

## Synthesis of $^{13}\text{C}$ -labeled carotenoids and retinoids\*

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*Abstract:* A three-part strategy has been developed to study molecular interactions in biological systems at the atomic level. First, isotopically labeled carotenoids and retinoids are prepared by organic total synthetic schemes with labels at predetermined atomic positions and combinations of positions. Subsequently, the labeled compounds are incorporated in the biological system. Finally, the system is studied by isotope sensitive spectroscopic techniques.

In this paper, the synthesis of 10-fold  $^{13}\text{C}$ -labeled retinal palmitate and  $\beta$ -carotene  $\alpha$ -crustacyanin carotene for nutritional studies is discussed. Also, the scheme to label the end positions of astaxanthin and canthaxanthin with  $^{13}\text{C}$  for spectroscopic investigations of  $\alpha$ -crustacyanin with isotope labels in the chromophore is given. The synthesis of 10-methyl retinal is discussed, starting from isotopically labeled synthons obtained via schemes to  $^{13}\text{C}$ -labeled natural retinal. Finally, the possibility for spectroscopic studies of caroteno and retino proteins via an expression of apoproteins by way of genetic techniques in the post-genomic era is discussed.

### INTRODUCTION

In excess of 600 carotenoids are found in plants and animals [1–4]. Carotenoids play an important role in the life processes in plants and photosynthetic bacteria. They are bound in the light harvesting complexes and, due to their absorption wavelengths, they absorb light which is not absorbed by chlorophyll and, in this way, enhance the efficiency of photosynthesis. An even more vital role in photosynthesis is carried out by the carotenoids that are bound in the photosynthetic reaction centers, where they protect these centers against light damage. The absence of carotenoids in the photosynthetic reaction centers leads to destruction of these centers in the presence of light, preventing photosynthesis from taking place. Without carotenoids, no photosynthesis could take place, such that life as we know it on earth would not be possible. Carotenoids are also vital in human and animal nutrition [5]. They are the source of vitamin A, which has important functions in animals. The most studied is vision, where the chromophore in visual pigments is derived from 11-*Z*-retinal. Due to insufficient vitamin A, many people have reduced eyesight or become totally blind. Another important role of carotenoids is the coloration of animals, flowers and fruit. A special group are the caroteno proteins, such as  $\alpha$ -crustacyanin, which give the coloration to marine animals such as lobsters and shrimps [6,7].

In order to study the role of carotenoids in photosynthetic reaction centers, in caroteno proteins and in rhodopsins,  $^2\text{H}$ - and  $^{13}\text{C}$ -enriched carotenoids and retinals are required to study the role of the carotenoid

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cofactor at the atomic level via isotope sensitive techniques. The strategy consists of three steps:

- (i) preparation of carotenoids (retinals) highly enriched with stable isotopes ( $^2\text{H}$ ,  $^{13}\text{C}$ ) at predetermined positions;
- (ii) reconstitution of the labeled carotenoid (retinal) with carotenoids (retinoid)-depleted protein complexes yielding the caroteno proteins (retinal proteins) with the specifically labeled chromophore;
- (iii) study of the isotopically labeled protein complexes by non-destructive, non-invasive isotope sensitive spectroscopic techniques, such as high resolution solid state  $^{13}\text{C}$  nuclear magnetic resonance (NMR) and resonance Raman spectroscopy.

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 labeling. As native protein complexes also contain carotenoids labeled at the natural abundance level (e.g. for  $^{13}\text{C}$ , 1.1%), this strategy has been successfully used in the elucidation of the detailed structure of retinal in bovine rhodopsin, central in the visual transduction mechanism, and in bacteriorhodopsin, the light-driven proton pump in *Halobacterium salinarium*. In the case of bovine rhodopsin, even the precise conformation of the isomerization region could be determined via isotope labeling, allowing structural information to be obtained in exquisite detail that cannot be obtained with other methods [8].

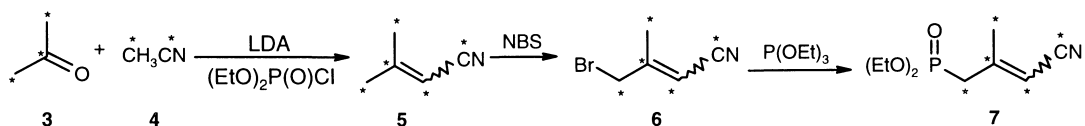
The blue shift of  $5500\text{ cm}^{-1}$  of the absorption spectrum of astaxanthin on binding to the lobster pigment  $\alpha$ -crustacyanin has been studied via  $^{13}\text{C}$  solid state NMR. It could be inferred that this bathochromic shift is caused by protonation of the 4 and 4' keto function of the astaxanthin chromophore in the protein [9].

