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Synthesis and Use of Deuterated Palmitic Acids to Decipher the Cryptoregiochemistry of a Δ^{13} Desaturation

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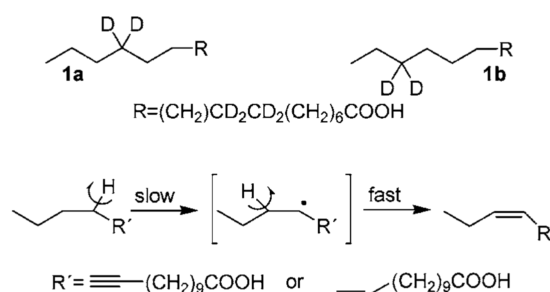

The synthesis of two hexadeuterated palmitic acids differing in the position of the diagnostic labels, and their use to decipher the cryptoregiochemistry of a Δ^{13} desaturation are described. A dithiane and a triple bond functionalities were used to introduce the diagnostic (C13 or C14) and tagging (C8 and C9) labels, respectively, in the palmitic acid skeleton. Using these probes, the cryptoregiochemistry of the Δ^{13} desaturation involved in the biosynthesis of *Thaumetopoea pityocampa* sex pheromone was studied by means of kinetic isotope effect determinations. Transformation of both (*Z*)-11-hexadecenoic and 11-hexadecynoic acids into (*Z, Z*)-11,13-hexadecadienoic and (*Z*)-13-hexadecen-11-ynoic acids, respectively, is initiated by abstraction of the hydrogen atom at the C13 position, followed by the fast elimination of the C14 hydrogen to give the double bond.

Introduction

Introduction of double bonds into a saturated aliphatic chain is an important biotransformation catalyzed by specific desaturases, which are present in all eukaryotic cells. In addition to the enzymes that act on saturated fatty acids to afford monounsaturated products, other desaturases that use unsaturated substrates to give conjugated or non-conjugated polyunsaturated fatty acids do also occur in nature. The several unsaturations can be either methylene interrupted in *cis*-configuration, as found in essential fatty acids such as linoleic and linolenic acids, or conjugated in either *cis*- or *trans*-configuration. Within the polyunsaturated compounds, enynes, either conjugated or not, have also been reported in a few plant species, such as the compositae *Crepis alpina*[1] and the moss *Ceratodon purpurea*.[2] Besides these plants, some lepidopteran species, including the moth *Heterocampa guttivitta*[3] and those belonging to the genus *Thaumetopoea,* biosynthesize (*Z,Z*)-11,13-hexadecadienyl or (*Z*)-13-hexadecen-11-ynyl acetates as their sex pheromone.[4] The biosynthetic pathways involve both Δ^{11} and Δ^{13} desaturations, as well as an acetylenation reaction in the case of the enyne structures (Figure 1).[5]

The mechanistics of both the Δ^{11} desaturation[6] and Δ^{11} acetylenation[7] have been investigated by use of pheromone gland metabolization studies with the properly deuterated probes. In the first case, experimental evidence was presented that the Δ^{11} desaturase of *Thaumetopoea pityocampa* (*T. pityocampa*) trans-

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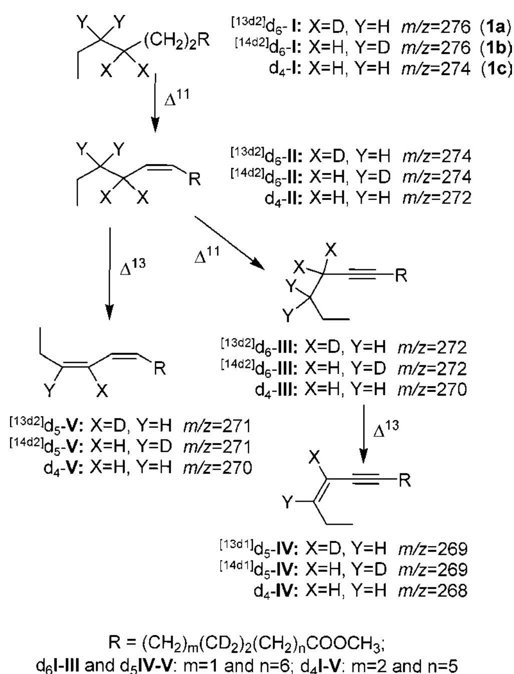


