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Synthesis of isotopically labelled carotenoids; investigations on structure and function of carotenoproteins at the atomic level

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Abstract - A three-part strategy has been developed to study molecular interactions in biological systems. Isotopically labelled carotenoids or other substances are prepared by organic total synthesis in specific isotopically labelled form. These small molecules are used to assemble the active biological system, which is then studied by physical techniques. A general synthetic scheme for the synthesis of carotenoids, both symmetrical and asymmetrical, selectively labelled with ^{13}C at predetermined positions in the C_{10}-central part, is presented. The synthetic scheme is based on the $C_{15} + C_{10} + C_{15}$ strategy. For symmetrical carotenoids the C_{10}-dialdehyde, 2,7-dimethylocta-2,4,6-triene-1,8-dial **1**, is used. For asymmetrical carotenoids two novel synthons are introduced: 2,7-dimethylocta-2,4,6-triene-8-alnitrile **2** and N-methoxy-N,2,7-trimethylocta-2,4,6-triene-8-alamide **3**. The use of these synthons is illustrated by the synthesis of labelled β-carotene **4**, astaxanthin **5** and spheroidene **6**. Some of the structural and functional results that have been obtained by this strategy in the fields of photosynthesis and vision, are given.

INTRODUCTION

In many of the important processes in which carotenoids are involved, the carotenoids are non-covalently bound to proteins as protein complexes. These complexes are known as carotenoproteins when the carotenoids are bound stoichiometrically to the protein. Two of the most important examples of carotenoproteins can be found in photosynthesis. A carotenoid protects the photosynthetic reaction centre by quenching singlet oxygen directly or by quenching the excited triplet state of the chlorophyll special pair (photoprotection) (ref. 1). In the light-harvesting or antenna complexes carotenoids act as accessory light-harvesting pigments (photoreception) (ref. 2). Another well-known class of carotenoproteins is found in marine organisms. An example of this group is α–crustacyanin, the blue 320 kDa carotenoprotein of the common lobster (ref. 3). A common feature of these carotenoproteins, of which nearly all have astaxanthin as the prosthetic group, is that binding induces a bathochromic shift in the absorption spectrum of the chromophore, leading to highly coloured purple, blue and green protein complexes. The mechanism of this shift has not yet been elucidated.

In order to understand fully the processes in which protein-bound carotenoids are involved, detailed information is needed on structure and electronic surroundings of the carotenoid in the carotenoprotein. A strategy has been developed by us in collaboration with others, to obtain information at the atomic level on intact and functionally active proteins (ref. 4). This strategy consists of three steps:

1) preparation of carotenoids highly enriched with stable isotopes (^2H and ^{13}C) at predetermined positions,

2) reconstitution of the labelled carotenoids with carotenoid-depleted protein and protein complexes, yielding the carotenoproteins with specifically labelled prosthetic groups,

3) study of the isotopically labelled protein complexes by non-destructive, non-invasive isotope-sensitive spectroscopic techniques like high resolution solid state NMR (refs. 4-9) and resonance Raman spectroscopy (refs. 4, 10, 11).

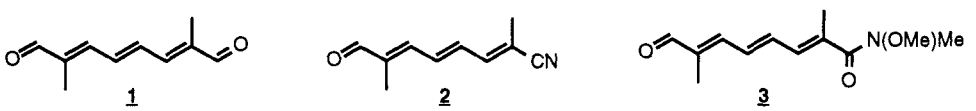
The great advantage of this strategy is that atomic resolution is achieved with intact and functionally active proteins. No changes in steric and electronic properties are introduced by isotope labelling, since native protein complexes also contain carotenoids labelled at the natural abundance level (e.g. for ^{13}C 1.1%).

This strategy was successfully used in the elucidation of the detailed structure of retinal in the visual pigment bovine rhodopsin and the light-driven proton pump bacteriorhodopsin. First, retinals were synthesized with high levels of isotopic enrichment at specific positions. These retinals were incorporated into the pigments by reconstitution of the retinals with the respective opsins. The labelled proteins were

subsequently studied by magic angle spinning (MAS) NMR and resonance Raman spectroscopy (refs. 4-11).
The three-step label strategy was successfully used for the first time with a labelled carotenoid in the study of the structure of the carotenoid spheroidene in the photosynthetic reaction centre of *Rhodobacter sphaeroides 2.4.1*. There was disagreement between the data obtained by X-ray crystallography of the crystallized membrane protein (refs. 12, 13) and data obtained by resonance Raman spectroscopy (refs. 14-16). Crystal structure data favoured an *E*-structure around the central 15,15'-double bond, while resonance Raman spectroscopy data favour a *Z*-structure. A labelled spheroidene ([14'-^{13}C]spheroidene) was synthesized and incorporated in the photosynthetic reaction centre. Studies with MAS NMR on the reconstituted system provided strong evidence for a *Z*-configuration of the 15,15'-double bond (refs. 17,18).
This approach can be applied generally. We need therefore a general method to prepare carotenoids that are isotopically labelled. Besides their utility in carotenoproteins, access to isotopically labelled carotenoids is also important to study the metabolism of carotenoids and their role in nutrition etc.

