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Synthesis and Use of Probes to Investigate the Cryptoregiochemistry of the First Animal Acetylenase

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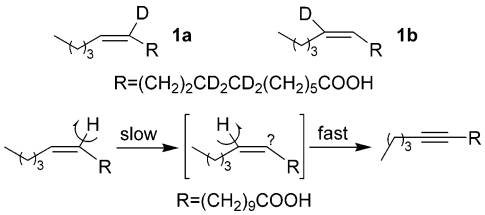

Thaumetopoea pityocampa pheromone glands contain an unusual Δ^{11} acetylenase that produces an alkynoic fatty acid intermediate in the sex pheromone biosynthetic pathway of this species. In this article, we describe the synthesis and use of the deuterated (*Z*)-11-hexadecenoic acid probes required to decipher the cryptoregiochemistry of this enzyme. The label in the olefinic bonds was introduced by Wittig reaction between the appropriate deuterated reagents. Besides the vinyl deuterium atoms, for reliable GC−MS analyses these compounds bear a tetradeuterium tag, which was introduced by deuteration of an alkyne intermediate in the presence of the Wilkinson catalyst. Pheromone gland metabolization studies of these probes provided experimental evidence that 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.

Introduction

Enzymatic desaturation of fatty acids is a key process in moth sex pheromone biosynthesis. This reaction is catalyzed by distinctive desaturases that, in contrast to the common desaturases of animal cells, afford a wide variety of unsaturated products with different position and configuration of unsaturations. Besides the desaturase-mediated formation of monoalkene and polyene fatty acids, the introduction of triple bonds by desaturases has also been reported in a few cases. Thus, some lepidopteran species belonging to the genus *Thaumetopoea* (*T. jordana*, *T. pinivora*, *T. wilkinsoni* or *T. pityocampa*), as well as the moth *Heterocampa guttivitta*,[1] use (*Z*)-13-hexadecen-11-ynyl acetate as their sex pheromone.[2] This semiochemical is biosynthesized by combined Δ^{11} and Δ^{13} desaturase reactions, which transform palmitic acid into (*Z*)-13-hexedecen-11-ynoic acid (**IV**) (Figure 1). The biosynthetic intermediate 11-hexadecynoic acid is produced by Δ^{11} desaturation of (*Z*)-11-hexadecenoic acid (**II**), which in turn arises from Δ^{11} desaturation of palmitic acid. The final enyne (**IV**) is synthesized by Δ^{13} desaturation of 11-hexadecynoic acid but not by Δ^{11}

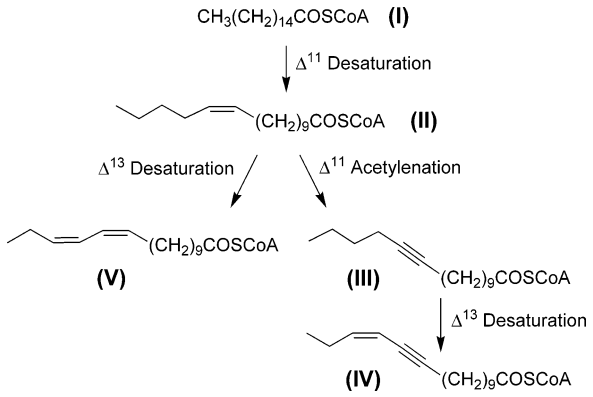

FIGURE 1. Desaturation reactions involved in the sex pheromone biosynthetic pathways of the *Thaumetopoea* genus.

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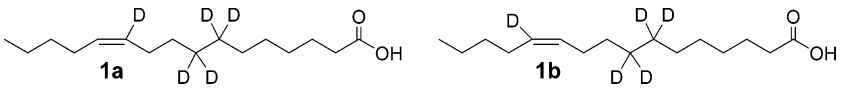
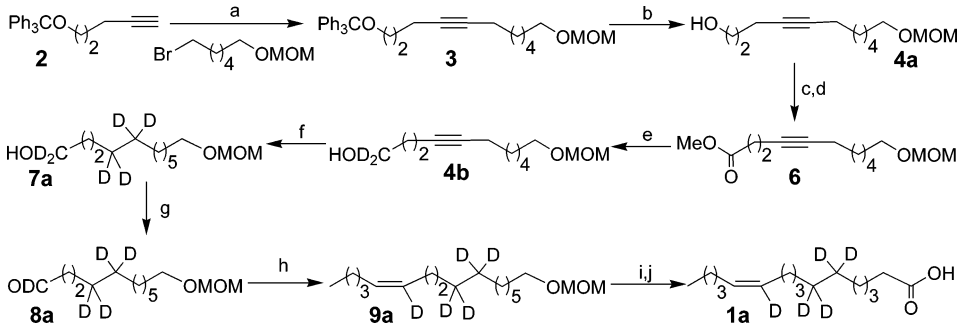


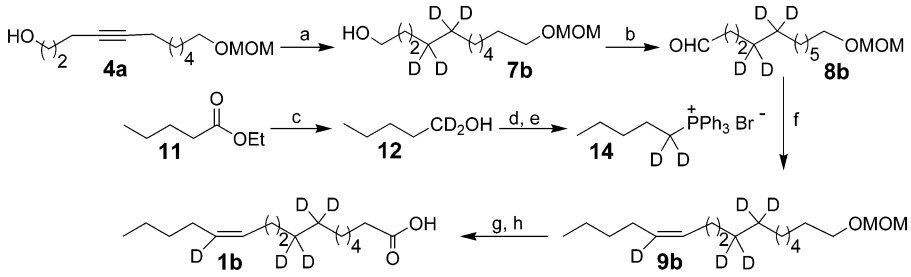
FIGURE 2. Tracers prepared to probe the cryptoregiochemistry of the Δ^{11} acetylenation.