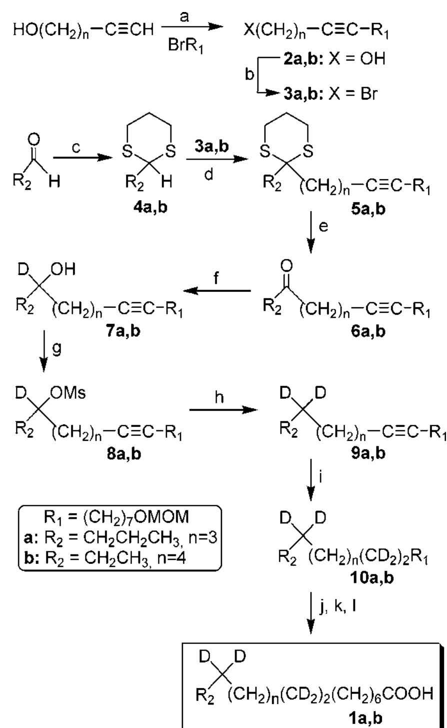
FIGURE 1. Deuterated probes **1a**−**c** and their consecutive desaturation products: Δ^{13}; Δ^{13}desaturase; Δ^{11}; Δ^{11}desaturase/acetylenase.

forms palmitic acid into (*Z*)-11-hexadecenoic acid by removal of the *pro*-(*R*)-hydrogen atoms from both C11 and C12, whereas the transformation of (*Z*)-11-hexadecenoic acid into 11-hexadecynoic acid by the Δ^{11} acetylenase takes place by a stepwise mechanism, in which a significant perturbation of the strong vinyl C11−H bond occurs prior to a fast elimination of the vinyl hydrogen at C-12. The products of those reactions, (*Z*)-11-hexadecenoic and 11-hexadecynoic acid, are then transformed into a conjugated diene or enyne fatty acid, respectively, by a Δ^{13} desaturation. The cryptoregiochemistry of this reaction is reported in this paper. Likewise, the preparation of the deuterated probes **1a**−**c** (Figure 1) required for this investigation is also described.

Results and Discussion

Preparation of Probes. The probes needed to investigate the cryptoregiochemistry of the Δ^{13} desaturation were prepared following the conventional reactions depicted in Scheme 1.[7,8] Thus, properly functionalized alkynyldithianes **5** were obtained by coupling reaction of bromoalkynyl derivatives **3** with the anion of dithiane **4** generated by reaction with BuLi.[9] Cleavage of the dithiane functionality with N-bromosuccinimide (NBS) afforded the corresponding ketones **6**, which were reduced to the monodeuterated alcohols **7** with $LiAlD_4$. Mesylation of the hydroxyl group and nucleophilic substitution with $LiAlD_4$ produced dideuterated compounds **9**. Introduction of the four deuterium atoms for tagging at C8 and C9 positions was achieved by nonscrambling deuteration of the protected hexadecanols **10** with the Wilkinson catalyst. Methoxymethane protective group removal in acid media gave the corresponding palmityl alcohols, which were oxidized to the final tri-*gem*-hexadeuterated palmitic acids **1** in two steps, involving oxidation with IBX in dimethyl sulfoxide (DMSO) and final Jones

SCHEME 1. Synthesis of Hexadeuterated Probes 1a,b[a]



[a] Reagents and conditions: (a) BuLi/THF/HMPA, 71%; (b) NBS/PPh_3/DMF, 87%; (c) $BF_3.Et_2O$/1,3-propanedithiol/$CH_3COOH/CHCl_3$, 88−92%; (d) BuLi/THF, 69−73%; (e) NBS/H_2O/acetone, 86%; (f) $LiAlD_4/Et_2O$, 95−98%; (g) $MsCl/Et_3N/CH_2Cl_2$, 92−95%; (h) $LiAlD_4/Et_2O$, 93%; (i) Wilkinson catalyst/D_2/benzene, 97%; (j) HCl/MeOH, 90%; (k) IBX/DMSO; (l) $H_2SO_4/CrO_3/H_2O$/acetone, 85%.

oxidation of the resulting aldehyde.[7] This two-step sequence gave significantly higher yields than direct oxidation of the alcohols to the acids.

Finally, probe **1c** used in the competition studies was prepared by coupling of the dithiane **4a** with $Br(CH_2)_4C{\equiv}C(CH_2)_6$-OMOM (**3c**) (Scheme 2). Cleavage of the resulting dithiane **5c** using the above conditions and reduction with $LiAlH_4$ gave the intermediate alcohol **7c**, which was treated with mesyl chloride to afford the corresponding ester **8c**. Mesyl group displacement with $LiAlH_4$ gave rise to the alkyne **9c**. The same final reaction sequence used for preparing compounds **1a,b** allowed obtaining the tetradeuterated pure acid **1c**.