SYNTHESIS OF ISOTOPICALLY LABELLED CAROTENOIDS

Chemical synthesis allows for labelling at specific positions with high isotopic enrichment. There are four constraints placed on the synthesis of labelled carotenoids:
1) there are very few isotopically enriched starting materials available that are economically feasible. These are simple C_1, C_2 and C_3 starting materials like paraformaldehyde, acetone, acetic acid, acetonitrile and methyl iodide. As ^{13}C labelled starting materials we use 99% ^{13}C enriched methyl iodide and [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]acetic acid, ethyl [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]bromoacetate and [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]acetonitrile. For ^{2}H enriched carotenoids, commercially available deuterated water, lithium aluminium deuteride, lithium deuteride and [2-$^{2}H_3$]acetonitrile can be used as starting materials.
2) incorporation of the label has to take place as late in the reaction scheme as possible to prevent loss of label during subsequent steps.
3) no scrambling of the label should occur: no exchange of label with other positions in the molecule is allowed.
4) no isotope dilution should occur: no exchange of label with solvent and/ or reagents is allowed to take place: the product should have the same isotopic enrichment as the starting materials.
For the synthesis of 14'-^{13}C and 15'-^{13}C spheroidene (the first ^{13}C-labelled carotenoid that has been reported) we used a consecutive synthetic method for the introduction of the ^{13}C label (refs. 17, 19). In this paper a general convergent synthetic method to introduce ^{13}C labels in the central part of carotenoids is discussed. This synthesis is based on the C_{15} + C_{10} + C_{15} scheme for the preparation of carotenoids. For the preparation of the central part of carotenoids the easily available 2,7-dimethylocta-2,4,6-triene-1,8-dial **1** is the synthon of choice. However, specifically ^{13}C labelled **1** can in general only be used for the preparation of central ^{13}C labelled carotenoids with identical end-groups. It is even not useful for the preparation of carotenoids with identical end-groups that are asymmetrically labelled in the central part and the end-groups.
In this paper we introduce two novel C_{10}-synthons: 2,7-dimethylocta-2,4,6-triene-8-alnitrile **2** and N-methoxy-N,2,7-trimethylocta-2,4,6-triene-8-alamide **3**. Specifically ^{13}C labelled **2** and **3** are the synthons of choice to prepare any carotenoid that is specifically labelled in the central part, and thus allow the preparation of both symmetrical and asymmetrical carotenoids with ^{13}C labels on any position and combination of positions in the carbon skeleton. The synthetic schemes have first been optimized by using non-labelled materials.



1 2 3

Fig.1: The three central C_{10}-subunits: 2,7-dimethylocta-2,4,6-triene-1,8-dial **1**, 2,7-dimethylocta-2,4,6-triene-8-alnitrile **2** and N-methoxy-N,2,7-trimethylocta-2,4,6-triene-8-alamide **3.**

In this paper we present the optimized routes by which it is possible to synthesize labelled C_{10}-central units. As examples of the synthesis of symmetric carotenoids labelled in the central part, the synthesis of [15-^{13}C]β-carotene **4a** and astaxanthin **5a** is presented. The synthesis of asymmetric carotenoids is illustrated by the synthesis of [15-^{13}C]spheroidene **6a** and [15'-^{13}C]spheroidene **6b**. One of the great advantages of this scheme is that two labelled carotenoids can be synthesized from one labelled asymmetric C_{10}-unit; in this case both of the labelled spheroidenes **6a** and **6b** were synthesized from [4-^{13}C]2,7-dimethylocta-2,4,6-triene-8-alnitrile **2a.**

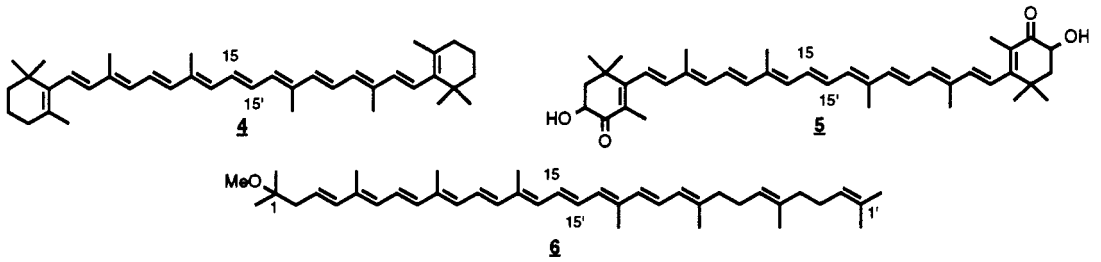
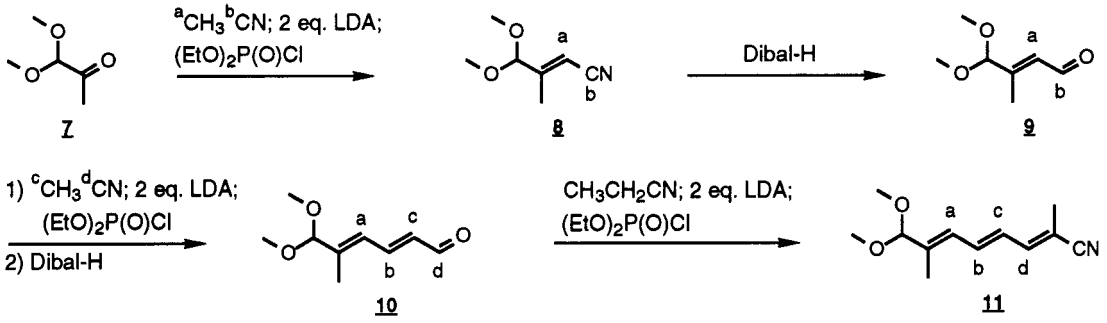


Fig.2: Structure of β-carotene **4**, astaxanthin **5** and spheroidene **6**.

