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The Preparation of All-Trans Uniformly ^{13}C-Labeled Retinal via a Modular Total Organic Synthetic Strategy. Emerging Central Contribution of Organic Synthesis toward the Structure and Function Study with Atomic Resolution in Protein Research

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Abstract: Uniformly [$^{13}C_{20}$]-labeled *all-trans*-retinal (**1**) has been prepared via a convergent modular total organic strategy with high isotope incorporation (>99%) and without isotope dilution starting from commercially available 99% enriched ^{13}C-labeled starting materials. For this purpose we have developed a strategy that is based on four different modules: [1,2,3,4,(3-CH_3)-$^{13}C_5$]-4-(diethylphosphono)-3-methyl-2-butenenitrile (**3**), [1,2,3,4-$^{13}C_4$]-ethyl acetoacetate (**7**), [U-$^{13}C_5$]-4-bromo-2-methyl-2-butene (**13**), and [U-$^{13}C_{10}$]-2,6,6-trimethylcyclohex-2-ene-1-ylcarbonitrile (**16**). This scheme permits the synthesis of the full cassette of all isotopomers with ^{13}C-labels at any position or combination of positions by using different ^{13}C-labeled starting materials. In addition, modifications of the synthesized modules will give access to a broad range of chemically modified ^{13}C-labeled retinoids and carotenoids. This modular strategy enables the synthesis of multifold and uniformly stable isotopically labeled (bio)macromolecules that can be used for studying proteins with atomic resolution, providing detailed functional information of the studied biological system.

Introduction

The primary structures of all human proteins are now available with the completion of the human genome project.[1] In the post-genomic era in a very rapid process, the total genomes of a plethora of other organisms are also becoming available in addition to mutants that lead to malfunctioning or nonfunctioning proteins leading to genetic diseases. Furthermore, efficient procedures are available via biotechnology to obtain the proteins using these genetic codes.[2,3] The fundamental challenge now is to study the chemical processes of these proteins involving (bio)macromolecules without perturbation in the native states at the atomic level in time scales ranging from femtoseconds up to days.

Nature provides us with the ultimate probe via stable isotopes. Isotopes combine the same chemistry with different physical properties.[4] Study of a system with site-directed isotope labeling with a high incorporation allows the determination of the whole force field via vibrational techniques such as FT infrared spectroscopy and (Resonance) Raman spectroscopy based on the difference in isotopic mass.[5−7] These techniques probe, for instance, the electron density in chemical bonds of the isotope-labeled molecule. Another spectroscopic method is solid-state magic angle spinning (MAS) NMR spectroscopy, which probes the electron density at the atoms. This technique allows the establishment of electronic charges in the atoms, protonation states, and configurations and conformations around bonds of the stable isotopically labeled molecule.[8,9]

Comparison of the structural parameters obtained via these techniques for intermediate I and intermediate I + 1 in the biochemical process of the studied system provides functional information, that is, changes in protonation states, bond lengths, configuration, and conformation around bonds on the time scale involved.[7,8] When sufficient structural and functional information at the atomic level of the native form has been obtained, a whole new dimension can be attained by studying in a similar fashion systems with mutations in the protein chain and systems with rationally designed chemical changes in the cofactors.[9,10] These studies will lead to an even deeper understanding of the biochemical process.

The implementation of the above-mentioned program has now utmost urgency. Without this program the now increasingly

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available genetic information in the post-genomic era cannot be translated into the required structural and functional information that will lead to the expected quantum jump in the understanding of the various processes in human (animal) health and diseases and the expected rational approach to treat these diseases.

The access to a full number of possible site-directed stable isotopically enriched building blocks (amino acids and cofactors) up to the uniformly labeled systems is a "condition sine qua non" for the proposed structural and functional investigations. All uniformly isotopically labeled amino acids and several cofactors are available via photosynthetic organisms that are grown in media containing $^{13}CO_2$ and $^{15}NH_3$.[11]

To start the above-mentioned program, access to building blocks with isotope labels at each defined atomic position and combination of positions up to the uniformly labeled form is required. The only way to obtain access to the whole cassette of desired isotopomers is a modular synthetic approach such that one synthetic scheme can give in a rational way the required building block as a cassette of all isotopomers. This approach may seem Herculean; however, only 20 different amino acids and a limited number of cofactors are required. The synthesis of these cassettes has to be based on a limited number of commercially available highly enriched stable isotopically labeled starting materials.

In this paper we describe the method to prepare the full cassette of all site-directed ^{13}C-labeled isotopomers of *all-trans*-retinal up to the uniformly ^{13}C-labeled form. The selection of [U-$^{13}C_{20}$]-labeled *all-trans*-retinal and all its isotopomers in the new modular approach is based on the fact that retinal and retinoids play a very important role in many life processes.[12−14] In addition, structural and functional studies with isotope-sensitive techniques have already been initiated in the field of rhodopsin.[8,9,15,16]

Rhodopsin serves as the paradigm for the superfamily of seven transmembrane helix G-protein coupled receptors (GPCRs).[17] The GPCRs mediate a broad array of important physiologically and pharmacologically signal transduction processes. GPCRs trigger a wide variety of physiological processes that involve signaling by neurotransmitters, hormones, and neuropeptides.[18] Consequently, GPCRs are the major pharmaceutical targets for pharmacological intervention in human (and veterinary) pathology. In rhodopsin the photoreactive ligand is 11-*cis*-retinal that is covalently bound in the interior of the protein via a protonated Schiff base linkage with lysine residue 296 to form the retinylidene chromophore.[12] Absorption of a photon leads to the ultrafast isomerization of the C11═C12 bond of the retinylidene ligand from the 11-cis to the all-trans configuration in only ∼200 fs, the fastest photochemical reaction on earth to date.[12,19]

The full vibration analysis of the chromophore of rhodopsin and its photoproduct, bathorhodopsin, has been reported via about 70 isotopomers.[6,20] Furthermore, the chemical shift values of the sp^2 carbons in the tail end of the chromophore have been reported.[9,15] The distances between $C_{10}-C_{20}$ and $C_{11}-C_{20}$ could be determined with very high precision (0.1 Å) via the 1-D rotational resonance solid-state ^{13}C NMR technique.[8] From these distance measurements the precise chromophore structure could be derived. The molecular torsional angle around a bond in the chromophore labeled with two ^{13}C isotopes could be directly established via this method.[21] Furthermore, we recently published the ultrahigh-field solid-state MAS NMR study on the [8,9,10,11,12,13,14,15,19,20-$^{13}C_{10}$]-11-*cis*-retinylidene chromophore in its natural lipid membrane environment.[9] This study showed that the use of multispin labeling in combination with 2-D solid-state MAS NMR correlation spectroscopy improves the relative accuracy of the shift measurements in solids. This allows the electronic structure of the retinylidene chromophore to be analyzed at high levels of understanding: (1) by specifying the interactions between the ^{13}C-labeled ligand and the G-protein coupled receptor target and (2) by making an assessment of the various factors contributing to the charge distribution in the chromophore. In one short experimental session, information of much higher quality about 10 carbon atoms at the same time was obtained that thus far has taken a decade to collect. Nevertheless, these results were obtained via studies of the almost unlimited supply of bovine rhodopsin from cattle eyes. However, cone pigments of man and other animals and the various opsins with site-directed mutations have to be obtained via biotechnological expression systems, which implies that now the availability is the limiting factor.[3,22] Recently we have published a solid-state ^{15}N MAS NMR study of rod visual pigment rhodopsin in which 99% ^{15}N enriched [α,ϵ-$^{15}N_2$]-L-lysine was incorporated by using the baculovirus/Sf9 cell expression system.[16]

It is clear that the demand for [U-$^{13}C_{20}$]-labeled *all-trans*-retinal, synthesized via a modular synthetic scheme, is urgently needed. In addition, this scheme must also allow an easy access to the complete cassette of all desired isotopomers. In this paper we describe a modular synthetic scheme, which enables us to prepare [U-$^{13}C_{20}$]-labeled *all-trans*-retinal and all isotopomers via an efficient and economic way.

Results and Discussion

Synthesis. For the synthesis of [U-$^{13}C_{20}$]-labeled *all-trans*-retinal (**1**) and all its isotopomers with ^{13}C-labels at any carbon position or combination of positions, we have developed a modular convergent synthetic scheme. This synthetic scheme has to meet several important requirements, which allows the introduction of the stable isotopes: (1) the synthesis should be based on starting compounds of low molecular weight (e.g. acetic acid, acetonitrile, acetone) that are commercially available

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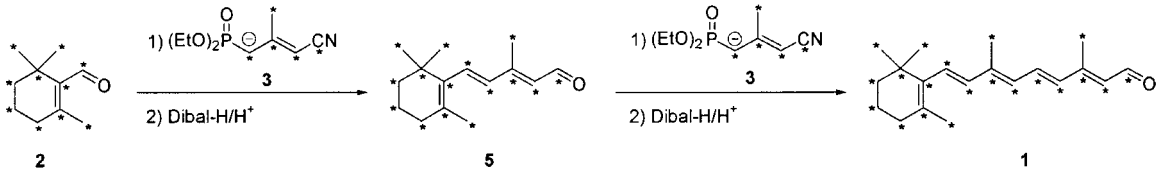
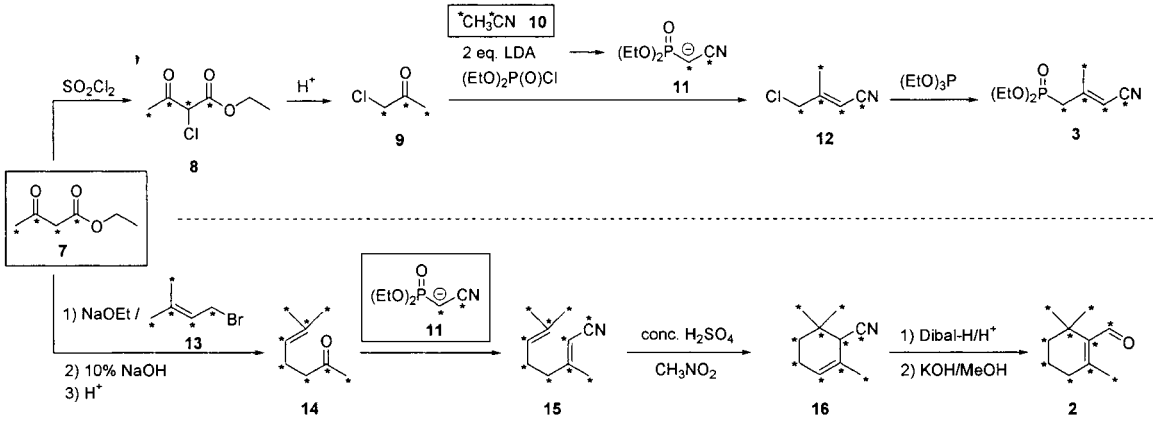
Scheme 1. Synthesis of [U-$^{13}C_{20}$]-*all-trans*-Retinal (**1**) via a Horner-Emmons Reaction Sequence Using the 5-Fold ^{13}C-Labeled C_5-Phosphonate Module, [1,2,3,4,(3-CH_3)-$^{13}C_5$]-4-(Diethylphosphono)-3-methyl-2-butene-nitrile (**3**)[a]



[a] The asterisks (*) indicate the positions of the ^{13}C-labels.