SCHEME 1. Synthesis of Tracer 1a[a]



[a] Reagents and conditions: (a) BuLi/THF/HMPA, 90%; (b) $ClCH_2COOH$/MeOH, 88%; (c) PDC/DMF, 71%; (d) BF_3/MeOH complex, 91%; (e) $LiAlD_4$/diethyl ether, 98%; (f) Wilkinson catalyst/D_2, 95%; (g) IBX/DMSO, 88%; (h) THF/BuLi/HMPA/ Ph_3P^+-$C_5H_{11}Br^-$ (Wittig reaction), 75%; (i) HCl/MeOH, 86%; (j) (1) IBX/DMSO; (2) CrO_3/H_2SO_4/acetone, 85%.

SCHEME 2. Synthesis of Tracer 1b[a]



[a] Reagents and conditions: (a) Wilkinson catalyst/D_2, 97%; (b) IBX/DMSO, 90%; (c) $LiAlD_4$/diethyl ether, 88%; (d) PPh_3/NBS/DMF, 82%; (e) PPh_3/CH_3CN; (f) THF/HMPA/BuLi (Wittig reaction), 73%; (g) HCl/MeOH, 92%; (h) (1) IBX/DMSO; (2) CrO_3/H_2SO_4/acetone, 87%.

desaturation of (*Z*,*Z*)-11,13-hexadecadienoic acid (**V**), which is a metabolic end product.[3] Both the cryptoregiochemistry[4] (site of initial oxidation) and stereochemistry of the Δ^{11} desaturation of palmitic acid have been reported in previous articles.[5−7] To gain some insight into the course of the acetylenation reaction, in this report we describe the preparation of pentadeuterated (*Z*)-11-hexadecenoic acids **1a** and **1b**, selectively monodeuterated at the C-11 or C-12 olefinic positions (Figure 2), and their use to investigate the cryptoregiochemistry of the acetylene formation. The results obtained agree with those previously reported on enzymes of the plant kingdom, specifically the compositae *Crepis alpina*[8] and the moss *Ceratodon purpurea*,[9] which contain bifunctional enzymes with desaturase/acetylenase activities.[10]

Results and Discussion

Preparation of the Labeled and Tagged Tracers. Most studies for unveiling the site of initial oxidation in enzymatic desaturations have relied on the use of substrates *gem*-dideuterated at each desaturation position. The amounts of desaturated product formed from each substrate, relative to a metabolic standard, indicate whether the desaturation rate is affected by the presence of the mass labels, and it allows detecting the isotope-sensitive cleavage step, which in turn reveals the site of initial oxidation in the desaturation reaction. Following this approach, the present investigation was carried out by using the pentadeuterated substrates **1a** and **1b**. These (*Z*)-11-alkene tracers bear a diagnostic deuterium atom attached at either the C-11 or C-12 vinyl position, as well as four deuterium atoms at C-7 and C-8 of the fatty acid chain. This last tetradeuterium tag was required for GC−MS analytical differentiation of the labeled compounds from the natural intermediates. The tetradeuterium moiety was introduced in a remote location from the site of dehydrogenation to avoid interferences with the desaturation process and the existence of secondary kinetic isotope effects.

Substrates **1a** and **1b** were prepared as depicted in Schemes 1 and 2. Thus, trityl-protected compound **2** was coupled with the methoxymethane derivative of 6-bromo-1-hexanol to afford compound **3**, which was selectively detritylated in acid media to obtain the common intermediate **4a**. This synthon had the proper functionalities needed for introduction of both label and

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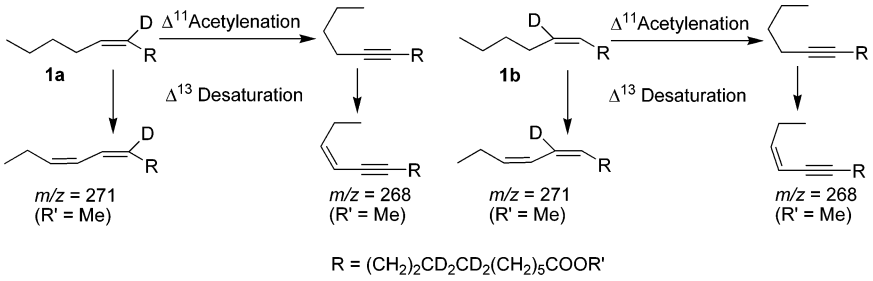

FIGURE 3. Deuterated probes and their desaturation products.

tag in the alcohol and alkyne positions, respectively, to prepare compound **1a**. Alcohol oxidation using PDC in DMF[11] afforded the corresponding acid **5** in high yields. However, to facilitate its purification, this acid was transformed into methyl ester **6** using boron trifluoride−methanol complex. Deuterium introduction at the carbinol position in compound **4b** was achieved by reduction of the methyl ester with $LiAlD_4$, which was transformed into the corresponding pentadeuterated aldehyde **8a** by nonscrambling deuteration with the Wilkinson catalyst followed by oxidation with IBX in DMSO. Wittig reaction with the phosphonium salt of 1-bromopentane gave rise to the *cis* olefin **9a** as the major product, with the aliphatic chain protected in the final alcohol position. Methoxymethane deprotection of derivative **9a** was accomplished by acid treatment with HCl/MeOH. The final acid **1a** was obtained from the resulting alcohol by IBX oxidation using DMSO as solvent to the corresponding aldehyde, which was directly oxidized using Jones conditions. These combined oxidation reactions afforded a higher final yield than that obtained using direct Jones[12] or Corey[13] conditions.

As shown in Scheme 2, a similar approach was attempted for the synthesis of compound **1b**. Thus, commercially available ethyl valerate was reduced to the dideuterated 1-pentanol **12** with $LiAlD_4$. Despite the different oxidation conditions used, isolation of the deuterated valeraldehyde was very difficult; therefore, we had to modify the strategy to obtain the labeled olefin **9b**. For this purpose, we transformed alcohol **12** into the dideuterated 1-bromopentane **13**, which was rapidly converted into the corresponding phosphonium salt **14**. Coupling of compound **8b** with **14** through a Wittig reaction allowed us to obtain as a major component the *cis* isomer of compound **9b** (8:2 *cis*:*trans*). Methoxymethane deprotection and final oxidation following the above conditions gave rise to acid **1b**.

