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Synthesis of isotopically labeled all-*trans* retinals for DNP-enhanced solid-state NMR studies of retinylidene proteins

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Three all-*trans* retinals containing multiple ^{13}C labels have been synthesized to enable dynamic nuclear polarization enhanced solid-state magic angle spinning NMR studies of novel microbial retinylidene membrane proteins including proteorhodpsin and channelrhodopsin. The synthetic approaches 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 synthesized in in 12, 8, and 7 linear steps from ethyl 2-oxocyclohexanecarboxylate (**5**) or β-ionone (**4**), respectively.

1 | 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 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; in contrast, the visual

receptor rhodopsin contains the polyene in its 11-Z configuration.

Much effort has been directed toward 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 analyzed 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 labeling, 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 3 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).

2 | RESULTS AND DISCUSSION

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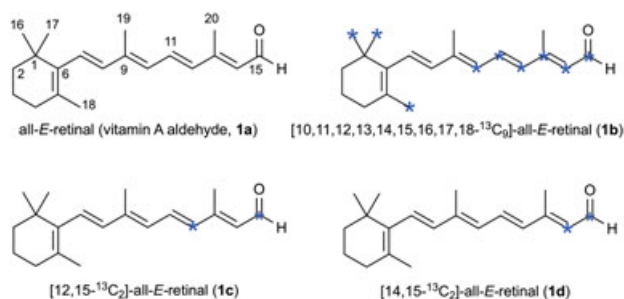
insight into light-driven conformational changes in retinylidene proteins by using DNP-enhanced solid-state NMR with ^{15}N-labeled and ^{13}C-labeled proteins.[12] The synthetic routes to polyenes **1b** and **1c** were planned to make use of inexpensive and readily available ^{13}C-enriched (99 atm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

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

The synthesis of the ^{13}C-labeled [10,11,12,13,14,15,16,17,18-[13]C$_9$]-all-*trans*-retinal (**1b**), containing 9 positions of ^{13}C-enrichment, began by trimethylation of ethyl 2-oxocyclohexanecarboxylate (**5**)[17] with [^{13}C]-CH$_3$I, to afford the β-ketoester **6** (Scheme 2). From **6**, acid-catalyzed 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-labeled β-ionone **[¹³C₃]-4**. The first olefination reaction using doubly ^{13}C-labeled triethyl phosphonoacetate (**[¹³C₂]-2**) yielded the trienoate **[¹³C₅]-8** as an unseparated mixture of E/Z isomers (~9:1 from ^{1}H NMR) in 78% yield.[19] Following LiAlH$_4$ reduction of the isomeric mixture to the allylic alcohols and subsequent TPAP oxidation, all-E-trienal stereoisomer **[¹³C₅]-9** was isolated by column chromatography.

Olefination of trienal **[¹³C₅]-9** using phosphonate **[¹³C₂]-2** for the second time introduced the C12 and C13 labeled carbon atoms, affording the tetraenoate as its all-E-stereoisomer. Formation of Weinreb amide **[¹³C₇]-10**
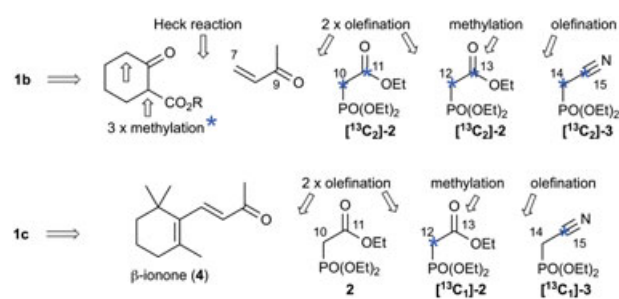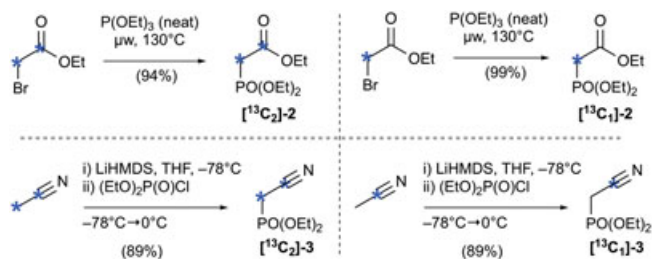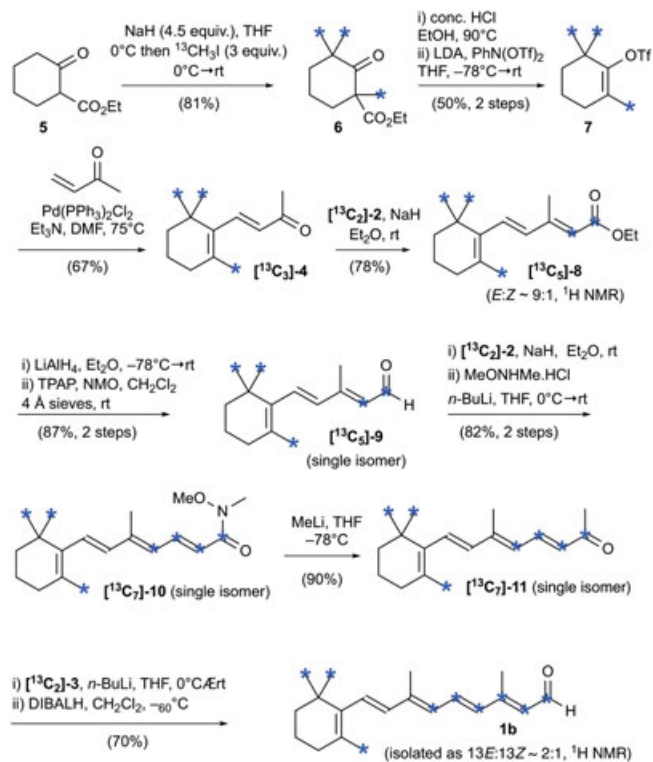

