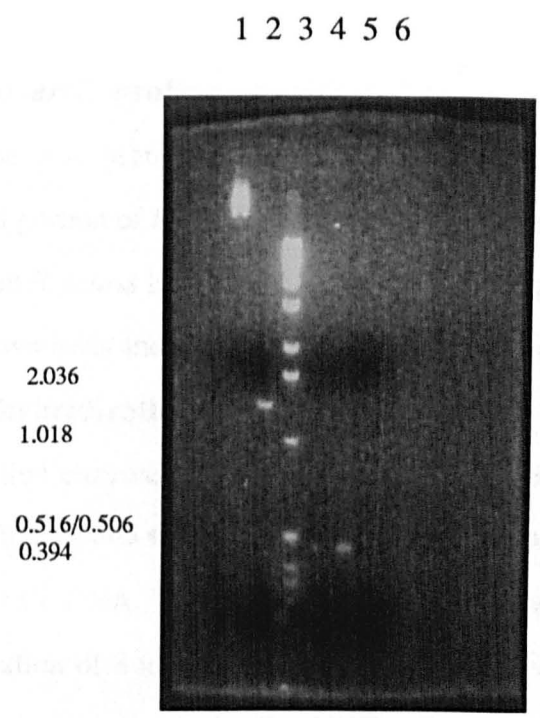


Figure 4.6: Agarose gel electrophoresis showing probe preparation from pAPU211

(1) pAPU211 uncut
(2) *crtBI* 1.271 kb
(3) 1 kb marker
(4) 1.271 kb from lane 2 cut with NcoI and PvuII
(5) *crtB* 468 base pairs
(6) *crtI* 230 base pairs

crtB and lane 6 was the 230 bp fragment of *crtI*. They were both used for probe preparation.

Therefore, three kinds of probe preparation were derived from the *E. herbicola* genes. They were *crtBI*, *crtB* and *crtI* probes which were all used for hybridization.

4.7.5.2 Preparation of *crtE* probe

The *crtE* probe was prepared by PCR from the primers which were designed from the central portion of *E. herbicola crtE*. A 20-mer primer was used for amplification of *crtE* from *R. maris* by PCR. The PCR product was 324 bp in length. Details of the *crtE* DNA synthesis and sequence will be given in Chapter 5.

4.7.6 Southern blot hybridization

The main restriction enzymes used for restriction of *R. maris* strain N1020 genomic DNA were BamHI, EcoRI and HindIII. Each reaction contained 10 μg of *R. maris* strain N1020 genomic DNA. The genomic DNA was cut with the restriction enzymes at the concentration of 8 u/ μg DNA in a total volume of 100 μl at 37°C overnight. The overnight restriction reaction product was partitioned by 1% agarose gel electrophoresis (50 v) overnight. The electrophoresis pattern of the fragmented DNA is shown in Figure 4.7. Lanes 1-3 represent the fragmented DNA distribution. These were cut with the single restriction enzymes BamHI, HindIII and EcoRI, respectively. The fragmented DNA migrated faster than intact genomic DNA (lane 11). The majority of the fragmented DNA in lane 2 migrated more slowly than that in the other two lanes. Lane 1, corresponding to genomic DNA which was restricted with BamHI, and the product showed a molecular weight less than the 23 kb marker. In lane 2, corresponding to genomic DNA digested with HindIII, the main products had lower mobility than 23 kb. Lane 3, contained genomic DNA cut with EcoRI, and the main product migrated between 9 and 23 kb. Lanes 4, 6 and 7 show fragmented DNA which was digested by two restriction enzymes. Lane 4 shows fragmented DNA cut with BamHI and EcoRI. The fragmented DNA migrated in a wide range between 2 and 12 kb, compared to the 1 kb marker represented in lane 5 and faster

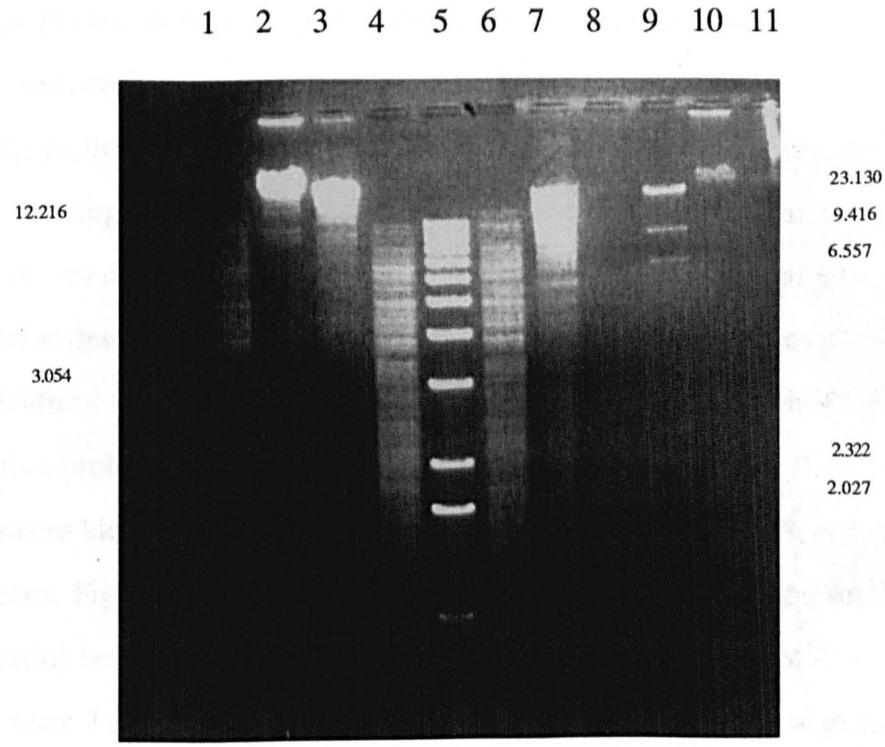


Figure 4.7: Agarose gel electrophoresis showing genomic DNA of *R. maris* strain N1020 cut with various restriction enzymes

(1) Genomic DNA cut with BamHI
(2) Genomic DNA cut with HindIII
(3) Genomic DNA cut with EcoRI
(4) Genomic DNA cut with BamHI and EcoRI
(5) 1 kb ladder
(6) Genomic DNA cut with BamHI and HindIII
(7) Genomic DNA cut with HindIII and EcoRI
(8) -
(9) λ HindIII marker
(10) Total genomic DNA of *E. coli*
(11) Total genomic DNA of *R. maris* strain N1020 without cutting

than the single digestion product from BamHI or EcoRI. Lane 6 shows genomic DNA cut with BamHI and HindIII, and the main fragmented DNA migrated faster than that from single digestion with BamHI or HindIII but the fragments were not much different from fragmented DNA derived from BamHI and EcoRI double digestion shown in lane 4. Lane 7 shows genomic DNA double restricted with HindIII and EcoRI the main fragmented DNA migrated between 9 and 23 kb, compared to the λ HindIII marker in lane 9 and migrated more rapidly than the products of single restriction with HindIII or EcoRI. The DNA was transferred to hybron N^+ membrane by capillary transfer with a mobile phase of 0.4 *M* NaOH overnight as described in Chapter 2 (II.10.1). The fixed membrane was prehybridized and hybridized with different probes. The protocol for southern hybridization with radioactive probes and markers has been described in Chapter 2 (II.10.2-II.10.4). The southern blot was hybridized with various probes which are *crtB*, *crtI*, *crtBI* and *crtE* probes. Figure 4.8 illustrates the electrophoretic behaviour of the southern blot hybridization between the *crtE* probe and fragmented genomic DNA of *R. maris* strain N1020. Lane 3 illustrates the positive region of genomic DNA cut with EcoRI; the mobility of their fragments was slower than that of the 12 kb marker. Lane 4 represents genomic DNA cut with BamHI and EcoRI; the positive band migrated a little more slowly than 3.054 kb. Lane 6 illustrates genomic DNA restricted with BamHI and HindIII, and has a positive band at a position of 12.216 kb. Lane 7 shows genomic DNA restricted with EcoRI and HindIII, and gives positive bands at about 12 kb. Lane 2 represents genomic DNA digested with HindIII; the positive band migrated more slowly than 12 kb. This highly positive region from the southern blot was in the same area with the *crtBI crtB* and *crtI* probes. The highly positive fragmented DNA regions from the southern blot were then considered for use in cloning.