Scheme 2. Synthetic Scheme for the Preparation of [1,2,3,4,(3-CH_3)-$^{13}C_5$]-4-(Diethylphosphono)-3-methyl-2-butenenitrile (**3**) (upper part) and [U-$^{13}C_{10}$]-2,6,6-Trimethylcyclohex-1-enylcarbaldehyde (**2**) (lower part)[a]



[a] Both compounds were prepared from the commercially available module [1,2,3,4-$^{13}C_4$]-ethyl acetoacetate (**7**). An important intermediate that is synthesized via this method is [U-^{13}C]-chloroacetone (**9**), which is not commercially available.

in specifically and high enriched (>99%) form; (2) since these starting materials are quite expensive, an efficient, convergent, and thoroughly optimized synthetic scheme is required; and (3) care should be taken that no scrambling or dilution of the ^{13}C-label can occur at any stage in the course of the synthesis. To meet these requirements, we have developed an economic and convergent scheme for the preparation of *all-trans*-retinal (**1**) that is based on four different modules: [1,2,3,4,(3-CH_3)-$^{13}C_5$]-4-(diethylphosphono)-3-methyl-2-butenenitrile (**3**), [1,2,3,4-$^{13}C_4$]-ethyl acetoacetate (**7**), [U-$^{13}C_5$]-4-bromo-2-methyl-2-butene (**13**), and [U-$^{13}C_{10}$]-2,6,6-trimethylcyclohex-2-ene-1-ylcarbonitrile (**16**). With the exception of β-keto ester **7**, which is commercially available, these modules are prepared in a small number of steps from commercially available 99% enriched ^{13}C-labeled starting materials of low molecular weight. Via these modules **1** can be obtained with ^{13}C-labels introduced at any carbon position or combination of positions in a highly enriched form. In addition, modification of the modules makes it possible to prepare a range of chemically modified retinoids and carotenoids. Furthermore, two modules in this scheme are used twice in the synthesis of **1**, leading to an efficient use of the relatively expensive isotopically enriched starting materials.

Via a Horner−Emmons reaction sequence using the 5-fold ^{13}C-labeled C_5-phosphonate module, [1,2,3,4,(3-CH_3)-$^{13}C_5$]-4-(diethylphosphono)-3-methyl-2-butenenitrile (**3**), we elongate in just four consecutive steps the polyene chain that is attached to the ring moiety, starting from [U-$^{13}C_{10}$]-2,6,6-trimethylcyclohex-1-enylcarbaldehyde (**2**) (Scheme 1). The unlabeled phosphonate has already been applied many times for the synthesis of several carotenoids and retinoids.[23,24] In the first chain elongation, the anion of phosphonate **3** was coupled with β-cyclocitral **2** affording the nitrile [U-$^{13}C_{15}$]-3-methyl-5-(2,6,6-trimethyl-cyclohex-1-enyl)penta-2,4-dienenitrile (**4**) in a yield of 81%. Subsequently, nitrile **4** was reduced with DIBAL-H to give the corresponding aldehyde [U-$^{13}C_{15}$]-3-methyl-5-(2,6,6-trimethyl-cyclohex-1-enyl)penta-2,4-dienal (**5**) in 79% yield. The same procedure was used to convert C_{15}-aldehyde **5** into [U-$^{13}C_{20}$]-3,7-dimethyl-9-(2,6,6-trimethyl-cyclohex-1-enyl)nona-2,4,6,8-tetraenal (**1**). Coupling of 5-fold labeled phosphonate **3** to aldehyde **5** via a second Horner−Emmons reaction gave [U-$^{13}C_{20}$]-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenenitrile (**6**) in 97% yield, which was reduced with DIBAL-H to obtain the final product, retinal **1**, as a mixture of isomers. This reaction sequence directly shows the potency of the use of modules for the preparation of multifold labeled molecules. In addition, chemically modified retinoids with various kinds of substituents introduced at positions C-10 and C-14 can be synthesized based on the methods described by Verdegem et al.[25] The aforementioned β-cyclocitral **2** and C_5-phosphonate **3** are both prepared from another important and commercially available module, [1,2,3,4-$^{13}C_4$]-ethyl acetoacetate (**7**) (Scheme 2).

The 5-fold ^{13}C-labeled C_5-phosphonate **3** was prepared in four steps from the commercially available β-keto ester module **7**. First, chlorination of **7** gives [1,2,3,4-$^{13}C_4$]-ethyl 2-chloro-acetoacetate (**8**) in a quantitative yield.[26] Subsequently, acid-catalyzed hydrolysis of the ester and decarboxylation of the acid group gave [U-$^{13}C_3$]-chloroacetone (**9**). This hydrolysis of ester **8** is the crucial step in the synthesis of the 5-fold ^{13}C-labeled phosphonate **3**. Generally, the hydrolysis of esters is carried

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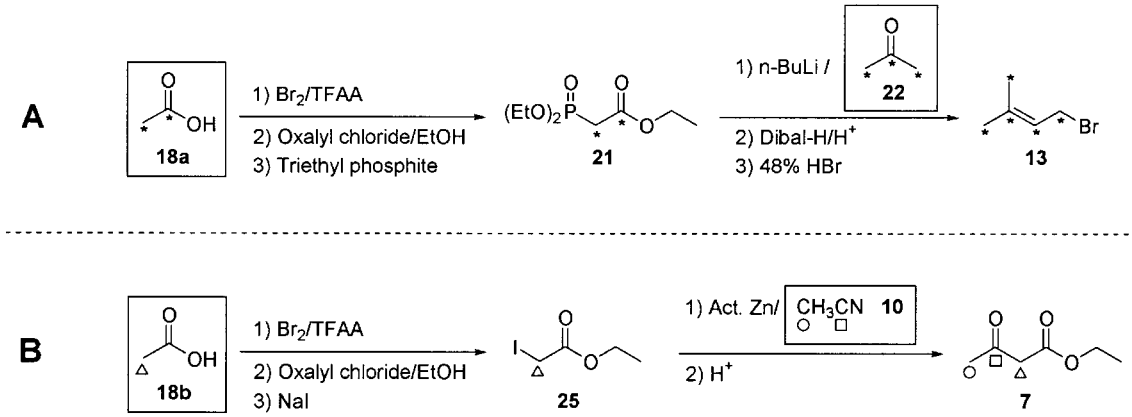
Scheme 3. (A) Synthetic Scheme for the Preparation of [U-$^{13}C_5$]-4-Bromo-2-methyl-2-butene (13) from the Commercially Available [$^{13}C_2$]-Acetic Acid (**18a**) and [$^{13}C_3$]-Acetone (**22**) and (B) Synthesis of [2,3,4-$^{13}C_3$]-Ethyl Acetoacetate (**7**)[a]



[a] The key step is a Blaise reaction where [$^{13}C_2$]-acetonitrile (**10**) is coupled to ester **25** to give in two steps β-keto ester **7**. The three ^{13}C-labeles are indicated with △, ○, and □ visualizing the three different positions of ^{13}C-labeling when different starting materials are used.

out with a basic solution. However, the hydrolysis of β-keto ester **8** cannot be carried out in an alkaline solution since under these conditions a retro-Claisen condensation occurs. Therefore, an acidic solution was used to hydrolyze the ester bond. Subsequently, the decarboxylation of the carboxyl group gave the uniformly ^{13}C-labeled ketone **9**. In addition, even though one ^{13}C-label is lost via this way because of the decarboxylation of the ester **8**, this method was used for the preparation of the phosphonate due to the fact that the 4-fold ^{13}C-labeled ethyl acetoacetate is commercial available.

Coupling of ketone **9** with the anion of the in situ prepared phosphonate **11** via a Horner−Emmons reaction gave [U-$^{13}C_5$]-4-chloro-3-methyl-2-butenenitrile (**12**) in a yield of 70% (two steps). This coupling is based on a reaction that has been used many times in our group.[24,27] After the deprotonation of [$^{13}C_2$]-acetonitrile (**10**) with 1 equiv of LDA, the formed anion reacted with diethyl chlorophosphate to give [$^{13}C_2$]-diethyl cyanomethylphosphonate (**11**), which was immediately deprotonated by the second equivalent of LDA. [$^{13}C_2$]-Acetonitrile (**10**) was in this way converted in situ into the reactive anion of the [$^{13}C_2$]-diethyl cyanomethylphosphonate (**11**), which reacted with ketone **9** to give nitrile **12**. The chlorine atom of nitrile **12** was substituted via an Arbuzov reaction with triethyl phosphite to afford the first module, [1,2,3,4,(3-CH_3)-$^{13}C_5$]-4-(diethylphosphono)-3-methyl-2-butenenitrile (**3**) in 95% yield.

The first step in the synthesis of β-cyclocitral **2** is an alkylation at the α-position by adding the third module, [U-$^{13}C_5$]-4-bromo-2-methyl-2-butene (**13**), to the anion of β-keto ester **7**, which was also used for the preparation of phosphonate **3**. Subsequently, saponification and acid-catalyzed decarboxylation gave [U-$^{13}C_8$]-6-methyl-5-hepten-2-one (**14**) in a yield of 75% based on bromide **13**. The Horner−Emmons reaction with the anion of the in situ prepared [$^{13}C_2$]-diethyl cyanomethylphosphonate (**11**) gave [U-$^{13}C_{10}$]-3,7-dimethylocta-2,6-dienenitrile (**15**) in 72% yield. Next, according to the method described by Jansen et al., the open-chain nitrile **15** was cyclized to give the cyclic compound [U-$^{13}C_{10}$]-2,6,6-trimethylcyclohex-2-ene-1-ylcarbonitrile (**16**).[28] In the workup procedure of this reaction it proved to be important to keep the conditions acidic to avoid a double bond shift to the β-position. Nitrile **16** might be seen as the fourth module, due to the fact that this intermediate can also be used for the synthesis of various carotenoids and retinoids containing chemical modifications in the ring moiety.[28,29] Reduction of nitrile **16** with DIBAL-H gave [U-$^{13}C_{10}$]-2,6,6-trimethylcyclohex-2-ene-1-ylcarbaldehyde (**17**) in a yield of 68%. Subsequently, isomerization of the double bond from the α-position to the β-position yielded β-cyclocitral **2** in a yield of 80%.

Bromide **13**, which was coupled to the β-keto ester **7**, was synthesized in 6 steps from the commercially available ^{13}C-labeled starting materials [$^{13}C_2$]-acetic acid (**18a**) and [$^{13}C_3$]-acetone (**22**) (Scheme 3A). Bromination of [$^{13}C_2$]-acetic acid (**18a**) with bromine and trifluoroacetic anhydride (TFAA) gave [$^{13}C_2$]-bromoacetic acid (**19a**) in an excellent yield of 93%.[30,31] Subsequently, acid **19a** was esterificated to give [$^{13}C_2$]-ethyl bromoacetate (**20a)** in 93% yield with oxalyl chloride and ethanol. Next, substitution of the bromine atom of ester **20a** via an Arbuzov reaction with triethyl phosphite gave [1,2-$^{13}C_2$]-ethyl-2-(diethylphosphono) acetate (**21**) in a quantitative yield. Coupling of phosphonate **21** with [$^{13}C_3$]-acetone (**22**) via a Horner−Emmons reaction gave [1,2,3,4,(3-CH_3)-$^{13}C_5$]-ethyl-3-methyl-2-butenoate (**23**) in a yield of 91%, based on acetone. The coupling was done by using an excess of 1.5 equiv of the phosphonate. Using an equimolar amount of **21** resulted in a much lower yield (∼30%) that stands for the loss of a large quantity of the expensive [$^{13}C_3$]-acetone. Ester **23** was reduced with DIBAL-H to give the corresponding alcohol [U-$^{13}C_5$]-3-methyl-2-buten-1-ol (**24**). Even though TLC analysis and change of color showed an instantaneous conversion of the ester group, the optimized yield of the reaction was only 55%. However, in the meantime a method is described by Vassilikogiannakis et al. using $LiAlH_4$ as reductor leading to an increase of the yield up to 90%.[32] Subsequently, alcohol **24** was converted into the bromide by using 47% hydrobromic acid to obtain the fourth module, bromide **13**.

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We have prepared [U-$^{13}C_{20}$]-retinal as a mixture of isomers via the above-described method. Through column chromatography the *all-trans*-retinal was isolated from the cis isomers. Subsequently, cis-to-trans isomerization was accomplished via irradiation of a solution of the cis isomers under a tungsten lamp in the presence of iodine crystals. Finally, a total amount of 125 mg of [U-$^{13}C_{20}$]-*all-trans*-retinal (**1**), which has identical chemical properties (λ_{max}, R_f value) as the natural abundance *all-trans*-retinal, was obtained via this modular synthetic scheme. In just two months time the [U-$^{13}C_{20}$]-*all-trans*-retinal (**1**) was synthesized from 2.0 g of [$^{13}C_2$]-acetic acid, 4.4 g of [1,2,3,4-$^{13}C_4$]-ethyl acetoacetate, 1.0 g of [$^{13}C_3$]-acetone, and 1.2 g of [$^{13}C_2$]-acetonitrile. These starting materials were a kind gift from Cambridge Isotope Laboratories.

