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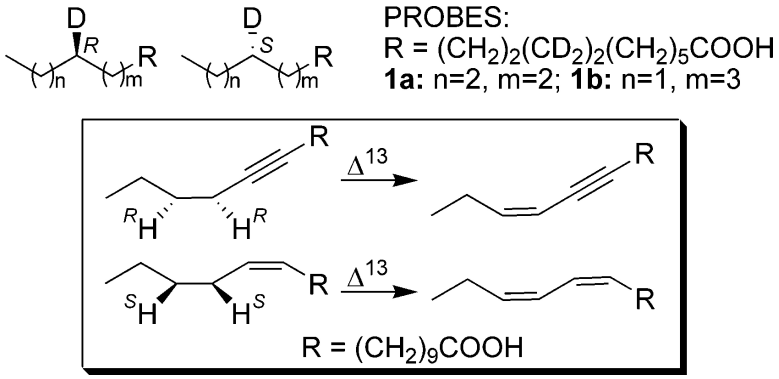

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Substrate-Dependent Stereochemical Course of the (*Z*)-13-Desaturation Catalyzed by the Processionary Moth Multifunctional Desaturase

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Abstract: The stereochemical course of the Δ^{13} desaturation involved in the biosynthesis of *Thaumetopoea pityocampa* sex pheromone was studied using stereotopically labeled and tagged palmitic acids as metabolic probes. In the synthetic pathway, a functionalized acetylene common synthon was used for introducing the four deuterium tags. Further coupling of the tetradeuterated synthon to the appropriated alkynol and a double chemoenzymatic strategy to resolve the alcohol functionality allowed one to obtain the enantiomerically enriched probes used in the mechanistic studies. Mass spectrometric analyses of extracts from tissues cultured with each probe revealed that removal of the C13 and C14 hydrogens in 11-hexadecynoate and (*Z*)-11-hexadecenoate are *pro*-(*R*)- and *pro*-(*S*)-specific *syn*-dehydrogenation processes, respectively. This finding constitutes the first example in the literature of an enzymatic (*Z*)-desaturation exhibiting a substrate-dependent stereochemical course.

Introduction

Desaturases are ubiquitous enzymes with a crucial role in the homeostasis of cell membranes. Besides this important function, desaturases are involved in cell signaling,[1] as well as in the biosynthesis of lipids and fatty acid-derived natural products.[2] In this last regard, fatty acyl CoA desaturases produce the unsaturated intermediates involved in moth sex pheromone biosynthetic pathways.[3−5] In these biological systems, desaturases catalyze the regio- and stereospecific introduction of double bonds into fatty acyl chains to afford a number of unsaturated fatty acids, both monoenoic and dienoic, either conjugated or not. In all cases, besides the usual *Z* isomer, the *E* isomer can also be produced. Because of their functional diversity, desaturases of sex pheromone producing cells constitute excellent models to gain insight into different aspects of biological desaturation. An interesting feature of moth desaturases is their multifunctionality, whereby a single enzyme can lead to different desaturated products depending on the substrate transformed. Thus, for example, the Δ^{11} desaturases of *Bombyx mori*[6] and *Spodoptera littoralis*[7,8] exhibit a bifunctional Δ^{11}/$\Delta^{10,12}$ desaturase activity, producing both C11-monoenoic and 10,12-dienoic acids with different double bond geometries depending on the substrate used. An even more extraordinary case has been recently discovered in the processionary moth, *Thaumetopoea pityocampa*. This species, along with other members of the Thaumetopoeidae family, produce a conjugated enyne acetate as its sex pheromone.[9] Other species of this family make a (*Z*,*Z*)-11,13-hexadecadienoate-derived aldehyde, alcohol, or acetate as pheromone constituents.[10] The (*Z*,*Z*)-11,13-hexadecadienoate intermediate is also biosynthesized by *T. pityocampa*,[11−14] although it is not further transformed into a pheromone component.[15] Both enyne and diene compounds are biosynthesized from palmitic acid by consecutive desaturation reactions, Δ^{11} desaturation, acetylenation, and Δ^{13} desaturation (Figure 1).[12−14]