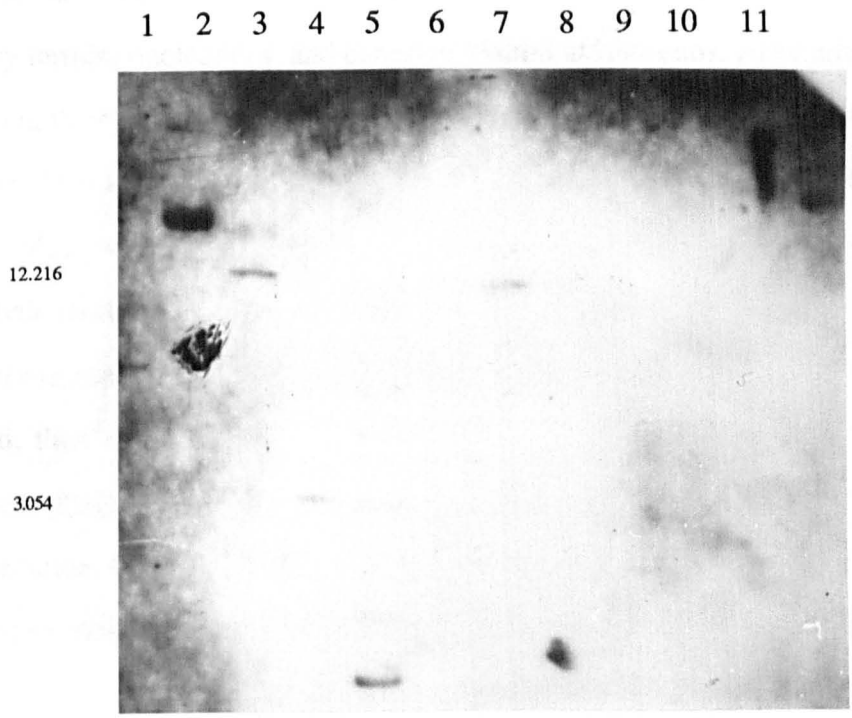


Figure 4.8: Southern blot of *R. maris* strain N1020 with *crtE* probe
(1) Genomic DNA cut with BamHI
(2) Genomic DNA cut with HindIII
(3) Genomic DNA cut with EcoRI
(4) Genomic DNA cut with BamHI and EcoRI
(5) 1 kb ladder
(6) Genomic DNA cut with BamHI and HindIII
(7) Genomic DNA cut with HindIII and EcoRI
(8) -
(9) λ HindIII marker
(10) Total genomic DNA of *E. coli*
(11) Total genomic DNA of *R. maris* strain N1020 (1 μg) without cutting

4.8 Construction and sceening of *Rhodococcus maris* strain N1020 genomic DNA library in λ bacteriophage

4.8.1 Introduction to bacteriophage

Lambda bacteriophage DNA gt11 is a linear DNA molecule approximately 50 kb in length, and is double stranded in the central portion and has single stranded complementary termini nucleotides, and cohesive termini at both ends. After entering a host bacterium, the cohesive termini are matched by base pairing to form a circular DNA molecule. This DNA molecule functions as a template for transcription in the early stage of infection. During infection, either lytic mode or lysogenic mode can occur. In the lytic mode, the circular viral DNA in the bacterial host is replicated and numerous genes of the bacteriophage are expressed. Progeny bacteriophage particles are assembled, then the cells lyse and many phage particles are released. In the lysogenic mode, phage DNA is integrated into the host chromosome at the specific site of recombination. It is subsequently replicated and transmitted to progeny bacteria like any other chromosomal gene. Only *c*I gene which codes for repressor of phage is expressed.

In order to clone larger sized DNA fragments, lambda bacteriophage GEM-11 has been applied as a cloning vector for genomic DNA of *R. maris* strain N1020. Lambda bacteriophage GEM-11 is 43 kb in length and is derived from EMBL3. This vector contains a multiple cloning site. When BamHI is used to restrict the vector, this gives three portions which are known as left arm, right arm and stuffer. Left arm is approximately 20 kb, stuffer is 14 kb and right arm is 9 kb. BamHI is an isochizomer enzyme with Sau3A which is the most common enzyme is applied for partial digestion of genomic DNA. Fragmented foreign DNA is then introduced as a replacement DNA in the central stuffer region. The successful recombinants should therefore contain left arm, fragmented foreign DNA and right arm. The left arm contains head and tail genes A-J, and the right arm contains genes from PpR through cosR sites which are essential for lytic growth of bacteriophage.

4.8.2 *Rhodococcus maris* strain N1020 genomic DNA library in bacteriophage GEM-11 and screening for carotenoid biosynthesis genes

DNA from *R. maris* strain N1020 (100 µg) was partially digested with Sau3A (0.001 u/ µg) for 30 minutes at 37°C. The digestion product was partitioned on 0.8% agarose gel. Fragmented DNA of sizes between 9 and 23 kb was then cut and embedded to the 1% agarose gel in the uv band elutor E91 gel tank. Gel electrophoresis was performed at 100 V. DNA solution was collected every 10 minutes from the focusing solution well which had been preloaded with focusing solution (20% v/v sucrose, 10% v/v 3 *M* sodium acetate, 1 x TAE). Aliquots (400 µl) of DNA solution were transferred to sterile eppendorf tubes and DNA in each eppendorf was precipitated with 40 µl of 3 *M* sodium acetate pH 6 and 1 ml of -20°C absolute ethanol at -20°C for 1 hour. After centrifugation, the pellet was washed with 0.5 ml 80% ethanol, dry at 50°C for 5 minutes and redissolved in 5 µl TE and accumulated for further cloning. 1 µg of fragmented DNA was ligated to Lambda GEM-11 BamHI arms in the proportion of 1:1 and 2:1. The ligation mixture, in a total volume of 10 µl, was composed of vector, fragmented DNA of *R. maris* strain N1020, 1 x ligation buffer and T4 DNA ligase (20 u). The reaction was left at 14°C overnight and the products were analysed by electrophoresis as shown in Figure 4.9. Lane 2 represents the three fragments of lambda GEM-11 BamHI arms. The smallest band migrated similar to 9.416 kb marker (lane 1) and should be the right arm which is 9 kb. The middle band was the stuffer fragment and the largest band which migrated was like the 23.130 kb lambda HindIII marker was the left arm (20 kb). Lane 5 represents purified 9-23 kb partial Sau3A *R. maris* strain N1020 genomic DNA which was used as the replacement fragment for the stuffer. The ligation products are shown in lanes 3 and 4. Lane 3 arose from the ligation of fragmented DNA vector in the proportion of 1:1; the ligation product migrated a little slower than purified fragmented DNA. Lane 4 represents the ligation products from the fragmented DNA and vector in the proportion of 2:1; the main ligation product

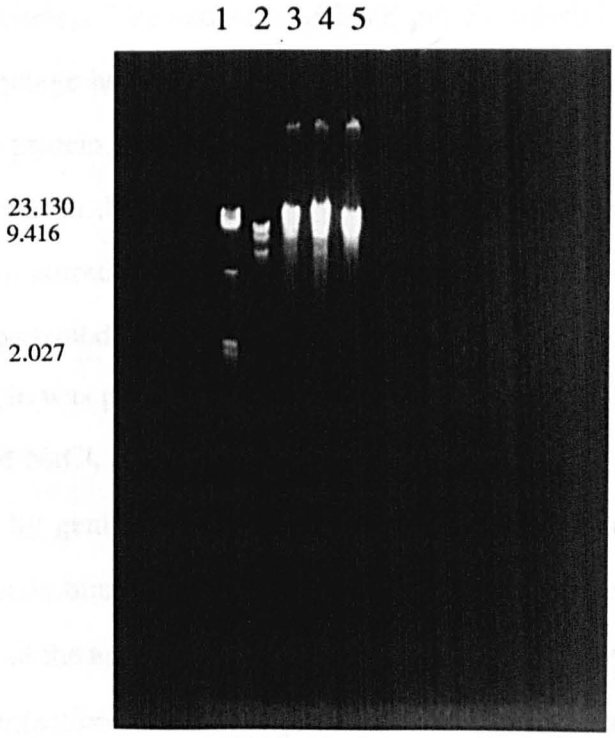


Figure 4.9: Agarose gel electrophoresis of the ligation mixture between lambda GEM-11 BamHI arms and *R. maris* strain N1020 DNA fragmented with Sau3A