SYNTHESIS OF THE ^{13}C LABELLED C_{10}-CENTRAL UNITS

For the synthesis of the three C_{10}-synthons labelled in the central part, we use one common scheme, starting from acetonitrile (scheme 1). This is dissolved in THF and deprotonated with one equivalent of lithium diisopropylamide (LDA) in the presence of another equivalent of LDA at -80°C. At the same temperature, one equivalent of diethyl chlorophosphate in THF is slowly added. The anion of acetonitrile replaces the chloro-group giving diethyl cyanomethylphosphonate, which is immediately deprotonated by the second equivalent of LDA. In this way, acetonitrile is efficiently converted *in situ* into the reactive anion of diethyl cyanomethylphosphonate (ref. 20). At 0°C, this reacts in a Horner-Wadsworth-Emmons (HWE) reaction with pyruvaldehyde dimethyl acetal **7** to give the C_5-nitrile **8**. After purification by flash chromatography, **8** is reduced with 1.1 equivalents of diisobutylaluminium hydride (Dibal-H) in petroleum ether at -70°C. After careful hydrolysis at 0°C with a silica/ water slurry and purification by flash chromatography, aldehyde **9** is obtained in a yield of 84% in two steps. This two-step procedure is repeated. Again, acetonitrile is converted *in situ* into the anion of diethyl cyanomethylphosphonate; this is coupled in a HWE reaction to aldehyde **9** giving the nitrile which, after purification, is reduced to aldehyde **10** with 1.1 equivalents of Dibal-H at -70°C. The yield of aldehyde **10** is 68% after two steps. With a slight modification this procedure is repeated. Instead of acetonitrile, propionitrile is used: this is deprotonated with one equivalent of LDA and is reacted with diethyl chlorophosphate at -80°C to give 2-(diethylphosphono)propionitrile, which is immediately deprotonated by the second equivalent of LDA similarly to the *in situ* formation of the anion of diethyl cyanomethylphosphonate described above. It is reacted in a HWE reaction with aldehyde **10** at 0°C. Work-up and purification gives the protected C_{10}-nitrile **11** in 85% yield.
By using [2-^{13}C]acetonitrile in the second coupling reaction, **11a** (c= ^{13}C) is obtained specifically labelled at the 3-position in an overall 58% yield relative to the labelled acetonitrile. This route gives access to **11** labelled at any of the central positions and any combination of positions when the corresponding labelled acetonitriles are used in the two coupling reactions.

Scheme 1

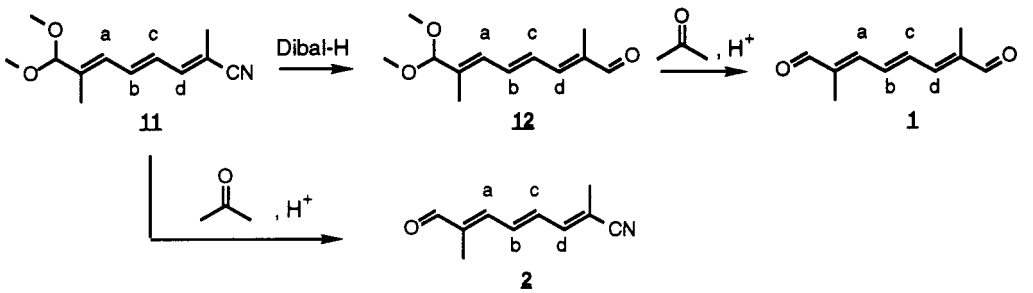


Synthesis of 2,7-dimethyl-8,8-dimethoxyocta-2,4,6-trienenitrile **11**.

For the synthesis of the symmetrical (either symmetrically or asymmetrically labelled) C_{10}-dialdehyde **1**, nitrile **11** is reduced with 1.1 equivalents of Dibal-H in petroleum ether at -70°C (scheme 2), giving the aldehyde in 82% yield after purification. In all the Dibal-H reduction reactions in this scheme, no more than 1.1 equivalents of Dibal-H are used and the reduction is carried out at low temperature; the reaction mixture is not allowed to warm up above 0°C. These mild conditions are necessary to prevent the reduction of the acetal group to a methoxy ether. As the length of the conjugated chain increases this side

reaction occurs more readily; especially in the last reduction step care has to be taken to prevent overreduction. The protected aldehyde **12** is deprotected by stirring for a few minutes in acetone acidified with one drop of hydrochloric acid, giving the aldehyde in a yield of 94% after purification by column chromatography. Until this point, mixtures of stereoisomers are obtained in every step. After the last step, **1** is obtained in predominantly the all-*E* form (>90%). It can be converted into the pure all-*E* form by irradiation with light in n-hexane in the presence of a trace of iodine.
This reaction sequence has been performed with **11a**, giving the [4-^{13}C]2,7-dimethylocta-2,4,6-triene-1,8-dial **1a** in overall 48% yield relative to the labelled acetonitrile. Because **11** can be obtained labelled at any of the four central positions and any combinations of these positions, **1** can now also be prepared labelled in any combination in the central part.

