Synthesis of Isotopically Labelled All-*Trans* Retinals for DNP-Enhanced Solid State NMR Studies of Retinylidene Proteins

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Abstract

Three all-*trans* retinals containing multiple ^{13}C labels have been synthesised to enable DNP enhanced solid-state MAS NMR studies of novel microbial retinylidene membrane proteins including proteorhodpsin and channelrhodopsin. The synthetic aproaches allowed specific introduction of ^{13}C labels in ring substituents and at different positions in the polyene chain to probe structural features such as ring orientation and interaction of the chromophore with the protein in the ground state and in photointermediates. [10-18-$^{13}C_9$]-All-*trans*-retinal (**1b**), [12,15-$^{13}C_2$]-all-*trans*-retinal (**1c**) and [14,15-$^{13}C_2$]-all-*trans*-retinal (**1d**) were synthesised in in twelve, eight and seven linear steps from ethyl 2-oxocyclohexanecarboxylate (**5**) or β-ionone (**4**) respectively.

Introduction

Retinoids, or vitamin A derivatives, are ubiquitous in nature and have been studied extensively due to their wide-ranging biological roles,[1] such as in vision,[2] immune function,[3] and tumor suppression.[4] The incorporation of retinals into rhodopsins, through a protonated Schiff's base linkage, is a fundamental requirement for the function of these light-sensitive membrane proteins, which are found in all phyla of life. A well-known example is the G-protein coupled receptor rhodopsin, which is responsible for dim light vision in mammals. The field of microbial rhodopsins, which act as sensors, ion pumps and channels, was for a long time dominated by extensive studies on bacteriorhodopsin. This field has attracted recent attention with the discovery of many new rhodopsins such as proteorhodopsin (PR), channelrhodopsin (ChR) and *Krokinobacter eikastus* rhodopsin 2 (KR2). PRs form a family of light-driven proton pumps,[5] ChRs function as light-gated ion channels,[6] and *Krokinobacter eikastus* rhodopsin 2 (KR2) is the first-discovered light-driven Na^+ pump.[7] These proteins have stimulated much recent interest, with possible applications in areas such as optogenetics and energy harvesting.[8-10] A significant structural feature of PR, ChR and KR2 is that they contain an all-*trans*-retinylidene chromophore in their unactivated states; by contrast, the visual receptor rhodopsin contains the polyene in its 11-*Z* configuration.

Much effort has been directed towards structural investigation of rhodopsins and their photocycle intermediates, to further advance understanding of how irradiation of the retinylidene chromophore induces structural changes that trigger the enzymatic cascade

ultimately leading to vision, or enable ion pumping or channel gating.[1] In this regard solid-state magic angle spinning (MAS) ^{13}C NMR at low temperatures has provided high resolution structural information such as bond lengths and torsion angles in the chromophore, which together with quantum chemical calculation, connects ground state crystallographic data and kinetic and functional data derived from optical spectroscopy and electrophysiology.[11]

One key advantage of solid-state NMR is certainly the fact that membrane proteins can be analysed directly embedded within a lipid bilayer, which is essential for mechanistic studies under conditions resembling their native environment.[12,13] However, the concentration of the protein in the lipid bilayer is restricted due to its stability and optical density of the sample, limiting the amount of observable protein for biophysical studies. Successful application of ^{13}C MAS NMR to these systems has therefore relied on strategically inserted ^{13}C labels within the retinylidene chromophore to achieve useful signal to noise ratios. Even with ^{13}C labelling, NMR signals remain fairly weak, especially for photocycle intermediates. Cross effect dynamic nuclear polarization (DNP)[14] has emerged as a technique to increase sensitivity in solid-state NMR, highlighting its value in the field of structural determination. Here we report the synthesis of three all-*trans* retinal isotopomers containing multiple ^{13}C labels at positions defined to enable DNP-enhanced solid-state NMR structural studies of retinylidene proteins such as PR, ChR and KR2 (Figure 1).

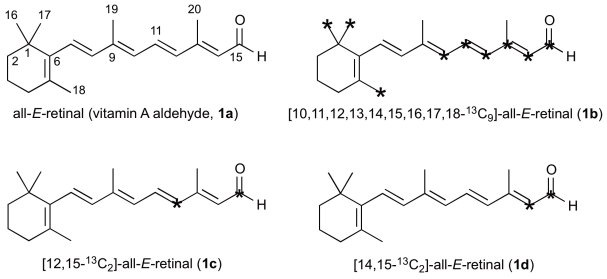


Figure 1. Retinal structures showing synthetic ^{13}C-labelling patterns
(* indicates position of ^{13}C)

Results and Discussion

Three all-*trans*-retinals, **1b–d** (Figure 1), containing multiple ^{13}C labels were designed to enable structural insight into light-driven conformational changes in retinylidene proteins by using DNP-enhanced solid-state NMR with ^{15}N-and ^{13}C labelled proteins.[12] The synthetic routes to polyenes **1b** and **1c** were planned to make use of inexpensive and readily available ^{13}C-enriched (99 atm % ^{13}C) building blocks, and ultimately permit flexible access to other labelling patterns (Figure 2). Stereocontrol in the final olefination was not critical here, due to the fact that labelled 13*E/Z* retinal isotopomers are separable, and the 13*Z* isomers also represent useful reference compounds when reconstituted into rhodopsins. A more convergent approach applied to the synthesis of **1d** (see Scheme 4 below) does allow for high levels of 13*E* selectivity, but was considered less convenient for the labelling patterns present in **1b** and **1c**.

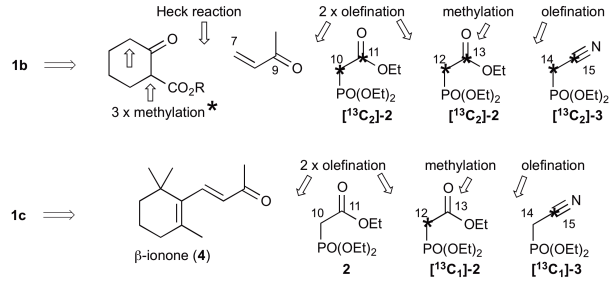
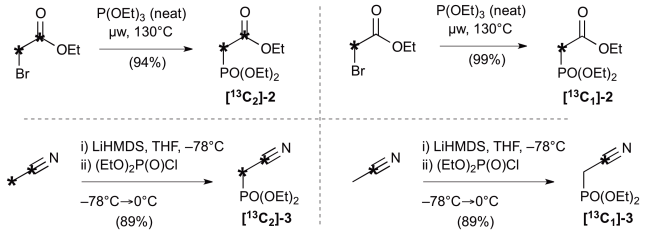


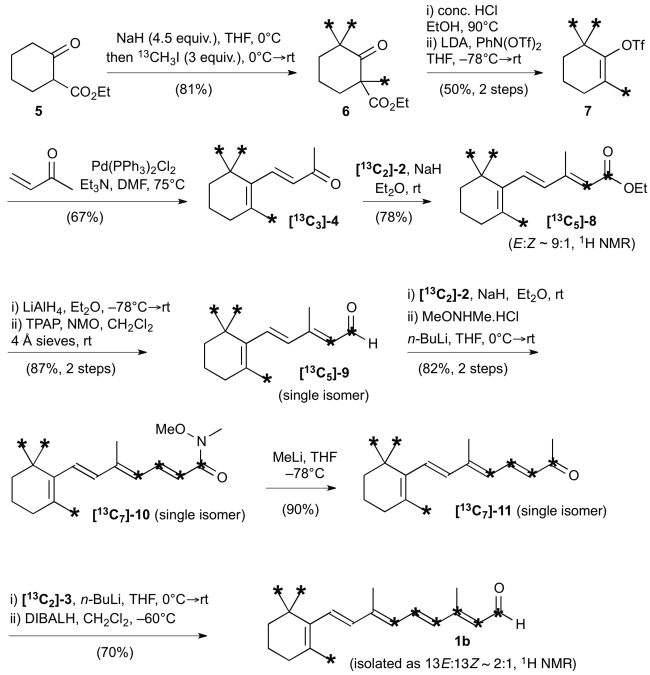
Figure 2. Synthesis plan for labelled retinal isotopomers **1b** and **1c**.
(* indicates position of ^{13}C)

Accordingly, singly and doubly ^{13}C-labelled diethyl (cyanomethyl)phosphonates **3** and triethyl phosphonoacetates **2** were prepared from labelled acetonitrile and ethyl bromoacetate respectively, and in high yields (Scheme 1).[15,16]



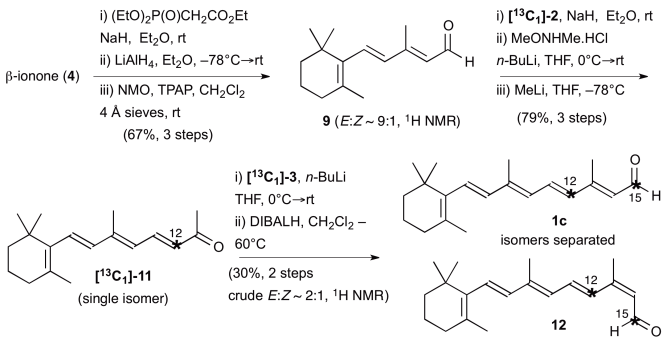
Scheme 1. Synthesis of ^{13}C-labelled phosphonates **[$^{13}C_2$]-2**, **[$^{13}C_1$]-2, [$^{13}C_2$]-3** and **[$^{13}C_1$]-3**.
(* indicates position of ^{13}C)

The synthesis of the ^{13}C-labelled [10,11,12,13,14,15,16,17,18-$^{13}C_9$]-all-*trans*-retinal (**1b**), containing nine positions of ^{13}C-enrichment, began by trimethylation of ethyl 2-oxocyclohexanecarboxylate (**5**)[17] with [^{13}C]-CH_3I, to afford the β-ketoester **6** (Scheme 2). From **6**, acid-catalysed hydrolysis and decarboxylation afforded a relatively volatile cyclohexanone, which was converted to vinyl triflate **7** prior to a Heck reaction with methylvinyl ketone[18] that delivered triply-labelled β-ionone **[$^{13}C_3$]-4**. The first olefination reaction using doubly ^{13}C-labelled triethyl phosphonoacetate (**[$^{13}C_2$]-2**) yielded the trienoate **[$^{13}C_5$]-8** as an unseparated mixture of *E*/*Z* isomers (~9:1 from ^1H NMR) in 78% yield.[19] Following $LiAlH_4$ reduction of the isomeric mixture to the allylic alcohols and subsequent TPAP oxidation the all-*E*-trienal stereoisomer **[$^{13}C_5$]-9** was isolated by column chromatography.