The deuterated compounds were characterized as previously reported for similar compounds.[6−8] Thus, the *gem*-dideuterated products showed IR symmetric and asymmetric stretching bands at frequency ranges of 2080−2095 and 2180−2195 cm^{-1}, respectively. On the other hand, the number and the presence of the deuterium labels were those expected as concluded from the analyses by ^{1}H and ^{13}C NMR. The characteristic ^{1}H NMR methyne hydrogen signals for compounds **7** (CHOH, ca. 3.60 ppm) and **8** (CHOMs, ca. 4.70 ppm) were not observed due to the presence of deuterium in the carbinolic center. On the other hand, ^{13}C NMR of compounds **7** and **8** showed the presence of a triplet at around 70 and 83 ppm, which corresponded to the trisubstituted carbon atoms C13/C14 of alcohols **7** and mesylates **8**, respectively (7**a**, 70.7 ppm; 7**b**, 72.0 ppm; **8a,b**, 83.0 ppm). These signals disappeared and became a quintuplet (CD_2) for compounds **9a,b**−**11a,b** and **1a,b**. In this case, ^{13}C NMR chemical shift differed between **a** and **b** series, being shifted at lower field in the second case (C13, 28.6 ppm; C14, 31.0 ppm).

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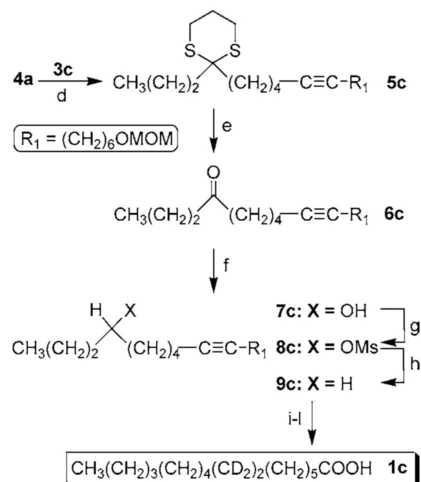
SCHEME 2. Synthesis of Competitor 1c[a]



[a] Reagents and conditions: (d) BuLi/THF, $Br(CH_2)_4-C\equiv C-(CH_2)_6OMOM$ (**3c**) 73%; (e) NBS/H_2O/acetone, 88%; (f) $LiAlH_4/Et_2O$, 97%; (g) $MsCl/Et_3N/CH_2Cl_2$, 92%; (h) $LiAlH_4/Et_2O$, 95%; (i) Wilkinson catalyst/D_2/benzene, 97%; (j) HCl/MeOH, 89%; (k) IBX/DMSO; (l) $H_2SO_4/CrO_3/H_2O$/acetone, 85%.

The final deuterium contents of the acids **1** were determined by GC-MS analysis of their respective methyl esters and under the optimum synthetic conditions, mean ratios of d_7, d_6, d_5, d_4, and d_3 fatty acids were respectively 12.5, 78.4, 6.6, 2.1, and 0.4 for compounds **1a**,**b** and 12.6, 78.4, 6.0, 2.2, and 0.8 for compound **1c**.

Cryptoregiochemistry Studies. As shown in Figure 1, the cryptoregiochemstry of the Δ^{13} desaturation could be investigated by determining the reaction rates of either the 11-hexadecynoate (**III**) into (*Z*)-13-hexadecen-11-ynoate (**IV**) biotransformation or that of (*Z*)-11-hexadecenoate (**II**) to (*Z*,*Z*)-11,13-hexadecadienote (**V**).

In the first case, the transformation of the acetylene metabolites $^{[13d2]}d_6$-**III** and $^{[14d2]}d_6$-**III**, with diagnostic labels at each oxidation site, into the corresponding enynes $^{[13d]}d_5$-**IV** and $^{[14d]}d_5$-**IV** was investigated. The experiments were carried out with the saturated probes **1a** ($^{[14d2]}d_6$-**I**) and **1b** ($^{[13d2]}d_6$-**I**), which were biotransformated *in situ* into the acetylene Δ^{13}desaturase substrates by subsequent Δ^{11}desaturation and acetylenation (Figure 1). The method of determining primary kinetic isotope effects from data obtained in competition experiments between each of the hexadeuterated probe ($^{[14d2]}d_6$-**I** and $^{[13d2]}d_6$-**I**) and that without deuteriums in the diagnostic positions (d_4-**I**) could not be followed here, since their transformation into the corresponding enynes (**IV**) was too low to allow reliable integrations. Furthermore, this problem was worsened by the presence of small impurities overlapping with the natural isotopomeric ($M^{\bullet+} + 1$, 269 for d_4-**IV** and 270 for d_5-**IV**) ions. Therefore, an alternative procedure was used, which, while not allowing the calculation of primary kinetic isotope effects, did allow clear deciphering, in the case of the enyne, the site of the initial oxidation of the Δ^{13} desaturation reaction. In this method, the amounts of every labeled intermediate (d_6-**II**, d_6-**III**, and d_5-**IV** from any d_6-**I**; d_4-**II**, d_4-**III**, and d_4-**IV** from d_4-**I**) relative to the respective endogenous counterpart (d_0-**II**, d_0-**III**, and d_0-**IV**) were determined by GC/MS analysis of methanolyzed lipid extracts (Figure 2) after treatment with each individual probe **1a** ($^{[14d2]}d_6$-**I**), **1b** ($^{[13d2]}d_6$-**I**), and **1c** (d_4-**I**), and the data were corrected for the corresponding probe incorporation.