FIGURE 1 Retinal structures showing synthetic ^{13}C-labeling patterns (* indicates position of ^{13}C)

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

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

SCHEME 2 Synthesis of $[10,11,12,13,14,15,16,17,18$-$^{13}C_9]$-all-trans-retinal (1b). (* indicates position of ^{13}C)

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 13E/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 13Z 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.

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-labeled phosphonates $[^{13}C_1]$-2 and $[^{13}C_1]$-3 (Scheme 3).[19] In this case, the 13Z
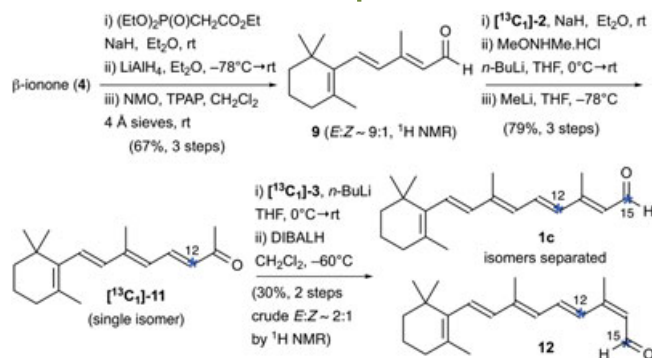
SCHEME 3 Synthesis of $[12,15$-$^{13}C_2]$-all-trans-retinal (1c) and $[12,15$-$^{13}C_2]$-(13Z)-retinal (12). (* indicates position of ^{13}C)

and the all-E isomers, 12 and 1c, respectively, were both isolated as pure compounds.

A more convergent strategy was applied to the synthesis of ^{13}C-labeled all-E-retinal 1d, by combining 2 major fragments 9 and 13 (Figure 3).[1b, 20, 21] The fragment containing 2 ^{13}C labels required the phosphonium ylide 14, which was combined with hydroxyacetone to give the E enoate 15 (Scheme 4). 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 ensuing reduction-oxidation sequence secured $[14,15$-$^{13}C_2]$-all-trans-retinal (1d).[21]

The labeled $[14,15$-$^{13}C_2]$-retinal (1d) has been incorporated in ^{15}N-labeled 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 6 labeled carbon
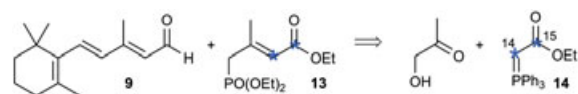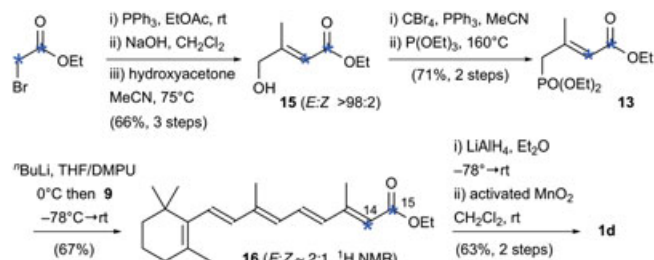

FIGURE 3 Synthesis plan for labeled retinal 1d. (* indicates position of ^{13}C)

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

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.