$\beta$ -Carotene and retinal and essential components in human and animal nutrition. Using 10-fold  $^{13}\text{C}$ -enriched  $\beta$ -carotene and retinal should allow the determination of the nutritional status of humans and animals in real time in individual cases. This is done by administration of precise amounts of  $^{13}\text{C}_{10}$   $\beta$ -carotene and  $^{13}\text{C}_{10}$  retinal in the food, and subsequent analysis of serum at predetermined times after ingestion with mass spectral techniques. We have a long-standing program for the synthesis of the systems in this paper; the newest synthetic developments will be discussed.

## SYNTHESIS

### Synthesis of [8,9,10,11,12,13,14,15,19,20- $^{13}\text{C}_{10}$ ]-retinyl palmitate and [12,12',13,13',14,14',15,15',20,20'- $^{13}\text{C}_{10}$ ]-all-*E*- $\beta$ -carotene

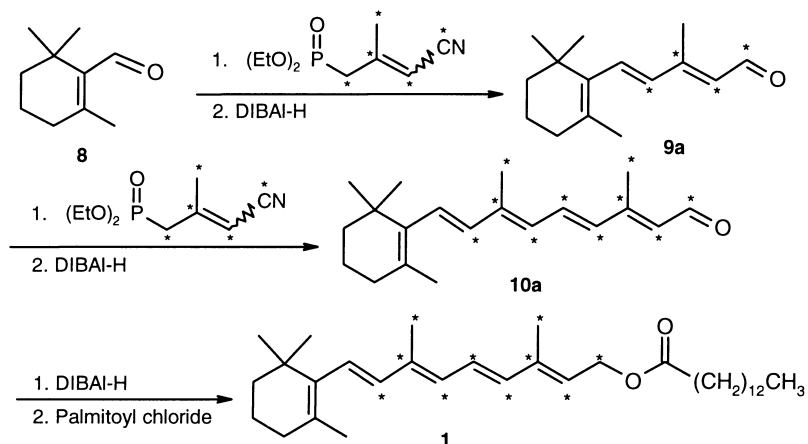
For these nutritional studies in collaboration with the research groups of C. E. West (Wageningen Agricultural University, The Netherlands) and R. B. van Breemen (University of Illinois, USA), we needed 1 g of each of the above-mentioned compounds with 99%  $^{13}\text{C}$  incorporation at each of the indicated positions. In order to optimize the synthesis and keep finances tolerable, we selected [ $^{13}\text{C}_5$ ]-4-(diethyl phosphono)-3-methyl-2-butenenitrile (**7**) as the synthon to introduce the  $^{13}\text{C}$  labels in [ $^{13}\text{C}_{10}$ ]-retinyl palmitate and [ $^{13}\text{C}_{10}$ ]-all-*E*- $\beta$ -carotene. In Scheme 1, we depict how this synthon was prepared starting from commercially available [ $^{13}\text{C}_3$ ]-acetone and [ $^{13}\text{C}_2$ ]-acetonitrile.



**Scheme 1** Synthesis of [ $^{13}\text{C}_5$ ]-4-(diethyl phosphono)-3-methyl-2-butenenitrile starting from commercially available [ $^{13}\text{C}_3$ ]-acetone and [ $^{13}\text{C}_2$ ]-acetonitrile.

[ $^{13}\text{C}_2$ ]-Acetonitrile (**4**) is treated with the strong non-nucleophilic base LDA (lithium diisopropylamide) and subsequently treated with diethyl chlorophosphate with an additional equivalent of base to give the anion of diethyl methyl cyanophosphonate. This anion is coupled with one equivalent of [ $^{13}\text{C}_3$ ]-acetone (**3**) to give [ $^{13}\text{C}_5$ ]-3-methyl-2-butenenitrile (**5**). The nitrile is treated with NBS (N-bromosuccinimide) to give [ $^{13}\text{C}_5$ ]-4-bromo-3-methyl-2-butenenitrile (**6**), which is subsequently converted by an Arbuzov reaction into the required [ $^{13}\text{C}_5$ ]-4-(diethyl phosphono)-3-methyl-2-butenenitrile (**7**).