(1) λ HindIII marker
(2) λ GEM-11 BamHI arms
(3) ligation mixture between *R. maris* strain N1020 Sau3A fragmented DNA and λ GEM-11 BamHI arms in the proportion of 1:1
(4) ligation mixture between *R. maris* strain N1020 Sau3A fragmented DNA and λ GEM-11 BamHI arms in the proportion of 2:1
(5) purified partial Sau3A DNA of *R. maris* strain N1020

migrated slower than the ligation product from the proportion of 1:1 in lane 3. These results show the success of the ligation procedure. The ligation mixture analysed in lane 4 (8 µl) was added to 50 µl of a fresh packagene extract thawed on ice. The reaction was left at 22°C for 4 hours. Packaging of bacteriophage *in vitro* used a mixture of extracts. The extract contains E protein which is the major component of the bacteriophage head and is required for the assembly of the earliest identifiable precursor D protein. D protein is localised on the outside of the bacteriophage head and is involved in the coupling process of the DNA insertion into the prehead and subsequent maturation of the head. The A protein is involved in the insertion of bacteriophage lambda DNA into the concatenated precursor DNA at the cos sites. The phage particle was purified by adding 445 µl of phage buffer (20 m*M* Tris.HCl pH 7.4, 100 m*M* NaCl, 10 m*M* $MgSO_4$) and 25 µl chloroform. The phage mixture was then mixed by gentle inversion and the phases were allowed to separate at 4°C. Packaged recombinant phage in the aqueous phase was serially diluted from 10^{-1}-10^{-4}. 100 µl of the appropriate dilution was added to 100 µl of prepared *E. coli* strain LE392 for infection and for the propagation of their recombinants. Infection took place at 37°C for 30 minutes. 200 µl of the infected cells were added to 3 ml of 50°C molten agarose (0.5% NaCl, 1% tryptone, 0.8% agarose) in a 30 ml sterilin tube. The suspension was shaken a few times and immediately poured onto LB plate prewarmed to 37°C. The plate was left to settle in the tissue culture cabinet, then inverted and incubated at 37°C overnight. Two different bacterial hosts were selected. Both strains are *hsdR*. LE392 is a *rec*$^+$ *hrdR* strain (Nader *et al.*, 1985). KW251 is a tetracycline resistant and F$^-$ strain.

Free phage particles can infect bacteria in the lawn and cause lysis of the host cell. The ratio between phage and bacteria in the infection procedure is important and the parameter of infection multiplicity (*m*) is taken from the formula

$$\text{multiplicity } (m) = \frac{\text{number of phage particles}}{\text{number of bacteria}}$$

as describe by Bainbridge (1987).

In order to get successful plaque development, serial dilutions of phage were manipulated for infection of various bacterial strains to give optimum plaque development per plate, *i.e.* about 200-300. Successful plaque development plates were accumulated. To the surface of each plate was added 3 ml of SM buffer (0.01% gelatin, 50 m*M* tris.HCl pH 7.5, 100 m*M* NaCl and 5 m*M* $MgSO_4$) and the top part of the agar was scraped off and collected in a sterilin tube. In the tube, the agar was cut into small pieces with a spatula and left at room temperature for 2 hours. The recombinant phage library was collected by centrifugation for 30 minutes at 9,000g at 4°C and purified by addition of 10 ml of chloroform and leaving at 4°C. After 1 hour, the organic phase was collected and another 5 ml of chloroform added. The library was then kept at 4°C for up to 6 months for further study. For one-year storage, the library was kept in 0.01% gelatin and 7% v/ v DMSO at -70°C. Two *R. maris* strain N1020 genomic library constructions were prepared. The first library was composed of 1,043 plaques and the other of 1,958 plaques. The efficiencies of recombinant phage packaging were 3.5×10^2 and 2.6×10^3 pfu/ μg, respectively. The control lambda *c*1857 *Sam*7 positive control DNA was 2×10^5 pfu/ μg. Genomic DNA libraries in phage were then amplified by serial dilution and used to infect the bacterial hosts LE392 and KW251. Well separated plaque formation plates (between 200-250 plaques per plate) were then transferred to membrane for screening with specific probes. The efficiency of recombinants was 5×10^3 pfu/ ml. Probes were prepared from pAPU211 DNA and *crtE* PCR product of *R. maris* strain N1020. The probe which was prepared from pAPU211 was composed partly of *crtB* and partly *crtI* as described in section 4.7.5.1. The probe was labelled with non radioactive digoxigenin-11-dUTP as detailed in Chapter 2 (II.11).

Each positive plaque was then lifted and redissolved in 1 ml SM buffer. The mixture was left at room temperature for 2 hours (Sambrook *et al.*, 1989). A few drops of chloroform were added and the mixture was kept at 4°C. Portions (1, 10 and 100 μl) of the aqueous phage preparation were then reinfected into *E. coli* strain

LE392 for further identification. No positive plaques were obtained from the reinfection. Approximately 9,000 plaque units were screened in 47 pieces of 66.5 cm^2 membranes.

4.9 Discussion

The colour of culture during cell growth ranged from yellow in the lag phase to orange in the stationary phase, suggesting that carotenoid accumulation was different in the different stages of cell growth.

For complement mutation of competent mutants containing pAPU211B with recombinant pACYC184 the antibiotic markers of both types of plasmids were present so transformants were grown in ampicillin and tetracycline plates. This shows the possibility of expressing genes from different species through these compatible plasmids.

From southern blot hybridization between probes derived from *E. herbicola* and *R. maris* strain N1020, positive bands were obtained in various fragmented DNA cut with different restriction enzymes. This showed homology between *R. maris* and *E. herbicola* in carotenoid biosynthesis genes *crtB* and *crtI*. The probe derived from *crtE* amplified from *R. maris* strain N1020 by PCR gave a positive region the same as with the *E. herbicola crtBI* probe. This would be expected if these genes are in the same cluster.

Molecular cloning of *R. maris* strain N1020 genomic DNA in the plasmids pACYC177, pACYC184 and pUC19 in order to clone the whole carotenoid biosynthesis gene cluster seems to be difficult as no coloured colonies were seen. This might be because

(1) the probability of cloning the whole gene cluster is low because of the fragment of *R. maris* DNA which had been cloned were relatively small therefore, the three genes (geranylgeranyl diphosphate synthase, phytoene synthase and phytoene dehydrogenase) which are essential and correspond to the early enzyme of carotenoid biosynthesis, were not completely cloned.

(2) the whole gene cluster responsible for the carotenoid biosynthesis which is cloned was not transformed to the host

(3) one or more parts of genes may have been cut off with restriction enzyme before cloning so that the residual gene encoded for a polypeptide which was then not able to function as an enzyme

(4) the clones of these three genes were not in the transcription frame so that the reading strand was converted to an antisense strand, or the orientation of plasmid RNA transcription was opposite to the reading strand of the insert *R. maris* strain N1020

(5) the genes in the cluster do not have the same orientation of transcription

In the molecular cloning of *R. maris* strain N1020 fragmented DNA in λ bacteriophage GEM-11 BamHI arms, the ligation mixture migrated more slowly than the arms and fragmented DNA. Therefore, ligation was successful. Plaque lift to hybron N^+ membrane and hybridization between *crtBI* probe and plaque gave no positive plaques. Approximately 9,000 plaque units may not have been enough to cover all possibilities of recombinant plaque formation.