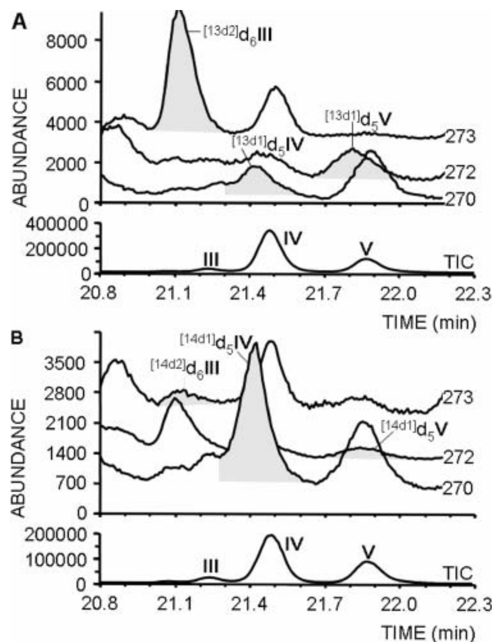


FIGURE 2. Formation of 11-hexadecynoic (**III**), (*Z*)-13-hexadecen-11-ynoic (**IV**), and (*Z,Z*)-11,13-hexadecadienoic (**V**) acids from **1a**,**b** in *T. pityocampa* pheromone glands. The traces correspond to GC-MS chromatograms of methanolyzed lipidic extract from *T. pityocampa* pheromone glands incubated with either **1a** (A) or **1b** (B) upon selection of ions at *m*/*z* 270 ($M^{\bullet+} + 1$ ion of d_5-**IV** methyl ester), *m*/*z* 272 ($M^{\bullet+} + 1$ ion of d_5-**V** methyl ester), and *m*/*z* 273 ($M^{\bullet+} + 1$ ion of d_6-**III** methyl ester). The total ion current chromatogram (TIC) showing the natural intermediates (**III**, **IV**, and **V** methyl esters) is depicted in the bottom of each figure. The experiments were performed as described in the Experimental Section. Compounds are the methyl esters of 11-hexadecynoic acid (**III**), (*Z*)-13-hexadecen-11-ynoic acid (**IV**), and (*Z,Z*)-11,13-hexadecadienoic acid (**V**). Labeled counterparts are indicated with $^{[xd2]}d_n$, where *n* is the number of deuterium atoms and [*x*d2] shows the diagnostic deuterium position.

As shown in Figure 3, similar relative amounts of labeled **II** were formed from the three probes. Likewise, proportions between the relative amounts of labeled **II**, **III**, and **IV** were similar after incubations with d_4-**I** and $^{[14d2]}d_6$-**I**. These results indicate that both d_4-**I** and $^{[14d2]}d_6$-**I** are metabolized to labeled **IV** with similar rates and, therefore, that the Δ^{13} desaturase activity is not sensitive to the presence of deuterium at C14 in $^{[14d2]}d_6$-**I**. In contrast, relative amounts of $^{[13d2]}d_6$-**III** found after treatment with $^{[13d2]}d_6$-**I** were significantly higher than those resulting from the other two probes. This result suggests that $^{[13d2]}d_6$-**III** is desaturated to the corresponding enyne at a lower rate than both $^{[14d2]}d_6$-**III** and d_4-**III**. In agreement, the relative amounts of $^{[13d1]}d_5$-**IV** produced from $^{[13d2]}d_6$-**I** were significantly lower than those found from d_4-**I** and $^{[14d2]}d_6$-**I**. Collectively, these data indicate that hydrogen abstraction at C13 by the Δ^{13} desaturase is isotope-sensitive and, therefore, that this is the rate-determining step of the desaturation reaction (C13 is the site of initial oxidation). Thus, in accordance with the general trend, conversion of acetylene **III** into enyne **IV** occurs by a slow removal of the hydrogen atom nearest to the substrate carboxy-terminal end (C13−H) followed by the fast elimination of the neighboring C14−H.