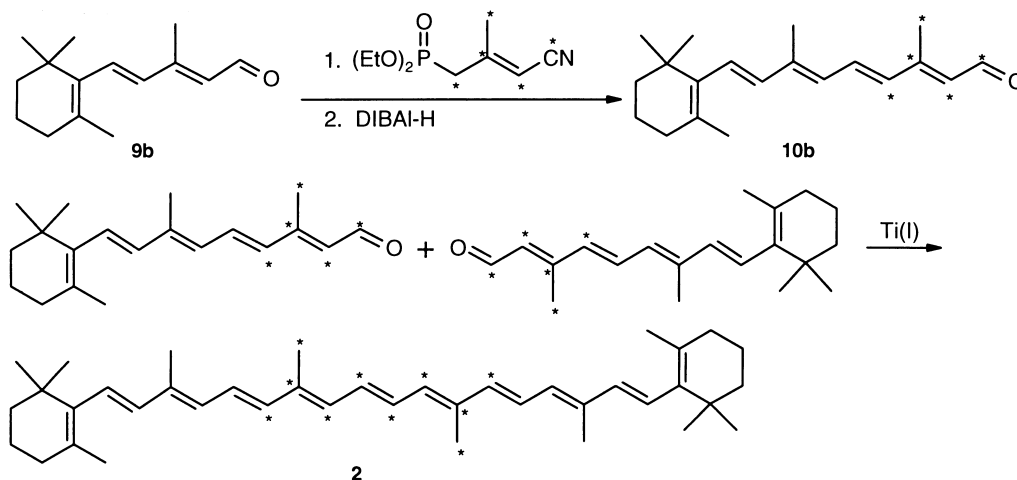
In Scheme 2, we indicate how this synthon is the base for the preparation of [ $^{13}\text{C}_{10}$ ]-retinyl palmitate.



**Scheme 2** Synthesis of [ $^{13}\text{C}_{10}$ ]-retinyl palmitate using [ $^{13}\text{C}_5$ ]-4-(diethyl phosphono)-3-methyl-2-butenenitrile.

$\beta$ -Cyclocitral (**8**) is coupled via a Wittig–Horner reaction with [ $^{13}\text{C}_5$ ]-4-(diethyl phosphono)-3-methyl-2-butenenitrile (**7**). The resulting nitrile is reduced with DIBAL-H (diisobutyl aluminium hydride) to form [ $^{13}\text{C}_5$ ]- $\beta$ -ionylidene acetaldehyde (**9a**) [10]. This sequence is then repeated to give [ $^{13}\text{C}_{10}$ ]-retinal (**10a**), which is subsequently reduced by DIBAL-H. The retinal is treated with palmitoyl chloride to give [8,9,10,11,12,13,14,15,19,20- $^{13}\text{C}_{10}$ ]-retinyl palmitate (**1**). We synthesized a total amount of 0.75 g of this compound.

For the synthesis of all-*E*-[12,12',13,13',14,14',15,15',20,20'- $^{13}\text{C}_{10}$ ]- $\beta$ -carotene, we used the reaction sequence in Scheme 3.



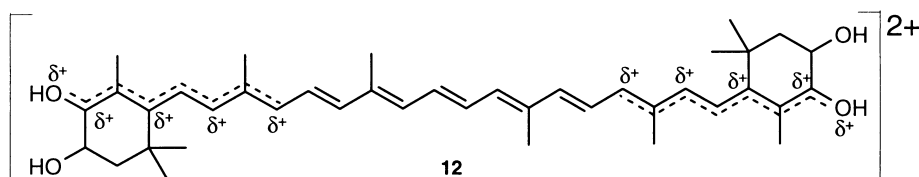
**Scheme 3** Synthesis of all-*E*-[12,12',13,13',14,14',15,15',20,20'- $^{13}\text{C}_{10}$ ]- $\beta$ -carotene from  $\beta$ -ionylidene acetaldehyde and [ $^{13}\text{C}_5$ ]-4-(diethyl phosphono)-3-methyl-2-butenenitrile.