3 | CONCLUSIONS

The synthesis of 3 all-*E* retinal isotopomers, **1b** to **1d**, containing multiple ^{13}C-labels, has been achieved in 12, 8, and 7 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, respectively, which were separated by preparative HPLC. The labeled chromophores have been incorporated in retinylidene proteins reconstituted as proteoliposomes to provide samples suitable for solid-state DNP-enhanced NMR studies. The biophysical studies of this intriguing group of light-responsive proteins containing site-specifically ^{13}C-labeled synthetic chromophores are ongoing[12] and will be disclosed in due course.

4 | EXPERIMENTAL

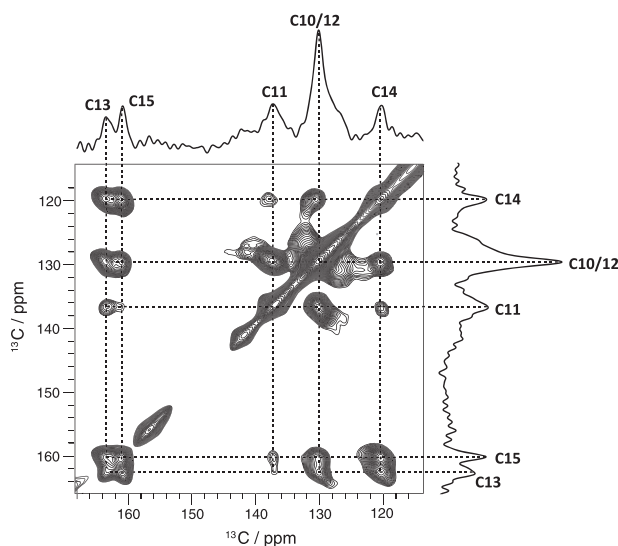Supporting information includes general experimental details and copies of ^1H and ^{13}C NMR spectra.

FIGURE 4 DNP-enhanced ^{13}C-^{13}C MAS-NMR spectrum of PR incorporating **1b** showing the 6 labeled carbon atoms in the chain. (Bruker 400 MHz DNP system with 3.2-mm HCN Cryo MAS probe, ~107 K, 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), pH 9, radical: 20 mM AMUpol, 30% d-6-glycerol, 60% D_2O, 10% H_2O (enhancement: ~32)

4.1 | 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 minutes. The mixture was stirred on ice for 30 minutes before addition of $^{13}CH_3I$ (6.47 mL, 104 mmol) dropwise over 20 minutes. The mixture was stirred at rt for 36 hours with the formation of a white precipitate after 1 hour. 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 × 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 colorless 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}. ^1H NMR (400 MHz, CDCl$_3$): δ 4.23 to 4.04 (m, 2H), 2.58 to 2.50 (m, 1H), 2.06 to 1.91 (m, 1H), 1.76 to 1.68 (m, 1H), 1.65 to 1.49 (m, 2H), 1.45 to 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 labeled 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.

4.2 | 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 neutralized with 1 M NaOH (170 mL). The aqueous layer was extracted with pentane (4 × 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 colorless 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 to 2.59 (m, 1H), 2.10 to 2.00 (m, 1H), 1.88 (qt, J_{HH} = 13.4, J_{HH} = 3.8 Hz, 1H), 1.81 to 1.72 (m, 1H), 1.69 to 1.61 (m, 1H), 1.60 to 1.49 (m, 1H), 1.38 to 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 labeled 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.

4.3 | 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 minutes. The mixture was stirred at −78°C for 2 hours 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 hours. The reaction was quenched with saturated aqueous NH_4Cl (80 mL) and the aqueous phase extracted with pentane (3 × 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 colorless 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}. ^{1}H NMR (CDCl$_3$, 400 MHz): δ 2.16 (t, J_{HH} = 5.9 Hz, 2H), 1.71 to 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 labeled carbons reported). LRMS (EI) m/z 275 [M]$^{+\bullet}$ (27%), 259 [M − 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.