As the Wittig reaction did not give the pure (*Z*)-stereoisomers, a final purification step of the wanted acids **1** was necessary. Removal of the (*E*)-stereoisomers was accomplished by preparation of the methyl esters and purification by column chromatography on silica gel impregnated with silver nitrate (10%). Methyl esters were transformed into the final pure (*Z*)-acids **1a** and **1b** by saponification with K_2CO_3/MeOH.

The final deuterium contents of the labeled probes were determined by GC−MS analysis of their respective methyl esters and were found to be as follows: **1a** 13% $^2H_6$, 68% $^2H_5$, 10% $^2H_4$, 7% $^2H_3$ and 3% $^2H_2$; **1b** 14% $^2H_6$, 65% $^2H_5$, 10% $^2H_4$, 8% $^2H_3$ and 3% $^2H_2$.

Characterization of the pentadeuterated compounds was carried out by ^1H and ^{13}C NMR, as previously reported.[5] The number and the presence of the deuterium labels was the expected as concluded from MS (the molecular ion mass was in accordance with the presence of five deuterium atoms) and ^{13}C NMR analyses (presence of two quintuplets for the CD_2 groups with slight upfield chemical shift changes at the 30−28.6 ppm range for compounds **7**−**10** and **1**, and an additional triplet for the olefinic CD in compounds **9**, **10**, and **1**). In addition, the presence of the olefinic deuterium atoms was also evidenced in the ^1H NMR spectra of **9**, **10**, and **1**, which showed simple olefinic hydrogen signals at ca. 5.3 ppm accounting for only one hydrogen. Furthermore, the integration of the signal at 1.25 ppm was in agreement with the presence of 2 CD_2 groups in the aliphatic chain.

Cryptoregiochemistry Studies. As shown in Figures 3 and 4, the labeled probes **1a** and **1b** follow two different pathways in the processionary moth pheromone gland, Δ^{11} acetylenation into 11-hexadecynoic acid and further Δ^{13} desaturation to (*Z*)-13-hexadecen-11-ynoic acid, and Δ^{13} desaturation to (*Z*,*Z*)-11,-13-hexadecadienoic acid. To determine the acetylenase cryptoregiochemistry, the elimination of the olefinic deuterium atoms of both deuterated alkenes **1a** and **1b** was investigated by GC−MS analysis. Accurate quantification of the resulting labeled 11-hexadecynoic acids produced from each probe was impracticable as a result of the low amounts of product occurring in the tissue. Since the Δ^{13} desaturase activity was not substantially affected by the presence of deuterium atoms in the probes, this problem was circumvented by measuring the quantities of labeled enynes, produced by Δ^{13} desaturation of the acetylenase product. The slight sensitivity of the Δ^{13} desaturase activity to the presence of labels was validated by measuring the levels of pentadeuterated methyl (*Z*,*Z*)-11,13-hexadecadienoate, synthesized by Δ^{13} desaturation of each probe. Thus, for equivalent substrate incorporations, as assessed from the labeled to natural methyl (*Z*)-11-hexadecenoate proportions (Table 1), similar methyl d_5/d_0 dienoate ratios resulted from each deuterated substrate (0.068 from **1a**; 0.058 from **1b**, Table 1). Therefore, monitoring of the deuterated enynes, produced by Δ^{13} desaturation of the acetylenase products, was considered appropriate to ascertain the site of initial oxidation in the acetylenase reaction. Furthermore, since conversion of both probes **1a** and **1b** into the dienoic acid was relatively insensitive to isotopic substitution, the diene intermediate serves as a metabolic standard for tracking the acetylenation of substrates bearing either hydrogen or deuterium at the vinyl positions. A reliable estimate of the KIE values can be obtained from the quotients between the d_5/d_0 diene and the d_4/d_0 enyne ratios with each probe. In all previous studies, the methodology for determining intermolecular primary deuterium KIE in desaturase-catalyzed

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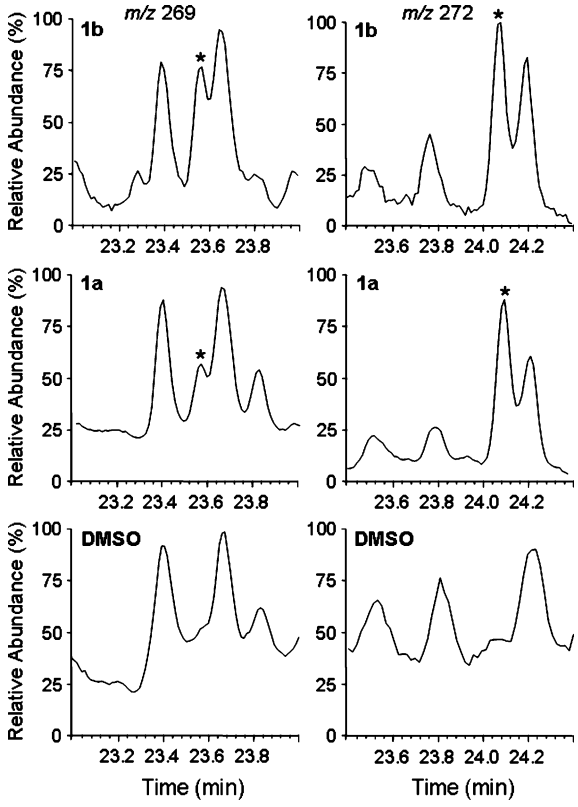

FIGURE 4. Metabolization of **1a** and **1b** in *T. pityocampa* pheromone glands. The traces correspond to GC−MS chromatograms of a methanolyzed lipid extract from *T. pityocampa* pheromone glands incubated with either **1b** (top), **1a** (middle), or DMSO (control, bottom). Left, chromatograms obtained by selection of ion at *m*/*z* 269 (molecular ion of methyl d_4(*Z*)-13-hexadecen-11-ynoate) in the total ion current chromatogram; right, chromatograms obtained by extraction of ion at *m*/*z* 272 (molecular ion of d_5(*Z*,*Z*)-11,13-hexadecadienoate) from the total ion current chromatogram. The experiments were performed as described in the Experimental Section. Asterisks indicate a labeled compound (left, methyl d_4(*Z*)-13-hexadecen-11-ynoate, and right, methyl d_5(*Z*,*Z*)-11,13-hexadecadienoate.)