To also have the ability to synthesize the complete cassette of isotopomers, a synthetic method has been developed for the preparation of ethyl acetoacetate (**7**), which is not commercially available with a ^{13}C-label at all position combinations. The β-keto ester **7** can be obtained via a Blaise reaction.[33,34] In this reaction a zinc ester enolate is coupled to a nitrile to give an enamino ester that is hydrolyzed to obtain the corresponding β-keto ester. Although the synthesis of [2,3,4-$^{13}C_3$]-ethyl acetoactetate is discussed in this paper, all combinations of the ^{13}C-labeled β-keto ester can be prepared via this method (Scheme 3B). For the synthesis of the 3-fold ^{13}C-labeled ester **7**, the zinc enolate was prepared from [2-^{13}C]-ethyl iodoacetate (**25**) that was coupled with ^{13}C-labeled acetonitrile (**10**). The use of ester **25** instead of ethyl bromoacetate, as is mentioned in the literature, enables us to lower the amount of **25** that is preferred due to the fact that ^{13}C-labeled starting compounds are relatively expensive.[34] Ester **25** was synthesized in three steps from [2-^{13}C]-acetic acid (**18b**). First, the acid was converted into [2-^{13}C]-ethyl bromoacetate (**20b**) in a yield of 88% (two steps), using the aforementioned method. Subsequently, the bromide was replaced by an iodide via an S_n2 reaction with NaI to give [2-^{13}C]-ethyl iodoacetate (**25**) in 98% yield. In the presence of activated zinc dust in refluxing THF, ester **25** was converted into the zinc enolate. Addition of [$^{13}C_2$]-acetonitrile (**10**) gave the enamino ester that was hydrolyzed to give [2,3,4-$^{13}C_3$]-ethyl acetoacetate (**7**) in a yield of 80% (two steps).

As depicted in Scheme 3B, all positions and combinations of positions of the β-keto ester can be labeled by using different ^{13}C-labeled starting materials. However, the labeling of both methyl groups C16 and C17 forms the exception. These labels are introduced by using [$^{13}C_3$]-acetone (**22**) (Scheme 3A), leading to the unfeasibility of the discrimination between these two positions. Nevertheless, the above-described schemes enable the synthesis of nearly the whole cassette of isotopomers of *all-trans*-retinal. Next to this, we are also able to prepare a broad range of chemically modified retinals via small modifications of the developed synthetic scheme .[28,29]

Characterization of [U-$^{13}C_{20}$]-*all-trans*-Retinal (1). The characterization of the compounds will be focused on the final product, [U-$^{13}C_{20}$]-*all-trans*-retinal (**1**), whereas the spectroscopic data of all other synthesized intermediates can be found in the Experimental Section. Preparative HPLC was used for the purification of *all-trans*-retinal. The purified all-trans isomer was subsequently characterized by using mass spectroscopy and high-resolution NMR spectroscopy.

Mass Spectrometry. The single focus electron-impact mass spectrum of the [U-$^{13}C_{20}$]-*all-trans*-retinal (**1**) appears as a typical retinoid mass spectrum with the base peak at *m*/*z* 304, the molecular ion peak. Compared to the natural abundance *all-trans*-retinal (*m*/*z* 284) the base peak has shifted 20 mass units.[35] This shift is due to the substitution of all 20 ^{12}C nuclei for ^{13}C nuclei. Next to the molecular ion peak a second large peak is observed at *m*/*z* 286. This peak is also observed in the mass spectrum of natural abundance *all-trans*-retinal and is due to the expulsion of water.

Double focus mass spectroscopy was used to determine the exact mass of the synthesized compounds. A mass of 304.28037 was determined for the [U-$^{13}C_{20}$]-retinal (**1**), whereas the natural abundance *all-trans*-retinal has a mass of 284.21412. These values are in close agreement with the calculated mass ($^{13}C_{20}H_{28}O$ = 304.28111; $C_{20}H_{28}O$ = 284.21402).

The degree of incorporation was determined by using the computer simulation program Isopro 3.0 (Cornell University). The isotope incorporation was determined via comparison of the exact mass of the $M^{+\bullet}$ peak with the $[M-1]^{+\bullet}$, $[M-2]^{+\bullet}$, and $[M-3]^{+\bullet}$ peak of the simulated mass spectrum and the spectrum of the [U-$^{13}C_{20}$]-retinal (**1**). On the basis of these results an isotope incorporation of ≥99% was found. Consequently, no isotope dilution took place during the synthesis of the retinal.

NMR Spectroscopy: (a) ^{1}H NMR Spectroscopy. ^{1}H NMR spectroscopy was used to determine the structure and purity of the synthesized products. Additional *J*-couplings occur due to the 99% enriched ^{13}C-label at each carbon position. However, these ^{13}C enrichments do not influence the chemical shifts of both the protons and carbons. In Figure 1 the most significant regions of the ^{1}H NMR spectrum (600 MHz, $CDCl_3$) of [U-$^{13}C_{20}$]-*all-trans*-retinal (Figure 1, spectra A and B) and natural abundance *all-trans*-retinal (Figure 1C) are shown. The additional *J*-couplings between the carbon nuclei and the protons result in complex splitting patterns (Figure 1A). However, by using ^{13}C decoupling these relatively strong carbon-proton couplings are eliminated, resulting in a simplification of the ^{1}H spectrum (Figure 1B). Due to the strong decoupling power, necessary for the ^{1}H−^{13}C spin-decoupling across the complete spectrum, line broadening occurs, which is caused by heating of the sample. Therefore, the *J*-couplings of the retinal where defined by using the lower spectrum whereas the ^{13}C decoupled proton spectrum was used for the assignment of all proton resonances. Comparison of the spectra of the [U-$^{13}C_{20}$]-*all-trans*-retinal and the unlabeled retinal directly proves that the introduction of the ^{13}C-labels does not affect the isotropic chemical shifts of the protons. These shifts and the relating *J*-couplings of [U-$^{13}C_{20}$]-retinal are summarized in Table 1. The determined spectral values are in complete agreement with the published data of Liu et al.[36]

In addition, the intensity of 15-H shows that a high isotope enrichment is achieved. The double-doublet resonance, marked with an asterisk (*), occurs as a result of isotope dilution: the incorporation of ^{13}C at position C15 can be estimated at ≥99%, within the experimental error.

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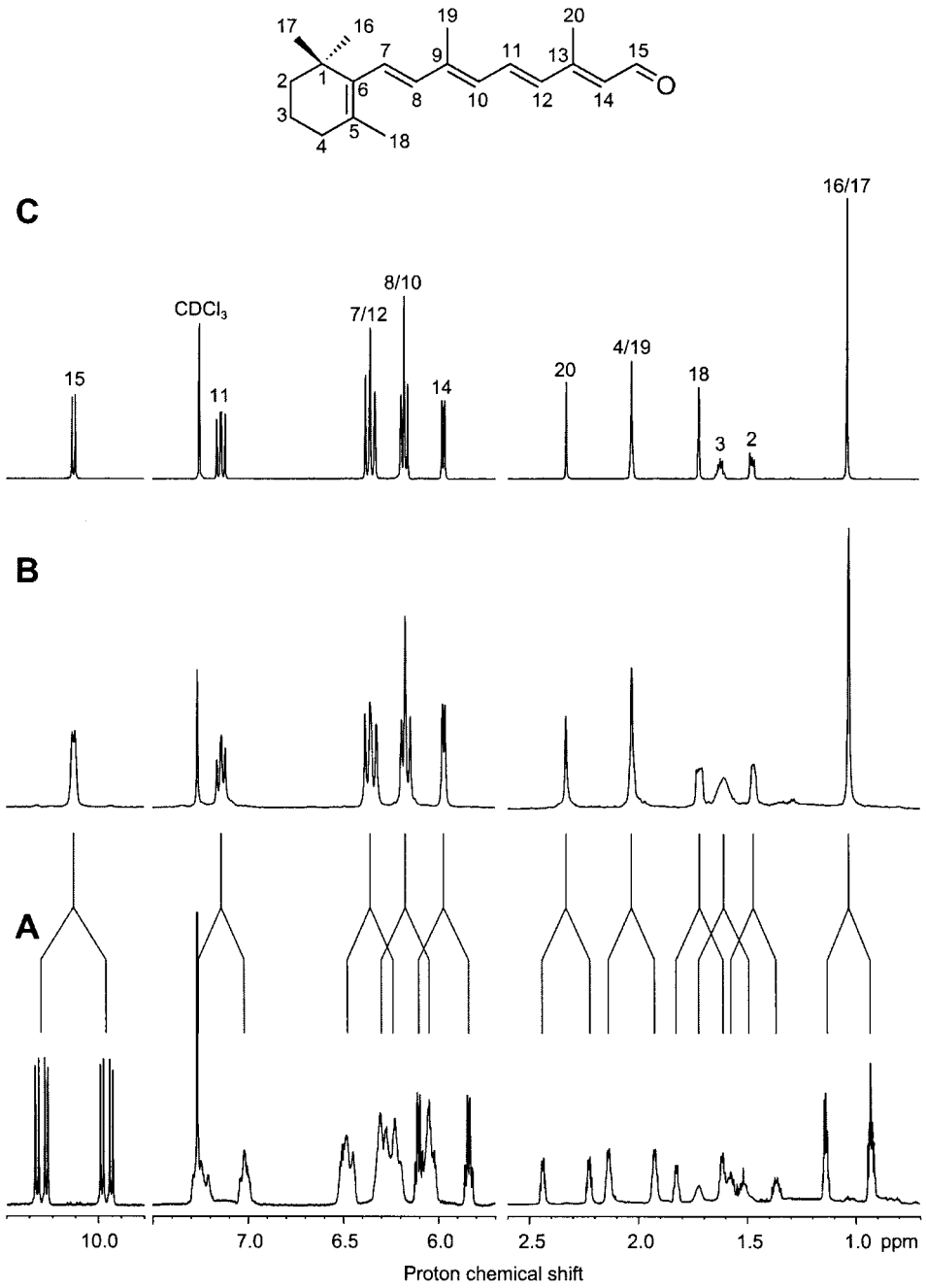

Figure 1. Significant regions of the ^{1}H NMR spectrum (600 MHz, $CDCl_3$) of the [U-$^{13}C_{20}$]-*all-trans*-retinal (**1**) (A + B) and the natural abundance *all-trans*-retinal (C). The additional *J*-couplings between the carbon nuclei and the protons result in complex splitting patterns (part A). However, by using ^{13}C decoupling these relatively strong carbon−proton couplings can be overcome, leading to a simplification of the ^{1}H spectrum (part B). Due to strong decoupling power line broadening occurs that is caused by heating of the sample. The lines demonstrate the large $^1J_{C-H}$ couplings. The doublet−doublet resonance, marked with an asterisk (*), occurs as a result of isotope dilution.