Scheme 2

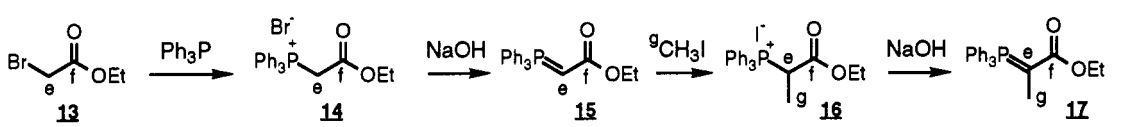


Synthesis of 2,7-dimethylocta-2,4,6-triene-1,8-dial **1** and 2,7-dimethylocta-2,4,6-triene-8-alnitrile **2**.

Alternatively, nitrile **11** can be directly deprotected by stirring in acidic acetone for a few minutes (scheme 2). The alnitrile **2** is obtained, after purification, in 95% yield as a mixture of two stereoisomers (2*E* and 2*Z*). These isomers can easily be separated by column chromatography, but the mixture can also be used directly in the subsequent steps. From **11a**, [4-^{13}C]2,7-dimethylocta-2,4,6-triene-8-alnitrile **2a** was obtained in an overall yield of 55% relative to the labelled acetonitrile. Again, because of the availability of **11** labelled in the central part, **2** can now be obtained labelled at any position or combination of positions in the central part.

Another target is to introduce labels at the 12, 13 and 13-Me positions in the carotenoids. For this, we first tried to prepare labelled 2-(diethylphosphono)propionitrile starting from acetonitrile which is *in situ* converted into the anion of diethyl cyanomethylphosphonate. Reacting this with methyl iodide, which is commercially available with 99% ^{13}C enrichment, should give the 2-(diethylphosphono)propionitrile. However, under all conditions (variation in temperature and number of equivalents of methyl iodide), a statistical mixture of non-, mono- and dimethylated phosphonate in a ratio of 1 : 2 : 1 was obtained as a consequence of the rapid proton exchange between the unreacted anion and the monomethylated product. Another route has been developed in which we start from ethyl bromoacetate **14**. This is commercially available with 99% ^{13}C enrichment on the 1 or 2 position and in the doubly labelled form. Alternatively, it can be synthesized starting from labelled acetic acid by a Hell-Vollhardt-Zelinsky reaction with phosphorous tribromide and bromine, followed by quenching of the acetyl bromide with ethanol, giving ethyl bromoacetate **14** in 86% yield. This is reacted, after aqueous work-up and extraction, without further purification, with triphenyl phosphine to give the phosphonium salt **14** in 97% yield after crystallization (scheme 3). This phosphonium salt **14** is monomethylated by first deprotonating it with NaOH in a two-layer system of dichloromethane and aqueous NaOH. After isolation of the phosphorane **15**, it is reacted with 1 equivalent of methyl iodide in dichloromethane at room temperature. The reaction is completed after 30 minutes. When an excess of unlabelled methyl iodide is used, the reaction requires only a few minutes for completion. The methylated phosphonium salt **16** is isolated and can be used without further purification. Less than 10% of dimethylation is observed, probably due to steric hindrance which prevents rapid hydrogen exchange. The C_3-phosphonium salt **16** is deprotonated with NaOH in the same way as described above. The product **17** can be isolated as a yellow solid which is stable at room temperature.

Scheme 3

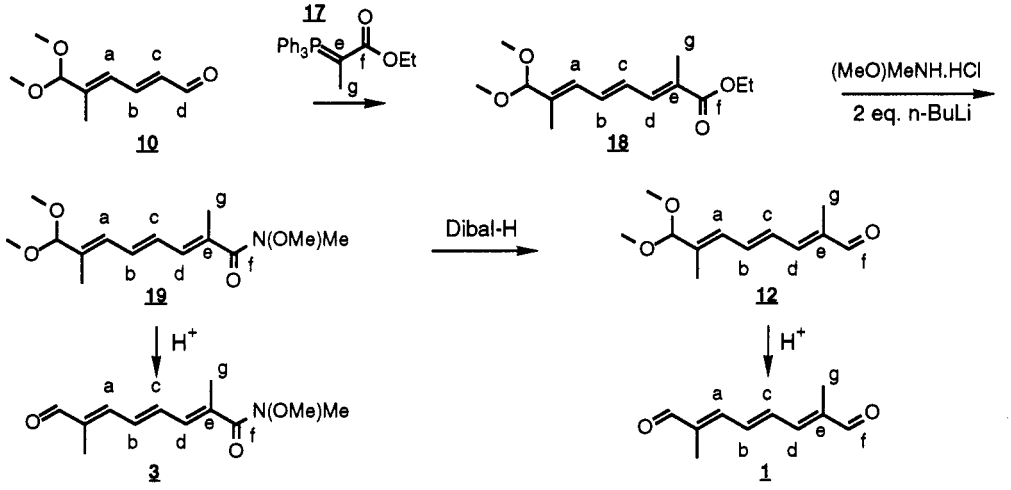


Synthesis of phosphorane **17** starting from ethyl bromoacetate **13**.

Ethyl bromoacetate is in this way quantitatively converted into the product **17**; this contains less than 10% of the dimethylated product, but since this does not react in the subsequent reactions, no further purification is necessary.