Chapter 5

PCR Cloning and Sequencing of *crtE* from *Rhodococcus maris*

5.1 Introduction to the polymerase chain reaction

The polymerase chain reaction (PCR) is an ideal method for amplifying *in vitro* a specific gene sequence of interest by simultaneous primer extension of the complementary strands. The procedure generates the product in a short time for further manipulation. Conditions such as template primer, reaction buffer, temperature and time for amplification need to be established in each case. DNA Taq polymerase is the most popular enzyme to use for amplification in the PCR (Taylor, 1993). It has thermostability and can still function at temperatures as high as 94°C so the temperature needed for the denaturation step in the PCR cycle will not affect the enzyme activity. It is an ideal enzyme for DNA sequencing as it reduces risk of premature termination at sequences with secondary structure and diminishes discrimination against dideoxy nucleotide analogues (Innis, *et al.*, 1988).

In most cases, the quantity and ratio of template and primer are crucial. Amplification of the gene of interest requires two primers. Each primer is antisense to the template. The annealed template-primer leaves the 3'OH of the primers to polymerize and the polymerization chain directions are opposite to one another but both chains polymerize toward the central portion of the amplifying gene. Ideally polymerization finishes at the end of the other primer. The amplified gene product from one primer is a single polynucleotide which is complementary to the other product from the other primer. Therefore, the end products from both primers are blunt double-stranded DNA. The PCR products can also themselves act as templates for the following cycles. Each primer length should be between 2 and 24 nucleotides, For complementarity between template and primer, at least three nucleotides at the 3' end should be matched completely with the template (Sommer and Tautz, 1989). Therefore, a mixed primer can be used to amplify the gene of interest. Degenerate

oligo-deoxyribonucleotide primers were used successfully to amplify immunoglobulin genes (Le Boeuf, *et al.*, 1989). From the known zeaxanthin diglucoside biosynthesis gene sequences in *E. herbicola* (Armstrong, *et al.*, 1992), *crtE* gene expression has been further studied with the substrate radiolabelled [^{14}C]-isopentenyl diphosphate. This gene was shown to be responsible for geranylgeranyl diphosphate synthase as labelled GGDP but not phytoene accumulated (Math *et al.*, 1992). In the present work, *crtE* amplified from *R. maris* strain N1020 by PCR was identified and used as a carotenoid biosynthesis probe to screen for a *crt* gene cluster from *R. maris* strain N1020. Primers for this amplification were designed from the central portion of the *E. herbicola crtE* gene; the sequences were

(a) reading strand

5'-GCCGAGCTGCGCCGCGGTCA-3'

(b) antisense strand

5'-CGGCGACGAGGCGGAAGCAA-3'

5.2 Results

5.2.1 Template and primers

A very small amount of template was needed for amplification in the reaction. Different amounts of genomic DNA of *R. maris* strain N1020 (100 ng, 50 ng and 25 ng template) were used for product yield comparison. All reactions were carried out in a total volume of 50 µl with other constant components, 200 n*M* of each primer, 1 x PCR buffer plus 15 m*M* $MgCl_2$ and 5 u Taq DNA polymerase. The highest PCR product yield was obtained when 25 ng of the template was used. As shown in Figure 5.1, the product displayed in lanes b and c showed two discrete bands at the migration position a little slower than 310 bp but faster than 603 bp of $\phi\chi$174 DNA HaeIII marker.

5.2.2 Reaction buffer for PCR

The standard buffer for the PCR reaction contains 100 m*M* Tris.HCl (pH 8.3), 500 m*M* KCl and 0.1% gelatin. In order to get the highest product yield from

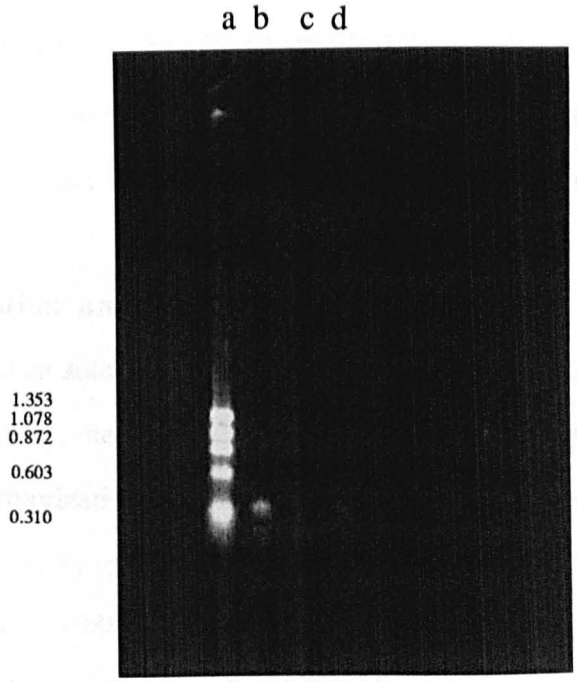


Figure 5.1: Gel electrophoresis showing the relationship between template concentration and *crtE* PCR product yield from *R. maris* strain N1020

(a) $\phi\chi$174 DNA HaeIII marker
(b) template 25 ng/ reaction
(c) template 50 ng/ reaction
(d) template 100 ng/ reaction

PCR, different $MgCl_2$ concentrations (5 m*M*, 15 m*M* and 25 m*M)* were tested for amplification of the *crtE* gene from *R. maris* strain N1020. Figure 5.2 represents the comparison of PCR product yield for the various concentrations of Mg^{2+}. Lanes b, c and d showed some PCR product with two bands at the same position as 310 bp and 234 bp compared to $\phi\chi174$ HaeIII marker. The highest yield was from the reaction which contained 25 m*M* $MgCl_2$ shown in lane b. The highest product yield amplified from pAPU211 was obtained when 15 m*M* $MgCl_2$ was used as shown in Figure 5.3, lane 2.

5.2.3 Temperature, duration and cycle

The thermocycler is an automatic machine which was used for amplification of the gene of interest. Normally, the reaction cycle consists of a denaturation stage, an annealing stage and a polymerization stage. Amplification was carried out for about 25-35 cycles. More cycles, *e.g.* 40 up to 60, can cause a multimeric high molecular weight product (Saiki, *et al.*, 1988; Wang, *et al.*, 1994). The temperature for denaturation of DNA strands was 94°C and the time used for this cycle was 30 seconds. The annealing temperature was 40-50°C and the time used for this stage was about 30-50 seconds to allow the primer to anneal with the complementary DNA sequence. The polymerization reaction was normally carried out at 72°C for 30-60 seconds. Successful *crtE* amplification from *R. maris* strain N1020 and pAPU211 gave products of 324 bp in length. This was achieved with a denaturation temperature of 94°C for 50 seconds, annealing temperature as 50°C for 45 seconds and polymerization at 72°C for 30 seconds. The result of electrophoresis of the PCR product is shown in Figure 5.3. Lane 3 represents the *crtE* PCR product yield from 10% of the total volume 50 µl reaction. The reaction mixture contained 0.2 µ*M* of each primer, 25 ng *R. maris* strain N1020, 100 n*M* dNTP mix, Taq polymerase 5 u, in standard buffer plus 25 m*M* $MgCl_2$. It gave a higher product yield than the one amplified from pAPU211 (shown in lane 2) and both gave only one clear single band that migrated a little slower than the migration position of $\phi\chi174$ HaeIII 310 bp. Lane 2 represents *crtE* PCR product yield from 10% of the reaction in 50 µl. The reaction