(b) ^{13}C NMR Spectroscopy. The significant regions of the ^{1}H-noise-decoupled ^{13}C NMR spectrum (150 MHz, $CDCl_3$) of [U-$^{13}C_{20}$]-*all-trans*-retinal (A) and natural abundance *all-trans*-retinal (B) are shown in Figure 2. Like the ^{1}H-spectrum, the ^{13}C spectrum of the ^{13}C-labeled retinal also shows similarity with the ^{13}C-spectrum of the natural abundance retinal. Due to all ^{13}C-labels, complex splitting patterns are observed in the ^{13}C NMR spectrum. Nevertheless, the chemical shift values of all carbon nuclei can be assigned. These shifts are in close agreement with the published data of Liu et al. and are summarized in Table 2.[36]

Conclusions

We have synthesized [U-$^{13}C_{20}$]-*all-trans*-retinal (**1**) via a modular strategy in an economic and convergent way with high isotope incorporation (>99%) and without isotope dilution. Using 2.0 g of [$^{13}C_2$]-acetic acid, 4.4 g of [1,2,3,4-$^{13}C_4$]-ethyl acetoacetate, 1.0 g of [$^{13}C_3$]-acetone, and 1.2 g [$^{13}C_2$]-acetonitrile we have prepared 125 mg of [U-$^{13}C_{20}$]-*all-trans*-retinal (**1**). The overall yield of the synthesis was 6% (17 steps) based on [$^{13}C_2$]-acetic acid (**18a**). After optimization of the scheme with unlabeled starting materials, the [U-$^{13}C_{20}$]-*all-trans*-retinal (**1**) was synthesized in just two months time. As far as we know

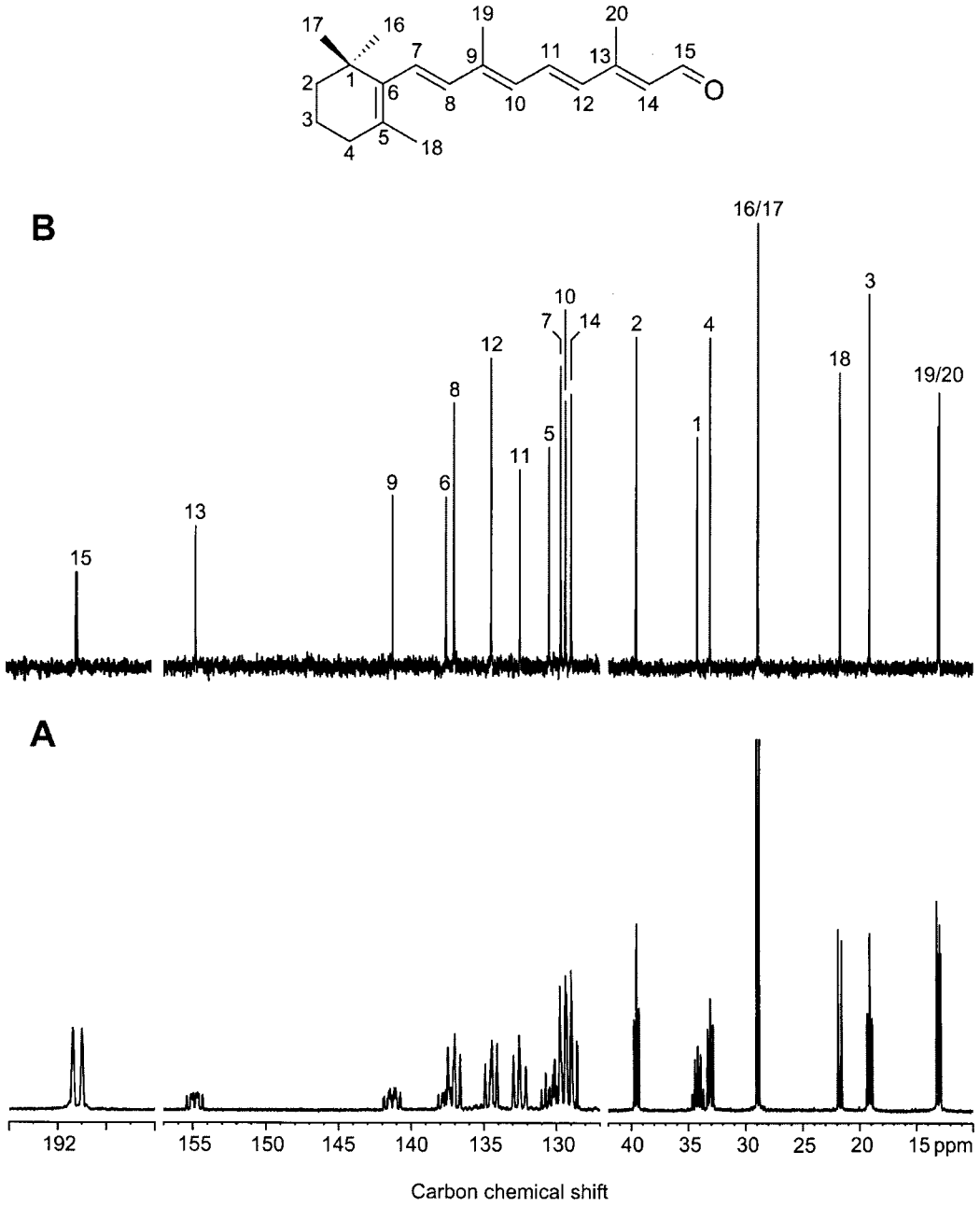

Figure 2. Significant regions of the ^{13}C NMR spectrum (150 MHz, $CDCl_3$) of the [U-$^{13}C_{20}$]-*all-trans*-retinal (**1**) (A) and the natural abundance *all-trans*-retinal (B). Due to all ^{13}C-labels, complex splitting patterns are observed in the ^{13}C NMR spectrum.

Table 1. ^1H NMR Data of [U-$^{13}C_{20}$]-*all-trans*-Retinal (**1**)[a]

δ (ppm)	H	multiplicity	J (Hz)	intensity
10.11	CHO	ddd	$^1J_{C-H} = 169.7$ $^2J_{C-H} = 24.5$ $^3J_{H-H} = 8.2$	1
7.14	H-11	dm	$^1J_{C-H} = 150.1$	1
6.36	H-7/H-12	m	–	2
6.17	H-8/H-10	m	–	2
5.97	H-14	dm	$^1J_{C-H} = 157.6$	1
2.33	13-CH_3	dm	$^1J_{C-H} = 127.8$	3
2.03	9-CH_3	dm	$^1J_{C-H} = 126.7$	3
2.01	H-4	m	–	2
1.72	5-CH_3	dm	$^1J_{C-H} = 125.7$	3
1.68	H-3	dm	$^1J_{C-H} = 127.5$	2
1.47	H-2	dm	$^1J_{C-H} = 128.2$	2
1.03	1-CH_3	dm	$^1J_{C-H} = 125.1$	6

[a] The ^1H chemical shift values are in complete agreement with the published data of Asato et al.[36]

this is the first organic system of average size where all carbons derive from a single carbon source. In addition, the developed scheme also enables the synthesis of the full cassette of site-directed ^{13}C-labeled isotopomers by using alternatively ^{13}C-labeled starting materials. In addition, modifications of the described modules give access to a broad range of chemically modified retinoids and carotenoids.[28,29] The [U-$^{13}C_{20}$]-labeled retinal was used for solid-state MAS NMR spectroscopic studies of the spatial and electronic structure of the 11-*cis*-retinylidene chromophore in rhodopsin and isorhodopsin. The structural information will lead to a deeper insight of the mechanism of the ultrafast photoconversion in rhodopsin.

On the basis of this work we think that organic synthesis is now able to supply in an efficient and economic way all cofactors and amino acids in a [U-^{13}C]-labeled form and as a

Table 2. ^{13}C NMR Data of [U-$^{13}C_{20}$]-*all-trans*-Retinal (**1**)[a]

δ (ppm)	C	multiplicity	J (Hz)
191.1	CHO	d	$^1J_{C-C} = 57.4$
154.9	C-13	m	
141.3	C-9	m	
137.5	C-6	m	
137.0	C-8	dd	$^1J_{C-C} = 72.1$
			$^1J_{C-C} = 58.4$
134.5	C-12	dddd	$^1J_{C-C} = 69.4$
			$^1J_{C-C} = 53.9$
			$^2J_{C-C} \approx {}^2J_{C-C} = 7.4$
132.5	C-11	ddm	$^1J_{C-C} = 69.9$
			$^1J_{C-C} = 53.9$
130.5	C-5	ddd	$^1J_{C-C} = 75.1$
			$^1J_{C-C} \approx {}^1J_{C-C} = 35.4$
129.2	C-7/C-10/C-14	m	
39.5	C-2	dd	$^1J_{C-C} \approx {}^1J_{C-C} = 35.4$
34.2	C-1	dddd	$^1J_{C-C} \approx {}^1J_{C-C} \approx {}^1J_{C-C} \approx {}^1J_{C-C} = 35.4$
33.1	C-4	dd	$^1J_{C-C} \approx {}^1J_{C-C} = 35.4$
28.9	1-CH_3	d	$^1J_{C-C} = 35.4$
21.8	5-CH_3	dm	$^1J_{C-C} = 41.9$
19.1	C-3	dd	$^1J_{C-C} \approx {}^1J_{C-C} = 35.4$
13.1	13-CH_3	dm	$^1J_{C-C} = 40.4$
13.0	9-CH_3	dm	$^1J_{C-C} = 40.4$

[a] The ^{13}C chemical shift values are in complete agreement with the published data of Liu et al.[36]

full cassette of site-directed isotopomers. The modular total organic synthetic strategy will make a central contribution to the already efficient expression methods. Together with the isotope-sensitive NMR and vibrational spectroscopic methods translation of the now available genetic information into structural and functional information at the atomic level of the proteins that are coded by the genes can be implemented. In addition, these studies can also be extended without any problem toward systems with site-directed modifications in the cofactors and in the protein chain. These structural changes can be compared with the native system at the atomic level leading to an even deeper understanding of the function of the protein system in question.

Experimental Section

General. All experiments were carried out in a dry nitrogen atmosphere, unless aqueous conditions were used. If necessary, reaction vessels were flame-dried under nitrogen prior to use. The following solvents were dried either by distillation (EtOH from Mg and light petroleum ether (low boiling petroleum ether 40−60 °C) from P_2O_5) or by storing over molecular sieves (4 Å) (THF). Saturated solutions of $Na_2S_2O_3$, NaOAc, $NaHCO_3$, and NH_4Cl refer to saturated solutions of the salt in water. Brine refers to a saturated solution of NaCl in water.

Reactions were monitored by using thin-layer chromatography (TLC, on Merck F_{254} silica gel 60 aluminum sheets, 0.2 mm; spots were visualized with UV-light (254 nm)) or treated with an oxidizing spray (2 g of $KMnO_4$ and 4 g of $NaHCO_3$ in 100 mL of water). Column chromatography was performed on Merck silica gel 60 (0.040−0.063 mm, 230−400 mesh).

Melting points were measured on a Büchi apparatus and are given uncorrected.

^{1}H NMR spectra were recorded on a Bruker AM-600 spectrometer or a Bruker WM-300 with tetramethylsilane (TMS; $\delta = 0.00$ ppm) as internal standard. ^{1}H noise-decoupled ^{13}C spectra were recorded on a Bruker AM-600 at 151 MHz with chloroform ($\delta = 77.0$ ppm) as an internal standard.

Mass spectra were recorded on a Finnigan MAT 900 equipped with a direct insertion probe (DIP) or on a Finnigan MAT 700-TSQ equipped with a custom-made electron-spray interface (ESI).

[2-^{13}C]-Acetic acid, [$^{13}C_2$]-acetic acid, [$^{13}C_2$]-acetonitrile, [$^{13}C_3$]-acetone, and [1,2,3,4-$^{13}C_4$]-ethyl acetoacetate were a kind gift from Cambridge Isotope Laboratories Inc., USA. All other chemicals were purchased from Aldrich, Fluka or Acros Chimica.

[U-$^{13}C_{15}$]-3-Methyl-5-(2,6,6-trimethylcyclohex-1-enyl)penta-2,4-dienenitrile (4). To a solution of 303 mg (1.4 mmol) of [1,2,3,4,(3-CH_3)-$^{13}C_5$]-4-(diethylphosphono)-3-methyl-2-butenenitrile (**3**) in 15 mL of dry THF was added at 0 °C via a syringe 0.75 mL (1.2 mmol) of *n*-BuLi (1.6 M solution in hexane). Stirring was continued for 15 min at room temperature. To the anion of the phosphonate was added 130 mg (0.80 mmol) of β-cyclocitral (**2**), dissolved in 10 mL of dry THF. The mixture was stirred for another 4 h at room temperature. Quenching of the reaction was accomplished by adding 50 mL of saturated NH_4-Cl solution. The aqueous layer was extracted three times with 50 mL of diethyl ether. The combined organic layers were washed with brine, dried with $MgSO_4$, and filtered. The product was purified by chromatography (silica gel, diethyl ether/40−60 light petroleum ether, 20:80) to give 150 mg (0.65 mmol, 81%) of **4** as a mixture of the 9-cis and all-trans compounds. For the analysis the all-trans isomer was isolated from the other isomer via HPLC purification.