Molecular cloning and heterologous functional expression in yeast have recently evidenced that the three desaturation reactions are catalyzed by a single enzyme.[17] A previous study showed that the (*Z*)-11 desaturation takes places by removal of both *pro*-(*R*) C11 and C12 hydrogen atoms from palmitate to

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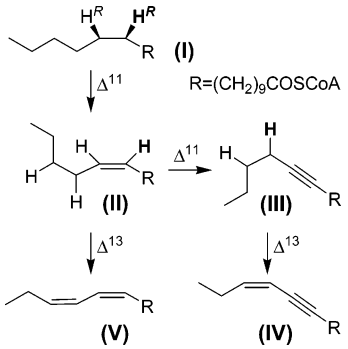

Figure 1. Desaturases of the *T. pityocampa* sex pheromone biosynthetic pathway. Bold hydrogens indicate first abstraction in the two-step desaturation reactions.[7,16] Among the different intermediates, only **IV** is reduced and acetylated to the pheromone. Desaturation reactions are abbreviated as: Δ^{11}, (*Z*)-11-desaturation or 11-acetylenation, Δ^{13}, (*Z*)-13-desaturation.

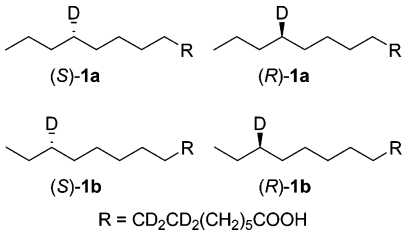

Figure 2. Pentadeuterated palmitic acids used in the stereochemistry study of the *T. pityocampa* Δ^{13} desaturation.

give (*Z*)-11-hexadecenoate.[18] Additionally, previous investigations reported that the three desaturations occur by initial oxidation of the carbon atom located nearest to the carboxyl functionality: C11 (Δ^{11} desaturation and Δ^{11} acetylenation)[7,16] and C13 (Δ^{13} desaturation).[19] To complete the mechanistic studies on this unique desaturase, the stereochemical course of the Δ^{13} desaturation of both (*Z*)-11-hexadecenoate and 11-hexadecynoate was undertaken by determining the fate of enantiomerically pure palmitic acids stereospecifically deuterated at C13 and C14 (Figure 2). We report and discuss the novel finding that the stereospecificity of this Δ^{13} desaturation depends on the enzyme substrate used, even though a (*Z*)-13 double bond is formed in both cases.

Results and Discussion

Synthesis of the Stereotopically Deuterated Tracers. As carried out in our previous studies, the stereochemistry of this Δ^{13} desaturation was analyzed through the use of multideuterated substrates bearing both an analytical tag and a stereospecifically deuterated stereogenic center of known configuration at the diagnostic site (C13 and C14 in this case). The mass of the resulting Δ^{13} desaturated products, as analyzed by GC/MS, indicates whether H or ^{2}H was lost to form the double bond. As rationalized in previous studies, the use of the specific Δ^{13} desaturase substrates ((*Z*)-11-hexadecenoic and 11-hexadecynoic acids) is not necessary, because these compounds are intracellularly biosynthesized from the saturated pentadeuterated palmitic acid probes by using the tissue culture assay,[19] thus circumventing a more complex preparation of these labeled unsaturated acid precursors.

The probes needed to investigate the stereochemistry of the Δ^{13} desaturation were prepared following the conventional reactions depicted in Schemes 2 (compound **1a**) and 3 (compound **1b**). Both synthetic pathways use protected [7,7,8,8-$^2H_4$]-10-bromodecan-1-ol (**4**) as common intermediate. This compound was obtained by coupling 3-butyn-1-ol with the bromomethoxymethane derivative of 1,6-hexanediol, reduction of the resulting alkynol with $^2H_2$ using the Wilkinson catalyst, and further bromination of the saturated tetradeuterated alcohol (Scheme 1).