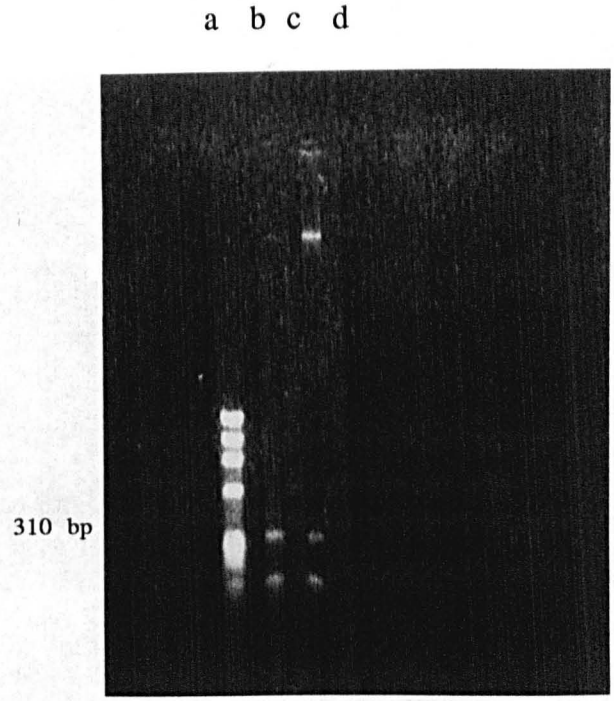


Figure 5.2: Gel electrophoresis showing effect of $MgCl_2$ concentrations on PCR product yield

(a) $\phi\chi$174HaeIII marker
(b) 25 m*M* $MgCl_2$
(c) 15 m*M* $MgCl_2$
(d) 5 m*M* $MgCl_2$

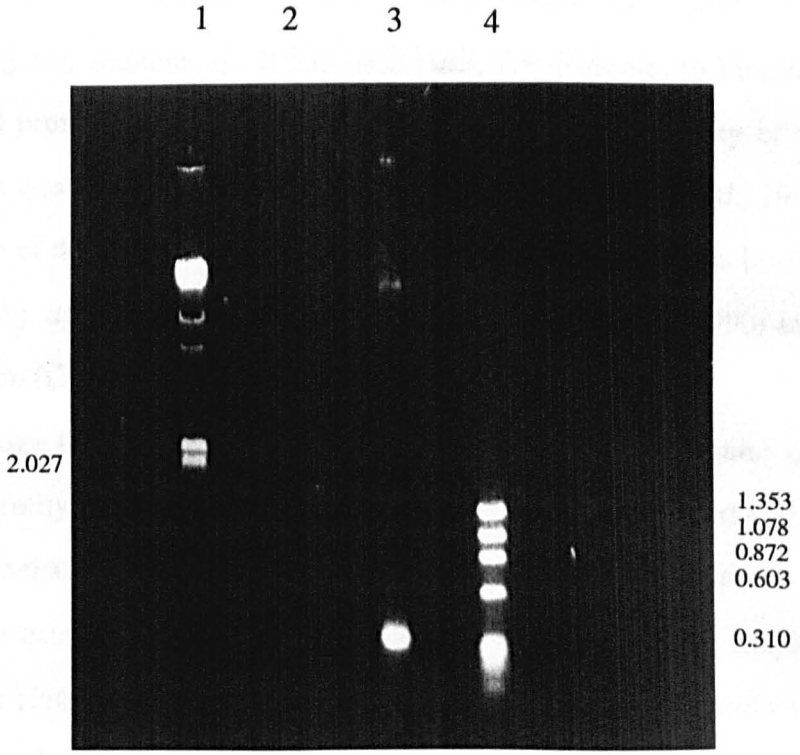


Figure 5.3: Gel electrophoresis showing the PCR product from the *crtE* genes

(1) λHindIII marker
(2) PCR product from pAPU211
(3) PCR product from *R. maris* strain N1020
(4) φχ174 HaeIII marker

contained 25 ng pAPU211, 0.2 μ*M* of each primer, 100 n*M* dNTP mix, Taq polymerase 5 u, in standard buffer plus 15 m*M* $MgCl_2$.

5.2.4 Cloning of the PCR product

PCR is an efficient method for amplification, isolation and identification of a gene by cloning and sequencing. It has been used, for example, to identify the human ribosomal protein S6 gene *hRPS6* (Pata *et al.*, 1992). Variability of rRNA genes in crustacea was studied by PCR cloning and sequencing (Kim, *et al.*, 1993).

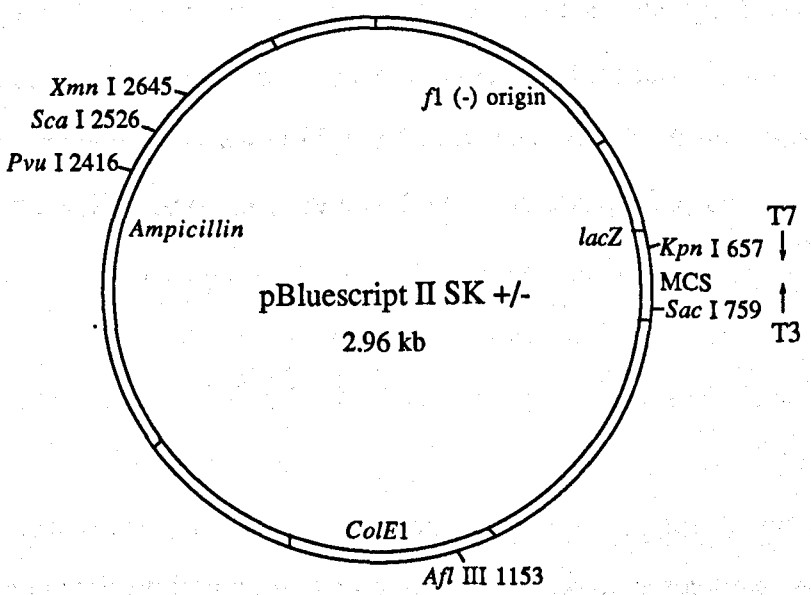
Cloning of the PCR products can be done by many methods such as blunt end ligation cloning, ligation independent cloning (Aslanidis and Jong, 1990) and the T-A cloning system (Clark, 1988; Hadjeb and Berkowitz, 1996).

In the alga *Cyanophora paradoxa,* the *crtE* nucleotide sequence was identified. The identity and similarity scores corresponding to amino acid sequence data compared with that of the photosynthetic bacterium *Rhodobacter capsulatus* were 28.6 and 68.5%, respectively (Michalowski *et al.*, 1991). Difficulties are frequently encountered when cloning PCR products because Taq polymerase can add a single nontemplate directed deoxyadenosine (A) residue to the 3' ends of duplex PCR product (Clark, 1988). A simple method using T4 DNA polymerase treatment after PCR amplification was one of the methods performed. Its 3',5' exonuclease activity eliminated the A overhang of the double-stranded DNA. However, self ligation was employed (Wang, *et al.*, 1994). To overcome the problem with cloning, a strategy for adding a T residue at the 3' hydroxy groups at both blunt ends of the vector was proposed. 3' ddT-tailed vector can be ligated directly to a PCR product that contains a 3' overhang A by forming a phosphodiester bond between the 5' phosphate of the vector and the 3'-OH group of the PCR product from the overhang A (Holton and Graham, 1990). An alternative technique, using XcmI to cut the vector to leave a T residue at the 3' end of both strands was then successfully used to clone *cop* (Copy number control) PCR amplified DNA from Staphylococcal plasmids (Kovalic, *et al.*, 1991).

5.2.5 Cloning of the PCR product by T-A cloning system

The *crtE* PCR product from *R. maris* strain N1020 was purified on 1% low melting point agarose gel. The band of interest was cut off and purified by means of a PCR Wizard column (Promega). pBluescript II SK +/- was chosen as a cloning vector for the *crtE* PCR product. This phagemid is of 2961 base pairs and is derived from pUC19. The SK designation indicates that the polylinker is oriented such that the transcription proceeds from SacI to KpnI. The multicloning site is flanked by T3 and T7 promoters. Ampicillin resistance gene is used as antibiotic selection marker. The *lac*Z gene provides α-complementation for blue/white colony selection of the recombinant phagemids. The map of this phagemid is shown in Figure 5.4. The plasmid was linearized with EcoRV in the multicloning site region to leave blunt ends. The linearized blunt ended plasmid was then added with thymidylic acid at the 3' ends to leave a T-3' overhang complementary to the A independent residue at the 3' end of the PCR product. The reaction consisted of EcoRV pBluescript II SK +/- 6 μg, 1 x PCR buffer, 1 m*M* dTTP and Taq polymerase 1 u/ μg in a total volume of 20 μl, and was incubated at 70°C for 2 hours (Marchuk, *et al.*, 1990). The purified T-overhang EcoRV pBluescript II SK +/- was then ligated with an excess amount of the purified PCR product in the ratio of 1:15. The ligation mixture was incubated at 14°C overnight. Portions (1, 3, or 5 μl) of the ligation mixture were then transformed to the *E. coli* XL1-Blue and selected for the antibiotic markers which were ampicillin and tetracycline. Ampicillin resistance was expressed from the plasmid and tetracycline resistance was expressed from the host. In the presence of IPTG/ X-gal, the white colonies represent the recombinants with their inserts in the cloning site.