^{1}H NMR (600 MHz, $CDCl_3$) of *all-trans*-[U-$^{13}C_{15}$]-3-methyl-5-(2,6,6-trimethylcyclohex-1-enyl)penta-2,4-dienenitrile (**4**): δ 6.55 (ddm, $^1J_{C-H} = 146.9$ Hz, $^3J_{H-H} = 15.2$ Hz, H-5, 1H), 6.13 (ddm, $^1J_{C-H} = 154.8$ Hz, $^3J_{H-H} = 15.2$ Hz, H-4, 1H), 5.15 (ddd, $^1J_{C-H} = 171.6$ Hz, $^2J_{C-H} \approx {}^2J_{C-H} = 7.4$ Hz, H-2, 1H), 2.20 (ddm, $^1J_{C-H} = 128.2$ Hz, $^2J_{C-H} = 9.5$ Hz, 3-CH_3, 3H), 2.04 (dm, $^1J_{C-H} = 127.3$ Hz, H-3′, 2H), 1.70 (dm, $^1J_{C-H} = 125.8$ Hz, 2′-CH_3, 3H), 1.61 (dm, $^1J_{C-H} = 130.6$ Hz, H4′, 2H), 1.43 (dm, $^1J_{C-H} = 126.2$ Hz, H-5′, 2H), 1.02 (dm, $^1J_{C-H} = 125.5$ Hz, 1′-CH_3, 6H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$) of *all-trans*-[U-$^{13}C_{15}$]-3-methyl-5-(2,6,6-trimethylcyclohex-1-enyl)penta-2,4-dienenitrile (**4**): δ 157.3 (dddm, $^1J_{C-C} = 73.8$ Hz, $^1J_{C-C} = 53.2$ Hz, $^1J_{C-C} = 42.2$ Hz, C-3), 136.2 (m, C-5/C-1′), 132.6 (m, C-4/C-2′), 118.1 (ddm, $^1J_{C-C} = 81.0$ Hz, $^2J_{C-C} = 8.8$ Hz, CN), 96.2 (ddm, $^1J_{C-C} = 81.0$ Hz, $^1J_{C-C} = 73.8$ Hz, C-2), 39.4 (ddm, $^1J_{C-C} \approx {}^1J_{C-C} = 32.9$ Hz, C-5′), 34.1 (m, C-6′), 33.1 (dd, $^1J_{C-C} \approx {}^1J_{C-C} = 36.8$ Hz, C-3′), 28.8 (d, $^1J_{C-C} = 35.6$ Hz, 6′-CH_3), 21.7 (dm, $^1J_{C-C} = 43.4$ Hz, 2′-CH_3), 19.0 (dd, $^1J_{C-C} \approx {}^1J_{C-C} = 36.8$ Hz, C-4′), 16.5 (d, $^1J_{C-C} = 42.2$ Hz, 3-CH_3) ppm.

HRMS (DIP − ESI): calcd for $^{13}C_{15}H_{22}N$ 231.2255; found 231.2234.

[U-$^{13}C_{15}$]-3-Methyl-5-(2,6,6-trimethylcyclohex-1-enyl)penta-2,4-dienal (5). A solution of 150 mg (0.65 mmol) of nitrile **4** in 25 mL of dry light petroleum ether was cooled to −60 °C after which 0.98 mL of 1 M (0.98 mmol) DIBAL-H was added via a syringe. The reaction mixture was allowed to warm to −40 °C in 1 h. Subsequently, a homogeneous mixture of 1.7 g of water absorbed on silica (0.3 g water/gram silica) was added and stirring was continued for another 2 h at 0 °C. After drying of the mixture by adding $MgSO_4$, all solids were filtered off and thoroughly washed with diethyl ether. Evaporation of the organic solvents in vacuo yielded a dark yellow liquid. The product was purified by chromatography (silica gel, diethyl ether/40−60 light petroleum ether, 5:95) to give 120 mg (0.51 mmol, 79%) of **5** as a mixture of the 9-cis and all-trans compound. For the analysis the all-trans isomer was isolated from the other isomer via HPLC purification.

^{1}H NMR (600 MHz, $CDCl_3$) of *all-trans*-[U-$^{13}C_{15}$]-3-methyl-5-(2,6,6-trimethylcyclohex-1-enyl)penta-2,4-dienal (**5**): δ 10.13 (ddd, $^1J_{C-H} = 169.7$ Hz, $^2J_{C-H} = 24.5$ Hz, $^3J_{C-H} = 8.1$ Hz, CHO, 1H), 6.74 (ddm, $^1J_{C-H} = 146.4$ Hz, $^3J_{H-H} = 15.0$ Hz, H-5, 1H), 6.21 (m, H-4, 1H), 5.93 (dddd, $^1J_{C-H} = 157.4$ Hz, $^2J_{C-H} \approx {}^2J_{C-H} \approx {}^3J_{H-H} = 7.4$ Hz, H-2, 1H), 2.31 (dm, $^1J_{C-H} = 127.7$ Hz, 3-CH_3, 3H), 2.04 (dm, $^1J_{C-H} = 125.2$ Hz, H-3′, 2H), 1.72 (dm, $^1J_{C-H} = 125.9$ Hz, 2′-CH_3, 3H), 1.62 (dm, $^1J_{C-H} = 128.7$ Hz, H-4′, 2H), 1.48 (dm, $^1J_{C-H} = 125.0$ Hz, H-5′, 2H), 1.04 (dm, $^1J_{C-H} = 125.5$ Hz, 6′-CH_3, 6H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$) of *all-trans*-[U-$^{13}C_{15}$]-3-methyl-5-(2,6,6-trimethylcyclohex-1-enyl)penta-2,4-dienal (**5**): δ 191.3 (dm, $^1J_{C-C} = 56.8$ Hz, CHO), 155.0 (m, C-3), 136.9 (m, C-1′), 135.6 (m, C-4/C-5), 132.7 (ddd, $^1J_{C-C} = 73.0$ Hz, $^1J_{C-C} \approx {}^1J_{C-C} = 41.7$ Hz,

C-2′), 128.7 (ddm, $^1J_{C-C}$ = 66.7 Hz, $^1J_{C-C}$ = 56.8 Hz, C-2), 39.5 (dd, $^1J_{C-C} \approx {}^1J_{C-C}$ = 34.7 Hz, C-5′), 34.2 (m, C-6′), 33.2 (dd, $^1J_{C-C} \approx {}^1J_{C-C}$ = 34.7 Hz, C-3′), 28.9 (d, $^1J_{C-C}$ = 35.5 Hz, 6′-CH_3), 21.7 (dm, $^1J_{C-C}$ = 74.2 Hz, 2′-CH_3), 19.0 (dd, $^1J_{C-C} \approx {}^1J_{C-C}$ = 34.7 Hz, C-4′), 12.9 (dm, $^1J_{C-C}$ = 40.3 Hz, 3-CH_3) ppm.

HRMS (DIP − ESI): calcd for $^{13}C_{15}H_{22}O$ 233.2174; found 233.2123.

[U-$^{13}C_{20}$]-3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenal (1). To a solution of 230 mg (1.0 mmol) of [1,2,3,4,-(3-CH_3)-$^{13}C_5$]-4-(diethylphosphono)-3-methyl-2-butenenitrile (**3**) in 15 mL of dry THF was added at 0 °C via a syringe 0.55 mL (0.9 mmol) of *n*-BuLi (1.6 M solution in hexane). Stirring was continued for 15 min at room temperature. To the anion of the phosphonate was added 120 mg (0.51 mmol) of aldehyde **5** dissolved in 10 mL of dry THF. The mixture was stirred for 1 h at room temperature. Quenching of the reaction was accomplished by adding 50 mL of saturated NH_4Cl solution. The aqueous layer was extracted three times with 30 mL of diethyl ether. The combined organic layers were washed with brine, dried with $MgSO_4$, and filtered. The product was purified by flash chromatography (silica gel, diethyl ether/40−60 light petroleum ether, 20:80) to give 150 mg (0.49 mmol, 97%) of [U-$^{13}C_{20}$]-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenenitrile (**6**) as a mixture of isomers. A solution of 150 mg (0.49 mmol) of nitrile **6** dissolved in 20 mL of dry light petroleum ether was cooled to −60 °C after which 0.75 mL of 1 M (0.75 mmol) DIBAL-H was added via a syringe. The reaction mixture was allowed to warm to −40 °C in 1 h. Subsequently, a homogeneous mixture of 1.3 g of water absorbed on silica (0.3 g water/gram silica) was added and stirring was continued for another 2 h at 0 °C. After the mixture was dried by adding $MgSO_4$, all solids were filtered off and thoroughly washed with diethyl ether. Evaporation of the organic solvents in vacuo yielded a dark orange liquid. The [U-$^{13}C_{20}$]-*all-trans*-retinal from the cis isomers was obtained via column chromatography (silica gel, diethyl ether/40−60 light petroleum ether, 20:80). Subsequently, a solution of the cis isomers in 40−60 light petroleum ether was irradiated for 2 h in the presence of iodine crystals under a tungsten lamp. The organic layer, containing the second batch of *all-trans*-retinal, was washed with saturated $Na_2S_2O_3$ and dried with $MgSO_4$. The *all-trans*-retinal was purified via column chromatography purification (silica gel, diethyl ether/40−60 light petroleum ether, 20:80). [U-$^{13}C_{20}$]-all-*trans*-retinal (**1**, 125 mg) was synthesized via this method in a yield of 83% based on aldehyde **5**.

^{1}H NMR (600 MHz, $CDCl_3$) of *all-trans*-[U-$^{13}C_{20}$]-retinal (**1**): δ 10.11 (ddd, $^1J_{C-H}$ = 169.7 Hz, $^2J_{C-H}$ = 24.5 Hz, $^3J_{H-H}$ = 8.2 Hz, CHO, 1H), 7.14 (dm, $^1J_{C-H}$ = 150.1 Hz, H-11, 1H), 6.36 (m, H-7/H-12, 2H), 6.17 (m, H-8/H-10, 2H), 5.97 (dm, $^1J_{C-H}$ = 157.6 Hz, H-14, 1H), 2.33 (dm, $^1J_{C-H}$ = 127.8 Hz, 13-CH_3, 3H), 2.03 (dm, $^1J_{C-H}$ = 126.7 Hz, 9-CH_3, 3H), 2.01 (m, H-4, 2H), 1.72 (dm, $^1J_{C-H}$ = 125.7 Hz, 5-CH_3, 3H), 1.68 (dm, $^1J_{C-H}$ = 127.5 Hz, H-3, 2H), 1.47 (dm, $^1J_{C-H}$ = 128.2 Hz, H-2, 2H), 1.03 (dm, $^1J_{C-H}$ = 125.1 Hz, 1-CH_3, 6H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$) of *all-trans*-[U-$^{13}C_{20}$]-retinal (**1**): δ 191.1 (d, $^1J_{C-C}$ = 57.4 Hz, CHO), 154.9 (m, C-13), 141.3 (m, C-9), 137.5 (m, C-6), 137.0 (dd, $^1J_{C-C}$ = 72.1 Hz, $^1J_{C-C}$ = 58.4 Hz, C-8), 134.5 (dddd, $^1J_{C-C}$ = 69.4 Hz, $^1J_{C-C}$ = 53.9 Hz, $^2J_{C-C} \approx {}^2J_{C-C}$ = 7.4 Hz, C-12), 132.5 (ddm, $^1J_{C-C}$ = 69.9 Hz, $^1J_{C-C}$ = 53.9 Hz, C-11), 130.5 (ddd, $^1J_{C-C}$ = 75.1 Hz, $^1J_{C-C} \approx {}^1J_{C-C}$ = 35.4 Hz, C-5), 129.2 (m, C-7/C-10/C-14), 39.5 (dd, $^1J_{C-C} \approx {}^1J_{C-C}$ = 35.4 Hz, C-2), 34.2 (dddd, $^1J_{C-C} \approx {}^1J_{C-C} \approx {}^1J_{C-C} \approx {}^1J_{C-C}$ = 35.4 Hz, C-1), 33.1 (dd, $^1J_{C-C} \approx {}^1J_{C-C}$ = 35.4 Hz, C-4), 28.9 (d, $^1J_{C-C}$ = 35.4 Hz, 1-CH_3), 21.8 (dm, $^1J_{C-C}$ = 41.9 Hz, 5-CH_3), 19.1 (dd, $^1J_{C-C} \approx {}^1J_{C-C}$ = 35.4 Hz, C-3), 13.1 (dm, $^1J_{C-C}$ = 40.4 Hz, 13-CH_3), 13.0 (dm, $^1J_{C-C}$ = 42.1 Hz, 9-CH_3) ppm.