The phosphorane **17** is coupled in a Wittig reaction to the labelled or unlabelled C_7-aldehyde **10** by stirring in dichloromethane at room temperature for 30 minutes (scheme 4). After work-up and flash chromatography, the C_{10}-ester **18** is obtained in a yield of 86%. This ester is converted into the N-methoxy-N-methyl amide **19** in 77% yield after purification, by reacting it with the anion of N,O-dimethylhydroxylamine (with BuLi as a base) in THF at -20°C. This amide group is the synthetic analogue of a nitrile group; it can also be selectively reduced by Dibal-H to an aldehyde. This is illustrated by the reaction of **19** with Dibal-H at -70°C in THF, giving the aldehyde **12** in 77% yield. Compound **12** can then be converted into the C_{10}-aldehyde **1** in the way described above, giving access to C_{10}-dialdehyde **1** labelled at the 1, 2 or 2-Me positions or combination of these positions. Alternatively, the protected aldehyde group of **19** can be deprotected by stirring in acidified acetone. This yields the C_{10}-alamide **3** in 98% yield.

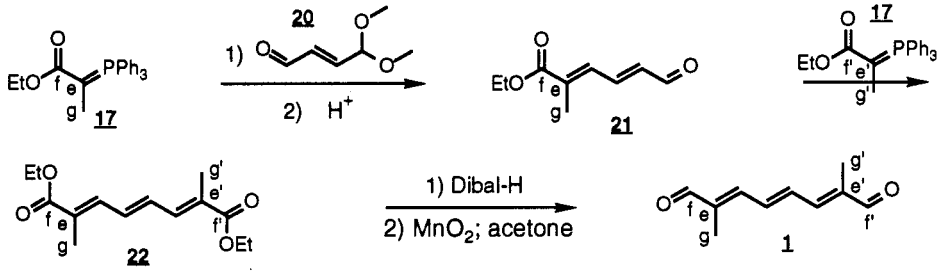
Scheme 4



Synthesis of 2,7-dimethylocta-2,4,6-triene-1,8-dial **1** and N-methoxy-N,2,7-trimethyl-octa-2,4,6-triene-8-alamide **3**. Isotope labels can be introduced at any of positions a to g.

For the preparation of symmetrical carotenoids symmetrically labelled at the 12, 12', 13, 13', 13-Me and 13'-Me positions, we developed another route to the C_{10}-dialdehyde **1** (scheme 5). This route starts from the C_3-phosphorane **17** which can be obtained labelled on any position and any combination of positions, as has been discussed above. It is coupled in a Wittig reaction to the monoacetal of fumaraldehyde **20**, by stirring it in dichloromethane. At room temperature, the reaction goes to completion after just a few minutes, giving the protected aldehyde. This acetal is not isolated, but directly deprotected by acidic work-up, giving the aldehyde **21** in 86% yield after flash chromatography. This aldehyde is coupled in a Wittig reaction with an equivalent of phosphorane **17** in dichloromethane, giving the C_{10}-diester **22** in 84% yield after purification. Compound **22** is then converted in two steps into the C_{10}-dialdehyde **1**: first it is reduced to the diol by reduction with five equivalents of Dibal-H at -70°C. Without purification, the

Scheme 5

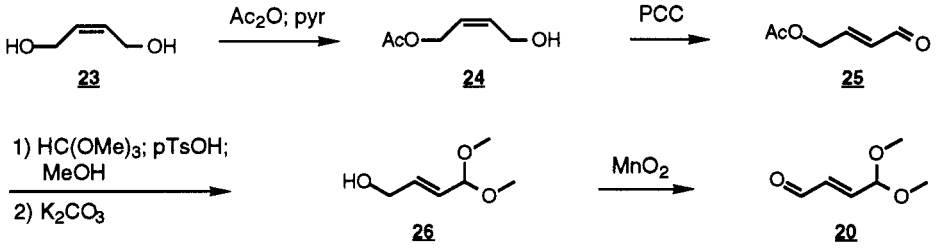


Synthesis of C_{10}-dialdehyde **1**. Labels can be introduced at positions f, e, g, f', e' and g'.

diol is converted into the C_{10}-dialdehyde by oxidizing it with 40-50 equivalents of MnO_2 in acetone. After 24 hours at room temperature, the reaction is complete, giving the dialdehyde **1** in 89% yield after purification. By using differently labelled phosphoranes **17** in the coupling reactions, the C_{10}-dialdehyde **1** can be labelled at any of positions 1, 2, 2-Me, 7, 8 and 7-Me, or combination of these positions.

For this synthetic scheme we need the monoprotected fumaraldehyde, which is not commercially available. For this C_4-synthon, we start from *cis*-2-buten-1,4-diol **23** (scheme 6). To a solution of **23** in dry toluene and 1.2 equivalents of pyridine at 0°C, one equivalent of acetic anhydride is slowly added. A statistical mixture of unreacted diol, monoacetate **24** and diacetate in a ratio of 1 : 2 : 1 is obtained. The diol can easily be removed by washing with water, the monoacetate **24** is separated from the diacetate by column chromatography. The free hydroxyl group of **24** is converted into the aldehyde by reacting it with pyridinium chlorochromate in dichloromethane at 0°C. The aldehyde **25** is obtained in a yield of 59% after purification. In a two-step one-pot reaction, the aldehyde is protected as an acetal and the acetate group is removed; **25** is dissolved in dry methanol with 2 equivalents of trimethylorthoformate. Addition of a catalytic amount of *p*-toluene sulphonic acid effects the conversion of the aldehyde into the dimethyl acetate. Subsequently, an excess of potassium carbonate is added: this removes the acid and removes the acetate group, giving the alcohol **26**, after purification, in 94% yield. This is then converted into the desired C_4-synthon by oxidizing the hydroxyl group with 10 equivalents of MnO_2 in acetone (ref. 21). The C_4-synthon **20** is obtained in 68% yield after purification by flash chromatography.