The *lac* promoter is part of the *lac* operon, a gene complex comprising three structural genes Z, Y and A coding for β-galactosidase, lactose permease and thiogalactoside transacetylase, respectively. In the presence of isopropylthiogalactoside (IPTG), which is a potent inducer of but not a substrate for β-galactosidase (*lacZ*), an induced cell can easily be distinguished from a repressed one by the blue colour derived from a chromogenic indicator which was derived from



5'-AATTAACCCTCACTAAAGGG
T3 primer

5-GTAATACGACTCACTATAGGGC
T7-primer

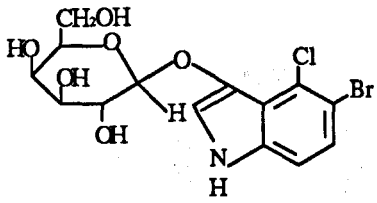
Figure 5.4: Map of pBluescript II SK +/-

non-inducing substrate analogues (X-gal). The colourless compound 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) is converted by β-galactosidase into its indoxyl derivative which in turn is oxidized to the blue dye 5,5'-dibromo-4-4'-dichloroindigo. Therefore, the colonies are blue. The reaction of colour development by β-galactosidase is shown in Figure 5.5. *E. coli* XL1-Blue transformed colonies which produce β-galactosidase are therefore easily screened by colour. Visible white colonies are obtained from insertional inactivation of the *lacZ* gene so that they contain foreign DNA in the cloning site. On the other hand, they are mutants which have recombinant plasmids (Miller, 1972). The cloning system for the *R. maris crtE* PCR product is shown in Figure 5.6.

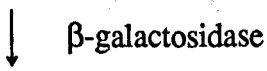
The white colonies should contain the recombinant the *crtE* gene. 10 of them were inoculated individually in 5 ml LB containing ampicillin (50 μg/ ml) and tetracycline (25 μg/ ml) and were incubated overnight at 37°C, 250 rpm. Overnight cultures were used for plasmid minipreparation. The *R. maris crtE* in pBluescript II SK +/- was then called pE. It was linearized with HindIII for size estimation by electrophoresis. Linear pE migrated a little slower than linear original pBluescript II SK +/- as shown in Figure 5.7. Lanes b and c represent pBluescript II SK +/- cut with EcoRI and HindIII. These migrated slower than 2.322 kb but faster than 4.361 kb when compared to lambda HindIII marker in lane a. Lane d represents linear HindIII pE the band position of which was a little slower than that of linear HindIII pBluescript II SK +/- in lane c. Lane e represents uncut pE, and showed two discrete bands, one migrating faster than 2.027 kb, the other slower than 2.322 kb. pE contains pBluescript and PCR insert. The former band was closed circular pE and the latter band was linear and/ or open circular pE.

5.2.6 Sequencing of the *Rhodococcus maris* strain N1020 *crtE* PCR product from the recombinant plasmid

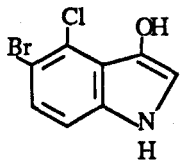
The concept of a universal primer with a combination of dideoxyribonucleotides can also be applied for sequencing double stranded DNA



5-Bromo-4-chloro-3-indolyl-β-D-galactoside
(X-gal)

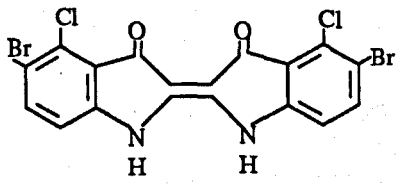


β-galactosidase



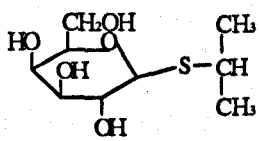
5-bromo-4-chloroindoxyl

Air



5,5'-Dibromo-4-4'-chloroindigo

Figure 5.5: Assay for β-galactosidase activity



Isopropylthiogalactoside
(IPTG)