HRMS (DIP − ESI): calcd for $^{13}C_{20}H_{28}O$ 304.2811; found 304.2804.

[1,2,3,4-$^{13}C_4$]-Ethyl 2-Chloroacetoacetate (8). To 2.86 g (21.3 mmol) of [1,2,3,4-$^{13}C_4$]-ethyl acetoacetate (**7**) was added at 0 °C via a dropping funnel 1.80 mL (22.4 mmol) of sulfuryl chloride. The mixture was stirred overnight at room temperature. The product was obtained by evaporation in vacuo of the formed SO_2 and HCl. **8** (3.53 g, 20.9 mmol, 98%) was synthesized via this method as a slightly yellow liquid.[26]

Both the ^{1}H NMR spectrum and the ^{13}C NMR spectrum showed a keto−enol mixture of [1,2,3,4-$^{13}C_3$]-ethyl 2-chloroacetoacetate.

^{1}H NMR (600 MHz, $CDCl_3$): δ 4.76 (ddd, $^1J_{C-H}$ = 160.0 Hz, $^2J_{C-H} \approx {}^2J_{C-H}$ = 5.6 Hz, H-2, 1H (keto)), 4.30 (m, H-1′, 4H (keto/enol)), 2.39 (dm, $^1J_{C-H}$ = 129.4 Hz, H-4, 3H (keto)), 2.19 (dm, $^1J_{C-H}$ = 129.5 Hz, H-4, 3H (enol)), 1.82 (s (br), OH, 1H (enol)), 1.32 (t, $^3J_{H-H}$ = 7.1 Hz, H-2′, 6H (keto/enol)) ppm.

^{13}C NMR (150 MHz, $CDCl_3$): δ 196.6 (dd, $^1J_{C-C}$ = 45.1 Hz, $^1J_{C-C}$ = 38.9 Hz, C-3 (keto)), 172.5 (ddd, $^1J_{C-C}$ = 86.1 Hz, $^1J_{C-C}$ = 50.8 Hz, $^2J_{C-C}$ = 4.7 Hz, C-3 (enol)), 169.3 (dm, $^1J_{C-C}$ = 86.1 Hz, C-1 (enol)), 164.9 (d, $^1J_{C-C}$ = 62.7 Hz, C-1 (keto)), 96.6 (ddd, $^1J_{C-C} \approx {}^1J_{C-C}$ = 86.1 Hz, $^2J_{C-C}$ = 4.3 Hz, C-2 (enol)), 63.1 (s, C-1′ (keto/enol)), 61.3 (ddd, $^1J_{C-C}$ = 62.7 Hz, $^1J_{C-C}$ = 38.9 Hz, $^2J_{C-C}$ = 15.4 Hz, C-2 (keto)), 26.2 (dd, $^1J_{C-C}$ = 45.1 Hz, $^2J_{C-C}$ = 15.4 Hz, C-4 (keto)), 19.7 (dm, $^1J_{C-C}$ = 50.8 Hz, C-4 (enol)), 13.9 (s, C-2′ (keto/enol)) ppm.

HRMS (DIP − ESI): calcd for $^{13}C_4C_2H_9ClO_3$ 168.0374; found 168.0432.

[U-$^{13}C_3$]-Chloroacetone (9). To 3.53 g (20.9 mmol) of [1,2,3,4-$^{13}C_4$]-ethyl 2-chloroacetoacetate (**8**) dissolved in 20 mL of THF was added 1.9 mL (0.1 mol) of water and 2.22 mL (41.8 mmol) of concentrated H_2SO_4. The reaction mixture was refluxed for 40 h. Subsequently, the mixture was cooled to room temperature followed by the addition of 15 mL of water and 25 mL of diethyl ether. The aqueous layer was extracted three times with 50 mL of diethyl ether. The combined organic layers were washed with saturated $NaHCO_3$ solution and brine. Drying with $MgSO_4$ and filtration yielded **9** dissolved in a mixture of diethyl ether and THF. Because of the volatility of the product, only the diethyl ether was removed via distillation at atmospheric pressure. The resulting solution of [1,2,3-$^{13}C_3$]-chloroacetone (**9**) in THF was used in the following step.

^{1}H NMR (600 MHz, $CDCl_3$): δ 4.09 (dd, $^1J_{C-H}$ = 149.0 Hz, $^2J_{C-H}$ = 4.1 Hz, H-1, 2H), 3.74 (m, 4H, THF), 2.31 (dd, $^1J_{C-H}$ = 128.6 Hz, $^2J_{C-H}$ = 4.1 Hz, H-3, 3H), 1.85 (m, 4H, THF) ppm.

^{13}C NMR (150 MHz, $CDCl_3$): δ 200.1 (dd, $^1J_{C-C}$ = 43.2 Hz, $^1J_{C-C}$ = 40.2 Hz, C-2), 67.7 (s, THF), 48.4 (dd, $^1J_{C-C}$ = 40.2 Hz, $^2J_{C-C}$ = 18.2 Hz, C-1), 26.7 (dd, $^1J_{C-C}$ = 43.2 Hz, $^2J_{C-C}$ = 18.2 Hz, C-3), 25.4 (s, THF) ppm.

[U-$^{13}C_5$]-4-Chloro-3-methyl-2-butenenitrile (12). A solution of 43.9 mmol of LDA was prepared at −60 °C from 6.42 mL (46.0 mmol) of diisopropylamine dissolved in 75 mL of dried THF and 27.4 mL (43.9 mmol) of *n*-butyllithium (1.6 M solution in hexane). Subsequently, 1.00 g (23.0 mmol) of [$^{13}C_2$]-acetonitrile (**10**) dissolved in 20 mL of THF was added dropwise. After the solution was stirred for 15 min at −60 °C, 3.62 mL (25.3 mmol) of diethyl chlorophosphate dissolved in 25 mL of THF was added slowly. The reaction mixture was allowed to warm to 0 °C in 1 h. The solution of [1,2,3-$^{13}C_3$]-chloroacetone (**9**) (maximum amount 20.9 mmol based on [1,2,3,4-$^{13}C_4$]-ethyl 2-chloroacetoacetate (**8**)) in THF was added slowly to the solution of the freshly prepared phosphonate anion. Stirring was continued for 90 min at room temperature followed by the addition of 50 mL of brine. The aqueous layer was extracted three times with 50 mL of diethyl ether. The combined organic layers were washed with brine, dried with $MgSO_4$, and filtered. Evaporation of the diethyl ether in vacuo yielded a yellow liquid. The product was purified by chromatography (silica gel, diethyl ether/40−60 light petroleum ether, 20:80) to give 1.76 g (14.7 mmol) of **12** as a mixture of the cis and trans compound. The overall yield starting from ester **8** was 70%.

^{1}H NMR (600 MHz, $CDCl_3$): δ 5.50 (dm, $^1J_{C-H}$ = 175.7 Hz, H-2 (trans), 1H), 5.27 (dm, $^1J_{C-H}$ = 174.4 Hz, H-2 (cis), 1H), 4.27 (dm, $^1J_{C-H}$ = 151.9 Hz, H-4 (cis), 2H), 4.08 (dm, $^1J_{C-H}$ = 151.3 Hz, H-4 (trans), 2H), 2.16 (dm, $^1J_{C-H}$ = 129.1 Hz, H-5 (trans), 3H), 2.07 (dm, $^1J_{C-H}$ = 128.9 Hz, H-5 (cis), 3H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$): δ 157.9 (m, C-3 (cis/trans)), 115.9 (ddd, $^1J_{C-C}$ = 80.2 Hz, $^2J_{C-C}$ = 8.5 Hz, $^3J_{C-C}$ = 3.7 Hz, C-1 (trans)), 115.1 (ddd, $^1J_{C-C}$ = 79.7 Hz, $^2J_{C-C}$ = 6.9 Hz, $^3J_{C-C}$ = 4.6 Hz, C-1 (cis)), 99.0 (ddm, $^1J_{C-C}$ = 79.7 Hz, $^1J_{C-C}$ = 75.6 Hz, C-2 (cis)), 98.7 (ddm, $^1J_{C-C}$ = 80.2 Hz, $^1J_{C-C}$ = 77.6 Hz, C-2 (trans)), 46.8 (dm, $^1J_{C-C}$ = 44.1 Hz, C-4 (trans)), 44.2 (dm, $^1J_{C-C}$ = 44.5 Hz, C-4 (cis)), 20.9 (ddm, $^1J_{C-C}$ = 43.5 Hz, $^2J_{C-C}$ = 7.1 Hz, C-5 (cis)), 19.0 (dm, $^1J_{C-C}$ = 43.0 Hz, C-5 (trans)) ppm.

HRMS (DIP − ESI): calcd for $^{13}C_5H_6NCl$ 120.0357; found 120.0378.

[1,2,3,4,(3-CH$_3$)-^{13}C$_5$]-4-(Diethylphosphono)-3-methyl-2-butenenitrile (3). To 1.76 g (14.7 mmol) of [1,2,3,4,(3-CH_3)-$^{13}C_5$]-4-chloro-3-methyl-2-butenenitrile (**12**) was added 3.31 mL (19.0 mmol) of triethyl phosphite. The mixture was heated at 180 °C for 6 h. The reaction was driven to completion by regular removal of the formed chloroethane with vacuum suction. The mixture was allowed to cool to room temperature and the product was isolated by using distillation at reduced pressure. The yield was 2.95 g (13.3 mmol, 91%) of the phosphonate **3** as a mixture of the cis and trans isomer (bp 118 °C, 0.2 mmHg).[37]

^{1}H NMR (600 MHz, $CDCl_3$): δ 5.28 (dm, $^1J_{C-H}$ = 173.3 Hz, H-2 (cis/trans), 2H), 4.14 (m, POC*H*$_2$CH$_3$, 4H), 2.87 (ddm, $^1J_{C-H}$ = 128.7 Hz, $^2J_{P-H}$ = 23.9 Hz, H-4 (cis), 2H), 2.83 (ddm, $^1J_{C-H}$ = 128.7 Hz, $^2J_{P-H}$ = 23.5 Hz, H-4 (trans), 2H), 2.20 (dm, $^1J_{C-H}$ = 128.8 Hz, H-5 (trans), 3H), 2.11 (dm, $^1J_{C-H}$ = 128.6 Hz, H-5 (cis), 3H), 1.36 (t, $^3J_{H-H}$ = 7.1 Hz, POCH$_2$C*H*$_3$, 6H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$): δ 155.5 (m, C-3 (cis/trans)), 116.3 (dm, $^1J_{C-C}$ = 80.0 Hz, C-1 (cis/trans)), 99.0 (m, C-2 (cis/trans)), 62.5 (d, $^2J_{C-P}$ = 6.8 Hz, POC*H*$_2$CH$_3$ (cis/trans)), 36.4 (ddm, $^1J_{C-P}$ = 135.9 Hz, $^1J_{C-C}$ = 40.6 Hz, C-4 (trans)), 34.6 (ddm, $^1J_{C-P}$ = 135.9 Hz, $^1J_{C-C}$ = 40.3 Hz, C-4 (cis)), 24.3 (dm, $^1J_{C-C}$ = 42.0 Hz, C-5 (cis)), 22.3 (dm, $^1J_{C-C}$ = 42.0 Hz, C-5 (trans)), 16.3 (d, $^3J_{C-P}$ = 6.1 Hz, POCH$_2$C*H*$_3$ (cis/trans)) ppm.

HRMS (DIP − ESI): calcd for $^{13}C_5C_4H_{16}O_3NP$ 222.1036; found 222.1002.