In Scheme 3,  $\beta$ -ionylidene acetaldehyde (**9b**) is the starting material (which can be easily prepared from  $\beta$ -cyclocitral or  $\beta$ -ionone). A Wittig–Horner coupling with [ $^{13}\text{C}_5$ ]-4-(diethyl phosphono)-3-methyl-2-butenenitrile (**7**) and subsequent DIBAL-H reduction gives [12,13,14,15,20- $^{13}\text{C}_5$ ]-retinal (**10b**). The retinal is reductively dimerized by low valent titanium to yield  $\beta$ -carotene (**2**) [11]. We prepared 1 g of the all-*E*-isomer of this compound.

### Synthesis of $^{13}\text{C}$ -labeled astaxanthins

To study the blue shift ( $5500\text{ cm}^{-1}$ ) in the absorption spectrum of astaxanthin on binding to the lobster

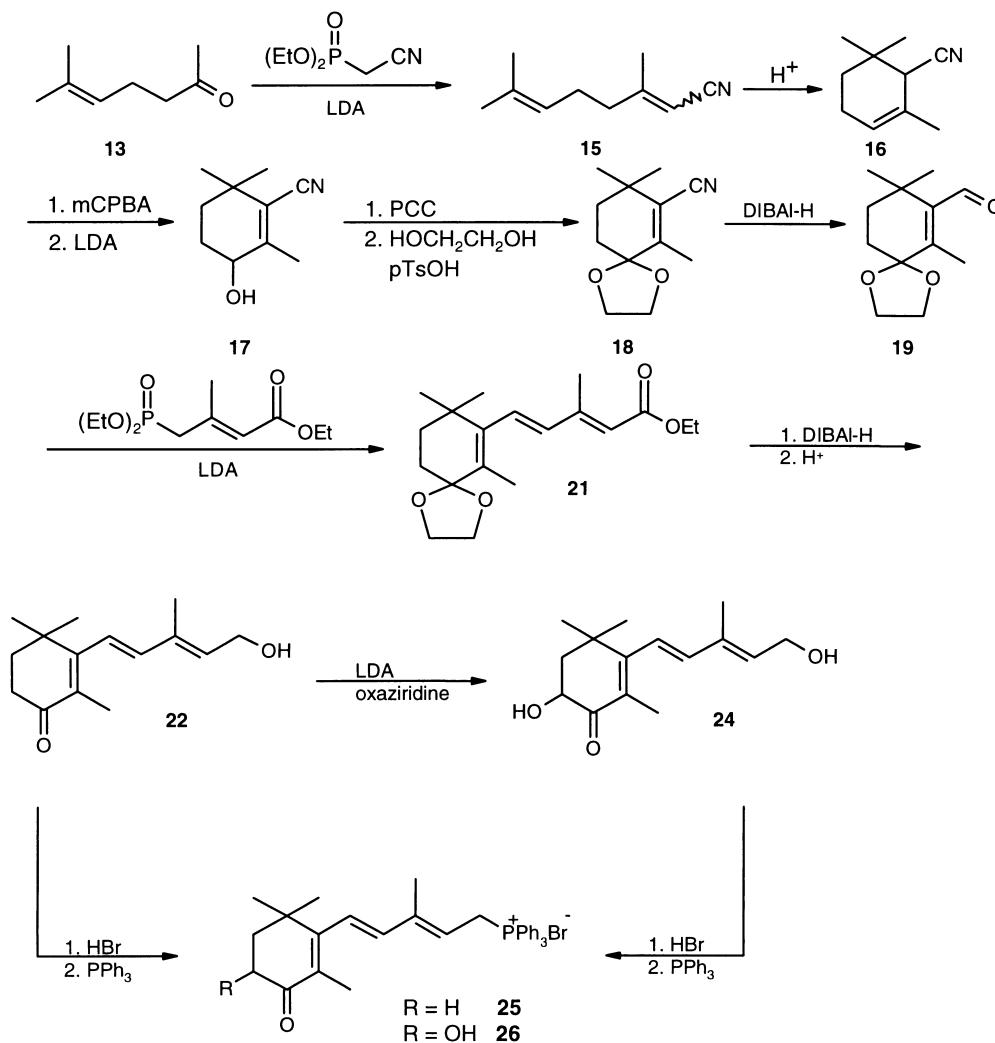
pigment  $\alpha$ -crustacyanin, we compared  $^{13}\text{C}$  chemical shift values of free astaxanthin and astaxanthin bound to the protein. From our study on  $[12,12'\text{-}^{13}\text{C}_2]$ -,  $[13,13'\text{-}^{13}\text{C}_2]$ -,  $[14,14'\text{-}^{13}\text{C}_2]$ -, and  $[15,15'\text{-}^{13}\text{C}_2]$ -astaxanthin, we inferred that the color shift and the chemical shift values can only be explained by the protonated model where both 4- and 4'-carbonyls of astaxanthin in  $\alpha$ -crustacyanin are protonated (see Fig. 1) [9].