Figure 5.5 A: Structure of isopropylthiogalactoside

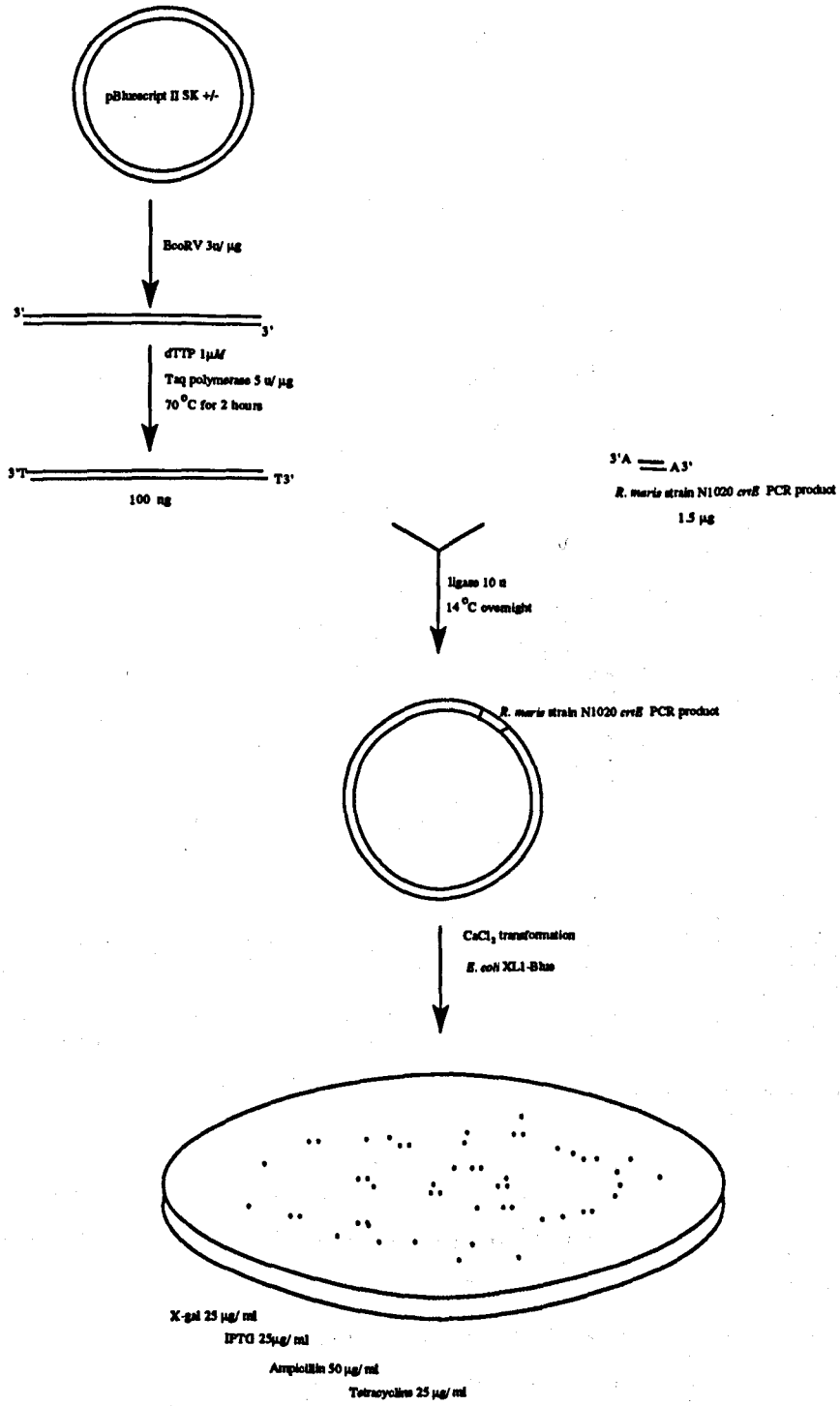


Figure 5.6: Cloning of *crtE* from PCR product

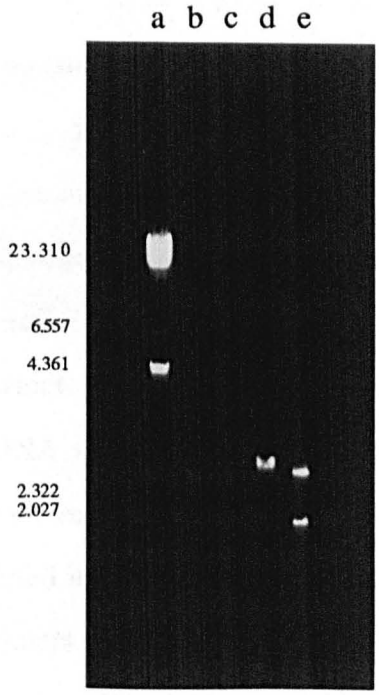


Figure 5.7: Agarose gel electrophoresis showing pE

(a) λ HindIII marker
(b) pBluescript II SK +/- cut with EcoRI
(c) pBluescript II SK +/- cut with HindIII
(d) pE cut with HindIII
(e) pE uncut

molecules. Specific DNA segments defined by the sequence of at least two oligonucleotides can be amplified by using the PCR technique. Sequencing templates can be generated from either cloned inserts or directly from the PCR product. Single stranded DNA template can be produced directly in the PCR or generated directly from double stranded DNA by enzymic treatment (Gyllensten, 1989) with the DNA holoenzyme which excludes 3'-5' exonuclease activity so that there is only one direction of DNA synthesis. The polymerization is terminated by dideoxyribonucleotides which occur randomly in the polynucleotide chains. The PCR product is ligated to the known sequence vector. Sequencing primers might be standard primers or ones which have been designed from the vector to use for sequencing the unknown DNA sequence which was slightly downstream to the primer at the cloning site of the recombinant (Reeves, *et al.*, 1995). The sequencing reaction was described in detail in Chapter 2 (II.14). The sequencing reaction was carried out by designing primers of standard T3 and T7. From the T3 primer, the possible sequence of the plasmid to be read before the inserted gene sequences at the cloning site is 5'-AACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGT GGATCCCCCGGGCTGCAGGAATTCGATT-3'. For the other primer, T7, the reading sequence of the plasmid before the cloning site is 5'-GAATTGGGTACCGGGCCCCCCCT CGAGGTCGACGGTATCGATAAGCTTGATT-3'.

The products of the sequencing reaction were partitioned by electrophoresis on 7% polyacrylamide gel. In order to get the optimal readable nucleotide sequence, the gel was electrophoresed for 2.5 hours, and 4 hours. The gel was photographed (Figure 5.8) and from this the *R. maris* strain N1020 *crtE* PCR sequence was deduced to be as follows:

10 20 30 40

5'-GCCGAGCTGC GCGGCCGTCA CCGTAGGCCT GAGAAGGTAT

50 60 70 80

CTTGATCGCG TATACGTGTT CCAAAGAGTC GAGTTCAAGC

90 100 110 120

CCAGTCTTGG TTCAACCTCG ATGGACATTA TTGCTTCCGC

130 140 150 160

TCGTCGCGAA TCGAATTCTG CAGCCGGGGG ATCCACTAGT

170 180 190 200

TCTAGAGCGG CGCACCGGGT GGAGCTCAGC TTTTGTTCCT

210 220 230 240

TTAGTGAGGG TTAATTCGAG CTTGGCGTAA TCATGGTCAT

250 260 270 280

AGCTGTTCGT GTGAATGTAT CGTCACATCA CACACATACG

290

GCGAGCATAA-3'

The nucleotide sequence from the T7 primer was also read in the *crtE* region from numbers 1- 290 and compared with the *E. herbicola crtE* sequence as follows

10 * 20 30 40

5'-GCCGAGCTGC GCGGCCGTCA CCGTAGGCCT GAGAAGGTAT

. . . . . .

5'- GCCGAGCTGC GCCGCGGTCA GCCCACTACC CACAAAAAAT

50 60 70 80

CTTGATCGCG TATACGTGTT CCAAAGAGTC GAGTTCAAGC

. . . .

TTGGTGAGAG CGTGGCGATC CTTGCCTCCG TTGGGCTGCT

90 100 110 120

CCAGTCTTGG TTCAACCTCG ATGGACATTA TTGCTTCCGC

. .. .

CTCTAAAGCC TTTGGTCTGA TCGCCGCCAC CGGCGATCTG

130 140 150 160

TCGTCGCGAA TCGAATTCTG CAGCCGGGGG ATCCACTAGT

. . . .

CCGGGGGAGA GGCGTGCCCA GGCGGTCAAC GAGCTCTCTA

170 180 190 200

TCTAGAGCGG CGCACCGGGT GGAGCTCAGC TTTTGTTCCT

. . ..

CCGCCGTGGG CGTGCAGGGC CTGGTACTGG GGCAGTTTCG

210 220 230 240

TTAGTGAGGG TTAATTCGAG CTTGGCGTAA TCATGGTCAT

. ...

CGATCTTAAC GATGCCGCCC TCGACCGTAC CCCTGACGCT

250 260 270 280

AGCTGTTCGT GTGAATGTAT CGTCACATCA CACACATACG

. . . . . .

ATCCTCAGCA CCAACCACCT CAAGACCGGC ATTCTGTTCA

290

GCGAGCATAA-3'

. .. .

GCGCGATGCT-3'

Note: the upper line sequence is from *R. maris*

the lower line sequence is from *E. herbicola*

bold letters indicate the primer sequence

dots represent homologous base pairs

star represents the misincorporation of base pairs

The base pair homology between *E. herbicola crtE* and *R. maris* strain N1020 *crtE* PCR product in the primer region is 95% (19 in 20). The comparison between these two sources started from number 21 to 290 because the sequence to be compared originated from the *R. maris* strain N1020 template. From base pair number 21 to 290, the homology between *crtE* of *E. herbicola* to the reading PCR sequence is 27% (72 in 270). The guanosine plus cytosine from the *R. maris* strain N1020 *crtE* PCR sequence is 50.7%.

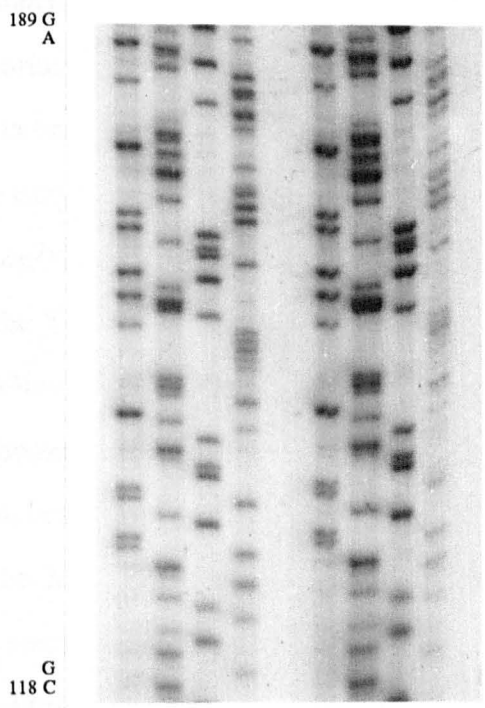


Figure 5.8: Partial nucleotide sequence of the *crtE* PCR product from *R. maris* strain N1020

5.3 Discussion

The *R. maris* strain N1020 genomic DNA which was used as template for amplification of *crtE* with the designed primer from *E. herbicola* by PCR gave a product indicating that it was satisfactory to use primers from the central portion of *crtE* to amplify. These gave high product yields compared with homologous *E. herbicola* itself. The highest yield was obtained from the lowest template DNA concentration. This may allow homologous primer to bind template in the later cycles were efficiently with low genomic DNA concentration than with excess genomic DNA which contained other genes unrelated to *crtE*. Template primer concentration is one of the factors to be considered in order to get good PCR product yield.