TABLE 1. Conversion of Probes 1a and 1b into Biosynthetic Intermediates[a]

	($d_5$diene/$d_0$diene)/(d_5Z11/d_0Z11)	($d_4$enyne/$d_0$enyne)/(d_5Z11/d_0Z11)	($d_5$diene/$d_0$diene)/($d_4$enyne/$d_0$enyne)
1a	0.068 ± 0.006	0.009 ± 0.003	7.55 ± 1.65
1b	0.058 ± 0.001	0.060 ± 0.007	0.97 ± 0.13

[a] Data are expressed as the mean ± SD of three replicates. Labeled-to-natural FAME ratios were determined from the areas of the $M^{\bullet+} + 1$ ions (d_5Z11, 274; d_0Z11, 269; $d_5$diene, 272; $d_0$diene, 267; $d_4$enyne, 269; $d_0$enyne, 265). Abbreviations are Z11, methyl (*Z*)-11-hexadecenoate; diene, methyl (*Z*,*Z*)-11−13-hexadecadienonate; enyne, methyl (*Z*)-13-hexadecen-11-ynoate.

reactions involved mass spectrometry analysis of the reaction products derived from direct competition between the appropriate regiospecifically dideuterated substrate and its nonlabeled analogue, both with extra-labels for analysis.[13,14] The different approach followed in this article, based on the use of a metabolic standard, avoids the need of synthesizing the competitor, as well as the data corrections derived from the competitor's use.

As summarized in Table 1, the (d_5/d_0 diene)/(d_4/d_0 enyne) ratio was close to 1 from **1b** and 7.5 from **1a**, which indicates that no isotope discrimination occurs in the removal of C12-H but the acetylenase reaction is sensitive to deuterium substitution at C11. The results obtained in these experiments clearly support a stepwise mechanism for the acetylenase-mediated dehydrogenation of (*Z*)-11-hexadecenoate. The very strong vinyl C−H bond at C-11 would be significantly perturbed first in an energetically demanding and therefore isotopically sensitive step. Loss of C12-H would occur next, although not necessarily after complete abstraction of C11-H. It is possible that base assistance is involved in the elimination of C12-H.

This cryptoregiochemistry is in accordance with that reported for a Δ^{12} acetylenase of *Crepis alpina*, in which a large KIE of 14.6[15] was observed for the homologous carbon, and it conforms to a general rule by which the desaturation is initiated at the carbon atom closest to the acid functionality.

The *Crepis* enzyme has been shown to have bifunctional oleate Δ^{12} desaturase and linoleate Δ^{12} acetylenase activity. An enzyme with similar bifunctional Δ^6 desaturase/Δ^6 acetylenase activity has also been cloned from the moss *Ceratodon purpureus*.[9] The acetylenase studied here is the first acetylenase reported in the animal kingdom. Whether it is also a bifunctional desaturase/acetylenase is currently being investigated.

Conclusions

In summary, we have reported the synthesis of pentadeuterared (*Z*)-11-hexadecenoic acids regiospecifically deuterated at each vinyl position and bearing a tetradeuterium tag distant from the site of desaturation. A novel methodological approach has been described through which, by quantifying the conversion of each probe into both tetradeuterated enyne and pentadeuterated diene intermediates, the site of initial oxidation in the Δ^{11}-acetylenation has been elucidated. This reaction involves an initial energetically demanding and thus isotope sensitive perturbation of the strong vinyl C11−H bond followed by the fast removal of the neighboring C12-H.

Experimental Section

Preparation of (Pent-4-ynyloxy)triphenylmethane (2). To a solution of 2.54 g (30 mmol) of 4-pentyn-1-ol in 60 mL of dry pyridine was added 8.92 g (32 mmol) of trityl chloride. The reaction mixture was stirred at room temperature for 36 h, and then 25 mL of water was added. The mixture was extracted with CH_2Cl_2, the organic layer was concentrated to dryness, and the residue was purified by flash chromatography on silica gel (0−10% MTBE/hexane) to give 9.2 g of a white solid (94% yield). Mp 79−80 °C; IR 3305, 3060, 3020, 2930, 2875, 1490, 1445, 1220, 1070, 760, 705 cm^{-1}; ^1H NMR δ 7.46−7.41 (6H), 7.32−7.20 (9H), 3.16 (t, J = 6.0 Hz, 2H), 2.39 (dt, J_1 = 7.0 Hz, J_2 = 2.5 Hz, 2H), 1.88 (t, J = 2.5 Hz, 1H), 1.81 (t, J = 7.0 Hz, 2H); ^{13}C NMR δ 144.2 (C), 128.6 (CH), 127.6 (CH), 126.8 (CH), 86.3 (C), 84.0 (C), 68.3 (CH), 61.8 (CH_2), 29.1 (CH_2), 15.5 (CH_2); MS *m*/*z* 326 (3, $M^{\bullet+}$), 283 (7), 271 (15), 249 (50), 243 (100), 167 (15). Anal. Calcd for $C_{24}H_{22}O$: C, 88.31; H, 6.79. Found: C, 88.20; H, 6.68.