**Fig. 1** Model of doubly protonated astaxanthin in  $\alpha$ -crustacyanin.

It is expected that the positive charge due to the protonation is mainly delocalized on atoms 4,4',6,6',8,8' and 10,10'. In order to test the amount of electric charge in these positions in  $\alpha$ -crustacyanin, we needed a synthetic scheme to label each mentioned position in the astaxanthin chromophore part of  $\alpha$ -crustacyanin.

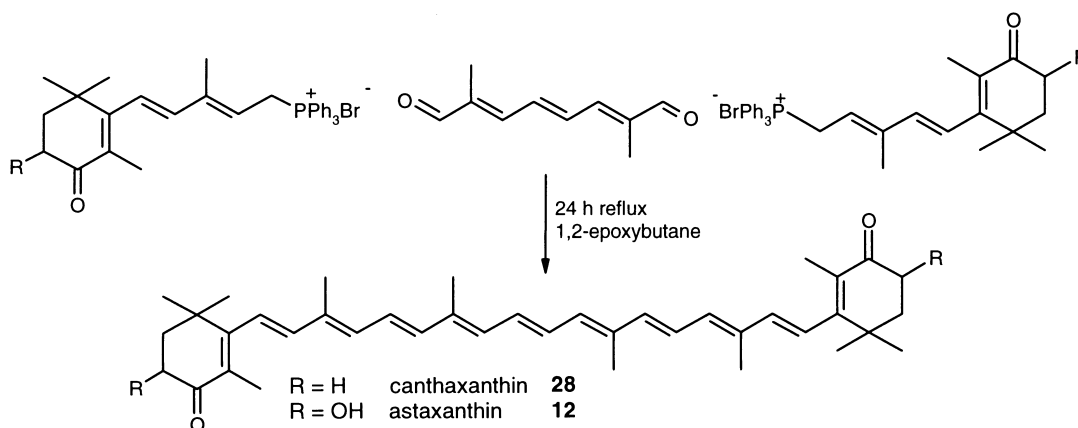
In Scheme 4, the reactions are given based on reagents in natural abundance that will be the basis for the preparation of astaxanthin labeled with  $^{13}\text{C}$  on each position or combinations of positions.



**Scheme 4** Synthesis of the  $\text{C}_{15}$ -Wittig salts for the preparation of labeled astaxanthin and canthaxanthin.

Commercially available 6-methyl-5-hepten-2-one (**13**) is treated with diethyl methyl cyanophosphonate in a Wittig–Horner reaction to give 3,7-dimethyl-2,6-octadienenitrile (**15**) in a mixture of *E*- and *Z*-isomers. Both synthons are available in all possible  $^{13}\text{C}$ -labeled forms. Treatment of this conjugated material with sulfuric acid in nitromethane gives 2,6,6-trimethyl-2-cyclohexenenitrile (**16**). Epoxidation of the cyclic nitrile gives the corresponding epoxide which, on treatment with base, gives 3-hydroxy-2,6,6-trimethyl-1-cyclohexenecarbonitrile (**17**). The alcohol can be oxidized by pyridium chlorochromate to the corresponding ketone and can be converted into the corresponding ethylene acetal (**18**). Subsequent reduction of the nitrile function gives the corresponding aldehyde (**19**). The aldehyde is converted via a Wittig–Horner reaction with triethyl 3-methyl-4-phosphonocrotonate into the 4-ethylene acetal of  $\beta$ -ionylidene acetic ester (**21**). Reduction of the ester and removal of the 4-acetal group gives 3-methyl-5-(2,6,6-trimethyl-3-oxo-1-cyclohexen-1-yl)-2,4-pentadienol (**22**). The primary alcohol is treated with two equivalents of LDA to form the enolate. The enolate is reacted with oxaziridine giving 3-methyl-5-(4-hydroxy-2,6,6-trimethyl-3-oxo-1-cyclohexen-1-yl)-2,4-pentadienol (**24**).