As shown in Scheme 2, crucial steps for the preparation of pentadeuterated probes **1a** were the double enzymatic resolutions of 1-hexyn-3-ol with *Candida antarctica* lipase (CALB) to give pure acetate (*S*)-**6a** and partially resolved alkynol (*R*)-**5a** (80% ee).[20] Likewise, a new enzymatic resolution with EP-100 immobilized *Thermomyces* (Humicola) *lanuginosus* (HLL) afforded enantiomerically pure propargyl alcohol (*R*)-**7a**. Conventional reactions such as non-scrambling deuteration with the Wilkinson catalyst, mesylation, nucleophilic substitution with $LiAl^2H_4$, methoxymethane deprotection, and two steps of oxidation[16] allowed the transformation of both alcohols (*R*)-**7a** and (*S*)-**7a** into the corresponding acids (*S*)-**1a** and (*R*)-**1a**.

A similar synthetic approach for preparation of **1b** enantiomers was unsuccessfully attempted by using 1-pentyn-3-ol as starting material. However, as depicted in Scheme 3, commercially available 5-hexyn-3-ol could be CALB resolved, and the absolute configurations of the isolated products assigned by NMR (see below) were in agreement with those predicted by the Kazlauskas rule.[21]

The resolved enantiomers of 5-hexyn-3-ol were transformed into the corresponding stereotopically deuterated fatty acids **1b** following the same sequence of reactions previously used in the synthesis of compound **1a**. The deuterated compounds were characterized as previously reported for similar compounds.[16,22,23]

On the other hand, the absolute configuration and enantiomeric excesses (ee) of the different alcohol intermediates were determined by NMR,[16,19] after derivatization with (*R*)-MPA ((*R*)-(−)-methoxyphenylacetic acid).[18,20,24] In this context, the sign of the optical rotation of resolved (*S*)-(−)-1-hexyn-3-ol agreed with that of similar propargylic alcohols (*S*)-(−)-1-octyn-3-ol (commercially available) and (*S*)-(−)-1-heptyn-3-ol. These compounds were synthesized in our laboratory by the same methodology.[18] Likewise, optical rotation signs of homopropargylic 5-hexyn-3-ol stereoisomers agreed with previously reported data.[25] Number and location of the deuterium tags and labels were as expected as concluded from the ^{1}H and ^{13}C NMR analyses. The final deuterium contents of the acids **1** were determined by GC−MS (EI) analysis of their respective methyl esters and under the optimum synthetic conditions, mean ratios of $[M + 1]^{\bullet+}$, $[M]^{\bullet+}$, $[M - 1]^{\bullet+}$, $[M - 2]^{\bullet+}$, and $[M - 3]^{\bullet+}$ fatty acids, were, respectively, 12.5, 78.4, 6.6, 2.1, and 0.4 for compound **1a** and 12.6, 78.4, 6.0, 2.2, and 0.8 for compound **1b**, respectively.