Scheme 2. Synthesis of [10,11,12,13,14,15,16,17,18-13C$_9$]-all-*trans*-retinal (**1b**). (* indicates position of ^{13}C)

Olefination of trienal **[^{13}C$_5$]-9** using phosphonate **[^{13}C$_2$]-2** for the second time introduced the C12 and C13 labelled carbon atoms, this time affording the tetraenoate as its all-*E*-stereoisomer. Formation of Weinreb amide **[^{13}C$_7$]-10** facilitated the subsequent high-yielding conversion to methyl ketone **[^{13}C$_7$]-11**.[15] The final olefination with diethyl (cyanomethyl)phosphonate **[^{13}C$_2$]-3** resulted in a mixture of 13*E*/*Z* isomers, which following DIBAL-H reduction and aqueous work-up,[15] delivered the [10-18-^{13}C$_9$]-all-*trans*-retinal as a 2:1 mixture with the minor 13*Z* isomer in 70% yield. This isomeric mixture was separated by column chromatography and preparative HPLC on silica gel to provide a pure sample of the all-*E* stereoisomer **1b**.



Scheme 3. Synthesis of [12,15-13C$_2$]-all-*trans*-retinal (**1c**) and [12,15-13C$_2$]-(13*Z*)-retinal (**12**). (* indicates position of ^{13}C)

The synthesis of [12,15-^{13}C$_2$]-all-*trans*-retinal (**1c**) was achieved similarly, but starting from commercial β-ionone and using the singly ^{13}C-labelled phosphonates **[^{13}C$_1$]-2** and **[^{13}C$_1$]-3** (Scheme 3).[19] In this case the 13*Z* and the all-*E* isomers, **12** and **1c** respectively, were both isolated as pure compounds.

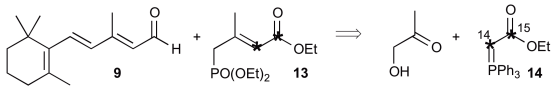
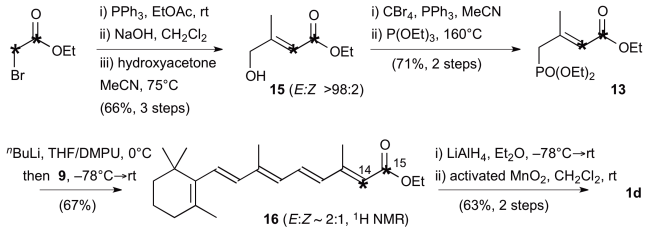


Figure 3. Synthesis plan for labelled retinal **1d**. (* indicates position of ^{13}C)

A more convergent strategy was applied to the synthesis of ^{13}C-labelled all-*E*-retinal **1d**, by combining two major fragments **9** and **13** (Figure 3).[1b,20,21] The fragment containing two ^{13}C labels required the phosphonium ylide **14**, which was combined with hydroxyacetone to give the *E* enoate **15** (Scheme 3). The allylic alcohol **15** was converted to the phosphonate **13** by bromination and Arbuzov reaction.[20] The final olefination proceeded with high *E* selectivity, and the subsequent reduction-oxidation sequence secured [14,15-$^{13}C_2$]-all-*trans*-retinal (**1d**).[21]



Scheme 4. Synthesis of [14,15-$^{13}C_2$]-all-*trans*-retinal (**1d**). (* indicates position of ^{13}C)

The labelled [14,15-$^{13}C_2$]-retinal (**1d**) has been incorporated in ^{15}N-labelled channelrhodopsin-2 (ChR2) reconstituted into lipid bilayers, and the results of DNP-enhanced solid-state NMR spectroscopy for the ground and intermediate states have been reported.[12] Proteorhodopsin containing [10,11,12,13,14,15,16,17,18-$^{13}C_9$]-all-*trans*-retinal (**1b**) was also reconstituted as proteoliposomes, and DNP-enhanced solid-state 2D ^{13}C-^{13}C correlation spectroscopy shows the six labelled carbon atoms present in the polyene chain (Figure 4). [12,15-$^{13}C_2$]-all-*trans*-retinal was incorporated into ChR2 and will be used for precise conformational analysis of the chromophore. Full assignments and biophysical studies of these and other retinylidene proteins will be reported elsewhere.

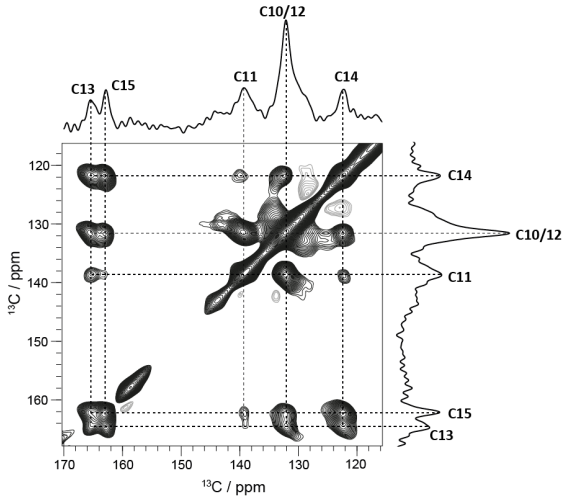


Figure 4. DNP-enhanced ^{13}C-^{13}C MAS-NMR spectrum of PR incorporating **1b** showing the six labeled carbon atoms in the chain. (Bruker 400 MHz DNP system with 3.2 mm HCN Cryo MAS probe, ~107K, PDSD mixing time 20 ms, 192 scans, 292 increments with a 33 μs delay, 1.5 mg protein in DMPC:DMPA lipid bilayers (lipid-to-protein ratio 2 w/w), pH9, radical: 20 mM AMUpol, 30% d-6-glycerol, 60% D_2O, 10% H_2O (enhancement: ~32)

Conclusions

The synthesis of three all-*E* retinal isotopomers, **1b**–**1d**, containing multiple ^{13}C-labels, have been achieved in twelve, eight and seven linear steps from ethyl 2-oxocyclohexanecarboxylate (**5**) or β-ionone (**4**) respectively. For **1b** and **1c** the final olefination delivered a 2:1 mixture of all-*trans*-retinals and their 13*Z* isomers, which were separated by preparative HPLC. The labelled chromophores have been incorporated in retinylidene proteins reconstituted into lipid bilayers to provide samples suitable for solid-state NMR studies. The biophysical studies of this intriguing group of light-responsive proteins containing site-specifically ^{13}C-labelled synthetic chromophores are ongoing,[12] and will be disclosed in due course.

Experimental

Ethyl 1,3,3-tri(methyl-^{13}C)-2-oxocyclohexane-1-carboxylate (6)

By adaption of a procedure by Stevans *et al*.,[17] to a suspension of NaH (60% in mineral oil, 5.60 g, 140 mmol) in THF (80 mL) under N_2 at 0°C was added a solution of ethyl 2-oxocyclohexane-1-carboxylate (5.91 mL, 34.5 mmol) in THF (80 mL) dropwise over 90 min. The mixture was stirred on ice for 30 min before addition of $^{13}CH_3I$ (6.47 mL, 104 mmol) dropwise over 20 min. The mixture was stirred at rt for 36 h with the formation of a white precipitate after 1 h. The mixture was diluted with Et_2O (100 mL) and cooled to 0°C before quenching with ice-cold H_2O (25 mL). The aqueous layer was extracted with Et_2O (3 x 30 mL) and the combined organic solution was washed with brine (30 mL), dried ($MgSO_4$) and the solvent removed under reduced pressure. Purification by silica gel chromatography (EtOAc:hexane = 2:98) afforded the product as a colourless oil (6.01 g, 27.9 mmol, 81%). FT-IR (neat) ν_{max} 2939 (m), 1732 (m), 1703 (s), 1423 (m), 1074 (m) cm^{-1}. ^{1}H NMR (400