[U-^{13}C$_8$]-6-Methyl-5-hepten-2-one (14). To 1.40 g (10.5 mmol) of [1,2,3,4-$^{13}C_4$]-ethyl acetoacetate (**7**) in 30 mL of dry ethanol was added 202 mg (8.8 mmol) of sodium in small portions. As soon as all sodium was dissolved the mixture was cooled to 0 °C and 1.23 g (8.0 mmol) of [U-$^{13}C_5$]-4-bromo-2-methyl-2-butene (**13**) dissolved in 25 mL of ethanol was added dropwise. The reaction mixture was stirred at room temperature for 2 h and subsequently refluxed for 4 h. During this time a brown solid (NaBr) was formed. After removal of the EtOH by evaporation, 20 mL of 10% NaOH solution was added. The mixture was stirred overnight at room temperature after which it was heated to 60 °C for another 3 h to complete the saponification. Decarboxylation was accomplished by acidification to a pH > 4. The aqueous layer was extracted three times with 50 mL of diethyl ether. The combined ether layers were washed with brine and dried over $MgSO_4$. Concentration in vacuo yielded a dark yellow liquid. The product was purified by chromatography on silica gel (diethyl ether/40−60 light petroleum ether, 10:90) to give 0.72 g (6.0 mmol, 75%) of **14** as a slightly yellow liquid.

^{1}H NMR (600 MHz, $CDCl_3$): δ 5.06 (dm, $^1J_{C-H}$ = 150.9 Hz, H-5, 1H), 2.46 (dm, $^1J_{C-H}$ = 128.3 Hz, H-4, 2H), 2.25 (dm, $^1J_{C-H}$ = 124.7 Hz, H-3, 2H), 2.14 (dd, $^1J_{C-H}$ = 127.0 Hz, $^2J_{C-H}$ = 5.7 Hz, H-1, 3H), 1.68 (dddd, $^1J_{C-H}$ = 125.3 Hz, $^2J_{C-H} \approx {}^3J_{C-H} \approx {}^3J_{C-H}$ = 5.7 Hz, H-7, 3H), 1.61 (dddd, $^1J_{C-H}$ = 125.3 Hz, $^2J_{C-H} \approx {}^3J_{C-H} \approx {}^3J_{C-H}$ = 5.7 Hz, H-8, 3H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$): δ 208.9 (ddm, $^1J_{C-C} \approx {}^1J_{C-C}$ = 38.7 Hz, C-2), 132.7 (dddd, $^1J_{C-C}$ = 73.9 Hz, $^1J_{C-C} \approx {}^1J_{C-C}$ = 42.3 Hz, $^2J_{C-C}$ = 5.1 Hz, C-6), 122.5 (ddm, $^1J_{C-C}$ = 73.9 Hz, $^1J_{C-C}$ = 44.7 Hz, C-5), 43.7 (dddm, $^1J_{C-C} \approx {}^1J_{C-C}$ = 38.7 Hz, $^2J_{C-C}$ = 13.8 Hz, C-3), 29.9 (dd, $^1J_{C-C}$ = 38.7 Hz, $^2J_{C-C}$ = 13.8 Hz, C-1), 25.6 (dm, $^1J_{C-C}$ = 42.3 Hz, C-7), 22.4 (ddm, $^1J_{C-C}$ = 44.7 Hz, $^1J_{C-C}$ = 38.7 Hz, C-4), 17.6 (dm, $^1J_{C-C}$ = 42.3 Hz, C-8) ppm.

HRMS (DIP − ESI): calcd for $^{13}C_8H_{14}O$ 134.1313; found 134.1351.

[U-^{13}C$_{10}$]-3,7-Dimethylocta-2,6-dienenitrile (15). A solution of 5.1 mmol of LDA was prepared at −60 °C from 1.48 mL (10.6 mmol) of diisopropylamine dissolved in 30 mL of dried THF and 6.42 mL (10.2 mmol) of *n*-butyllithium (1.6 M solution in hexane). Subsequently, 0.22 g (5.1 mmol) of [$^{13}C_2$]-acetonitrile (**10**) dissolved in 5 mL of THF was added dropwise. After the mixture was stirred for 15 min at −60 °C, 0.74 mL (5.1 mmol) of diethyl chlorophosphate dissolved in 5 mL of THF was added slowly. The reaction mixture was allowed to warm to 0 °C in 1 h. A solution of 0.47 g (3.9 mmol) of [U-$^{13}C_8$]-6-methyl-5-hepten-2-one (**14**) in 5 mL of THF was added slowly to the solution of the phosphonate anion. Stirring was continued for 2 h at room temperature. Adding 25 mL of brine quenched the reaction mixture. The aqueous layer was extracted three times with 30 mL of diethyl ether. The combined organic layers were washed with brine, dried with $MgSO_4$, and filtered. Evaporation of the diethyl ether in vacuo yielded a yellow liquid. The product was purified by chromatography on silica gel (diethyl ether/40−60 light petroleum ether, 10:90) to give 450 mg (2.8 mmol, 72%) of **15** as a mixture of the cis and trans isomers.

^{1}H NMR (600 MHz, $CDCl_3$): δ 5.10 (dm, $^1J_{C-H}$ = 170.3 Hz, H-6 (cis/trans), 2H), 5.02 (dm, $^1J_{C-H}$ = 149.9 Hz, H-2 (trans), 1H), 4.93 (dm, $^1J_{C-H}$ = 147.1 Hz, H-2 (cis), 1H), 2.55 (dm, $^1J_{C-H}$ = 129.1 Hz, H-5 (cis), 1H), 2.42 (dm, $^1J_{C-H}$ = 129.3 Hz, H-5 (trans), 1H), 2.20 (dm, $^1J_{C-H}$ = 128.8 Hz, H-9 (trans), 3H), 2.16 (dm, $^1J_{C-H}$ = 128.0 Hz, H-9 (cis), 3H), 2.05 (dm, $^1J_{C-H}$ = 127.8 Hz, H-4 (trans), 2H), 1.90 (dm, $^1J_{C-H}$ = 129.3 Hz, H-4 (cis), 2H), 1.69 (dm, $^1J_{C-H}$ = 125.9 Hz, H-8 (cis/trans), 6H), 1.60 (dm, $^1J_{C-H}$ = 120.0 Hz, H-10, 6H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$): δ 164.9 (m, C-3 (cis/trans)), 135.0 (dddm, $^1J_{C-C}$ = 74.2 Hz, $^1J_{C-C} \approx {}^1J_{C-C}$ = 42.4 Hz, C-7 (cis)), 133.0 (m, C-7 (trans)), 122.0 (m, C-6 (cis/trans)), 117.1 (dm, $^1J_{C-C}$ = 74.6 Hz, C-1 (cis)), 116.8 (dm, $^1J_{C-C}$ = 74.9 Hz, C-1 (trans)), 95.7 (ddm, $^1J_{C-C} \approx {}^1J_{C-C}$ = 74.9 Hz, C-2 (trans)), 95.0 (ddm, $^1J_{C-C} \approx {}^1J_{C-C}$ = 74.6 Hz, C-2 (cis)), 38.4 (ddm, $^1J_{C-C} \approx {}^1J_{C-C}$ = 38.2 Hz, C-4 (trans)), 36.1 (ddm, $^1J_{C-C} \approx {}^1J_{C-C}$ = 36.2 Hz, C-4 (cis)), 25.7 (m, C-5 and C-8 (cis/trans)), 22.7 (dm, $^1J_{C-C}$ = 41.3 Hz, C-9 (cis)), 20.8 (dm, $^1J_{C-C}$ = 50.3 Hz, C-9 (trans)), 17.5 (m, C-10 (cis/trans)) ppm.

HRMS (DIP − ESI): calcd for $^{13}C_{10}H_{15}N$ 159.1540; found 159.1519.

[U-^{13}C$_{10}$]-2,6,6-Trimethylcyclohex-2-ene-1-ylcarbonitrile (16). To a solution of 1.43 mL of concentrated sulfuric acid in 15 mL of nitromethane was added at 0 °C via a dropping funnel 450 mg (2.8 mmol) of [U-$^{13}C_{10}$]-3,7-dimethylocta-2,6-dienenitrile (**15**) dissolved in 5 mL of nitromethane. After the mixture was stirred for 45 min at 0 °C the reaction was quenched by adding 25 mL of ice−water. The aqueous layer was extracted three times with 30 mL of diethyl ether. The combined organic layers were washed with brine and saturated NaOAc solution, dried with $MgSO_4$, and filtered. Evaporation of the diethyl ether in vacuo yielded a slightly yellow liquid. The product was purified by chromatography (silica gel, diethyl ether/40−60 light petroleum ether, 10:90) to give 372 mg (2.3 mmol, 83%) of **16** as a mixture of [U-$^{13}C_{10}$]- 2,6,6-trimethylcyclohex-2-ene-1-ylcarbonitrile (**16a**) and [U-$^{13}C_{10}$]-2,6,6-trimethylcyclohexene-1-ylcarbonitrile (**16b**) (97:3).[28]

^{1}H NMR (600 MHz, $CDCl_3$) of [U-$^{13}C_{10}$]-2,6,6-trimethylcyclohex-2-ene-1-ylcarbonitrile (**16a**): δ 5.58 (dm, $^1J_{C-H}$ = 152.8 Hz, H-4, 1H), 2.76 (dm, $^1J_{C-H}$ = 132.2 Hz, H-2, 1H), 2.08 (dm, $^1J_{C-H}$ = 127.6 Hz, H-5, 2H), 1.83 (dm, $^1J_{C-H}$ = 126.2 Hz, 3-CH_3, 3H), 1.55 (dm, $^1J_{C-H}$ = 127.5 Hz, H-6, 1H), 1.33 (dm, $^1J_{C-H}$ = 128.9 Hz, H-6, 1H), 1.13 (dm, $^1J_{C-H}$ = 125.8 Hz, 1-CH_3, 3H), 1.09 (dm, $^1J_{C-H}$ = 125.6 Hz, 1-CH_3, 3H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$) of [U-$^{13}C_{10}$]-2,6,6-trimethylcyclohex-2-ene-1-ylcarbonitrile (**16a**): δ 126.4 (dddm, $^1J_{C-C}$ = 73.3 Hz, $^1J_{C-C}$ = 43.5 Hz, $^1J_{C-C}$ = 39.9 Hz, C-3), 124.5 (ddm, $^1J_{C-C}$ = 73.3 Hz, $^1J_{C-C}$ = 39.8 Hz, C-4), 119.6 (d, $^1J_{C-C}$ = 55.2 Hz, CN), 43.8 (dddm, $^1J_{C-C}$ = 55.2 Hz, $^1J_{C-C}$ = 43.5 Hz, $^1J_{C-C}$ = 34.3 Hz, C-2), 32.7 (dd, $^1J_{C-C}$

(37) Gebhard, R.; Van der Hoef, K.; Lefeber, A. W. M.; Erkelens, C.; Lugtenburg, J. *Recl. Trav. Chim. Pays-Bas* **1990**, *109*, 378−387.

$\approx {}^1J_{C-C} = 34.3$ Hz, C-6), 31.8 (dddd, ${}^1J_{C-C} \approx {}^1J_{C-C} \approx {}^1J_{C-C} \approx {}^1J_{C-C} = 34.3$ Hz, C-1), 26.8 (d, ${}^1J_{C-C} = 34.3$ Hz, 1-CH_3), 25.5 (d, ${}^1J_{C-C} = 34.3$ Hz, 1-CH_3), 22.4 (m, C-5 and 3-CH_3) ppm.

HRMS (DIP − ESI): calcd for $^{13}C_{10}H_{15}N$ 159.1540; found 159.1562.

[U-$^{13}C_{10}$]-2,6,6-Trimethyl-cyclohex-2-ene-1-yl-carbaldehyde (17). A solution of the mixture of both the α-nitrile **16a** and *β*-nitrile **16b** (330 mg, 2.1 mmol) in 30 mL of dry light petroleum ether was cooled to −60 °C and 3.15 mL of 1 M (3.2 mmol) DIBAL-H was added via a syringe. The reaction mixture was allowed to warm to −40 °C in 1 h. TLC analysis showed complete conversion of α-nitrile **16a** whereas *β*-nitrile **16b** was unaffected. After addition of a homogeneous mixture of 5.5 g of water adsorbed on silica (0.3 g of water/g of silica), the reaction mixture was stirred for 2 h at 0 °C. Subsequently, $MgSO_4$ was added and the solids were filtered off and thoroughly washed with diethyl ether. Evaporation of the organic solvents in vacuo yielded a dark yellow liquid. The product was purified by chromatography (silica gel, diethyl ether/40−60 light petroleum ether, 10:90) to give 249 mg (1.5 mmol, 73%) of aldehyde **17** and 12 mg (0.08 mmol, 4%) of *β*-nitrile **16b**.