All DNA polymerase enzymes require the presence of divalent metal cations for activation of the reaction. Mg^{2+} was considered to be important for the enzyme in the polymerization reaction. The 5'-3' DNA polymerase activity required Mg^{2+}. The *R. maris crtE* PCR product on low Mg^{2+} salt concentration gave low yield and was hardly seen in the gel electrophoresis. Medium Mg^{2+} salt gave a higher product yield than lower Mg^{2+} salt but the highest product yield was from high salt concentration. pAPU211*crtE* PCR gave the highest product yield from medium Mg^{2+} salt concentration. Taq DNA polymerase is sensitive to free Mg^{2+} concentration. Low Mg^{2+} concentrations have an additive effect on the fidelity of DNA synthesis *in vitro* (Eckert, and Kunkel, 1990).

The PCR product gave a background which was in the low molecular weight band. This may be because the annealing of primer to target DNA was downstream to the replication complex in the later PCR cycles when amplified template is abundant for replication to take place and there is more chance to obtain the annealing complex at positions downstream from the replication complex. Therefore, PCR should not be carried out for more than 30 cycles.

From the reading sequence of the *crtE* PCR product, the primer sequence, which should be totally the same as the design primer, showed 95% homology. This may be because Taq DNA polymerase is an enzyme which lacks 3'-5' exonuclease

activity so that it has no proofreading activity. Therefore, the misincorporation of dNTP can occur without editing. The undesirable mutation is caused by substitution (Ho, *et al.*, 1989). By direct sequencing of the PCR amplified DNA from different individuals, three point mutations have been found in a 220 base pairs fragment from the promoter region of the human *cystatinC* gene (Balbin and Abrahamson, 1991).

The similarity of the nucleotide sequence between *R. maris* strain N1020 and *E. herbicola* was 27% which is low. It may be that the central portion of the enzyme polypeptide is not important, so the primer did not represent a consensus part of the *crtE* gene. The homology of *crtE* between *E. herbicola* and *R. maris* strain N1020 is low. The *crtE* gene from *E. herbicola* was isolated by PCR and cloned into *E. coli* expression vector pARC306N. *E. coli* JM101 was transformed with these expression recombinant plasmids and the DNA sequence of this gene was identified. The deduced amino acid sequence was compared with that from other species. The putative coding sequences of geranylgeranyl diphosphate synthase *crtE* are similar to those of *E. uredovora* and *Neurospora crassa* (Math, *et al.*, 1992), whilst the deduced amino acid sequence of *Cyanophora paradoxa* showed about 29% identity and 69% similarity with the amino acid sequence of the *crtE* gene from *R. capsulatus* (Michalowski, *et al.*, 1991). However, the similarity of *crtE* between *R. maris* strain N1020 and *E. herbicola* which is 0.267 is significantly higher than the probability of obtaining the same level of homology with 270 base pairs by random occurrence, which is very low $(0.25)^{270}$. Therefore, it can be concluded that this PCR product can be used as a probe for the *crtE* gene.

The percentage G-C content from the *R. maris crtE* PCR product was 50.7. This is not as high as the previously reported *Rhodococcus* DNA base composition for which G-C content is 61-72% (Zakrzewska-Czerwinska, *et al.*, 1988). This may be because the G-C content was calculated from the PCR product which was from only 270 base pairs, *i.e.* very few compared to the total genomic DNA. On the other hand, the *R. maris* strain N1020 G-C content may be different from the majority of DNA in the genus *Rhodococcus*.

Plan of future work

There are a few possible ways in which to extend this project making use of the part of the nucleotide sequence of *crtE* of *Rhodococcus maris* strains N1020 which has already been isolated and identified. The most likely way of isolating the canthaxanthin biosynthesis gene cluster is using the linker ligation technique. The sequence of the polylinker on either side of the gene can be used for the design of one of the primers for amplification of the unknown regions of the *crtE* gene by PCR. The other primers can be designed for synthesis in both directions from the known sequence of *crtE*. If the PCR reaction gives products, they will be cloned and the nucleotide sequenced extended especially upstream of the start codon for promotor identification. The promoter region will provide information for the criteria of the most suitable cloning vectors for canthaxanthin biosynthesis gene expression. The oxygenase gene will then be isolated and cloned in the pACYC type plasmids for complementation of pAPU211 in the *E. coli* system for astaxanthin production.

The oxygenase gene from *R. maris* strains N1020 will be important in research aimed at the elucidation of the mechanism of astaxanthin configuration formation in the microbiology system.

Appendix

1) oligolabelling buffer contained solution A:B:C = 2:5:3

solution A:

2 *M* tris.HCl pH 8	625 µl
5 *M* $MgCl_2$	25 µl
H_2O	350 µl
2-mercaptoethanol	18 µl
100 m*M* dATP	5 µl
100 m*M* dGTP	5 µl
100 m*M* dTTP	5 µl

solution B:

2 *M* HEPES pH 6.6

solution C:

Hexadeoxyribonucleotides at 90 OD unit/ ml

2) 100 x Denhardts solution:

2% (w/v) bovine serum albumin

2% (w/v) ficoll

2% (w/v) polyvinylpyrrolidone

3) 10 x PCR buffer

100 m*M* tris.HCl

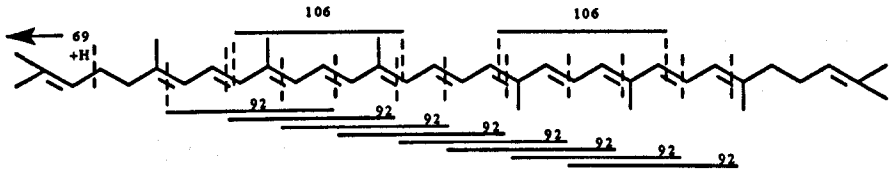
500 m*M* KCL

5 m*M* $MgCl_2$ (low concentration)

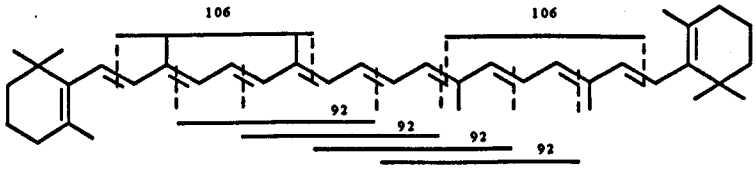
15 m*M* $MgCl_2$ (medium concentration)

25 m*M* $MgCl_2$ (high concentration)

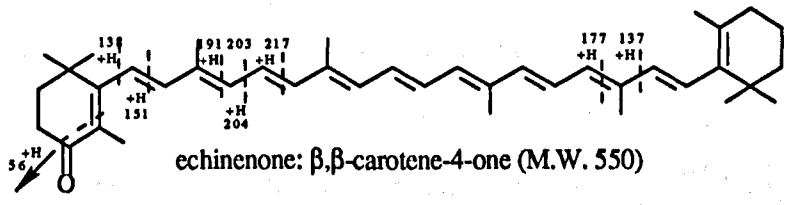
1 mg/ ml gelatin



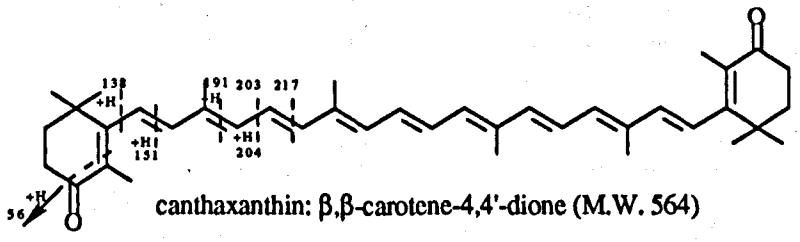
lycopene (M.W. 536)



β carotene: β,β-carotene (M.W. 536)



echinenone: β,β-carotene-4-one (M.W. 550)



canthaxanthin: β,β-carotene-4,4'-dione (M.W. 564)

Mass spectrometric fragmentation of some carotenoids

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