MHz, $CDCl_3$): δ 4.23–4.04 (m, 2H), 2.58–2.50 (m, 1H), 2.06–1.91 (m, 1H), 1.76–1.68 (m, 1H), 1.65–1.49 (m, 2H), 1.45–1.35 (m, 2H), 1.29 (d, J_{CH} = 131.0 Hz, 3H), 1.23 (t, J_{HH} = 7.1 Hz, 3H), 1.09 (dd, J_{CH} = 128.0, J_{CH} = 5.0 Hz, 3H), 1.07 (dd, J_{CH} = 127.7, J_{CH} = 5.0 Hz, 3H) ppm (Only peaks for ^{13}C labelled carbons reported). LRMS (ES^+) *m/z* 216 $[M+H]^+$, 238 $[M+Na]^+$. HRMS (ES^+) For $C_9{}^{13}C_3H_{20}O_3Na^+$ calculated 238.1405, found 238.1402 Da

2,2,6-Tri(methyl-^{13}C)cyclohexan-1-one

A solution of ethyl 1,3,3-tri(methyl-^{13}C)-2-oxocyclohexane-1-carboxylate (6) (5.48 g, 25.5 mmol) and conc. HCl (40 mL) in EtOH (80 mL) was heated under reflux for 3 days. The mixture was then cooled and diluted with H_2O (20 mL) and neutralised with 1 M NaOH (170 mL). The aqueous layer was extracted with pentane (4 x 50 mL) and the combined organic solution washed with brine and dried ($MgSO_4$). The solvent was removed under reduced pressure (400 mBar, 20°C) to afford the product as a colourless oil (3.64 g, 32.1 mmol, 100%). FT-IR (neat) ν_{max} 2967 (w), 2929 (m), 2868 (w), 1704 (s), 1454 (m) cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 2.72–2.59 (m, 1H), 2.10–2.00 (m, 1H), 1.88 (qt, J_{HH} = 13.4, J_{HH} = 3.8 Hz, 1H), 1.81–1.72 (m, 1H), 1.69–1.61 (m, 1H), 1.60–1.49 (m, 1H), 1.38–1.22 (m, 1H), 1.18 (dd, J_{CH} = 127.0, J_{CH} = 4.9 Hz, 3H), 1.04 (dd, J_{CH} = 126.4, J_{CH} = 4.9 Hz, 3H), 0.99 (dd, J_{CH} = 126.8, J_{HH} = 6.4 Hz, 3H) ppm. ^{13}C NMR ($CDCl_3$, 101 MHz): δ 25.6 ($^{13}CH_3$), 25.3 ($^{13}CH_3$), 15.0 ($^{13}CH_3$) ppm (Only peaks for ^{13}C labelled carbons reported). LRMS (EI) m/z 143 $[M]^{+\bullet}$ (33%), 84 (100%). HRMS (EI) For $C_6{}^{13}C_3H_{16}O^{+\bullet}$ calculated 143.1296, found 143.1300 Da.

2,6,6-Tri(methyl-^{13}C)cyclohex-1-en-1-yl trifluoromethanesulfonate (7)

By adaption of a procedure by Breining *et al.*,[18] to a solution of LDA (1.90 M, 21.3 mL, 40.5 mmol) in THF (35 mL) under N_2 at −78°C was added 2,2,6-tri-(methyl-^{13}C)cyclohexan-1-one (3.62 g, 25.3 mmol) in THF (35 mL) dropwise over 45 min. The mixture was stirred at −78°C for 2 h before dropwise addition of a solution of *N*-phenyl-bis(trifluoromethanesulfonimide) (9.02 g, 25.3 mmol) in THF (40 mL). The mixture was allowed to warm to rt over 24 h. The reaction was quenched with saturated aqueous NH_4Cl (80 mL) and the aqueous phase extracted with pentane (3 x 40 mL). The combined organic solution was washed with brine, dried ($MgSO_4$) and the solvent removed under reduced pressure (200 mbar, 23°C) to give an orange oil. Purification by silica gel chromatography (pentane) afforded the vinyl triflate as a colourless oil (3.47 g, 12.6 mmol, 50%). FT-IR (neat) ν_{max} 2940 (w), 1458 (w), 1400 (s), 1243 (s), 1201 (s), 1139 (s), cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 2.16 (t, J_{HH} = 5.9 Hz, 2H), 1.71–1.58 (m, 4H), 1.76 (d, J_{CH} = 128.0 Hz, 3H), 1.16 (dd, J_{CH} = 126.9, J_{CH} = 4.7 Hz, 6H) ppm. ^{13}C NMR ($CDCl_3$, 101 MHz): δ 26.4 ($^{13}CH_3$), 17.6 ($^{13}CH_3$) ppm (Only peaks for ^{13}C labelled carbons reported). LRMS (EI) m/z 275 $[M]^{+\bullet}$ (27%), 259 $[M\text{-}CH_3]^+$ (33%), 69 $[CF_3]^+$ (100%). HRMS (EI) For $C_7{}^{13}C_3H_{15}F_3O_3S^{+\bullet}$ calculated 275.0789, found 275.0790 Da.

(*E*)-4-(2,6,6-Tri(methyl-^{13}C)cyclohex-1-en-1-yl)but-3-en-2-one ([$^{13}C_3$]-4)

By adaption of a procedure by Breining *et al.*,[18] a solution of vinyl triflate 7 (3.33 g, 12.1 mmol), NEt_3 (6.75 mL, 48.4 mmol), methyl vinyl ketone (1.97 mL, 48.4 mmol) and $[Pd(PPh_3)_2Cl_2]$ (425 mg, 0.605 mmol) in DMF (60 mL) under N_2 was heated at 75°C for 18 h. The reaction was quenched with H_2O (50 mL) and the aqueous phase extracted with

pentane (4 x 50 mL). The combined organic solution was washed with brine, dried (Na_2SO_4) and the solvent removed under reduced pressure. Purification by silica gel chromatography (Et_2O:hexane = 3:97) afforded the ketone as a pale yellow oil (1.58 g, 8.09 mmol, 67%). FT-IR (neat) ν_{max} 2930 (m), 2863 (m), 1692 (w), 1670 (s) cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 7.28 (d, J_{HH} = 16.5 Hz, 1H), 6.12 (d, J_{HH} = 16.5 Hz, 1H), 2.30 (s, 3H), 2.05–2.12 (unresolved t, 2H), 1.77 (d, J_{CH} = 126.1 Hz, 3H), 1.62–1.67 (m, 2H), 1.46–1.53 (m, 2H), 1.08 (dd, J_{CH} = 125.4, J_{CH} = 4.9 Hz, 6H) ppm. ^{13}C NMR ($CDCl_3$, 101 MHz) δ 28.7 ($^{13}CH_3$), 21.7 ($^{13}CH_3$) ppm (Only peaks for ^{13}C labelled carbons reported). LRMS (ES^+) m/z = 196 $[M+H]^+$. HRMS (ES^+) For $C_{10}{}^{13}C_3H_{21}O^+$ calculated 196.1688, found 196.1692 Da.

Triethylphosphonoacetate-$^{13}C_2$ ([$^{13}C_2$]-2)

By adaption of a procedure by Kiddle *et al.*,[16] a solution of 2-^{13}C-ethyl bromoacetate (2.152 g, 12.74 mmol) and $P(OEt)_3$ (2.05 g, 12.4 mmol) under N_2 was heated at 130°C in a microwave for 10 min. Purification by silica gel chromatography (EtOAc:hexane = 85:15) afforded the product as a colourless oil (2.62 g, 11.6 mmol, 94%). ^1H and ^{13}C NMR spectroscopic data was consistent with those reported.[22] FT-IR (neat) ν_{max} 2980 (m), 1692 (s), 1252 (s), 1020 (s), 963 (s) cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 4.29-4.09 (m, 6H), 2.96 (ddd, J_{CH} = 129.9, J_{HP} = 21.5, J_{HH} = 7.3 Hz, 2H), 1.36 (t, J_{HH} = 7.2 Hz, 6H), 1.29 (t, J_{HH} = 7.2 Hz, 3H) ppm. ^{13}C NMR ($CDCl_3$, 101 MHz): δ 165.7 (^{13}C, dd, J_{CC} = 58.7, J_{CP} = 5.9 Hz), 34.3 ($^{13}CH_2$ dd, J_{CC} = 134.3, J_{CP} = 59.4 Hz) ppm. (Only peaks for ^{13}C labelled carbons reported) LRMS (ES^+) m/z = 227 $[M+H]^+$.