4.4 | (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$_3$)$_2$Cl$_2$] (425 mg, 0.605 mmol) in DMF (60 mL) under N_2 was heated at 75°C for 18 hours. The reaction was quenched with H_2O (50 mL) and the aqueous phase extracted with pentane (4 × 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$_2$O: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}. ^{1}H 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 to 2.12 (unresolved t, 2H), 1.77 (d, J_{CH} = 126.1 Hz, 3H), 1.62 to 1.67 (m, 2H), 1.46 to 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 labeled 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.

4.5 | 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 minutes. Purification by silica gel chromatography (EtOAc:hexane = 85:15) afforded the product as a colorless oil (2.62 g, 11.6 mmol, 94%). ^{1}H and ^{13}C NMR spectroscopic data were consistent with those reported.[22] FT-IR (neat) ν_{max} 2980 (m), 1692 (s), 1252 (s), 1020 (s), 963 (s) cm^{-1}. ^{1}H NMR (CDCl$_3$, 400 MHz): δ 4.29 to 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 labeled carbons reported) LRMS (ES$^+$) m/z = 227 [M + H]$^+$.

4.6 | Ethyl (2E,4E)-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$_2$O (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$_2$O (8 mL) dropwise, and the solution was stirred for 2 hours 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$_2$O (8 mL) was added dropwise, and the resulting solution was stirred for 96 hours. The reaction was quenched with H_2O (10 mL) and the aqueous phase was extracted with Et$_2$O (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$_2$O: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%, 9E:9Z = 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}. ^{1}H 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 to 2.00 (m, 2H), 1.70 (d, J_{CH} = 125.8 Hz, 3H), 1.66 to 1.58 (m, 2H), 1.52 to 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 labeled carbons reported) LRMS (ES$^+$) m/z = 262 [M + H]$^+$. HRMS (ES$^+$) For C$_{12}$^{13}C$_5$H$_{26}$NaO$_2^+$ calculated 290.1993, found 290.2000.

4.7 | (2E,4E)-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$_2$O (15 mL) under N$_2$ at −78°C was added a solution of ethyl (2E,4E)-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$_2$O (30 mL) dropwise and stirred at −78°C for 30 minutes. The mixture was then warmed to rt and stirred for 20 minutes. The reaction was quenched with H$_2$O (6 mL), 1 M NaOH (2 mL) and H$_2$O (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 colorless oil which was re-dissolved in CH$_2$Cl$_2$ (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 minutes. 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}. ^{1}H 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 to 2.02 (m, 2H), 1.73 (d, J_{CH} = 125.8 Hz, 3H), 1.68 to 1.59 (m, 2H), 1.53 to 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 labeled carbons reported) LRMS (ES$^+$) m/z 224 [M + H]$^+$. HRMS (ES$^+$) For C$_{10}$^{13}C$_5$H$_{22}$ONa$^+$ calculated 246.1731, found 246.1739 Da.

4.8 | Ethyl (2E,4E,6E)-5-methyl-7-(2,6,6-tri(methyl-^{13}C)cyclohex-1-en-1-yl)hepta-2,4,6-trien oate-1,2,3,4-^{13}C$_4$

To a suspension of NaH (60% in mineral oil, 354 mg, 8.86 mmol) in Et$_2$O (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$_2$O (5 mL) dropwise and the suspension was stirred for 1 hour at rt. A solution of (2E,4E)-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$_2$O (5 mL) was added dropwise and stirred at rt for 18 hours. The reaction was then quenched with H$_2$O (6 mL) and the aqueous phase was extracted with Et$_2$O (3 × 6 mL). The combined organic solution was washed with brine, dried (Na$_2$SO$_4$) 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}. ^{1}H NMR (CDCl$_3$, 400 MHz): δ 7.92 to 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 to 1.57 (m, 2H), 1.50 to 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 labeled carbons reported) LRMS (ES$^+$): m/z = 296 [M + H]$^+$. HRMS (ES$^+$) For C$_{12}$^{13}C$_7$H$_{28}$O$_2$Na$^+$ calculated 318.2216, found 318.2218 Da.

4.9 | (2E,4E,6E)-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 minutes. After stirring at −15°C for 1 hour a solution of ethyl (2E,4E,6E)-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 minutes at −15°C. After 30 minutes at −15°C, the reaction was quenched with saturated aqueous NH$_4$Cl and the aqueous phase was extracted with Et$_2$O (3 × 10 mL). The combined organic solution was washed with brine, dried (Na$_2$SO$_4$) 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 to 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 to 2.00 (m, 5H), 1.72 (d, J_{CH} = 125.6 Hz, 3H), 1.66 to 1.58 (m, 1H), 1.53 to 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 labeled carbons reported) LRMS (ES$^+$) m/z 311 [M + H]$^+$. HRMS (ES$^+$) For C$_{12}$^{13}C$_7$H$_{30}$NO$_2$$^+$ calculated 311.2506, found 311.2513 Da.