1,1,1-Triphenyl-2,14,16-trioxaheptadec-6-yne (3). To a mixture of **2** (8.15 g, 25 mmol), 40 mL of dry HMPA, and 40 mL of anhydrous THF was added dropwise a solution of butyllitium (1.6 M) in hexanes (18 mL, 29 mmol) at −15° C. The resulting pale red mixture was stirred for 10 min, and a solution of $BrCH_2(CH_2)_4$-CH_2OMOM (4.48 g, 20 mmol) in 10 mL of dry THF was added dropwise at that temperature. Stirring was continued overnight at

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0 °C. The reaction mixture was then poured into saturated $NaHCO_3$ (5 mL) and extracted with hexane (3 × 40 mL). Solvent was evaporated, and the residue was purified by flash chromatography on silica gel using a gradient hexane/MTBE (0−30%) to afford 8.46 g of the expected alcohol **3** in 90% yield. IR 3475, 3060, 3030, 2935, 2865, 1595, 1490, 1450, 1150, 1110, 1070, 1045, 915, 760, 705 cm^{-1}; ^1H NMR (500 MHz) δ 7.46−7.41 (3H), 7.34−7.18 (12H), 4.60 (s, 2H), 3.51 (t, J = 6.5 Hz, 2H), 3.36 (s, 3H), 3.14 (t, J = 6.0 Hz, 2H), 2.30 (m, 2H), 2.09 (m, 2H), 1.78 (t, J = 2.5 Hz, 2H), 1.58 (m, 2H), 1.50−1.30 (6H); ^{13}C NMR δ 144.2 (C), 128.6 (CH), 127.6 (CH), 126.7 (CH), 93.3 (CH_2), 86.2 (C), 80.3 (C), 79.6 (C), 67.7 (CH_2), 62.1 (CH_2), 55.0 (CH_3), 29.6 (CH_2), 29.5 (CH_2), 28.9 (CH_2), 28.5 (CH_2), 25.7 (CH_2), 18.6 (CH_2), 15.8 (CH_2); MS *m*/*z* 470 (2, $M^{\bullet+}$), 393 (5), 283 (5), 271 (9), 243 (100), 197 (10), 167 (70). Anal. Calcd for $C_{32}H_{38}O_3$: C, 81.66; H, 8.14. Found: C, 81.47; H, 8.16.

12,14-Dioxapentadec-4-yn-1-ol (4a). Trityl deprotection of **3** was achieved by treatment of 7.05 g (15 mmol) of the protected alkyne diol with 200 mL of MeOH/$Cl_2HCCOOH$ solution (2%) for 36 h at room temperature. The resulting mixture was neutralized with saturated $NaHCO_3$ aqueous solution and concentrated, and the crude was extracted with CH_2Cl_2, dried, and concentrated to a residue that was purified by flash chromatography on silica gel using a gradient of 0−20% AcOEt in hexane to give rise 3.2 g of an oil (88% yield) corresponding to alcohol **4**. IR 3340, 2935, 2865, 1725, 1465, 1440, 1390, 1215, 1145, 1110, 1045, 920 cm^{-1}; ^1H NMR δ 4.62 (s, 2H), 3.75 (t, J = 6.0 Hz, 2H), 3.52 (t, J = 6.5 Hz, 2H), 3.36 (s, 3H), 2.27 (m, 2H), 2.15 (m, 2H), 1.73 (m, 2H), 1.60 (t, J = 7.0 Hz, 1H), 1.49−1.38 (bb, 8H); ^{13}C NMR δ 96.3 (CH_2), 80.9 (C), 79.4 (C), 67.7 (CH_2), 61.9 (CH_2), 55.1 (CH_3), 31.5 (CH_2), 29.5 (CH_2), 28.9 (CH_2), 28.5 (CH_2), 25.7 (CH_2), 18.6 (CH_2), 15.4 (CH_2); MS *m*/*z* 229 (3, $M^{\bullet+}$ +1), 197 (100), 179 (15), 161 (12) 135 (10), 111 (30) 97 (20), 85 (12), 71 (14). Anal. Calcd for $C_{13}H_{24}O_3$: C, 68.38; H, 10.69. Found: C, 68.47; H, 10.53.

12,14-Dioxapentadec-4-ynoic acid (5). According to the procedure reported by Corey and Schmidt,[11] 2.74 g (12 mmol) of the previously obtained alcohol was stirred in a solution of 180 mL of DMF containing 3 equiv of PDC for 3 days. After this time, the reaction mixture was treated with a $Na_2S_2O_3$ solution to give a green color mixture, and then 12 mL of HCl (1 M) was added, extracted with CH_2Cl_2, dried, and concentrated to a residue that was purified by flash chromatography on silica gel using hexane/MTBE 85:15 to give 2.05 g (71% yield) of the corresponding acid. IR 2935, 2860, 1740, 1465, 1440, 1360, 1250, 1165, 1115, 1045, 995 cm^{-1}; ^1H NMR (500 MHz) δ 4.64 (s, 2H), 3.53 (t, J = 6.5 Hz, 2H), 3.37 (s, 3H), 2.55 (m, 2H), 2.47 (m, 2H), 2.13 (m, 2H), 1.60 (m, 2H), 1.47 (m, 2H), 1.38 (bb, 4H); ^{13}C NMR δ 177.4 (C), 96.2 (CH_2), 81.1 (C), 77.9 (C), 67.7 (CH_2), 55.0 (CH_3), 33.8 (CH_2), 29.4 (CH_2), 28.7 (CH_2), 28.4 (CH_2), 25.6 (CH_2), 18.5 (CH_2), 14.5 (CH_2); Anal. Calcd for $C_{13}H_{22}O_4$: C, 64.44; H, 9.15. Found: C, 64.59; H, 9.00.