Ethyl (2*E*,4*E*)-3-methyl-5-(2,6,6-tri(methyl-^{13}C)cyclohex-1-en-1-yl)penta-2,4-dienoate - 1,2-$^{13}C_2$ ([$^{13}C_5$]-8)

Following a procedure by McLean *et al.*,[19] to a suspension of NaH (630 mg, 15.7 mmol) in Et_2O (12 mL) under N_2 at 0°C was added a solution of triethylphosphonoacetate-$^{13}C_2$ ([$^{13}C_2$]-2) (3.51 mL, 15.5 mmol) in Et_2O (8 mL) drop-wise and the solution was stirred for 2 h at rt. A solution of (*E*)-4-(2,6,6-tri(methyl-^{13}C)cyclohex-1-en-1-yl)but-3-en-2-one ([$^{13}C_3$]-4) (1.54 g, 7.87 mmol) in Et_2O (8 mL) was added drop-wise and the resulting solution was stirred for 96 h. The reaction was quenched with H_2O (10 mL) and the aqueous phase was extracted with Et_2O (3 x 10 mL). The combined organic solution was washed with brine and dried (Na_2SO_4) before concentrating under reduced pressure. Purification by silica gel chromatography (Et_2O:hexane = 0.5:99.5) afforded the product as a pale yellow oil and a mixture of inseparable isomers (1.645 g, 6.153 mmol, 78%, 9*E*:9*Z* = 9:1). FT-IR (neat) ν_{max} 2957 (m), 2928 (m), 2865 (m), 1709 (s), 1606 (m), 1443 (m), 1233 (m) 1155 (s), 1047 (m), 967 (m), 875 (w) cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 7.64 (dd, J_{HH} = 16.4, J_{HH} = 2.3 Hz, 1H, *Z*), 6.56 (d, J_{HH} = 16.1 Hz, 1H, *E*), 6.10 (dd, J_{HH} = 16.1, J_{CH} = 5.3 Hz, 1H, *E*), 5.74 (d, J_{CH} = 159.3 Hz, 1H, *E*), 5.65 (d, J_{CH} = 160.3 Hz, 1H, *Z*), 4.18 (qd, J_{HH} = 7.1, J_{CH} = 3.1 Hz, 2H), 2.34 (d, J_{CH} = 4.6 Hz, 3H), 2.07-2.00 (m, 2H), 1.70 (d, J_{CH} = 125.8 Hz, 3H), 1.66-1.58 (m, 2H), 1.52-1.43 (m, 2H), 1.29 (t, J_{HH} = 7.1 Hz, 3H), 1.07 (dd, J_{CH} = 125.3, J_{CH} = 4.8 Hz, 6H, *Z*), 1.02 (dd, J_{CH} = 125.3, J_{CH} = 4.8 Hz, 6H, *E*) ppm. ^{13}C NMR ($CDCl_3$, 101 MHz): δ 167.3 (^{13}C, d, J_{CC} = 77.0 Hz), 166.3 (^{13}C, d, J_{CC} = 77.0 Hz), 118.0 (^{13}CH, d, J_{CC} = 77.0), 116.2 (^{13}CH, d, J_{CC} = 77.0), 28.9 ($^{13}CH_3$), 28.9 ($^{13}CH_3$), 21.8 ($^{13}CH_3$), 21.6 ($^{13}CH_3$) ppm. (Only

peaks for ^{13}C labelled carbons reported) LRMS (ES^+) m/z = 262 $[M+H]^+$. HRMS (ES^+) For $C_{12}{}^{13}C_5H_{26}NaO_2^+$ calculated 290.1993, found 290.2000

(2*E*,4*E*)-3-Methyl-5-(2,6,6-tri(methyl-^{13}C)cyclohex-1-en-1-yl)penta-2,4-dienal-1,2-$^{13}C_2$ ([$^{13}C_5$]-9)

Following a procedure by McLean *et al.*,[19] To a $LiAlH_4$ (1 M in THF, 7.15 mL, 7.15 mmol) in Et_2O (15 mL) under N_2 at −78°C was added a solution of ethyl (2*E*,4*E*)-3-methyl-5-(2,6,6-tri(methyl-^{13}C)cyclohex-1-en-1-yl)penta-2,4-dienoate-1,2-$^{13}C_2$ ([$^{13}C_5$]-8) (1.59 g, 5.95 mmol) in Et_2O (30 mL) dropwise and stirred at −78°C for 30 min. The mixture was then warmed to rt and stirred for 20 min. The reaction was quenched with H_2O (6 mL), 1 M NaOH (2 mL) and H_2O (2 mL) sequentially and the white solid was filtered through celite. The filtrate was dried ($MgSO_4$) and the solvent removed under reduced pressure to afford a colourless oil which was re-dissolved in CH_2Cl_2 (40 mL). Crushed molecular sieves (2.00 g), NMO (1.40 g, 11.9 mmol) and TPAP (105 mg, 2.98 mmol) was added and stirred at rt for 30 min. The black mixture was concentrated and the resulting black oil purified by silica gel chromatography (EtOAc:hexane = 4:96) to afford the product as a yellow oil (1.16 g, 5.17 mmol, 87%). FT-IR (neat) ν_{max} 2928 (m), 2861 (m), 1627 (s), 1601 (s), 1442 (m) cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 10.13 (ddd, J_{CH} = 169.7, J_{CH} = 24.6, J_{HH} = 8.1 Hz, 1H), 6.74 (d, J_{HH} = 16.1 Hz, 1H), 6.22 (dd, J_{HH} = 16.1, J_{CH} = 4.9 Hz, 1H), 5.94 (dd, J_{CH} = 157.5, J_{HH} = 8.1 Hz, 1H), 2.32 (d, J_{CH} = 4.0 Hz, 3H), 2.09-2.02 (m, 2H), 1.73 (d, J_{CH} = 125.8 Hz, 3H), 1.68-1.59 (m, 2H), 1.53-1.45 (m, 2H), 1.05 (dd, J_{CH} = 125.3, J_{CH} = 4.9 Hz, 6H) ppm. ^{13}C NMR ($CDCl_3$, 101 MHz): δ 191.3 (^{13}CH, d, J_{CC} = 77.0 Hz), 128.7 (^{13}CH, d, J_{CC} = 77.0 Hz), 28.9 ($^{13}CH_3$), 21.7 ($^{13}CH_3$) ppm. (Only peaks for ^{13}C labelled carbons reported) LRMS (ES^+) m/z 224 $[M+H]^+$. HRMS (ES^+) For $C_{10}{}^{13}C_5H_{22}ONa^+$ calculated 246.1731, found 246.1739 Da.

Ethyl (2*E*,4*E*,6*E*)-5-methyl-7-(2,6,6-tri(methyl-^{13}C)cyclohex-1-en-1-yl)hepta-2,4,6-trienoate-1,2,3,4-$^{13}C_4$

To a suspension of NaH (60% in mineral oil, 354 mg, 8.86 mmol) in Et_2O (5 mL) under N_2 at 0°C was added a solution of $^{13}C_2$-triethylphosphonoacetate ([$^{13}C_2$]-2) (2.01 g, 8.86 mmol) in Et_2O (5 mL) dropwise and the suspension was stirred for 1 h at rt. A solution of (2*E*,4*E*)-3-methyl-5-(2,6,6-tri(methyl-^{13}C)cyclohex-1-en-1-yl)penta-2,4-dienal-1,2-$^{13}C_2$ ([$^{13}C_5$]-9) (1.03 g, 4.65 mmol) in Et_2O (5 mL) was added dropwise and stirred at rt for 18 h. The reaction was then quenched with H_2O (6 mL) and the aqueous phase was extracted with Et_2O (3 x 6 mL). The combined organic solution was washed with brine, dried (Na_2SO_4) and the solvent removed under reduced pressure. Purification by silica gel chromatography (EtOAc:hexane = 2:97) afforded the product as a yellow/green oil (1.24 g, 4.19 mmol, 90%). FT-IR (neat) ν_{max} 2927 (m), 2864 (m), 2361 (m), 1707 (s), 1619 (m), 1597 (m) cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 7.92-7.52 (m, 1H), 6.40 (d, J_{HH} = 16.2 Hz, 1H), 6.16 (br dd, J_{HH} = 16.2, J_{CH} = 5.3 Hz, 1H), 6.15 (dd, J_{CH} = 153.9, J_{HH} = 12.5 Hz, 1H), 5.87 (dddd, J_{CH} = 162.3, J_{HH} = 15.0, J_{CH} = 7.0, J_{CH} = 2.9 Hz, 1H), 4.22 (qd, J_{HH} = 7.1, J_{CH} = 3.1 Hz, 2H), 2.05 (d, J_{CH} = 4.9 Hz, 3H), 2.03 (t, J_{HH} = 6.2 Hz, 2H), 1.72 (d, J_{CH} = 125.7 Hz, 3H), 1.66-1.57 (m, 2H), 1.50-1.45 (m, 2H), 1.31 (t, J_{HH} = 7.1 Hz, 3H), 1.03 (dd, J_{CH} = 125.3, J_{CH} = 4.8 Hz, 6H) ppm. ^{13}C NMR ($CDCl_3$, 101 MHz): δ 167.5 (ddd, J_{CC} = 76.5, J_{CC} = 7.9, J_{CC} = 1.5 Hz, ^{13}C), 140.6 (ddd, J_{CC} =

70.0, J_{CC} = 57.6, J_{CC} = 1.5 Hz, ^{13}CH), 127.2 (ddd, J_{CC} = 57.6, J_{CC} = 8.1, J_{CC} = 1.0 Hz, ^{13}CH), 120.0 (ddd, J_{CC} = 76.5, J_{CC} = 70.0, J_{CC} = 1.5 Hz, ^{13}CH), 28.9 ($^{13}CH_3$), 21.7 ($^{13}CH_3$) ppm. (Only peaks for ^{13}C labelled carbons reported) LRMS (ES^+): m/z = 296 $[M+H]^+$. HRMS (ES^+) For $C_{12}{}^{13}C_7H_{28}O_2Na^+$ calculated 318.2216, found 318.2218 Da.