Scheme 6



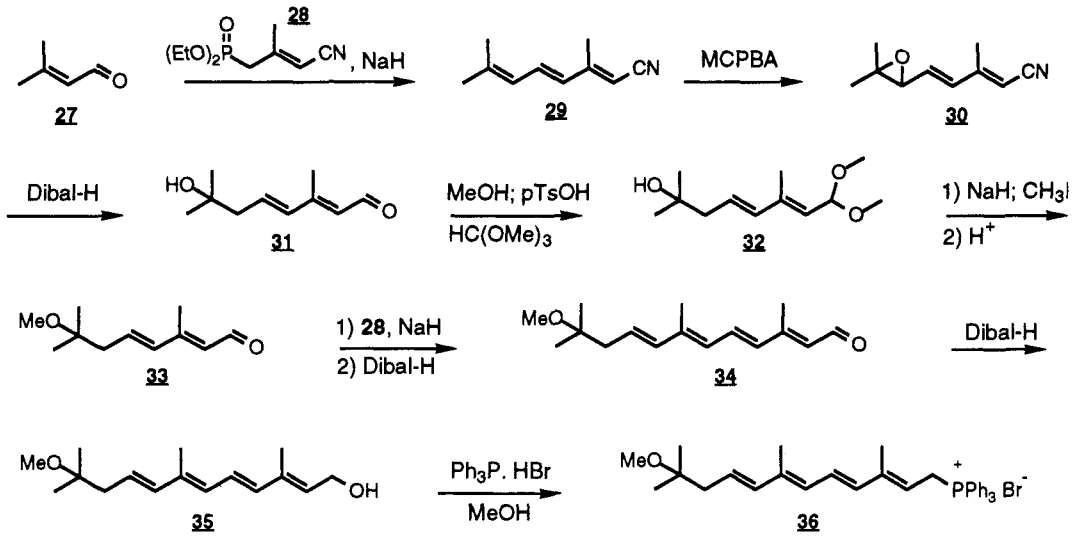
Synthesis of the monoacetal of fumaraldehyde **20**.

SYNTHESIS OF C_{15}-SYNTHONS

The above section describes the preparation of the C_{10}-synthons for use in the $C_{15} + C_{10} + C_{15}$ strategy. The C_{15}-parts are used in the form of phosphonium salts. For nearly all the end-groups of carotenoids, the syntheses of the corresponding C_{15}-phosphonium salts have been reported (refs. 22-25). The C_{15}-end-groups which are used for the synthesis of β-carotene **4** and astaxanthin **5** have been synthesized according to literature procedures starting from the commercially available β-ionone (refs. 23, 24). The same is true for the 1'-unsubsituted end-group of spheroidene, which is synthesized from commercially available *trans, trans*-farnesol (ref. 25). The synthesis of the C_{10}-phosphonium salt for the 1-methoxy end-group of spheroidene was described by our group (ref. 26). Based on this synthetic scheme, we developed a route for the preparation the C_{15}-phosphonium salt **36** which is used for this end-group.
This scheme starts from the commercially available 3-methyl-2-butenal **27**, which is coupled with the C_5-phosphonate **28** in a HWE reaction using NaH as a base, giving the C_{10}-nitrile **29** in 92% yield after column chromatography (scheme 7). The nitrile **29** can be oxidized selectively by adding 1.1 equivalents of *m*-chloroperoxybenzoic acid (MCPBA) at 0°C and stirring overnight at room temperature. Only the electron-rich end double bond is attacked and oxidized to the epoxide **30**. The yield after purification is 93%. In the next step both this epoxide and the nitrile are reduced with 3 equivalents of Dibal-H in petroleum ether at -60°C: the epoxide is reductively opened to the alcohol and the nitrile is converted into the aldehyde, yielding the C_{10}-hydroxylaldehyde **31** (56%). Compound **31** is converted into the corresponding C_{10}-methoxyaldehyde **33**. First, the aldehyde is quantitatively protected as the acetal **32** by reacting it with dry methanol in the presence of trimethylorthoformate and a trace of acid. The tertiary alcohol can now be methylated in a Williamson reaction with 5 equivalents of methyl iodide and an excess of NaH as base. Complete conversion is achieved after refluxing in THF for three hours. Deprotection of the acetal is accomplished by acidic work-up of the reaction mixture, yielding the methoxyaldehyde **33** in 75% yield relative to **31**. The protection-methylation-deprotection sequence proved to be necessary, as attempts to convert the C_{10}-hydroxylaldehyde **31** into the C_{15} 1,11-diol, followed by methylation of the hydroxyl groups gave only very poor yields. The C_{10}-methoxyaldehyde **33** is separated into its isomers by column chromatography and the all-*E* isomer is coupled in a HWE

reaction with the C_5-phosphonate **28** and NaH as base, yielding the C_{15}-nitrile which, after reduction with Dibal-H, gives the C_{15}-methoxyaldehyde **34** in 73% yield after two steps. The aldehyde **34** is separated into the isomers by column chromatography. The less abundant *Z*-isomers can be converted by irradiation in hexane in the presence of a trace of iodine into the all-*E* isomer. The all-*E* aldehyde **34** is converted in two steps into the corresponding C_{15}-methoxyphosphonium salt **36**: reduction with Dibal-H affords the alcohol **35** in 86% yield after purification. This is reacted with triphenylphosphine hydrobromide in methanol to give **36** in 95% yield. The overall yield of **36** is 20%.