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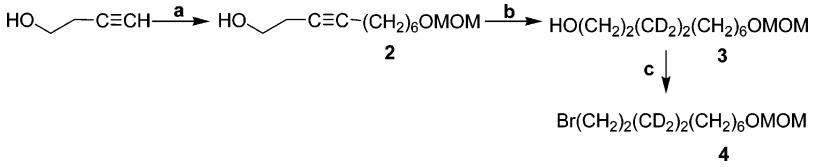
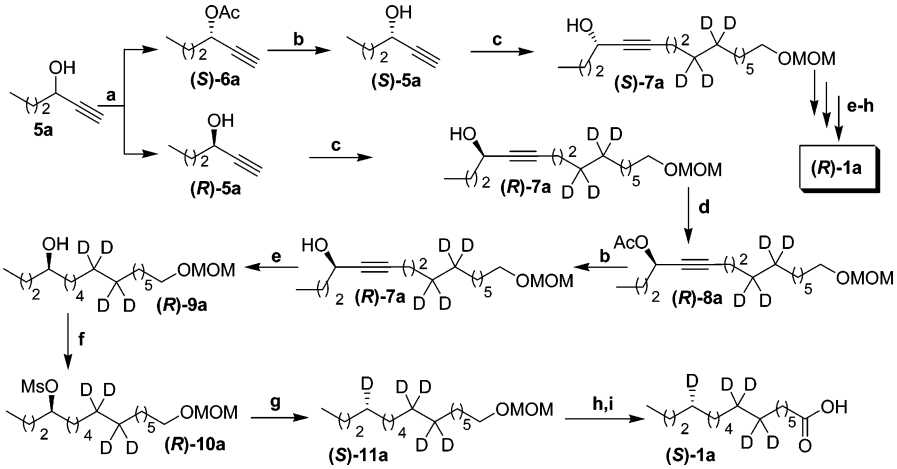
Scheme 1. Synthesis of Labeled Intermediate **4**[a]



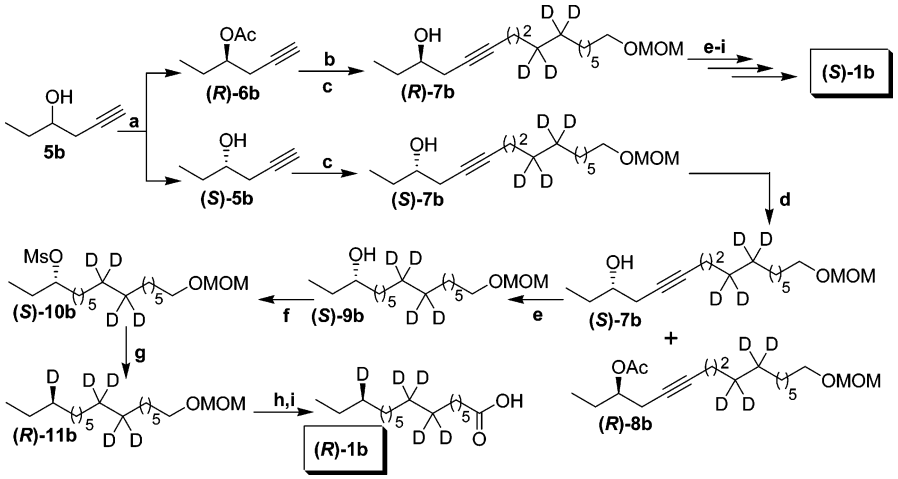
[a] Reagents and conditions: (a) BuLi/HMPA/THF/$BrCH_2(CH_2)_4CH_2OMOM$, 78%; (b) WC/D_2/benzene, 75%; (c) PPh_3/NBS/DMF, 79%.

Scheme 2. Synthesis of the Stereotopically Pentadeuterated Probes **1a**[a]



[a] Reagents and conditions: (a) CALB/DIPE/$AcOCH{=}CH_2$ (conversion 45% and 90%, two rounds, ee 96%); (b) K_2CO_3/MeOH, 82−93%; (c) BuLi/HMPA/THF, **4**, 71%; (d) HLL, DIPE, $AcOCH{=}CH_2$, 78%; ee 96%; (e) WC/H_2/benzene, 66%; (f) MsCl/NEt_3/CH_2Cl_2, 92%; (g) $LiAlD_4$/THF, 81%; (h) HCl//MeOH, 85%; (i) (1) IBX/DMSO; (2) CrO_3/H_2SO_4/acetone, 86%.