This result was confirmed when the formation of dienes $^{[13d1]}d_5$-**V** and $^{[14d1]}d_5$-**V** from their respective alkene precursors $^{[13d2]}d_6$-**II** and $^{[14d2]}d_6$-**II**, which were in turn produced *in situ* from the saturated probes $^{[14d2]}d_6$-**I** and $^{[13d2]}d_6$-**I**, respectively,

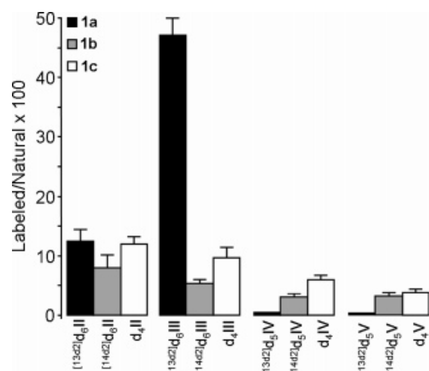


FIGURE 3. Conversion of tracers **1a**−**c** into biosynthetic intermediates. Data correspond to the mean ± SD of three replicates. Labeled natural fatty acid methyl ester ratios were determined from the areas of the specific $M^{\bullet+} + 1$ ions in the GC-MS chromatograms (labeled: $^{[13d2]}d_6$-**I** and $^{[14d2]}d_6$-**I**, 277; $^{[13d2]}d_6$-**II** and $^{[14d2]}d_6$-**II**, 275; d_4-**II**, 273; $^{[13d2]}d_6$-**III** and $^{[14d2]}d_6$-**III**, 273; d_4-**III**, 271; $^{[13d1]}d_5$-**V** and $^{[14d1]}d_5$-**V**, 272; $^{[13d1]}d_5$-**IV** and $^{[14d1]}d_5$-**IV**, 270; d_4-**IV**, 269; natural and **II**, 269; **III** and **V**, 267; **IV**, 265). The resulting data were corrected for the respective levels of incorporation of each specific probe, which were administered individually (probe/Σ(**II** + **III** + **IV** + **V**): **1a**, 1.3 ± 0.21; **1b**, 1.4 ± 0.22; **1c**, 0.8 ± 0.12). Compounds are the methyl esters of (*Z*)-11-hexadecenoic acid (**II**), 11-hexadecynoic acid (**III**), (*Z*)-13-hexadecen-11-ynoic (**IV**), and (*Z*,*Z*)-11,13-hexadecadienoic acids (**V**). Isotopic labeling is indicated with $^{[xd2]}d_n$, where *n* is the number of deuterium atoms and [*x*d2] shows the diagnostic deuterium position.

TABLE 1. Transformation of 1a−c into d_4 and d_5 (Z,Z)-11,13-Hexadecadienoic Acids in Competitive Experiments

substrates (ratio)[a]	products (ratio)[a]	KIE[b]
1c/**1a** (1.1)	d_4-**V**/$^{[13d2]}d_5$-**V** (7.4 ± 1.1)	6.7 ± 1.2
1c/**1b** (1.2)	d_4-V/$^{[14d2]}d_5$-V (1.4 ± 0.2)	1.2 ± 0.1

[a] Ratios between d_4 and d_6 probes in the mixture used and ratios between d_4 and d_5 (*Z*,*Z*)-11,13-hexadecadienoic acids produced. For the probes, the ratio corresponds to a single determination with a BF_3−MeOH-derivatized sample of the mixture used. For the dienes, data are mean ± standard deviation of four different experiments. [b] Product KIEs were calculated using the equation: [% d_4(product)/% d_5(product)]/[% d_4(substrate)/% d_6(substrate)].

were investigated (Figure 3). In this case, we were able to probe the mechanism using kinetic isotope effect (KIE) measurements from data obtained in competitive experiments with probe **1c**, which is labeled with the tetradeuterium tag, but lacks the diagnostic dideuterium units. As shown in Figure 3, the three compounds are transformed at equal rates into the respective (*Z*)-11-hexadecenoic acids, which are the actual Δ^{13} desaturation substrates. The base methanolyzed lipidic extracts obtained after incubation of tissues with an approximately 1:1 mixture of **1c** and each of the hexadeuterated substrates **1a** or **1b**, were analyzed by GC-MS under the selected ion monitoring mode. Integration of peaks corresponding to the tetradeuterated and pentadeuterated methyl (*Z*, *Z*)-11,13-hexadecadienoates formed from each mixture afforded the data required to determine the KIEs. As shown in Table 1, a large isotope effect was observed for the carbon−hydrogen bond cleavage at C13, while the C14−H bond breaking step was shown to be essentially insensitive to deuterium substitution.

The magnitude of the obtained KIE effects correlates well with others previously reported in other biological models.[10,11] These results strongly suggest that dienoate production is initiated by an energetically difficult and hence isotopically sensitive hydrogen abstraction at C13 and completed by a second facile and kinetically unimportant hydrogen abstraction at C14.