(2*E*,4*E*,6*E*)-*N*-methoxy-*N*,5-dimethyl-7-(2,6,6-tri(methyl-^{13}C)cyclohex-1-en-1-yl)hepta-2,4,6-trienamide-1,2,3,4-$^{13}C_4$ ([$^{13}C_7$]-10)

By adaption of a procedure by Groesbeek *et al.*,[15] to a solution of *N*,*O*-dimethylhydroxylamine hydrochloride (1.83 g, 18.7 mmol) in THF (30 mL) under N_2 at −15°C was added ^{n}BuLi (2.27 M in hexanes, 16.1 mL, 36.5 mmol) dropwise over 15 min. After stirring at −15°C for 1 h a solution of ethyl (2*E*,4*E*,6*E*)-5-methyl-7-(2,6,6-tri(methyl-^{13}C)cyclohex-1-en-1-yl)hepta-2,4,6-trienoate-1,2,3,4-$^{13}C_4$ (1.23 g, 4.25 mmol) in THF (20 mL) was added dropwise over 15 min at −15°C. After 30 min at −15°C, the reaction was quenched with saturated aqueous NH_4Cl and the aqueous phase was extracted with Et_2O (3 x 10 mL). The combined organic solution was washed with brine, dried (Na_2SO_4) and the solvent removed under reduced pressure. Purification by silica gel chromatography (EtOAc:hexane = 15:85) afforded the product as a yellow/green oil (1.13 g, 3.72 mmol, 88%). FT-IR (neat) ν_{max} 2932 (m), 2861 (w), 1603 (s), 1537 (s), 1364 (m) cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.02-7.52 (m, 1H), 6.47 (dddd, J_{CH} = 161.6, J_{HH} = 14.9, J_{CH} = 7.0, J_{CH} = 3.7 Hz, 1H), 6.37 (br d, J_{HH} = 16.5 Hz, 1H), 6.22 (br dd, J_{CH} = 152.7, J_{HH} = 12.0 Hz, 1H), 6.16 (dd, J_{HH} = 16.5, J_{CH} = 5.4 Hz, 1H), 3.72 (s, 3H), 3.27 (d, J_{CH} = 2.0 Hz, 3H), 2.08-2.00 (m, 5H), 1.72 (d, J_{CH} = 125.6 Hz, 3H), 1.66-1.58 (m, 1H), 1.53-1.42 (m, 2H), 1.03 (dd, J_{CH} = 125.3, J_{CH} = 5.1 Hz, 6H) ppm. ^{13}C NMR ($CDCl_3$, 101 MHz): δ 167.6 (dd, J_{CC} = 71.2, J_{CC} = 7.0 Hz, ^{13}C), 139.5 (ddd, J_{CC} = 71.2, J_{CC} = 58.0, J_{CC} = 1.5 Hz, ^{13}CH), 127.9 (ddd, J_{CC} = 71.2, J_{CC} = 69.0, J_{CC} = 2.2 Hz, ^{13}CH), 117.8 (ddd, J_{CC} = 71.2, J_{CC} = 69.0, J_{CC} = 2.2 Hz, ^{13}CH), 28.9 ($^{13}CH_3$), 21.7 ($^{13}CH_3$) ppm. (Only peaks for ^{13}C labelled carbons reported) LRMS (ES^+) m/z 311 $[M+H]^+$. HRMS (ES^+) For $C_{12}{}^{13}C_7H_{30}NO_2^+$ calculated 311.2506, found 311.2513 Da.

(3*E*,5*E*,7*E*)-6-Methyl-8-(2,6,6-tri(methyl-^{13}C)cyclohex-1-en-1-yl)octa-3,5,7-trien-2-one-2,3,4,5-$^{13}C_4$ ([$^{13}C_7$]-11)

By adaption of a procedure by Groesbeek *et al.*,[15] To a solution of (2*E*,4*E*,6*E*)-*N*-methoxy-*N*,5-dimethyl-7-(2,6,6-tri(methyl-^{13}C) cyclohex-1-en-1-yl)hepta-2,4,6-trienamide-1,2,3,4-$^{13}C_4$ ([$^{13}C_7$]-10) (289 mg, 0.93 mmol) in THF (15 mL) at −78°C was added methyl lithium (1.48 M in Et_2O, 0.75 mL, 1.12 mmol) dropwise over 10 min. After 10 min at −78°C the reaction was quenched by addition of a slurry of silica (1 g) and H_2O (2 mL) and the resulting suspension was stirred for 15 min. The mixture was dried (Na_2SO_4), the solids filtered and the filtrate concentrated under reduced pressure. Purification by silica gel chromatography (EtOAc:hexane = 1:9) afforded the product as a viscous green oil (222 mg, 8.37 mmol, 90%). ^{1}H NMR (C_6D_6, 400 MHz): δ 7.70-7.29 (m, 1H), 6.34 (d, J_{HH} = 16.0 Hz, 1H), 6.18 (dd, J_{HH} = 16.0, J_{CH} = 5.4 Hz, 1H), 6.05 (dddd, J_{CH} = 156.7, J_{HH} = 15.0, J_{CH} = 7.2, J_{CH} = 3.3 Hz, 1H), 6.01-5.53 (m, 1H), 1.93 (m, 2H), 1.79 (d, J_{CH} = 125.3 Hz, 3H), 1.71 (d, J_{CH} = 5.0 Hz, 3H), 1.60-1.52 (m, 2H), 1.47-1.41 (m, 2H), 1.06 (dd, J_{CH} = 125.3, J_{CH} = 4.8 Hz, 6H) ppm. ^{13}C NMR (C_6D_6, 101 MHz): δ 196.55 (ddd, J_{CC} = 54.3, J_{CC} = 6.6, J_{CC} = 1.5 Hz, ^{13}C), 138.4 (dd,

J_{CC} = 68.0, J_{CC} = 57.2 Hz, ^{13}CH), 130.4 (dd, J_{CC} = 68.0, J_{CC} = 54.3 Hz, ^{13}CH), 129.0 (dd, J_{CC} = 57.2, J_{CC} = 6.6 Hz, ^{13}CH), 29.4 ($^{13}CH_3$), 22.2 ($^{13}CH_3$) ppm. (Only peaks for ^{13}C labelled carbons reported) LRMS (ES^+) m/z 266 $[M+H]^+$. HRMS (ES^+) For $C_{11}{}^{13}C_7H_{26}NaO^+$ calculated 288.2111, found 288.2115 Da.

Diethyl ((cyano-^{13}C)methyl-^{13}C)phosphonate ($[^{13}C_2]$-3)

By adaption of a procedure by Groesbeek *et al.*,[15] to a solution of *n*-BuLi (2.32 M in hexanes, 4.76 mL, 11.0 mmol) in THF (8 mL) under N_2 at −78°C was added a solution of HMDS (2.45 mL, 11.6 mmol) in THF (6 mL) drop-wise over 15 min. After stirring for 15 min a solution of $^{13}CH_3{}^{13}CN$ (250 mg, 5.81 mmol) in THF (6 mL) was added drop-wise over 15 min and the solution was stirred for a further 40 min. A solution diethylchlorophosphate (0.92 mL, 6.40 mmol) in THF (6 mL) was added drop-wise over 10 min and then stirred for a further 40 min. The mixture was allowed to warm to rt and stirred for 40 min before pouring into a stirred mixture of 2 M HCl (10 mL) and CH_2Cl_2 (15 mL). The aqueous phase was extracted with CH_2Cl_2 (3 x 10 mL) and then the combined organic solution was washed with water before drying ($MgSO_4$) and concentrating under reduced pressure to afford an orange oil. Purification by silica gel chromatography (EtOAc:hexane = 7:3 to 100:0) afforded the product as an orange oil (926 mg, 5.17 mmol, 89%). Spectroscopic data were consistent with those reported.[23] FT-IR (neat) ν_{max} 2984 (w), 2909 (w), 1260 (m), 1012 (s) cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.30-4.20 (m, 4H), 2.87 (ddd, J_{CH} = 135.0, J_{HP} = 21.0, J_{CH} = 10.5 Hz, 2H), 1.39 (t, J_{HH} = 7.1 Hz, 6H) ppm. ^{13}C NMR ($CDCl_3$, 101 MHz): δ 112.6 (^{13}C, d, J_{CC} = 57.9, J_{CC} = 11.4 Hz), 16.5 ($^{13}CH_2$, d, J_{CC} = 143.8, J_{CP} = 60.2) ppm. (Only peaks for ^{13}C labelled carbons reported) LRMS (ES^+): m/z 179 $[M+H]^+$.

[10,11,12,13,14,15,16,17,18-$^{13}C_9$]-All-*trans*-retinal (1b)

By adaption of a procedure by Groesbeek *et al.*,[15] to a solution of diethyl ((cyano-^{13}C)methyl-^{13}C)phosphonate ($[^{13}C_2]$-3) (205 mg, 1.14 mmol) in THF (1.5 mL) under N_2 atm. at 0°C was added *n*-BuLi (2.32 M in hexanes, 0.45 mL, 1.00 mmol) drop-wise over 5 min and the solution was stirred at rt for 90 min. A solution of (3*E*,5*E*,7*E*)-6-methyl-8-(2,6,6-tri(methyl-^{13}C)cyclo hex-1-en-1-yl)octa-3,5,7-trien-2-one-2,3,4,5-$^{13}C_4$ ($[^{13}C_7]$-11) (138 mg, 0.52 mmol) in THF (5 mL) was added drop-wise and stirred at rt for 18 h. The reaction was quenched with saturated aqueous NH_4Cl and the aqueous phase extracted with EtOAc (3 x 8 mL). The combined organic solution was washed with brine, dried (Na_2SO_4) and concentrated under reduced pressure to afford an orange oil. Purification by silica gel chromatography (EtOAc:hexane = 4:96, silica deactivated with NEt_3) afforded the impure nitrile intermediate as an orange oil (145 mg).