4.10 | (3E,5E,7E)-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 (2E,4E,6E)-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$_2$O, 0.75 mL, 1.12 mmol) dropwise over 10 minutes. After 10 minutes at −78°C the reaction was quenched by addition of a slurry of silica (1 g) and H$_2$O (2 mL) and the resulting suspension was stirred for 15 minutes. The mixture was dried (Na$_2$SO$_4$), 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$_6$D$_6$, 400 MHz): δ 7.70 to 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 to 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 to 1.52 (m, 2H), 1.47 to 1.41 (m, 2H), 1.06 (dd, J_{CH} = 125.3, J_{CH} = 4.8 Hz, 6H) ppm. ^{13}C NMR (C$_6$D$_6$, 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 labeled carbons reported) LRMS (ES$^+$) m/z 266 [M + H]$^+$. HRMS (ES$^+$) For C$_{11}$^{13}C$_7$H$_{26}$NaO$^+$ calculated 288.2111, found 288.2115 Da.

4.11 | 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) dropwise over 15 minutes. After stirring for 15 minutes a solution of ^{13}CH$_3$^{13}CN (250 mg, 5.81 mmol) in THF (6 mL) was added dropwise over 15 minutes and the solution was stirred for a further 40 minutes. A solution diethylchlorophosphate (0.92 mL, 6.40 mmol) in THF (6 mL) was added dropwise over 10 minutes and then stirred for a further 40 minutes. The mixture was allowed to warm to rt and stirred for 40 minutes before pouring into a stirred mixture of 2 M HCl (10 mL) and CH$_2$Cl$_2$ (15 mL). The aqueous phase was extracted with CH$_2$Cl$_2$ (3 × 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 to 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 labeled carbons reported) LRMS (ES$^+$): m/z 179 [M + H]$^+$.

4.12 | [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) dropwise over 5 minutes and the solution was stirred at rt for 90 minutes. A solution of (3E,5E,7E)-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 dropwise and stirred at rt for 18 hours. The reaction was quenched with saturated aqueous NH$_4$Cl and the aqueous phase extracted with EtOAc (3 × 8 mL). The combined organic solution was washed with brine, dried (Na$_2$SO$_4$) 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 dropwise at −60°C. After stirring at −60°C for 15 minutes, a slurry of SiO_2 and H_2O was added and stirred for 15 minutes 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%, 13E:13Z = 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 to 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 to 5.83 (m, 1H), 5.96 (dt, $J_{CH} = 157.1$, $J_{HH} = 7.8$ Hz, 1H), 1.99 to 1.93 (m, 2H), 1.78 (d, $J_{CH} = 4.4$ Hz, 3H), 1.75 to 1.70 (m, 3H), 1.76 (d, $J_{CH} = 125.6$ Hz, 3H), 1.62 to 1.55 (m, 2H), 1.50 to 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 to 129.6 (m, ^{13}CH), 130.0 to 128.8 (m, ^{13}CH), 28.8 ($^{13}CH_3$), 21.6 ($^{13}CH_3$) ppm. (Only peaks for ^{13}C labeled 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.

4.13 | 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 minutes. Purification by silica gel chromatography (EtOAc:hexane = 85:15) afforded the product as a colorless 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 labeled carbons reported) ^{31}P NMR ($CDCl_3$, 162 MHz): δ 19.81 (d, $J_{CP} = 134.6$ Hz) ppm. LRMS (ES^+) m/z = 226 $[M + H]^+$.

4.14 | Ethyl (2E,4E,6E)-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 minutes 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 hours. The reaction was then quenched with H_2O (10 mL), and the aqueous phase was extracted with Et_2O (3 × 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 to 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 to 1.59 (m, 2H), 1.52 to 1.45 (m, 2H), 1.32 (t, $J_{HH} = 7.1$ Hz, 3H), 1.04 (s, 6H) ppm. ^{13}C NMR ($CDCl_3$, 101 MHz): δ 120.1 ($^{13}CH_2$) ppm. (Only peaks for ^{13}C labeled 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.