Both **22** and **24** are converted in two steps in the corresponding Wittig salts and condensed with half an equivalent of 2,7-dimethylocta-2,4,6-triene-1,8-dial (**27**) to give, respectively, canthaxanthin (**28**) and astaxanthin (**12**) (Scheme 5).



**Scheme 5** Formation of canthaxanthin and astaxanthin via  $\text{C}_{15} + \text{C}_{10} + \text{C}_{15}$  Wittig coupling.

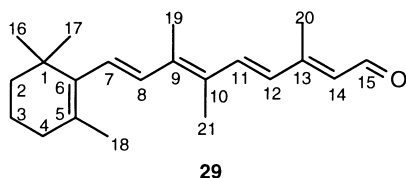
The synthesis of 3-hydroxy-2,6,6-trimethyl-1-cyclohexenecarbonitrile is based on 6-methyl-5-hepten-2-one and acetonitrile. Incorporation of  $^{13}\text{C}$  labels will be effectuated by using commercially available  $^{13}\text{C}$ -labeled acetonitrile. The synthesis of 2,7-dimethylocta-2,4,6-triene-1,8-dial, the synthon of the central part of all carotenoids in almost all possible  $^{13}\text{C}$ -labeled forms, has already been described in the literature [12]. The 8,8',9,9',10,10',-11,11',19,19' positions in canthaxanthin and astaxanthin derive from triethyl-3-methyl-4-phosphonocrotonate [13]. For the various isotopically labeled forms of this synthon, a scheme can be devised similar to that given for the corresponding nitrile in Scheme 1.

### Synthesis of $[10,20\text{-}^{13}\text{C}_2]$ and $[13,21\text{-}^{13}\text{C}_2]$ -10-methyl retinal

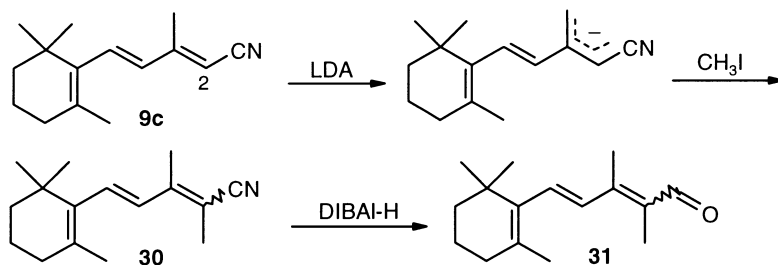
For one-dimensional (1D) rotational  $^{13}\text{C}$  solid state spectroscopy, we needed  $[10,20\text{-}^{13}\text{C}_2]$ -10-methyl retinal and  $[13,21\text{-}^{13}\text{C}_2]$ -10-methyl retinal to determine the precise conformation of 10-methyl rhodopsin and compare this with the geometry of the natural system in the active site (Fig. 2).

In the past, we have worked out schemes to  $^{13}\text{C}$  label any position and combination of positions in the conjugated part of the retinal molecule (**10**). An optimal strategy to  $^{13}\text{C}$  label chemically modified retinals at various positions is in the use of isotopically labeled intermediates in retinal synthesis via which the chemical modification is introduced.  $\beta$ -Ionylidene acetonitrile is the synthon of choice to introduce the additional 10-methyl group in retinal via the reactions depicted in Scheme 6.

$\beta$ -Ionylidene acetonitrile (**9c**) is treated with LDA at low temperature to quantitatively form the anion by abstraction of a proton of the  $\beta$ -methyl group with respect to the nitrile function. Treatment with 1.1



**Fig. 2** Structure and numbering of 10-methyl retinal.



**Scheme 6** Synthesis of [(2-CH<sub>3</sub>)-<sup>13</sup>C]-2-methyl-β-ionylidene acetaldehyde.

equivalent of methyl iodide gives a quantitative amount of 2-methyl-β-ionylidene acetonitrile (**30**). The electrophile CH<sub>3</sub>I attacks the anion selectively at the carbon position next to the nitrile; no unreacted or dimethylated product is formed. The nitrile group is subsequently reduced to the aldehyde as a mixture of *E/Z*-isomers, which can be converted into the corresponding retinal (**31**). By starting with [2-<sup>13</sup>C]-2-methyl-β-ionylidene acetonitrile, in this way the [2-<sup>13</sup>C]-2-methyl-β-ionylidene acetaldehyde could be made, which was subsequently converted into [10,20-<sup>13</sup>C<sub>2</sub>]-10-methyl retinal. By carrying out this reaction with <sup>13</sup>CH<sub>3</sub>I and application of <sup>13</sup>C-labeled 2-methyl-β-ionylidene acetaldehyde, in a further synthesis [13,21-<sup>13</sup>C<sub>2</sub>]-10-methyl retinal could be made.