The orange oil was taken up in CH_2Cl_2 (3.5 mL) under N_2 and DIBAL (1 M in CH_2Cl_2, 0.76 mL, 0.76 mmol) was added drop-wise at −60°C. After stirring at −60°C for 15 min, a slurry of SiO_2 and H_2O was added and stirred for 15 min at rt. The solids were filtered and the filtrate dried twice (Na_2SO_4) before concentrating under reduced pressure to afford an orange oil. Purification by silica gel chromatography (EtOAc:hexane = 4:96 to 1:9, silica deactivated with NEt_3) afforded the product as an orange oil (106 mg, 0.36 mmol, 70%, 13*E*:13*Z* = 2:1). The isomers were separated by preparative HPLC (EtOAc:pet. ether = 3.5:96.5). FT-IR (neat)

ν_{max} 2928 (w), 1716 (w), 1662 (w), 971 (m) cm^{-1}. ^1H NMR (C_6D_6, 500 MHz): δ 10.01 (ddd, J_{CH} = 169.1, J_{CH} = 25.4, J_{HH} = 7.8 Hz, 1H), 7.03-6.62 (m, 1H), 6.37 (br d, J_{HH} = 16.1 Hz, 1H), 6.26 (dd, J_{HH} = 16.1, J_{CH} = 4.9 Hz, 1H), 6.21-5.83 (m, 1H), 5.96 (dt, J_{CH} = 157.1, J_{HH} = 7.8 Hz, 1H), 1.99-1.93 (m, 2H), 1.78 (d, J_{CH} = 4.4 Hz, 3H), 1.75-1.70 (m, 3H), 1.76 (d, J_{CH} = 125.6 Hz, 3H), 1.62-1.55 (m, 2H), 1.50-1.44 (m, 2H), 1.12 (dd, J_{CH} = 125.2, J_{CH} = 4.7 Hz, 6H) ppm. ^{13}C NMR (C_6D_6, 126 MHz): δ 189.4 (ddd, J_{CC} = 56.7, J_{CC} = 6.8, J_{CC} = 2.9 Hz, ^{13}C), 152.6 (dddd, J_{CC} = 67.1, J_{CC} = 54.2, J_{CC} = 6.8, J_{CC} = 2.9 Hz, ^{13}C), 135.2 (ddddd, J_{CC} = 67.1, J_{CC} = 54.2, J_{CC} = 6.8, J_{CC} = 3.3, J_{CC} = 1.7 Hz, ^{13}CH), 131.2 (ddd, J_{CC} = 67.1, J_{CC} = 58.4, J_{CC} = 7.6 Hz, ^{13}CH), 130.3-129.6 (m, ^{13}CH), 130.0-128.8 (m, ^{13}CH), 28.8 ($^{13}CH_3$), 21.6 ($^{13}CH_3$) ppm. (Only peaks for ^{13}C labelled carbons reported) LRMS (ES^+) m/z 294 $[M+H]^+$. HRMS (ES^+) For $C_{11}{}^{13}C_9H_{29}NaO^+$ calculated 294.2515, found 294.2519 Da.

Triethylphosphonoacetate-2-^{13}C ([$^{13}C_1$]-2)

By adaption of a procedure by Kiddle *et al.*,[16] a solution of 2-^{13}C-ethyl bromoacetate (1.77 g, 10.5 mmol) and $P(OEt)_3$ (1.70 g, 10.3 mmol) under N_2 was heated at 130°C in a microwave for 10 min. Purification by silica gel chromatography (EtOAc:hexane = 85:15) afforded the product was afforded as a colourless oil (2.28 g, 10.1 mmol, 99%). ^1H and ^{13}C NMR spectroscopic data were consistent with those reported.[24] FT-IR (neat) ν_{max} 2983 (m), 1773 (s), 1253 (s), 1017 (s), 963 (s) cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 4.18 (dq, J_{HP} = 14.6, J_{HH} = 7.2 Hz, 6H), 2.95 (dd, J_{CH} = 130.0, J_{HP} = 21.5 Hz, 2H), 1.34 (t, J_{HH} = 7.2 Hz, 6H), 1.28 (t, J_{HH} = 7.2 Hz, 3H) ppm. ^{13}C NMR ($CDCl_3$, 101 MHz): δ 34.3 ($^{13}CH_3$, d, J_{CP} = 134.3 Hz) ppm. (Only peaks for ^{13}C labelled carbons reported) ^{31}P NMR ($CDCl_3$, 162 MHz): δ 19.81 (d, J_{CP} = 134.6 Hz) ppm. LRMS (ES^+) m/z = 226 $[M+H]^+$.

Ethyl (2*E*,4*E*,6*E*)-5-methyl-7-(2,6,6-trimethylcyclohex-1-en-1-yl)hepta-2,4,6-trienoate-2-^{13}C

To a suspension of NaH (60% in mineral oil, 427 mg, 10.7 mmol) in Et_2O (8 mL) under N_2 at 0 °C was added a solution of triethylphosphonoacetate-2-^{13}C ([$^{13}C_1$]-2) (2.25 g, 10.0 mmol) in Et_2O (4 mL) dropwise and the solution was stirred for 90 min at rt. A solution of dienal 9 (1.10 g, 5.04 mmol) in Et_2O (4 mL) was added dropwise and the solution was stirred at rt for 2 h. The reaction was then quenched with H_2O (10 mL) and the aqueous phase was extracted with Et_2O (3 x 8 mL). The combined organic solution was washed with brine, dried (Na_2SO_4) and the solvent removed under reduced pressure. Purification by silica gel chromatography (EtOAc:hexane = 2:97) afforded the product as a yellow/green oil (1.37 g, 4.73 mmol, 94%). FT-IR (neat) ν_{max} 2929 (m), 2861 (m), 1663 (s), 1565 (m) cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.72 (ddd, J_{HH} = 15.0, J_{HH} = 12.1, J_{CH} = 2.5 Hz, 1H), 6.40 (br d, J_{HH} = 16.0 Hz, 1H), 6.20-6.12 (m, 2H), 5.88 (dd, J_{CH} = 162.4, J_{HH} = 15.0 Hz, 1H), 4.22 (d, J_{HH} = 7.1 Hz, 2H), 2.05 (s, 3H), 2.03 (t, J_{HH} = 5.9 Hz, 2H), 1.72 (s, 3H), 1.67-1.59 (m, 2H), 1.52-1.45 (m, 2H), 1.32 (t, J_{HH} = 7.1 Hz, 3H), 1.04 (s, 6H) ppm. ^{13}C NMR ($CDCl_3$, 101MHz): δ 120.1 ($^{13}CH_2$) ppm. (Only peaks for ^{13}C labelled carbons reported) LRMS (ES^+) m/z 290 $[M+H]^+$. HRMS (ES^+) For $C_{18}{}^{13}CH_{28}NaO_2^+$ calculated 312.2015, found 312.2018 Da.

(2*E*,4*E*,6*E*)-*N*-methoxy-*N*,5-dimethyl-7-(2,6,6-trimethylcyclohex-1-en-1-yl)hepta-2,4,6-trienamide-2-^{13}C

By adaption of a procedure by Grosbeek *et al.*,[15] To a solution of *N*,*O*-dimethylhydroxylamine hydrochloride (1.83 g, 18.7 mmol) in THF (30 mL) under N_2 at −15°C was added *n*-BuLi (2.27 M in hexanes, 16.1 mL, 36.5 mmol) dropwise over 15 min. After stirring the solution at −15°C for 1 h, a solution of ethyl (2*E*,4*E*,6*E*)-5-methyl-7-(2,6,6-trimethylcyclohex-1-en-1-yl)hepta-2,4,6-trienoate-2-^{13}C (1.23 g, 4.25 mmol) in THF (20 mL) was added dropwise over 15 min at −15°C. After 30 min at −15 °C, the reaction was quenched with saturated aqueous NH_4Cl and the aqueous phase was extracted with Et_2O (3 x 10 mL). The combined organic solution was washed with brine, dried (Na_2SO_4) and the solvent removed under reduced pressure. Purification by silica gel chromatography (EtOAc:hexane = 15:85) afforded the product as a yellow/green oil (1.13 g, 3.72 mmol, 88%). FT-IR (neat) ν_{max} 2928 (w), 2963 (m), 1645 (s), 1589 (m), 1408 (m), 1371 (s) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (ddd, J_{HH} = 14.9, J_{HH} = 12.0, J_{CH} = 2.9 Hz, 1H), 6.47 (br dd, J_{HH} = 161.9, J_{HH} = 14.9 Hz, 1H), 6.37 (br d, J_{HH} = 16.1 Hz, 1H), 6.22 (dd, J_{HH} = 12.0, J_{HH} = 4.4 Hz, 1H), 6.15 (d, J_{HH} = 16.1 Hz, 1H), 3.71 (s, 3H), 3.27 (s, 3H), 2.06 (s, 3H), 2.03 (t, J_{HH} = 6.0 Hz, 2H), 1.71 (s, 3 H), 1.67-1.58 (m, 2H), 1.51-1.45 (m, 2H), 1.03 (s, 6H) ppm. ^{13}C NMR ($CDCl_3$, 101 MHz): δ = 117.9 (^{13}CH) ppm. (Only peaks for ^{13}C labelled carbons reported) LRMS (ES^+) m/z 305 $[M+H]^+$. HRMS (ES^+) For $C_{18}{}^{13}CH_{30}NO_2^+$ calculated 305.2305, found 305.2303 Da.