Scheme 3. Synthesis of the Stereospecifically Deuterated Probes **1b**[a]



[a] Reagents and conditions: (a) CALB/DIPE/$AcOCH{=}CH_2$ (conversion 45% and 90%, two rounds, ee 94%); (b) K_2CO_3/MeOH, 82−93%; (c) BuLi/HMPA/THF, **4**, 70−72%; (d) HLL, DIPE, $AcOCH{=}CH_2$, 78−80%; ee 94% (acetate discarded); (e) WC/H_2/benzene, 66%; (f) MsCl/NEt_3/CH_2Cl_2, 90−92%; (g) $LiAlD_4$/THF, 81%; (h) (1) HCl//MeOH, 85%; (i) (1) IBX/DMSO; (2) CrO_3/H_2SO_4/acetone, 80−85%.

Stereospecificity Studies. To determine the stereospecificity of the Δ^{13} desaturation, the destiny of the C13 and C14 hydrogen/deuterium atoms of **1a** and **1b**, respectively, was investigated. To this aim, pheromone glands of *T. pityocampa* females were cultured with each one of the prepared probes, tissues were extracted and methanolyzed, and the resulting extracts were analyzed by chemical ionization GC/MS with methane as ionization gas (Figure 3). This procedure affords the $[M + 1]^{\bullet+}$ ion as the most abundant fragment in the mass spectra (Table 1).

In the experiments with (*R*)-**1b**, the $[M + 1]^{\bullet+}$ ion clusters of the resulting methyl hexadecadienoate and methyl hexadecenynoate were centered at *m*/*z* 272 and 269, respectively. In contrast, (*S*)-**1b** gave rise to methyl hexadecadienoate and methyl hexadecaenynoate in which the most abundant $[M + 1]^{\bullet+}$ ions in the clusters were those at *m*/*z* 271 and 270, respectively. These results imply that conversion of (*R*)-**1b** into the enyne occurred by loss of deuterium from C14, whereas diene formation took place by hydrogen loss. Conversely, transformation of (*R*)-**1b** into the enyne and the diene occurred by loss of C14−H and C14−^{2}H, respectively (Figure 4).

In parallel experiments, incubations with (*R*)-**1a** gave a major methyl hexadecadienoate $[M + 1]^{\bullet+}$ isotopomer at *m*/*z* 272, but deuterated methyl hexadecenynoate ($[M + 1]^{\bullet+}$ at *m*/*z* 269) was not detected. On the other hand, (*S*)-**1a** afforded the main methyl hexadecenynoate $[M + 1]^{\bullet+}$ isotopomer at *m*/*z* 270, whereas