Methyl 12,14-Dioxapentadec-4-ynoate (6). To a solution of 2.05 g of acid **5** (8.5 mmol) in 40 mL of methanol was added 10 mL of boron trifluoride methanol complex (20%). The reaction mixture was vigorously stirred until reaction was completed (TLC monitoring) and then neutralized with saturated $NaHCO_3$ aqueous solution, concentrated at reduced pressure, and extracted with hexane, and the crude obtained after evaporation of the solvent was purified by flash chromatography on silica gel using a gradient hexane/MTBE (5%) to give 1.98 g (91% yield) of the corresponding ester. IR 2930, 2855, 1735, 1455, 1440, 1360, 1250, 1190 cm^{-1}; ^1H NMR (500 MHz) δ 4.61 (s, 2H), 3.69 (s, 3H), 3.51 (t, J = 6.5 Hz, 2H), 3.36 (s, 3H), 2.49 (m, 4H), 2.12 (t, J = 7.0 Hz, 2H), 1.59 (m, 2H), 1.47 (m, 2H), 1.37 (bb, 4H); ^{13}C NMR (125 MHz) δ 172.6 (C), 96.3 (CH_2), 81.0 (C), 78.0 (C), 67.7 (CH_2), 55.0 (CH_3), 51.6 (CH_3), 33.8 (CH_2), 29.6 (CH_2), 28.8 (CH_2), 28.5 (CH_2), 25.7 (CH_2), 18.6 (CH_2), 14.6 (CH_2); MS *m*/*z* 284 (15, $M^{\bullet+}$ + 29), 257 (100, $M^{\bullet+}$ + 1), 225 (32). Anal. Calcd for $C_{14}H_{24}O_4$: C, 65.60; H, 9.44. Found: C, 65.47; H, 9.42.

[1,1-$^2H_2$]-12,14-Dioxapentadec-4-yn-1-ol (4b). A solution of methyl ester **6** (1.54 g, 6 mmol) in 20 mL of Et_2O, maintained under nitrogen atmosphere and at room temperature, was treated with 4.5 molar equiv of $LiAlD_4$. The mixture was stirred until the reaction was completed. After the usual workup by careful addition of water, aluminum salts were filtered off, and the organic solution was concentrated to a residue and then purified by flash chromatography using as eluent hexanes/MTBE 75:25 to afford 1.35 g of deuterated alcohol **4b** (98% yield). IR 3340, 2935, 2865, 2210, 2100, 1725, 1440, 1390, 1210, 1145, 1110, 1045, 965, 920 cm^{-1}; ^1H NMR (500 MHz) δ 4.62 (s, 2H), 3.53 (t, J = 6.5 Hz, 2H), 3.36 (s, 3H), 2.27 (m, 2H), 2.15 (m, 2H), 1.95 (d, J = 7.0 Hz, 1H), 1.72 (t, J = 7.0 Hz, 2H), 1.60 (quint, J = 6.5 Hz, 2H), 1.49 (quint, J = 7.0 Hz, 2H), 1.45−1.34 (bb, 6H); ^{13}C NMR δ 96.2 (CH_2), 80.7 (C), 79.3 (C), 67.6 (CH_2), 60.8 (CD_2, quint, J = 21.5 Hz), 55.0 (CH_3), 31.3 (CH_2), 29.4 (CH_2), 28.8 (CH_2), 28.4 (CH_2), 25.6 (CH_2), 18.5 (CH_2), 15.2 (CH_2); MS *m*/*z* 231 (2, $M^{\bullet+}$ + 1), 199 (100), 181 (24), 163 (15), 154 (14), 137 (13), 113 (35), 99 (18), 87 (15), 73 (20). Anal. Calcd for $C_{13}H_{22}{}^2H_2O_3$: C, 67.59; H, 10.51. Found: C, 67.60; H, 10.49.

Reduction with Wilkinson Catalyst. To a mixture of compound **4** and 0.1 equiv of $RhCl(PPh_3)_3$ (prepared according to the procedure previously reported)[13] was added degassed benzene under argon atmosphere to get a reddish solution. The system was purged by applying several times vacuum and then a stream of D_2 through. Finally a D_2 atmosphere was kept from a balloon and the solution was stirred for 48 h. The brown mixture was filtered through a bed of Celite, and the solvent was evaporated. The residue was purified by flash chromatography on silica gel (0−20% MTBE/hexane) to obtain saturated alcohols **7** (95−98% yield).

[1,1,4,4,5,5-$^2H_6$]-12,14-Dioxapentadecan-1-ol (7a). This compound (904 mg, 95% yield) was obtained from 915 mg (4 mmol) of alkyne **4b**. IR 3340, 2930, 2855, 2185, 2090, 1725, 1460, 1385, 1215, 1145, 1110, 1045, 965, 920 cm^{-1}; ^1H NMR (500 MHz) δ 4.62 (s, 2H), 3.51 (t, J = 7.0 Hz, 2H), 3.36 (s, 3H), 1.56 (m, 5H), 1.36−1.26 (bb, 10H); ^{13}C NMR δ 96.2 (CH_2), 67.7 (CH_2), 61.9 (CD_2, quint, J = 21.0 Hz), 54.9 (CH_3), 32.4 (CH_2), 29.6 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.1 (CH_2), 28.4 (CD_2, quint, J = 18.5 Hz), 28.3 (CD_2, quint, J = 18.5 Hz), 26.0 (CH_2), 25.3 (CH_2); MS *m*/*z* 239 (1, $M^{\bullet+}$ + 1), 235 (5, $M^{\bullet+}$ − 3), 207 (100), 177 (38), 156 (12). Anal. Calcd for $C_{13}H_{22}{}^2H_6O_3$: C, 65.50; H, 11.85. Found: C, 65.60; H, 11.79.

Aldehydes Preparation. These compounds were prepared by using the procedure reported by Frigerio et al.[16] To a solution of compound **7** (3 mmol), in 20 mL of DMSO was added 3 equiv of freshly prepared IBX[17] with vigorous stirring under nitrogen atmosphere at room temperature for 4 h. Then 50 mL of water was added, and the organic mixture was extracted with hexane/MTBE 1:1 (3 × 20 mL). The combined organic fractions were dried, concentrated to dryness, and purified by flash chromatography on silica gel (0−10% MTBE/hexane) obtaining the pure aldehyde (88−91% yield).