Scheme 7



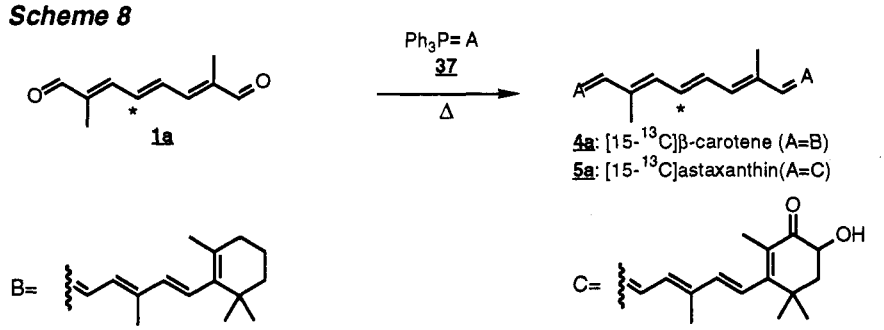
Synthesis of the C_{15}-methoxy phosphonium salt **36**.

SYNTHESIS OF CAROTENOIDS LABELLED IN THE CENTRAL PART

The synthesis of the labelled C_{10}-central units and the C_{15}-phosphonium salts has been discussed. From these synthons, symmetrical carotenoids can be synthesized in a one-step procedure, asymmetrical carotenoids in a three-step procedure.

The one-step conversion of the C_{10}-dialdehyde **1** into the symmetrical carotenoid by a double Wittig reaction with 2.5 equivalents of the corresponding C_{15}-phosphonium salt is most conveniently performed by refluxing the two compounds in 1,2 epoxybutane (b.p. 63°C), which acts both as a solvent and as a hydrogen bromide scavenger (ref. 21). The synthesis of labelled symmetrical carotenoids from labelled C_{10}-dialdehyde **1** is illustrated by the synthesis of [15-^{13}C]β-carotene **4a** and astaxanthin **5a** from [4-^{13}C]2,7-dimethylocta-2,4,6-triene-1,8-dial **1a** (scheme 8). The dialdehyde **1a** is refluxed in 1,2-epoxybutane for 24 h in the presence of 2.5 equivalents of the corresponding C_{15}-phosphonium salt **37a** (A=B) or **37b** (A=C). The carotenoid is isomerized by refluxing in n-hexane and purified by repeated crystallization. (all-*E*)-β-Carotene **4a** was obtained in 56% yield after crystallization from dichloro-

Scheme 8

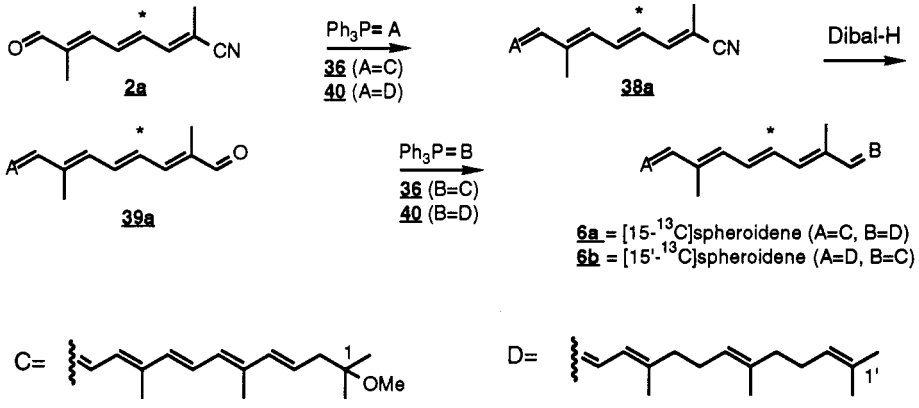


Synthesis of symmetrical carotenoids labelled in the central part starting from 2,7-dimethylocta-2,4,6-triene-1,8-dial **1**: β-carotene **4** (A=B) and astaxanthin **5** (A=C).

methane/ methanol and petroleum ether. Pure (all-*E*)-astaxanthin **5a** was obtained in 42% yield after crystallization from dichloromethane/ methanol and dichloromethane/ n-hexane. Spectra (^{1}H-NMR, ^{13}C-NMR, UV/VIS and mass spectrometry) were in complete agreement with literature data, showing high levels of ^{13}C enrichment (>95%, within the limits of the techniques used) at the correct position (refs. 28, 29).

As an example of the synthesis of an asymmetric carotenoid, we have synthesized [15-^{13}C]- and [15'-^{13}C]spheroidene. The versatility of this method is illustrated by the fact that from one labelled C_{10}-synthon, [4-^{13}C]2,7-dimethylocta-2,4,6-triene-8-alnitrile **2a**, the two species, [15-^{13}C]- and [15'-^{13}C]-spheroidene, were synthesized (scheme 9).
In the first step, 2,7-dimethylocta-2,4,6-triene-8-alnitrile **2** is coupled to a C_{15}-phosphonium salt in a Wittig reaction by refluxing **2** and 1.5 equivalents of phosphonium salts in 1,2-epoxybutane, to give the C_{25}-nitrile **38** as a mixture of stereoisomers. After purification by flash chromatography, **38** is reduced with Dibal-H in petroleum ether at -70°C, giving the C_{25}-aldehyde **39**. The aldehyde is easily separated into its stereoisomers by column chromatography. The all-*E* isomer is converted into spheroidene **6** by reacting it in a Wittig reaction with the other phosphonium salt by refluxing in 1,2-epoxybutane. The all-*E* spheroidene **6** is obtained by purification by column chromatography followed by repeated crystallization from petroleum ether.