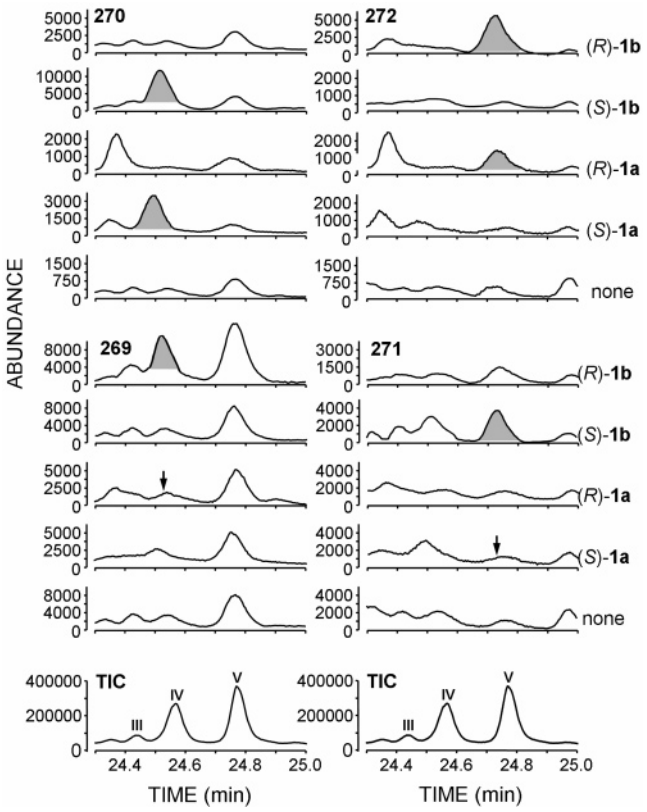


Figure 3. Isotopomers of (*Z*)-13-hexadecen-11-ynoic (**IV**) and (*Z*,*Z*)-11,-13-hexadecadienoic (**V**) acids formed by Δ^{13} desaturation of (*R*)- and (*S*)-**1a**, and (*R*)- and (*S*)-**1b**. The traces correspond to GC/MS chromatograms of methanolyzed lipidic extracts from *T. pityocampa* pheromone glands cultured without (none) or with the indicated probe upon selection of ions at *m*/*z* 269, 270, 271, and 272 (see Table 1). The total ion current chromatogram (TIC) showing the methyl esters derived from the natural acylCoA biosynthetic intermediates (**III**, **IV**, and **V**, see Figure 1) is depicted at the bottom of the figure. In the mass spectra of unlabeled **IV** and **V**, abundance of ions are: **IV** *m*/*z*, 265, 100%; 266, 20%; 267, 12%; 268, 3%; 269, 5%; 270, 2%; 271, 2%; 272, 2%; 273, 1%; 274, 1%; 275, 1%; 276, 1%; **V** *m*/*z*, 267, 100%; 268, 18%; 269, 11%; 270, 3%; 271, 4%; 272, 2%; 273, 3%; 274, 1%; 275, 1%; 276, 1%. Shadowed peaks correspond to the most abundant isotopomer formed from each probe; peaks marked with an arrow indicate the retention time of isotopomers formed by loss of C13−^{2}H, which are barely detected because of the existence of primary isotope effect.[19] The results obtained by integration of ions are summarized in Table 1.

Table 1. Stereochemistry of the Δ^{13} Desaturations[a]

		isotopomer $[M+1]^{\bullet+}$ mass		
probe	product	d_4	d_5	d_5/d_4 ratio
(*R*)-**1a**	**V**	271	272	8.3 ± 2.7
	IV	269	270	bg
(*S*)-**1a**	**V**	271	272	bg
	IV	269	270	9.1 ± 4.9
(*R*)-**1b**	**V**	271	272	11.1 ± 3.7
	IV	269	270	0.1 ± 0.0
(*S*)-**1b**	**V**	271	272	0.1 ± 0.0
	IV	269	270	8.3 ± 2.7

[a] Percentages of isotopomers correspond to the mean ± standard deviation of two different experiments with duplicates and have been corrected for the abundance of the $[M+1]^{\bullet+}$ and $[M-1]^{\bullet+}$ ions in the probes, in which the mean d_5/d_4 ratios are 11.9 (acid **1a**) and 13.1 (acid **1b**). **V** refers to methyl (*Z*,*Z*)-11,13-hexadecadienoate and **IV** to methyl (*Z*)-13-hexadecen-11-ynoate. bg, not determined because peak abundances are not different from background.

levels of labeled methyl hexadecadienoate ($[M+1]^{\bullet+}$ at *m*/*z* 271) were negligible in this case. These results imply that the

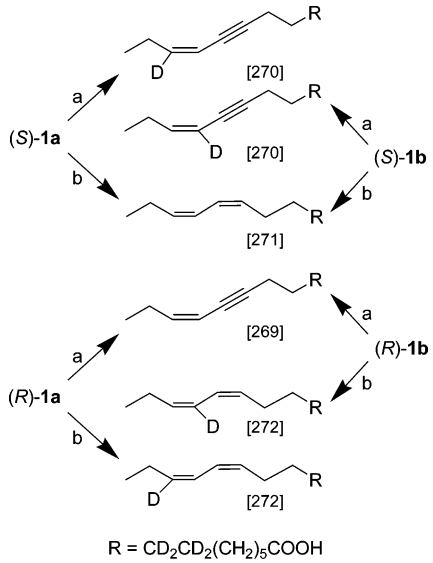


Figure 4. Isotopomeric multidesaturation products of the pentadeuterated palmitic acids used in this study. Numbers between brackets correspond to the *m*/*z* values of the $[M+1]^{\bullet+}$ ions. (a) Δ^{11}, Δ^{11}, and Δ^{13}; (b) Δ^{11} and Δ^{13}.