Both data indicate that enynoic **IV** and dienoic **V** acids are produced via initial H-atom abstraction at C13 of an alkyne and alkene substrates, respectively, and a second fast elimination of hydrogens at C14 and support the hypothesis that this transformation could represent a variation of the same enzyme for two different substracts. Confirmation of this hypothesis awaits cloning and functional expression of this enzyme, which are currently ongoing in our laboratories.

Conclusions

In summary, we have reported the preparation of novel deuterated palmitic acids and their use to decipher the site of initial oxidation of a Δ^{13} desaturation reaction that transforms 11-hexadecynoic and (*Z*)-11-hexadecenoic acids into (*Z*)-13-hexadecen-11-ynoic and (*Z,Z*)-11,13-hexadecadienoic acids, respectively. In agreement with other previously reported acyl CoA desaturase reactions, both substrates are first oxidized at the position closest to the acid functionality (C13) in the rate-determining step of the reaction, which is then followed by the fast removal of the hydrogen atom located at the neighboring position (C14).

Experimental Section

Preparation of Deuterated Alcohols 7. The reaction was accomplished according to a previously reported procedure.[8] Thus, treatment of ketone **6** with $LiAlD_4$ in Et_2O, under argon atmosphere and at room temperature, followed by the usual workup, allowed one to obtain an oily residue that was purified by flash chromatography on silica gel using 80:20 hexane/MTBE to give the corresponding pure deuterated alcohols **7** in 96−98% yields.

[4-^{2}H]-17,19-Dioxaicos-8-yn-4-ol (7a). This deuterated alcohol was isolated (882 mg, 98% yield) starting from 3 mmol (888 mg) of the corresponding ketone. IR 3425, 2935, 2860, 2210, 1465, 1410, 1145, 1110, 1045, 940, 920 cm^{-1}; ^{1}H NMR δ 4.62 (s, 2H), 3.52 (t, *J* = 6.5 Hz, 2H), 3.36 (s, 3H), 2.18 (m, 2H), 2.14 (m, 2H), 1.28−1.68 (19H), 0.93 (t, *J* = 7 Hz, 3H); ^{13}C NMR 96.3 (CH_2), 80.5 (C), 79.9 (C), 70.7 (CD, t, *J* = 21 Hz), 67.8 (CH_2), 55.1 (CH_3), 39.5 (CH_2), 36.4 (CH_2), 29.6 (CH_2), 29.0 (CH_2), 28.9 (CH_2), 28.7 (CH_2), 26.1 (CH_2), 25.2 (CH_2), 18.8 (CH_2), 18.7 (CH_2), 18.7 (CH_2), 14.1(CH_3); MS *m/z* 300 (2, $M^{\bullet+} + 1$), 298 (5, $M^{\bullet+} - 1$), 268 (90), 250 (100), 238 (28), 232 (25), 220 (20), 194 (15), 164 (10), 150 (15), 124 (20), 109 (25); Anal. Calcd for $C_{18}H_{33}{}^2HO_3$: C, 72.19; H, 11.44. Found: C, 72.18; H, 11.39.

Preparation of Mesyl Esters 8. General Procedure. These products were prepared by the procedure described by Abad et al.[8] A solution of alcohol (2 mmol) and Et_3N (830 μL, 6 mmol) in 20 mL of CH_2Cl_2 was treated with CH_3SO_3Cl (170 μL, 2.2 mmol), and the mixture was stirred under argon for 2 h at room temperature. Then, the reaction mixture was washed with H_2O (2 × 20 mL), dried, concentrated, and purified by flash chromatography on silica gel using 85:15 hexane/MTBE to give the expected products in 95% yields.

[4-^{2}H]-17,19-Dioxaicos-8-yn-4-yl methanesulfonate (8a). (685 mg, 95% yield). IR 2935, 2860, 1465, 1355, 1175, 1145, 1115, 1045, 910 cm^{-1}; ^{1}H NMR δ 4.62 (s, 2H), 3.52 (t, *J* = 6.5 Hz, 2H), 3.36 (s, 3 H), 3.01 (s, 3H), 2.20 (t, *J* = 7 Hz, 2H), 2.13 (t, *J* = 7 Hz, 2H), 1.52−1.86 (10H), 1.28−1.52 (12H), 0.95 (t, *J* = 7 Hz, 3H); ^{13}C NMR δ 96.3 (CH_2), 83.0 (CD, t, *J* = 22.5 Hz), 81.0 (C),

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79.1 (C), 67.7 (CH_2), 55.0 (CH_3), 38.7 (CH_3), 36.4 (CH_2), 33.3 (CH_2), 29.6 (CH_2), 28.9 (CH_2), 28.9 (CH_2), 28.8 (CH_2), 26.1 (CH_2), 24.3 (CH_2), 18.6 (CH_2), 18.4 (CH_2), 18.2 (CH_2), 13.8 (CH_3).