4.15 | (2E,4E,6E)-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 minutes. After stirring the solution at −15°C for 1 hour, a solution of ethyl (2E,4E,6E)-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 minutes at −15°C. After 30 minutes at −15 °C, the reaction was quenched with saturated aqueous NH_4Cl, and the aqueous phase was extracted with Et_2O (3 × 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}. ^1H 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 to 1.58 (m, 2H), 1.51 to 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 labeled 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.

4.16 | (3E,5E,7E)-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 (2E,4E,6E)-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$_2$O, 2.62 mL, 3.80 mmol) dropwise over 10 minutes. After 10 minutes at −78°C, the reaction was quenched by addition of a slurry of silica (2 g) and H$_2$O (10 mL) and the resulting suspension was stirred for 15 minutes 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 to 1.59 (m, 2H), 1.51 to 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.

4.17 | 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) dropwise over 15 minutes. After stirring for 15 minutes, a solution of $^{13}CH_3CN$ (500 mg, 11.89 mmol) in THF (12 mL) was added dropwise over 15 minutes and then stirred for a further 40 minutes. A solution diethylchlorophosphate (1.89 mL, 13.1 mmol) in THF (12 mL) was added dropwise over 10 minutes, and the solution was stirred for a further 40 minutes. The mixture was allowed to warm to rt and stirred for 40 minutes

before pouring into a stirred mixture of 2 M HCl (20 mL) and CH$_2$Cl$_2$ (30 mL). The aqueous phase was extracted with CH$_2$Cl$_2$ (3 × 20 mL), and then the combined organic solution washed with H$_2$O 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 to 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]$^+$.

4.18 | [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) dropwise over 5 minutes, and the solution was stirred at rt for 75 minutes. A solution of (3E,5E,7E)-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 dropwise and stirred at rt for 18 hours. The reaction was quenched with saturated aqueous NH$_4$Cl and the aqueous phase extracted with EtOAc (3 × 8 mL). The combined organic solution was washed with brine, dried (Na$_2$SO$_4$), 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$_2$Cl$_2$ (3 mL) under N$_2$, and DIBAL (1 M in CH$_2$Cl$_2$, 0.64 mL, 0.64 mmol) was added dropwise at −60°C. After stirring at −60°C for 1 hour, a slurry of SiO$_2$ and H$_2$O was added and stirred for 15 minutes at rt. The solids were filtered and the filtrate dried twice (Na$_2$SO$_4$) 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%, 13E:13Z = 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$_6$D$_6$, 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 to 1.55 (m, 2H), 1.51 to 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 labeled 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.

13Z- (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 to 1.75 (m, 6H), 1.59 (br d, J_{CH} = 4.0 Hz, 3H), 1.62 to 1.55 (m, 2H), 1.50 to 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 labeled 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.

4.19 | 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 hours. 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 labeled 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.

4.20 | 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 hours. 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 colorless 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 labeled carbons reported). LRMS (ES[+]) m/z 226 [M + NH$_4$][+].

4.21 | 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 hours. 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} = 160.5 Hz, 1H), 4.15 to 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 to 2.30 (m, 3H), 1.35 to 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 labeled 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.

4.22 | [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 minutes 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 hours. The reaction was quenched with saturated aqueous NH_4Cl and

the aqueous layer extracted with EtOAc (× 2). The combined organic solution was washed with water, brine, and then dried (MgSO₄) before concentrating under reduced pressure. Careful purification of the mixture of olefin isomers (13E/13Z, estimated 9:1 by ^{1}H NMR spectroscopy) by silica gel chromatography (EtOAc:hexane = 3:97) afforded the ester (ethyl (2E,4E,6E,8E)-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 LiAlH₄ in THF (0.97 mL, 0.97 mmol) in Et₂O (1.5 mL) under N₂ was added a solution of ethyl ester 16 (200 mg, 0.61 mmol) in Et₂O (6 mL) dropwise at −78°C and stirred at −78°C for 30 minutes. The mixture was warmed to rt and stirred for 20 minutes. 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 Et₂O. The filtrate was dried (MgSO₄) and concentrated under reduced pressure to afford a pale yellow oil (189 mg) that was re-dissolved in CH₂Cl₂ (8 mL). Powered, activated MnO₂ (0.530 g, 6.00 mmol) was added, and the reaction stirred at rt for 1 hour 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 (C₆D₆, 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 to 1.54 (m, 2H), 1.50 to 1.43 (m, 2H), 1.12 (s, 6H) ppm; 13C NMR (CDCl₃, 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 labeled 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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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.