[1,4,4,5,5-$^2H_5$]-12,14-Dioxapentadecanal (8a). In this case 620 mg (88% yield) of aldehyde **8a** were obtained from 715 mg (3 mmol) of initial alcohol **7a**. IR 2930, 2855, 2190, 2085, 1715, 1455, 1390, 1215, 1150, 1110, 1045, 920 cm^{-1}; ^1H NMR (500 MHz) δ 4.62 (s, 2H), 3.52 (t, J = 6.5 Hz, 2H), 3.36 (s, 3H), 2.41 (t, J = 7.5 Hz, 2H), 1.62−1.57 (m, 4H), 1.36−1.27 (bb, 8H); ^{13}C NMR δ 202.6 (CD, t, J = 26.0 Hz), 96.3 (CH_2), 67.7 (CH_2), 55.0 (CH_3), 43.6 (CH_2, t, J = 3.5 Hz), 29.6 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.0 (CH_2), 28.4 (CD_2, quint, J = 18.5 Hz), 28.3 (CD_2, quint, J = 18.5 Hz), 26.1 (CH_2), 21.7 (CH_2); MS *m*/*z* 235 (3, $M^{\bullet+}$), 234 (5, $M^{\bullet+}$-1), 215 (25), 187 (100), 169 (25), 151 (15), 137 (10), 123 (15). Anal. Calcd for $C_{13}H_{21}{}^2H_5O_3$: C, 66.34; H, 11.14. Found: C, 66.25; H, 11.05.

(16) Frigerio, M.; Santagostino, M.; Sputore, S.; Palmisano, G. *J. Org. Chem.* **1995**, *60*, 7272−7276.

(17) Frigerio, M.; Santagostino, M.; Sputore, S. *J. Org. Chem.* **1999**, *64*, 4537−4538.

Preparation of [1,1-$^2H_2$]-1-Pentanol (12) A solution of 5.2 g (40 mmol) of ethyl valerate (**11**) in 50 mL of dry Et_2O, maintained under argon and at room temperature, was treated with 1.52 g of $LiAlD_4$ (40 mmol), and the mixture was stirred until the reaction was completed (TLC monitoring). After careful addition of deuterated water to the reaction mixture, the white solid was filtered off and the solvent carefully evaporated to dryness and then the residue obtained was purified by flash chromatography on silica gel using a gradient of pentane/diethyl ether (0−50%) to give 3.24 g of the pure dideuterated alcohol **12** (88%). IR 3345, 2925, 2855, 2190, 2090, 1460, 1170, 1135, 1085, 1065, 970 cm^{-1}; ^1H NMR (500 MHz) δ 1.56 (t, $J = 6.5$ Hz, 2H, CH_2), 1.46 (sa, 1H), 1.38−1.30 (4H, CH_2), 0.91 (t, $J = 6.5$ Hz, 3H, CH_3); ^{13}C NMR (125 MHz) δ 62.1 (CD_2, quint, $J = 21$ Hz), 32.3 (CH_2), 27.8 (CH_2), 22.4 (CH_2), 14.0 (CH_3).

Preparation of [1,1-$^2H_2$]-1-Bromo-pentane (13) This reaction was accomplished with minor modifications according to the procedure described by Bates et al.[18] A solution of NBS (36 mmol, 6.1 g in 18 mL of DMF) was added dropwise at 0 °C to a stirred DMF solution (24 mL) containing 3.24 g (35.2 mmol) of alcohol **12** and 9.47 g (36 mmol) of triphenylphosphine. Stirring was continued for 1 h, and 250 μL of methanol was added to the pale yellow mixture to quench the reagent excess. After 5 min, water was added (240 mL), the mixture was extracted with pentane (3 × 20 mL), and the organic layers were combined and distilled to obtain 4.17 g (78%) of the dideuterated bromoderivative **13** (bp 129 °C). IR 2925, 2855, 1720, 1460, 1375 cm^{-1}; ^1H NMR δ 1.85 (t, $J = 6.5$ Hz, 2H, CH_2), 1.41 (m, 2H, CH_2), 1.35 (m, 2H, CH_2), 0.92 (t, $J = 6.5$ Hz, 3H, CH_3); ^{13}C NMR δ 33.4 (CD_2, quint, $J = 23$ Hz), 32.3 (CH_2), 30.2 (CH_2), 21.8 (CH_2), 13.8 (CH_3).

Wittig Reaction. A mixture of (455 mg, 3 mmol) of 1-bromopentane **13** and 0.9 g (3.25 mmol) of triphenylphosphine was refluxed in 20 mL of anhydrous CH_3CN under argon atmosphere for 36 h. Solvent was evaporated, and residue was suspended in dry pentane (3 × 20 mL), decanted, and dried at reduced pressure to give a white solid corresponding to the triphenylphosphonium bromide **14**.

To a mixture of the triphenylphosphonium salt **14** (650 mg, 1.5 mmol) previously obtained, 5 mL of anhydrous THF, and 1 mL of dry HMPA was added 1.2 mL of a butyllitium solution in hexane (1.2 equiv, 1.5 M) dropwise at −50°C under nitrogen atmosphere to afford a pale orange color suspension. The reaction mixture was stirred at that temperature for 30 min and then cooled at −78 °C. Stirring was continued for 10 additional min, and aldehyde **8 (**1 mmol, ca. 234 mg**)** dissolved in 4 mL of anhydrous THF was added dropwise and stirred for 1 h at −78 °C and 3 h at room temperature. The reaction mixture was poured into water and extracted with hexane. The combined organic fractions were dried, concentrated to dryness, and purified by flash chromatography on silica gel impregnated with $AgNO_3$ (10%) using as eluent a gradient hexane/MTBE (5%) to give a 9/1 mixture of *Z*/*E* isomers (70−75% yield).