Reduction of Mesylates 8. General Procedure. A solution of the mesyl derivative **8** (650 mg, 1.8 mmol) in Et_2O (20 mL) was treated with $LiAlD_4$ (6 molar equiv) for 16 h at 20 °C. H_2O was added dropwise to the crude reaction mixture, and the resulting white precipitate was filtered through a bed of Celite. Solvent was concentrated to give a residue that, after purification by flash chromatography on silica gel using a gradient of 0−10% MTBE in hexane, gave the corresponding pure deuterated products in 90−93% yields.

[4,4-$^2H_2$]-17,19-Dioxa-8-icosyne (9a). (475 mg, 93% yield). IR 2930, 2855, 2180, 2100, 1440, 1145, 1110, 1050, 920 cm^{-1}; ^{1}H NMR δ 4.62 (s, 2H), 3.52 (t, J = 6.5 Hz, 2H), 3.36 (s, 3H), 2.14 (m, 4H), 1.59 (quint, J = 7.0 Hz, 2H), 1.48 (m, 4H), 1.20−1.42 (12H), 0.88 (t, J = 6.5 Hz, 3H); ^{13}C NMR δ 96.3 (CH_2), 80.3 (C), 80.1 (C), 67.8 (CH_2), 55.0 (CH_3), 31.5 (CH_2), 29.7 (CH_2), 29.1 (CH_2), 29.0 (CH_2), 28.9 (CH_2), 28.7 (CH_2), 28.6 (CH_2), 28.0 (CD_2, quint, J = 19 Hz), 26.1 (CH_2), 22.5 (CH_2), 18.7 (CH_2), 18.7 (CH_2), 14.1 (CH_3); MS m/z 285 (15, $M^{•+}$ + 1), 253 (100), 235 (30); Anal. Calcd for $C_{18}H_{32}{}^2H_2O_2$: C, 76.00; H, 12.05. Found: C, 75.95; H, 11.99.

Deuteration of Compounds 9. To a mixture of 426 mg (1.5 mmol) of **9** and 93 mg (0.1 mmol) of $RhCl(PPh_3)_3$,[12] 20 mL of degassed benzene was added under argon atmosphere to get a reddish solution. The system was purged by a combination of vacuum and passing a D_2 stream throughout, then D_2 atmosphere was kept from a balloon, and the solution was stirred for 36 h. The mixture was filtered through a bed of Celite and the solvent evaporated. Residue was purified by flash chromatography on silica gel (0−3% MTBE/hexane) to give product **10**.

[12,12,13,13,17,17-$^2H_6$]-2,4-Dioxaicosane (10a). (429 mg, 98% yield). IR 2920, 2855, 2185, 2100, 1465, 1150, 1110, 1050, 920 cm^{-1}; ^{1}H NMR δ 4.62 (s, 2H), 3.52 (t, J = 6.5 Hz, 2H), 3.36 (s, 3H), 1.59 (quint, J = 6.5 Hz, 2H), 1.20−1.40 (20H), 0.88 (t, J = 7.0 Hz, 3H); ^{13}C NMR δ 96.3 (CH_2), 67.8 (CH_2), 55.0 (CH_3), 31.7 (CH_2), 29.7 (CH_2), 29.6 (CH_2), 29.6 (CH_2), 29.4 (CH_2), 29.4 (CH_2), 28.7 (CD_2, quint, J = 19 Hz), 28.6 (CD_2, quint, J = 19 Hz), 26.2 (CH_2), 22.6 (CH_2), 14.1 (CH_3); MS m/z 293 (5, $M^{•+}$ + 1), 291 (30, $M^{•+}$ − 1), 261 (100), 259 (90), 247 (25), 231 (40); Anal. Calcd for $C_{18}H_{32}{}^2H_6O_2$: C, 73.90; H, 13.10. Found: C, 73.92; H, 13.02.

Alcohol Deprotection. General Procedure. The methoxymethane protecting group of alcohols **11** were removed by acid treatment with a MeOH/HCl solution (1 M) for 36 h at room temperature. Solvent was evaporated, and the crude product was washed with saturated $NaHCO_3$ solution, extracted with CH_2Cl_2, dried, and purified by flash chromatography on silica gel using a gradient of 0−35% MTBE in hexane, for obtaining the corresponding pure deuterated alcohols **11** in 83−90% yields.