(3*E*,5*E*,7*E*)-6-Methyl-8-(2,6,6-trimethylcyclohex-1-en-1-yl)octa-3,5,7-trien-2-one-3-^{13}C ([^{13}C$_1$]-11)

To a solution of (2*E*,4*E*,6*E*)-*N*-methoxy-*N*,5-dimethyl-7-(2,6,6-trimethylcyclohex-1-en-1-yl)hepta-2,4,6-trienamide-2-^{13}C (964 mg, 3.18 mmol) in THF (50 mL) at −78°C was added methyl lithium (1.45 M in Et_2O, 2.62 mL, 3.80 mmol) dropwise over 10 min. After 10 min at −78°C the reaction was quenched by addition of a slurry of silica (2 g) and H_2O (10 mL) and the resulting suspension was stirred for 15 min at rt. The mixture was dried ($MgSO_4$), the solids filtered and the filtrate concentrated under reduced pressure to afford the product as viscous green oil (788 mg, 3.04 mmol, 96%). FT-IR (neat) ν_{max} 2927 (m), 2864 (w), 1657 (s), 1585 (s), 1445 (w), 1359 (m), 1020 (s) cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.58 (ddd, J_{HH} = 15.1, J_{HH} = 11.9, J_{CH} = 1.2 Hz, 1H), 6.43 (br d, J_{HH} = 16.0 Hz, 1H), 6.18 (br d, J_{HH} = 16.0 Hz, 1H), 6.17 (br dd, J_{HH} = 11.9, J_{CH} = 4.5 Hz, 1H), 6.17 (dd, J_{CH} = 157.5, J_{HH} = 15.1 Hz, 1H), 2.30 (d, J_{CH} = 1.0 Hz, 3H), 2.07 (s, 3H), 2.04 (br t, J_{HH} = 6.2 Hz, 2H), 1.72 (s, 3H), 1.67-1.59 (m, 2H), 1.51-1.45 (m, 2H), 1.04 (s, 6H) ppm. ^{13}C NMR ($CDCl_3$, 101 MHz): δ 129.3 (^{13}CH) ppm. LRMS (ES^+) m/z = 260 $[M+H]^+$. HRMS (ES^+) For $C_{17}{}^{13}CH_{26}NaO^+$ calculated 282.1909, found 282.1918 Da

Diethyl ((cyano-^{13}C)methyl)phosphonate ([^{13}C$_1$]-3)

By adaption of a procedure by Groesbeek *et al.*,[15] to a solution of *n*-BuLi (2.17 M in hexanes, 10.79 mL, 23.42 mmol) in THF (16 mL) under N_2 at −78°C was added a solution of HMDS (5.02 mL, 23.78 mmol) in THF (12 mL) drop-wise over 15 min. After stirring for 15 min a solution of $^{13}CH_3CN$ (500 mg, 11.89 mmol) in THF (12 mL) was added drop-wise over 15

min and then stirred for a further 40 min. A solution diethylchlorophosphate (1.89mL, 13.1 mmol) in THF (12 mL) was added drop-wise over 10 min and the solution was stirred for a further 40 min. The mixture was allowed to warm to rt and stirred for 40 min before pouring into a stirred mixture of 2 M HCl (20 mL) and CH_2Cl_2 (30 mL). The aqueous phase was extracted with CH_2Cl_2 (3 x 20 mL) and then the combined organic solution washed with H_2O before drying ($MgSO_4$) and concentrating under reduced pressure to afford an orange oil. Purification by silica gel chromatography (EtOAc:hexane = 7:3 to 100:0) afforded the product as an orange oil (1.885 mg, 10.58 mmol, 89%). ^{1}H and ^{13}C NMR spectroscopic data were consistent with those reported.[15] FT-IR (neat) ν_{max} 2984 (w), 2909 (w), 2256 (w), 1260 (m), 1012 (s) cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.31-4.19 (m, 4H), 2.87 (dd, J_{HP} = 21.1, J_{CH} =10.6 Hz, 2H), 1.39 (t, J_{HH} = 7.1 Hz, 6H) ppm. ^{13}C NMR ($CDCl_3$, 101 MHz): δ 112.6 (^{13}C, d, J_{CP} 11.0 Hz) ppm. LRMS (ES$^+$) m/z 178 [M+H]$^+$.

[12-15-$^{13}C_2$]-all-*trans*-retinal (1c)

By adaption of a procedure by Groesbeek *et al.*,[15] to a solution of diethyl ((cyano-^{13}C)methyl)phosphonate ([$^{13}C_1$]-3) (250 mg, 1.41 mmol) in THF (1.5 mL) under N_2 atm. at 0°C was added *n*-BuLi (2.31 M, 0.55 mL, 1.3 mmol) drop-wise over 5 min and the solution was stirred at rt for 75 min. A solution of (3*E*,5*E*,7*E*)-6-methyl-8-(2,6,6-trimethylcyclohex-1-en-1-yl)octa-3,5,7-trien-2-one-3-^{13}C ([$^{13}C_1$]-11) (165 mg, 0.64 mmol) in THF (5 mL) was added drop-wise and stirred at rt for 18 h. The reaction was quenched with saturated aqueous NH_4Cl and the aqueous phase extracted with EtOAc (3 x 8 mL). The combined organic solution was washed with brine, dried (Na_2SO_4) and concentrated under reduced pressure to afford an orange oil. Purification by silica gel chromatography (EtOAc:hexane = 3:97) afforded the impure nitrile intermediate as an orange oil (121 mg). The orange oil was taken up in CH_2Cl_2 (3 mL) under N_2 and DIBAL (1 M in CH_2Cl_2, 0.64 mL, 0.64 mmol) was added drop-wise at −60°C. After stirring at −60°C for 1 h, a slurry of SiO_2 and H_2O was added and stirred for 15 min at rt. The solids were filtered and the filtrate dried twice (Na_2SO_4) before concentrating under reduced pressure to afford an orange oil. Purification by silica gel chromatography (EtOAc:hexane = 4:96 to 1:9, silica deactivated with NEt_3) afforded the product as an orange oil (120 mg, 0.42 mmol, 30%, 13*E*:13*Z* = 2:1). The isomers were separated by preparative HPLC (EtOAc:pet. ether = 3.5:96.5). All-*trans*- (1c): FT-IR (neat) ν_{max} 2928 (w), 1716 (w), 1662 (w), 971 (m) cm^{-1}. ^{1}H NMR (C_6D_6, 400 MHz): δ 10.01 (dd, J_{CH} = 169.2, J_{HH} = 8.1 Hz, 1H), 6.83 (dd, J_{HH} = 15.2, J_{HH} = 11.5 Hz, 1H), 6.37 (d, J_{HH} = 16.1 Hz, 1H), 6.26 (d, J_{HH} = 16.1 Hz, 1H), 6.02 (dd, J_{HH} = 11.5, J_{CH} = 4.8 Hz, 1H), 6.02 (dd, J_{CH} = 154.4, J_{HH} = 15.2 Hz, 1H), 5.96 (apparent t, $J_{HH/CH}$ = 7.9 Hz, 1H), 1.96 (t, J_{HH} = 6.0 Hz, 2H), 1.78 (s, 3H), 1.77 (s, 3H), 1.73 (d, J_{CH} = 4.0 Hz, 3H), 1.62-1.55 (m, 2H), 1.51-1.44 (m, 2H), 1.12 (s, 6H) ppm. ^{13}C NMR (C_6D_6, 101 MHz): δ 190.1 (^{13}CH, d, J_{CC} = 7.0 Hz), 135.9 (^{13}CH, d, J_{CC} = 7.0 Hz) ppm. (Only peaks for ^{13}C labelled carbons reported) LRMS (ES$^+$) m/z = 287 [M+H]$^+$. HRMS (ES$^+$) For $C_{18}{}^{13}C_2H_{28}NaO^+$ calculated 309.2099, found 309.2102 Da.

13*Z*- (12): ^{1}H NMR (C_6D_6, 400 MHz): δ 10.15 (dd, J_{CH} = 169.7, J_{HH} = 7.2 Hz, 1H), 7.07 (dd, J_{CH} = 153.8, J_{HH} = 15.0 Hz, 1H), 6.74 (dd, J_{HH} = 15.0, J_{HH} = 11.4 Hz, 1H), 6.37 (d, J_{HH} = 16.1 Hz, 1H), 6.28 (d, J_{HH} = 16.1 Hz, 1H), 6.05 (dd, J_{HH} = 11.4, J_{CH} = 4.9 Hz, 1H), 5.76 (apparent t, $J_{CH/HH}$ = 8.3 Hz, 1H), 1.96 (br t, J_{HH} = 6.1 Hz, 2H), 1.82-1.75 (m, 6H), 1.59 (br d,

J_{CH} = 4.0 Hz, 3H), 1.62-1.55 (m, 2H), 1.50-1.45 (m, 2H), 1.13 (s, 6H) ppm. ^{13}C NMR (C_6D_6, 101 MHz): δ = 189.0 (d, J_{CC} = 5.1 Hz, ^{13}C), 127.9 (d, J_{CC} = 5.1 Hz, ^{13}CH) ppm (Only peaks for ^{13}C labelled carbons reported) LRMS (ES^+) m/z = 287 $[M+H]^+$. HRMS (ES^+) For $C_{18}{}^{13}C_2H_{28}NaO^+$ calculated 309.2099, found 309.2102 Da.