[6,9,9,10,10-$^2H_5$]-(*Z*)-17,19-Dioxaeicos-5-ene (9a). This compound was isolated (219 mg, 75% yield) starting from 235 mg of aldehyde **8a**. IR 2925, 2855, 2185, 2090, 1725, 1460, 1385, 1145, 1105, 1045, 965, 920 cm^{-1}; ^1H NMR (500 MHz) δ 5.35 (t, $J = 7.5$ Hz, 1H), 4.62 (s, 2H), 3.52 (t, $J = 7.0$ Hz, 2H), 3.36 (s, 3H), 2.01 (m, 4H), 1.59 (m, 2H), 1.35−1.28 (bb, 14H), 0.89 (t, $J = 7.0$ Hz, 3H); ^{13}C NMR δ 129.6 (CH), 129.4 (CD, t, $J = 22.5$ Hz), 96.3 (CH_2), 67.8 (CH_2), 54.9 (CH_3), 31.9 (CH_2), 29.7 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 29.4 (CH_2), 29.2 (CH_2), 28.2 (CD_2, quint, $J = 18.0$ Hz), 27.0 (CH_2), 26.8 (CH_2), 26.1 (CH_2), 22.3 (CH_2), 13.9 (CH_3); MS *m*/*z* 290 (15, $M^{\bullet+} + 1$), 258 (100), 240 (25), 214 (10). Anal. Calcd for $C_{18}H_{31}{}^2H_5O_2$: C, 74.68; H, 12.54. Found: C, 74.53; H, 12.37.

(18) Bates, H. A.; Farina, J.; Tong, M. *J. Org. Chem.* **1986**, *51*, 2637−2641.

Methoxymethane Deprotection. General Procedure. Products **9** were deprotected to the corresponding alcohols **10** by treatment with a MeOH/HCl solution (0.5 M) for 24 h at room temperature. Solvent was concentrated, and the crude was neutralized with a saturated $NaHCO_3$ aqueous solution, extracted with CH_2Cl_2, dried, and concentrated to a residue that was finally purified by flash chromatography on silica gel using a gradient of AcOEt in hexanes (10−25%) to obtain an oil (86−92% yield).

[7,7,8,8,11-$^2H_5$]-(*Z*)-Hexadec-11-en-1-ol (10a). This alcohol was isolated (106 mg, 86%) starting from 145 mg (0.5 mmol) of **9a**. IR 3350, 2925, 2855, 2185, 2090, 1725, 1460, 1045 cm^{-1}; ^1H NMR (500 MHz) δ 5.34 (t, $J = 7.0$ Hz, 1H), 3.63 (t, $J = 6.5$ Hz, 2H), 2.01 (m, 4H), 1.56 (m, 2H), 1.43 (s, 1H), 1.35−1.25 (bb, 14H), 0.89 (t, $J = 7.5$ Hz, 3H); ^{13}C NMR δ 129.6 (CH), 129.5 (CD, t, $J = 23.0$ Hz), 62.9 (CH_2), 32.7 (CH_2), 31.9 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 28.4 (CD_2, quint, $J = 18.5$ Hz), 28.2 (CD_2, quint, $J = 18.5$ Hz), 27.0 (CH_2), 26.8 (CH_2), 25.7 (CH_2), 22.3 (CH_2), 13.9 (CH_3); MS *m*/*z* 246 (100, $M^{\bullet+} + 1$), 226 (35), 202 (15), 184 (10), 170 (12), 156 (20), 142 (30), 128 (42), 114 (50), 99 (70), 85 (50), 71 (40). Anal. Calcd for $C_{16}H_{27}{}^2H_5O$: C, 78.29; H, 13.15. Found: C, 78.23; H, 13.07.

Carboxylic Acids Preparation (1). These compounds were prepared by reaction of alcohol **10** 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 was 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 solution of H_2SO_4/CrO_3. Chromatography on silica gel using hexane/MTBE 85:15 afforded the corresponding acids in 85−87% yield.

[7,7,8,8,11-$^2H_5$]-(*Z*)-Hexadec-11-enoic Acid (1a). This product (88 mg, 85%) was isolated as oil from 98 mg (0.4 mmol) of **10a**. IR 3210, 2925, 2855, 2190, 2090, 1725, 1460, 1270, 965 cm^{-1}; ^1H NMR (500 MHz) δ 5.34 (t, $J = 7.0$ Hz, 1H), 2.34 (t, $J = 7.5$ Hz, 2H), 2.01 (m, 4H), 1.63 (m, 2H), 1.32−1.26 (bb, 12H), 0.89 (t, $J = 7.0$ Hz, 3H); ^{13}C NMR (125 MHz) δ 179.7 (C), 129.7 (CH), 129.5 (CD, t, $J = 23.0$ Hz), 33.9 (CH_2), 31.9 (CH_2), 31.5 (CH_2), 29.4 (CH_2), 29.1(CH_2), 29.1 (CH_2), 29.0 (CH_2), 28.2 (CD_2, quint, $J = 19.0$ Hz), 27.0 (CH_2), 26.9 (CH_2), 26.8 (CH_2), 24.6 (CH_2), 22.3 (CH_2), 14.0 (CH_3); MS *m*/*z* (OCH_3), 302 (20, $M^{\bullet+} + 29$), 274 (100, $M^{\bullet+} + 1$), 242 (32). Anal. Calcd for $C_{16}H_{25}{}^2H_5O_2$: C, 74.07; H, 11.66. Found: C, 74.13; H, 11.59.

***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 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 that 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 $M^{\bullet+} + 1$ of the resulting isotopomers.

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Supporting Information Available: ^{1}H and ^{13}C NMR and DEPT spectra for compounds **1**−**10** and **12**−**13**. This material is available free of charge via the Internet at http://pubs.acs.org.

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