Scheme 9



Synthesis of [15-^{13}C]spheroidene **6a** (A=C, B=D) and [15'-^{13}C]-spheroidene **6b** (A=D, B=C) starting from [4-^{13}C]2,7-dimethylocta-2,4,6-triene-8-alnitrile **2a.**

It is possible to perform this sequence in two ways. Phosphonium salt **40** (A=D) can first be coupled and in the second coupling step the phosphonium salt **36** (B=C) or the order of coupling of **40** and **36** can be reversed. When using unlabelled **2** the products are of course identical. When starting from [4-^{13}C]2,7-dimethylocta-2,4,6-triene-8-alnitrile **2a** two different products are obtained. When first coupling **40** (A=D), giving the C_{25}-nitrile **38a** in 92% yield after purification, reduction with Dibal-H, giving the C_{25}-aldehyde **39a** in 82% yield, and coupling with **36** (B=C), [15-^{13}C]spheroidene **6a** is obtained in 75% yield. When reversing the order of coupling, the yields are for the C_{25}-nitrile **38b** 84%, C_{25}-aldehyde **39b** 80%. [15'-^{13}C]Spheroidene **6b** is then obtained in 78% yield. Spectra (^{1}H-NMR, ^{13}C-NMR, UV/VIS and mass spectrometry) were in complete agreement with literature data, showing high levels of ^{13}C enrichment (>95%, within the limits of the techniques used) at the correct position (ref. 17).

SOME RESULTS

Photosynthesis - The photosynthetic reaction centres of *Rhodobacter sphaeroides* specifically labelled at the C-14' and C-15' positions of the spheroidene chromophore were studied by solid state ^{13}C NMR. The spheroidene isomerizes upon binding and forms a 15*Z* configuration (the chemical shift values can discriminate between a *Z* or *E* configuration) (refs. 17, 18). Similarly, the 28 tyrosine residues in the photosynthetic reaction centre of *Rhodobacter sphaeroides* R26 could be selectively enriched with ^{13}C in the 4'-positions. The NMR spectra consist of at least seven narrow lines superimposed on a broad doublet. The chemical shift anisotropy is similar to that for crystalline tyrosine. The two narrowest resonances correspond to signals of individual tyrosines with 28 ± 5 Hz width, comparable to what is observed for quaternary carbons in linearly elastic organic solids. This provides strong evidence for an unusually

ordered, well-shielded, and structurally, electrostatically, and thermodynamically stable interior of the protein complex without structural inhomogeneities (ref. 30).

Vision - Assignment of the fingerprint vibrations of the chromophore in bovine rhodopsin and bathorhodopsin has been accomplished (ref. 31). The complete assignment of the hydrogen out-of-plane (HOOP) vibrations of the chromophore in bovine rhodopsin has been realized (ref. 32). The analysis of the HOOP vibrations of the chromophore of octopus rhodopsin indicates a less perturbed all-*E* retinylidene structure than in the bovine case (refs. 33, 34).

The chemical shift values of the carbon atoms in the chromophore of bovine opsin and bathorhodopsin have been measured (ref. 4). These results show that photoisomerization does not result in electrostatic energy storage (ref. 35).

Bacteriorhodopsin - Light-adapted bacteriorhodopsin contains a perturbed 6-s-*trans* chromophore (ref. 36). The photochemical step in the proton pump action is a 13-*trans-cis* isomerization, whereas light-dark adaptation is a 13*E*,15*E* to 13*Z*,15*Z* thermal isomerization (ref. 37). Bacteriorhodopsin has a complex counterion near the protonated Schiff base (ref. 38).

The dipole-dipole interaction between two spins is distance dependent. With the rotational resonance NMR technique, distances between two ^{13}C labels can be accurately measured (ref. 9). In bacteriorhodopsin an internuclear distance of 4.2 Å between C-8 and C-18 has been found, in good agreement with the 6-s-*trans* conformation (ref. 9). The internuclear distance measured between C-14 and [ε-^{13}C]Lys established the *E* Schiff base configuration in the M photointermediate (ref. 39). These results show that rotational resonance can be used for structural studies in membrane proteins and other situations where diffraction and solution NMR techniques yield limited or no information.

CONCLUDING REMARKS

Isotopic labelling, combined with isotope-sensitive spectroscopy, provides a very powerful method for obtaining information about biologically active systems at the atomic level.

We have developed a route to label any carotenoid, both symmetrical and asymmetrical, with ^{13}C at any position and combination of positions in the central part. From one labelled asymmetric synthon **2** or **3**, two labelled asymmetric carotenoids can be synthesized. All of the labelled carotenoids can be synthesized on the gram scale. Based on the work in our group on the synthesis of labelled vitamin A and vitamin A derivatives (ref. 40), many of the end-groups can also be labelled with ^{13}C, giving access to a large number of carotenoids labelled at specific positions in the carbon skeleton.

These labelled carotenoids are of vital importance in determining the detailed structure and electronic surroundings of carotenoids in carotenoproteins, as was demonstrated earlier in our group by the elucidation of the 15,15'-double bond configuration of spheroidene bound in the reaction centre of *Rhodobacter sphaeroides* 2.4.1 (refs. 17, 18). Labelled carotenoids in combination with incorporation into active biomolecules and studies with non-invasive isotope-sensitive techniques give access to information with atomic resolution that is otherwise not easily or not at all accessible.

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