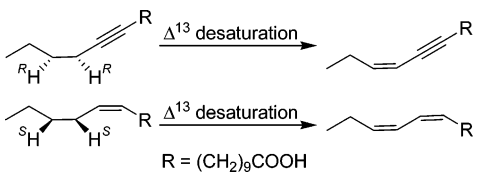


Figure 5. Stereospecificity of the Δ^{13} desaturation.

diene and the enyne are formed by loss of C13−H and C13−^{2}H from (*R*)-**1a** and loss of C13−^{2}H and C13−H from (*S*)-**1a**, respectively.

The insignificant levels of labeled methyl (*Z*)-13-hexadecen-11-yonate and methyl (*Z*,*Z*)-11,13-hexadecadienoate formed from (*R*)-**1a** and (*S*)-**1a**, respectively, were not unexpected in the light of the cryptoregiochemistry found for the Δ^{13} desaturation, which occurs with the slow initial oxidation of C13 and subsequent fast elimination of C14−H.[19] As an experimental proof of this mechanism, the Δ^{13} desaturation is sensitive to isotope substitution at C13, but not C14, and, therefore, the loss of C13−^{2}H should occur at a lower rate than that of the geminal hydrogen atom, which should have a final influence on the levels of the resulting desaturation products. Furthermore, it is noteworthy that the ratios of labeled to natural methyl (*Z*)-13-hexadecen-11-yonate resulting from either (*R*)-**1b** or (*S*)-**1b** were similar and approximately 6 times lower than that produced from (*S*)-**1a**. Because the desaturation rate of (*S*)-**1a** to the diene is lowered by the kinetic isotope effect, the pathway is shifted toward formation of the enyne. In agreement, the ratios of labeled to natural methyl (*Z*,*Z*)-11,13-hexadecadienoate resulting from either (*R*)-**1b** or (*S*)-**1b** were similar and approximately 8 times lower than that produced from (*R*)-**1a**. In that case, the isotope effect affects the enyne formation, and desaturation to the diene is favored.

The overall data obtained in the above biochemical studies indicate that the Δ^{13} desaturation studied here follows different sterochemical courses depending on the substrate converted. Thus, whereas the Δ^{13} desaturation of 11-hexadecynoic occurs with removal of both *pro*-(*R*) C13−H and C14−H, that of (*Z*)-11-hexadecenoic acid takes place with loss of both *pro*-(*S*)