^{1}H NMR (600 MHz, $CDCl_3$) of [U-$^{13}C_{10}$]-2,6,6-trimethylcyclohex-2-ene-1-ylcarbaldehyde (**17**): δ 9.47 (ddd, ${}^1J_{C-H} = 171.2$ Hz, ${}^2J_{C-H} = 20.9$ Hz, ${}^3J_{H-H} = 5.2$ Hz, CHO, 1H), 5.72 (dm, ${}^1J_{C-H} = 151.9$ Hz, H-3, 1H), 2.36 (dm, ${}^1J_{C-H} = 130.3$ Hz, H-1, 1H), 2.14 (dm, ${}^1J_{C-H} = 127.4$ Hz, H-4, 2H), 1.59 (dm, ${}^1J_{C-H} = 127.2$ Hz, 2-CH_3, 3H), 1.49 (dm, ${}^1J_{C-H} = 125.1$ Hz, H5, 2H), 0.99 (dm, ${}^1J_{C-H} = 124.8$ Hz, 1-CH_3, 3H), 0.91 (dm, ${}^1J_{C-H} = 125.3$ Hz, 1-CH_3, 3H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$) of [U-$^{13}C_{10}$]-2,6,6-trimethylcyclohex-2-ene-1-ylcarbaldehyde (**17**): δ 202.4 (d, ${}^1J_{C-C} = 38.4$ Hz, CHO), 127.1 (dm, ${}^1J_{C-C} = 72.5$ Hz, C-2), 125.4 (ddm, ${}^1J_{C-C} = 72.5$ Hz, ${}^1J_{C-C} = 38.4$ Hz, C-3), 63.3 (m, C-1), 31.6 (m, C-5/C-6), 27.4 (d, ${}^1J_{C-C} = 34.1$ Hz, 6-CH_3), 26.9 (d, ${}^1J_{C-C} = 33.4$ Hz, 6-CH_3), 23.0 (m, C-4), 22.5 (d, ${}^1J_{C-C} = 42.4$ Hz, 2-CH_3) ppm.

HRMS (DIP − ESI): calcd for $^{13}C_{10}H_{16}O$ 162.1537; found 162.1573.

[U-$^{13}C_{10}$]-2,6,6-Trimethylcyclohex-1-enylcarbaldehyde (2). To a solution of aldehyde **17** (180 mg, 1.1 mmol) in 5 mL of MeOH was added at 0 °C 1.5 mL of 5% KOH in MeOH. After the mixture was stirred for 45 min at 0 °C the reaction was quenched by adding 5 mL of brine. The aqueous layer was extracted three times with 15 mL of diethyl ether. The combined organic layers were washed with brine, dried with $MgSO_4$, and filtered. Evaporation of the diethyl ether in vacuo yielded **2**: 142 mg (0.86 mmol, 80%) of a slightly yellow liquid.

^{1}H NMR (300 MHz, $CDCl_3$): δ 10.13 (ddm, ${}^1J_{C-H} = 169.8$ Hz, ${}^2J_{C-H} = 21.5$ Hz, CHO, 1H), 2.19 (dm, ${}^1J_{C-H} = 127.3$ Hz, H-3, 2H), 2.09 (ddm, ${}^1J_{C-H} = 127.1$ Hz, ${}^2J_{C-H} = 10.0$ Hz, 2-CH_3, 3H), 1.62 (dm, ${}^1J_{C-H} = 129.6$ Hz, 4-H, 2H), 1.44 (dm, ${}^1J_{C-H} = 125.1$ Hz, H-5, 2H), 1.19 (dm, ${}^1J_{C-H} = 125.6$ Hz, 6-CH_3, 6H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): δ 192.2 (d, ${}^1J_{C-C} = 52.4$ Hz, CHO), 159.9 (ddd, ${}^1J_{C-C} = 68.4$ Hz, ${}^1J_{C-C} \approx {}^1J_{C-C} = 40.2$ Hz, C-2), 140.5 (ddd, ${}^1J_{C-C} = 68.4$ Hz, ${}^1J_{C-C} = 52.4$ Hz, ${}^1J_{C-C} = 41.5$ Hz, C-1), 40.5 (dd, ${}^1J_{C-C} \approx {}^1J_{C-C} = 33.2$ Hz, C-5), 35.7 (dd, ${}^1J_{C-C} \approx {}^1J_{C-C} = 40.2$ Hz, C-3), 33.0 (m, C-6), 27.7 (d, ${}^1J_{C-C} = 35.1$ Hz, 6-CH_3), 18.8 (m, C-4/2-CH_3) ppm.

HRMS (DIP − ESI): calcd for $^{13}C_{10}H_{16}O$ 162.1537; found 162.1569.

[1,2,3,4,(3-CH_3)-$^{13}C_5$]-Ethyl 3-Methyl-2-butenoate (23). To 5.56 g (24.6 mmol) of ethyl [1,2-$^{13}C_2$]-2-(diethylphosphono)acetate (**21**) dissolved in 75 mL of dry of THF was added at 0 °C via a syringe 14.3 mL (22.9 mmol) of *n*-BuLi (1.6 M solution in hexane). Stirring was continued for 15 min at room temperature. To the anion of the phosphonate was added 1.00 g (16.4 mmol) of [1,2,3-$^{13}C_3$]-acetone (**22**), dissolved in dry THF. The mixture was stirred for another 3 h at room temperature. Quenching of the reaction was accomplished by adding 50 mL of saturated NH_4Cl solution. The aqueous layer was extracted three times with 50 mL of diethyl ether. The combined organic layers were washed with brine, dried with $MgSO_4$, and filtered. Evaporation of the diethyl ether in vacuo yielded **23**: 1.99 g (14.9 mmol, 91%) of a slightly yellow liquid.

^{1}H NMR (600 MHz, $CDCl_3$): δ 5.65 (dm, ${}^1J_{C-H} = 159.7$ Hz, H-2, 1H), 4.14 (m, H-1′, 2H), 2.16 (dm, ${}^1J_{C-H} = 127.6$ Hz, H-4 (Z), 3H), 1.89 (dm, ${}^1J_{C-H} = 126.7$ Hz, H-4 (E), 3H), 1.27 (t, ${}^3J_{H-H} = 7.1$ Hz, H-2′, 3H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$): δ 166.7 (ddm, ${}^1J_{C-C} = 76.0$ Hz, ${}^2J_{C-C} = 5.5$ Hz, C-1), 156.3 (dddd, ${}^1J_{C-C} = 72.1$ Hz, ${}^1J_{C-C} \approx {}^1J_{C-C} = 40.2$ Hz, ${}^2J_{C-C} = 5.5$ Hz, C-3), 116.0 (dd, ${}^1J_{C-C} = 76.0$ Hz, ${}^1J_{C-C} = 72.1$ Hz, C-2), 59.4 (s, C-1′), 27.3 (dm, ${}^1J_{C-C} = 40.2$ Hz, C-4 (E)), 20.1 (dm, ${}^1J_{C-C} = 40.2$ Hz, C-4 (Z)), 14.3 (s, C-2′) ppm.

HRMS (DIP − ESI): calcd for $^{13}C_5C_2H_{12}O_2$ 133.1005; found 133.1047.

[U-$^{13}C_5$]-3-Methyl-2-buten-1-ol (24). A solution of 1.85 g (13.9 mmol) of [1,2,3,4,(3-CH_3)-$^{13}C_5$]-ethyl-3-methyl-2-butenoate (**23**) in 50 mL of dry light petroleum ether was cooled to −60 °C and 30.6 mL of 1 M (30.6 mmol) DIBAL-H was added via a syringe. Stirring was continued for 30 min at −60 °C, followed by the addition of a homogeneous mixture of 53.5 g of water adsorbed on silica (0.3 g of water/g of silica). The mixture was stirred for 2 h at 0 °C. Subsequently, $MgSO_4$ was added and the solids were filtered off and thoroughly washed with diethyl ether. Evaporation of the organic solvents in vacuo yielded **24**: 1.62 g of a slightly yellow liquid. The product was used without further purification.

^{1}H NMR (600 MHz, $CDCl_3$): δ 5.40 (dm, ${}^1J_{C-H} = 153.2$ Hz, H-2, 1H), 4.12 (dm, ${}^1J_{C-H} = 141.7$ Hz, H-1, 2H), 1.74 (dddd, ${}^1J_{C-H} = 125.7$ Hz, ${}^2J_{C-H} \approx {}^3J_{C-H} \approx {}^3J_{C-H} = 5.1$ Hz, H-4 (E), 3H), 1.68 (dddd, ${}^1J_{C-H} = 125.7$ Hz, ${}^2J_{C-H} \approx {}^3J_{C-H} \approx {}^3J_{C-H} = 5.1$ Hz, H-4 (Z), 3H), 1.54 (s (br), OH, 1H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$): δ 136.3 (ddd, ${}^1J_{C-C} = 72.5$ Hz, ${}^1J_{C-C} = 42.8$ Hz, ${}^1J_{C-C} = 41.4$ Hz, C-3), 123.5 (ddm, ${}^1J_{C-C} = 72.5$ Hz, ${}^1J_{C-C} = 47.5$ Hz, C-2), 59.3 (dm, ${}^1J_{C-C} = 47.5$ Hz, C-1), 25.7 (dm, ${}^1J_{C-C} = 42.8$ Hz, C-4 (E)), 17.7 (dm, ${}^1J_{C-C} = 41.4$ Hz, C-4 (Z)) ppm.

HRMS (DIP − ESI): calcd for $^{13}C_5H_{10}O$ 91.0914; found 91.0898.

[U-$^{13}C_5$]-4-Bromo-2-methyl-2-butene (13). A solution of 1.62 g of [1,2,3,4,(3-CH_3)-$^{13}C_5$]-3-methyl-2-buten-1-ol (**24**) in 15 mL of dichloromethane was cooled to 0 °C and 6.63 mL of 47% hydrobromic acid dissolved in dichloromethane was added slowly. Stirring was continued for 2 h with exclusion of light. To the mixture was added 25 mL of a saturated $NaHCO_3$ solution followed by some drops of 1,2-epoxybutane. The aqueous layer was extracted three times with 50 mL of diethyl ether. The combined organic layers were washed with brine, dried with $MgSO_4$, and filtered. Because of the volatility of the product, most of the organic solvent was removed by distillation at atmospheric pressure. Only the last part of the solvent was removed by evaporation in vacuo yielding **13**: 1.23 g (8.0 mmol) of a yellow liquid. The overall yield starting from ester **23** was 57%. The product was used without further purification.

^{1}H NMR (600 MHz, $CDCl_3$): δ 5.53 (dm, ${}^1J_{C-H} = 158.7$ Hz, H-2, 1H), 4.01 (dm, 152.8 Hz, H-1, 2H), 1.78 (dddd, ${}^1J_{C-H} = 126.1$ Hz, ${}^2J_{C-H} \approx {}^3J_{C-H} \approx {}^3J_{C-H} = 5.6$ Hz, H-4 (E), 3H), 1.73 (dddd, ${}^1J_{C-H} = 126.1$ Hz, ${}^2J_{C-H} \approx {}^3J_{C-H} \approx {}^3J_{C-H} = 5.6$ Hz, H-4 (Z), 3H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$): δ 140.1 (ddd, ${}^1J_{C-C} = 73.5$ Hz, ${}^1J_{C-C} \approx {}^1J_{C-C} = 42.5$ Hz, C-3), 120.7 (ddm, ${}^1J_{C-C} = 73.5$ Hz, ${}^1J_{C-C} = 47.7$ Hz, C-2), 29.7 (dm, ${}^1J_{C-C} = 47.7$ Hz, C-1), 25.8 (dm, ${}^1J_{C-C} = 42.5$ Hz, C-4 (E)), 17.5 (dm, ${}^1J_{C-C} = 42.5$ Hz, C-4 (Z)) ppm.

HRMS (DIP − ESI): calcd for $^{13}C_5H_9Br$ 153.0055; found 153.0100.

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Supporting Information Available: Additional experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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