[8,8,9,9,13,13-$^2H_6$]-1-Hexadecanol (11a). Starting from 146 mg (0.5 mmol) of protected alcohol **10a**, this compound was isolated as a white solid (108 mg, 88% yield). mp 48−50 °C; IR 3250, 2955, 2915, 2850, 2180, 2090, 1465, 1215, 1065, 760 cm^{-1}; ^{1}H NMR δ 3.64 (t, J = 6.5 Hz, 2H), 1.57 (quint, J = 8.0 Hz, 2H), 1.18−1.44 (21H), 0.88 (t, J = 7 Hz, 3H); ^{13}C NMR δ 62.9 (CH_2), 32.8 (CH_2), 31.7 (CH_2), 29.6 (CH_2), 29.6 (CH_2), 29.6 (CH_2), 29.4 (CH_2), 29.4 (CH_2), 28.6 (CD_2, quint, J = 19 Hz), 28.5 (CD_2, quint, J = 19 Hz), 25.7 (CH_2), 22.6 (CH_2), 14.1 (CH_3); MS m/z 247 (35, $M^{•+}$ − 1), 231 (100), 191 (25), 177 (20), 163 (28), 151 (25), 137 (30), 123 (35), 109 (45), 97 (60); Anal. Calcd for $C_{16}H_{28}{}^2H_6O$: C, 77.34; H, 13.80. Found: C, 77.21; H, 13.82.

Carboxylic Acids Preparation (1). These compounds were prepared by reaction of alcohol with a 0.2 M (6 equiv) solution of IBX in DMSO at room temperature for 12 h to afford the aldehyde intermediate. After this time (4 × DMSO volume) mL of H_2O were added, and the reaction mixture was extracted with diethyl ether/hexanes mixture (1:1), dried, and concentrated to a residue that was solubilized in 10 mL of acetone and then treated dropwise with a 1 M aqueous solution of H_2SO_4/CrO_3. Chromatography on silica gel using 85:15 hexane/MTBE afforded the corresponding acids **1** in 85−87% yields.

[8,8,9,9,13,13-$^2H_6$]-1-Hexadecanoic acid (1a). This product (88 mg, 85%) was isolated from 98 mg (0.4 mmol) of **11a**. IR 3000, 2955, 2915, 2850, 2180, 2075, 1700, 1460, 1410, 1315, 1090, 940 cm^{-1}; ^{1}H NMR δ 2.35 (t, J = 7.5 Hz, 2H), 1.63 (quint, J = 7.5 Hz, 2H), 1.18−1.38 (18H), 0.88 (t, J = 7.0 Hz, 3H); ^{13}C NMR δ 180.6 (C), 34.1 (CH_2), 31.7 (CH_2), 29.6 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.3 (CH_2), 29.1 (CH_2), 28.6 (CD_2, quint, J = 19.0 Hz), 28.5 (CD_2, quint, J = 19.0 Hz), 24.7 (CH_2), 22.6 (CH_2), 14.1 (CH_3); MS m/z (methyl ester), 305 (100, $M^{•+}$ + 28), 277 (100, $M^{•+}$ + 1); Anal. Calcd for $C_{16}H_{26}{}^2H_6O_2$: C, 73.22; H, 12.29. Found: C, 73.13; H, 12.19.

In Vivo Gland Culture Procedure. In these experiments, newly emerged virgin *T. pityocampa* females were briefly anesthetized on ice and pheromone glands were everted and impregnated (1 μL every 3 h × 4 times) with the DMSO solutions of the corresponding deuterated probes **1** (10 mg/mL each). The *in vivo* incubation proceeded for 36 h. To obtain the methyl ester derivatives of the gland lipids for analysis, the pheromone glands were excised and soaked in chloroform methanol (2:1) at 25 °C for 1 h and base methanolized in 0.5 M KOH for 1 h. After this time, the organic solution was neutralized with 1 N HCl, washed with saturated $NaHCO_3$ solution, and extracted with hexane. Ten glands were used for each assay.

Instrumental Analysis of the Biological Extracts. The GC-MS analysis of biological extracts was performed by Chemical Ionization (CI) using methane as ionization gas. The system was equipped with a nonpolar HP5-MS capillary column (30 m × 0.25 mm i.d., 0.25 μm stationary phase thickness) using the following program: from 120 to 180 °C at 5 °C/min and then 260 °C at 2 °C/min after an initial delay of 2 min. Analyses were carried out on methanolyzed lipidic extracts from pheromone glands using the equipment and conditions described above. KIEs were calculated from the ratios of formed products from each probe, which afforded a cluster of ions, analyzed as methyl ester, and are based in the abundance of the respective molecular ions in the range m/z 265−272 in which the most abundant corresponded to the molecular ion of the resulting isotopomers.

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Supporting Information Available: ^{1}H and ^{13}C NMR and DEPT spectra for compounds **1**−**3** and **5**−**11**. This material is available free of charge via the Internet at http://pubs.acs.org.

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