Ethyl (*E*)-4-hydroxy-3-methylbut-2-enoate-1,2-$^{13}C_2$ (15)

By adaption of a procedure by Magoulas *et al.*,[20] a suspension of hydroxyacetone (0.38 g, 5.1 mmol) and [1,2-$^{13}C_2$](ethoxycarbonylmethylene) triphenylphosphorane[25] (1.87 g, 5.34 mmol) in MeCN (10 mL) was heated under reflux for 12 h. The reaction mixture was allowed to cool, concentrated to a yellow oil and triturated with Et_2O and placed in the freezer overnight. The resultant white solid was removed by filtration and discarded. The filtrate was concentrated under reduced pressure and purification by silica gel column chromatography (EtOAc:hexane = 4:1) afforded the title compound as a pale yellow oil (0.540 g, 3.72 mmol, 70%, *E*:*Z* > 98:2). FT-IR (neat) ν_{max} 3373 (m), 1651 (s), 1631 (s), 1209 (m) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.98 (ddt, J_{HH} = 1.5, 3.1 Hz, J_{CH} = 161.1 Hz, 1H), 4.20 - 4.11 (m, 4H), 2.09 (d, J_{CH} = 4.6 Hz, 3H), 1.99 (br t, J_{HH} = 5.7 Hz, 1H), 1.73 (br s, 1H), 1.29 (t, J_{HH} = 7.2 Hz, 3H) ppm. ^{13}C NMR ($CDCl_3$, 101 MHz): δ 166.84 (^{13}CO, d, J_{CC} = 76 Hz), 113.82 (^{13}CH, d, J_{CC} = 76 Hz) ppm. (Only peaks for ^{13}C labelled carbons reported). LRMS (ES^+) *m/z* 185 $[M+K]^+$. HRMS (ESI^+) for $C_5{}^{13}C_2H_{13}O_3$ calculated 147.0926, found 147.0927 Da.

Ethyl (*E*)-4-bromo-3-methylbut-2-enoate-1,2-$^{13}C_2$

By adaption of a procedure by Magoulas *et al.*,[20] to a suspension of ethyl (*E*)-4-hydroxy-3-methylbut-2-enoate-1,2-$^{13}C_2$ (15) (0.450 g, 3.10 mmol) and Ph_3P (0.815 g, 3.10 mmol) in MeCN (1 mL), was added CBr_4 (1.03 g, 3.10 mmol) portionwise and the resulting solution was stirred at rt for 2 h. The reaction mixture was concentrated under reduced pressure and purification by silica gel column chromatography (CH_2Cl_2:hexane = 0:1 to 1:1) afforded the title compound as a clear colourless oil (0.480 g, 2.30 mmol, 74%, *E*:*Z* > 98:2). FT-IR (neat) ν_{max} 1732 (m), 1674 (m), 1221 (m), 1143 (m) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.96 (d, J_{CH} = 161.1 Hz, 1H), 4.18 (qd, J_{HH} = 7.1, J_{CH} = 3.1 Hz, 2H), 3.95 (dd, J_{HH} = 5.7, J_{HH} = 0.7 Hz, 2H), 2.28 (dt, J_{CH} = 4.6, J_{CH} = 1.2 Hz, 3H), 1.27 - 1.23 (t, J_{HH} = 7.1 Hz, 3H) ppm. ^{13}C NMR ($CDCl_3$, 101 MHz): δ 165.92 (^{13}CO, d, J_{CC} = 76 Hz), 119.52 (^{13}CH, d, J_{CC} = 76 Hz) ppm. (Only peaks for ^{13}C labelled carbons reported). LRMS (ES^+) *m/z* 226 $[M+NH_4]^+$.

Ethyl (*E*)-4-(diethoxyphosphoryl)-3-methylbut-2-enoate-1,2-$^{13}C_2$ (13)

By adaption of a procedure by Magoulas *et al.*,[20] neat $(EtO)_3P$ (0.42 mL, 2.47 mmol) and ethyl (*E*)-4-bromo-3-methylbut-2-enoate-1,2-$^{13}C_2$ (463 mg, 2.22 mmol) were heated in a sealed pressure vial at 160°C for 4 h. After cooling the reaction mixture was purified by silica gel column chromatography (EtOAc) to afford the title compound as a pale yellow oil (0.572 g, 2.15 mmol, 96%, *E*:*Z* > 98:2). FT-IR (neat) ν_{max} 1670 (m), 1619 (m), 1242 (m), 1129 (m) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.79 (dd, J_{PH} = 5.1 Hz, J_{CH} = 160.5Hz, 1H), 4.15-4.09 (m, 6H), 2.68 (ddd, J_{HH} = 0.7 Hz, J_{PH} = 6.1 Hz, J_{CH} = 23.5 Hz, 2H), 2.32-2.30 (m, 3H), 1.35-1.26 (m, 9H) ppm. ^{13}C NMR ($CDCl_3$, 101 MHz): δ 166.0 (^{13}CO, dd, J_{CC} = 76 Hz, J_{PC} = 4

Hz), 120.0 (^{13}CH, dd, J_{CC} = 76 Hz, J_{PC} = 12.5 Hz) ppm. (Only peaks for ^{13}C labelled carbons reported). LRMS (ES$^+$) *m/z* 289 [M+Na]$^+$. HRMS (ESI$^+$) for $C_9{}^{13}C_2H_{21}NaO_5P^+$ calculated 289.1086, found 289.1085 Da.

[14,15 -$^{13}C_2$]-Trans-retinal (1d)

By adaption of a procedure by Wada *et al.*,[21b] to a solution of ethyl (*E*)-4-(diethoxyphosphoryl)-3-methylbut-2-enoate-1,2-$^{13}C_2$ (13) (268 mg, 1.00 mmol) in THF (2 mL) at 0°C was added DMPU (0.48 mL, 0.51 g, 4.0 mmol) followed by ^{n}BuLi (2.3 M in hexanes, 0.52 mL, 1.20 mmol) dropwise. The reaction was kept at 0°C for 40 min then cooled to –78°C. Aldehyde (9) (200 mg, 0.916 mmol) in THF (1 mL) was added dropwise slowly and the reaction was allowed to warm to 0°C and maintained below 5°C for 2 h. The reaction was quenched with saturated aqueous NH_4Cl and the aqueous layer extracted with EtOAc (x 2). The combined organic solution was washed with water, brine and then dried ($MgSO_4$) before concentrating under reduced pressure. Careful purification of the mixture of olefin isomers (13*E*/13*Z*, estimated 9:1 by ^{1}H NMR spectroscopy) by silica gel chromatography (EtOAc:hexane = 3:97) afforded the ester (ethyl (2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoate-1,2-$^{13}C_2$)[26] (16) as a pale yellow oil (203 mg, 0.614 mmol, 67%).

To a 1 M solution of $LiAlH_4$ in THF (0.97 mL, 0.97 mmol) in Et_2O (1.5 mL) under N_2 was added a solution of ethyl ester 16 (200 mg, 0.61 mmol) in Et_2O (6 mL) dropwise at –78°C and stirred at –78°C for 30 min. The mixture was warmed to rt and stirred for 20 min. The reaction was quenched with water (0.2 mL), 15% NaOH solution (0.2 mL) and water (0.3 mL) sequentially and the white solid was filtered through celite and washed with Et_2O. The filtrate was dried ($MgSO_4$) and concentrated under reduced pressure to afford a pale yellow oil (189 mg) that was re-dissolved in CH_2Cl_2 (8 mL). Powered, activated MnO_2 (0.530 g, 6.00 mmol) was added and the reaction stirred at rt for 1 h and then concentrated. Purification by column chromatography on aluminia (gradient 2:98 to 5:95 = EtOAc:hexane) afforded the product as a yellow oil (109 mg, 0.38 mmol, 63%). Spectroscopic data were consistent with those reported.[27] FT-IR (neat) ν_{max} 2931 (m), 1680 (m), 1615 (s), 968 (m) cm^{-1}. 1H NMR (C_6D_6, 400 MHz): δ 10.00 (ddd, J_{CH} = 169.2, J_{CH} = 25.6, J_{HH} = 7.9 Hz, 1H), 6.83 (dd, J_{HH} = 15.1, J_{HH} = 11.3 Hz, 1H), 6.37 (d, J_{HH} = 16.2 Hz, 1H), 6.26 (d, J_{HH} = 16.2 Hz, 1H), 6.02 (dd, J_{HH} = 15.4, J_{HH} = 4.9 Hz, 2H), 5.95 (dd, J_{CH} = 7.9 Hz, J_{CH} = 157.0 Hz, 1H), 1.96 (t, J_{HH} = 6.0 Hz, 2H), 1.78 (s, 3H), 1.77 (s, 3H), 1.73 (d, J_{CH} = 4.1 Hz, 3H), 1.62-1.54 (m, 2H), 1.50-1.43 (m, 2H), 1.12 (s, 6H) ppm; 13C NMR ($CDCl_3$, 101 MHz): δ 191.2 (^{13}CO, d, J_{CC} = 57 Hz), 128.9 (^{13}CH, d, J_{CC} = 57 Hz) ppm. (Only peaks for 13C labelled carbons reported); LRMS (ES$^+$) m/z = 287 [M+H]$^+$ (100%). HRMS (EI) For $C_{18}{}^{13}C_2H_{28}O^{+\bullet}$ calculated 286.2202 found 286.2190 Da.

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