It is to be expected that, besides <sup>13</sup>CH<sub>3</sub>I, many other electrophiles can be used, which opens up an easy way to prepare a host of 10-modified retinals <sup>13</sup>C isotopically labeled at each carbon position and any combinations of positions. We found earlier that the corresponding unsaturated aldehyde cannot be used in this reaction, because these systems undergo self-condensation such that no substitution products are formed.

## CONCLUSION AND OUTLOOK

In this paper, the synthesis of [<sup>13</sup>C<sub>10</sub>]-retinyl palmitate and [<sup>13</sup>C<sub>10</sub>]-β-carotene has been described with 99% <sup>13</sup>C incorporation at each predetermined position at the gram level. Starting with 7.0 g [U-<sup>13</sup>C<sub>10</sub>]-acetone and 5.0 g [U-<sup>13</sup>C<sub>10</sub>]-acetonitrile, we synthesized 0.75 g of 10-fold-labeled retinyl palmitate (**1**) and 1 g of 10-fold-labeled all-*E*-β-carotene (**2**). Access to these systems allows a study of the role of vitamin A palmitate and carotene in nutrition in exquisite detail in real time at the individual level. It will also provide detailed information about the metabolic changes of these compounds in the body. Some 10 other carotenoids are introduced into the human body via the food. Access to these other carotenoids, with 10-fold isotope labels in the central part, will extend nutrition studies to all important carotenoids and their possible role in the formation of vitamin A in metabolism. Other carotenoids with 10-fold <sup>13</sup>C labels can efficiently be prepared starting from [<sup>13</sup>C<sub>10</sub>]-2,7-dimethyl-2,4,6-octatriene-1,8-dial, from which almost all known carotenoids can be prepared. Studies to prepare this <sup>13</sup>C<sub>10</sub>-labeled synthon at the gram level in an economical fashion are currently being investigated.

Carotenoids and retinoids are cofactors of many important colored membrane proteins, such as photosynthetic reaction centers, antenna pigments and rod and cone visual pigments. By exchange of the natural carotenoids and retinoids in these proteins with U-<sup>13</sup>C carotenoids and U-<sup>13</sup>C retinoids, these proteins become accessible to non-invasive isotope sensitive spectroscopic techniques for research of structure–function relations. Solid state NMR techniques now exist to obtain the chemical shift values of each of the carbon atoms in the unitarily labeled cofactor which provides an insight into the electronic

configuration of the cofactor in the protein. While U- $^{13}\text{C}$   $\beta$ -carotene can be obtained via growth of photosynthetic organisms on media containing  $^{13}\text{CO}_2$  as the sole carbon source, many functional molecules cannot be obtained in this way. We believe that our schemes provide access to unitarily  $^{13}\text{C}$ -labeled systems that can be tailor-made via organic total synthesis in an economical fashion.

Within a few years the human genome will be finished and the sequences of all proteins in the human body will be known. About 40% of these 400 000 human proteins are membrane proteins and many of these have important functions in signal transduction pathways. Knowledge of their structure is important to understand their mechanisms; however, obtaining structural information about membrane proteins is still a challenge.

Already, water-soluble proteins can be obtained routinely with isotope labels in the peptide chain by expressing them in media containing isotopically labeled amino acids. Recently, systems have been developed to express eukaryotic membrane proteins, which means that all amino acids in membrane proteins can be labeled in a similar manner. Labeled cofactors can be introduced in the active site as has been discussed for caroteno and retino proteins. The post-genomic era that is now about to start will allow the tailor-made production of (caroteno and retino) membrane proteins from humans, animals and plants with site-directed isotope labels in the cofactor and peptide chain. The study of these systems will allow structural and functional information to be obtained at the atomic level about the cofactor, its interaction with the protein and the interpeptide interactions.

The integration of organic total synthesis, biotechnology and spectroscopy on isotope-labeled membrane proteins will allow an understanding of biological function at the atomic level in exquisite detail. It is a happy thought that investigations on isotopically labeled carotenoids and retinoids have been a fundamental step for investigations at the atomic level of functional biological systems in the post-genomic era.

## ACKNOWLEDGEMENTS

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