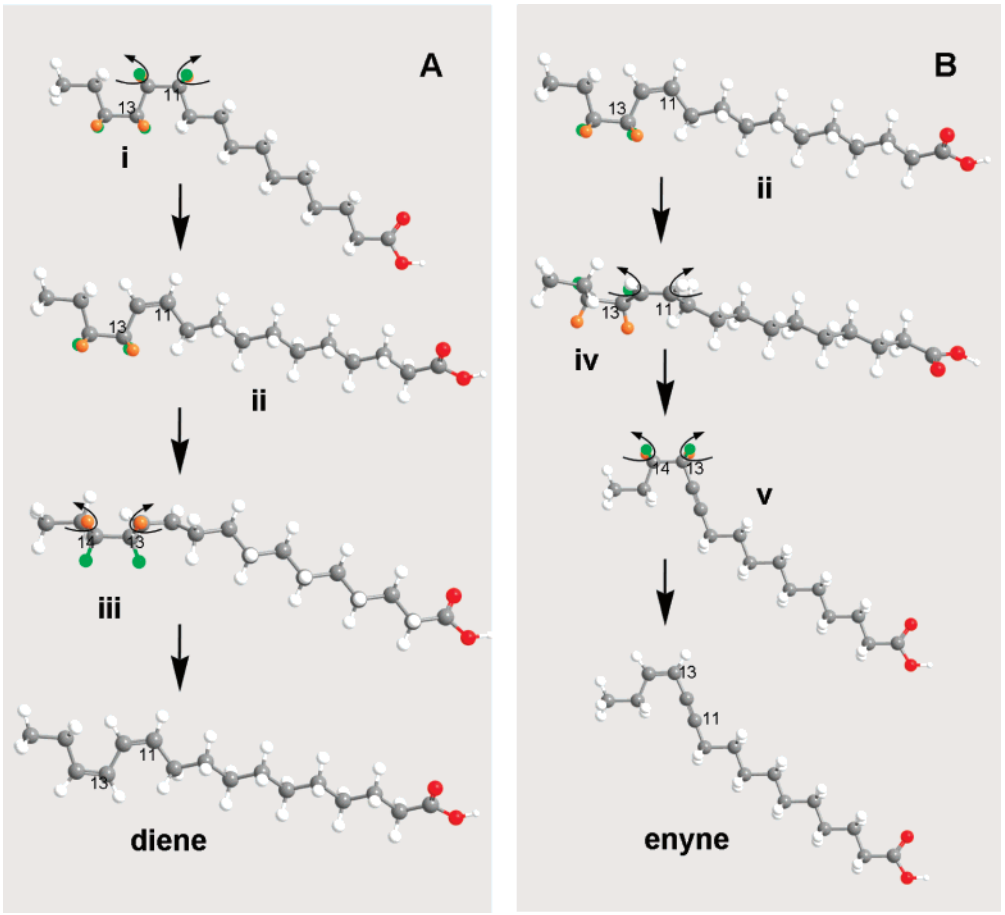


Figure 6. Transformation of palmitic acid into both (*Z*,*Z*)-11,13-hexadecadienoic (A) and (*Z*)-13-hexadecen-11-ynoic (B) acids. In the models, the hydrogen atoms removed to give the unsaturations are syn and facing toward the reader, wherein the oxidizing site is considered to reside. Rotation of substrates occurs whenever necessary to place the hydrogens for removal in the right orientation (i.e., **ii** → **iii**, **ii** → **iv**, and **iv** → **v**). In (A), the stereochemical outcome of the overall conversion is determined by the transoid conformation of the palmitate C10−C15 bonds at the enzyme active site (**i**). In the enyne case (B), rotation of the hexadecenoate substrate (**ii** → **iv**) locates the two sp^2 hydrogens in the appropriate spatial arrangement for elimination. The resulting acetylene adopts a conformation **v** similar to that initially assumed by palmitate (**i**), leaving the two *pro*-(*R*) C13−H and C14−H properly oriented for Δ^{13} desaturation. In the models, light gray indicates C, and red shows oxygen. The *pro*-(*R*) and *pro*-(*S*) hydrogen atoms at C11, C12, C13, and C14 are shown in green and orange, respectively. The models were obtained with the ChemBats3D 9.0 program. The dihedral angles C10−C11−C12−C13 and C11−C12−C13−C14 were set to 0°.

C13−H and C14−H (Figure 5). In the first case, the stereochemistry is the same reported for other (*Z*)-desaturation reactions, including those catalyzed by the ubiquitous (*Z*)-9 desaturase,[26,27] several moth (*Z*)-11 desaturases,[28−30] as well as a monoene desaturase.[24]

However, the Δ^{13} desaturation of (*Z*)-11-hexadecenoate represents the first case of rupture of the stereochemical trend of (*Z*)-desaturation. Taking into account that desaturation reactions are *syn* elimination processes, the different stereochemical courses of this Δ^{13} desaturation can be explained assuming that, as occurring in multifunctional enzymes with different active site domains,[31] the consecutive reactions occur without dissociation of each product into the bulk solvent.[32] In that case, the palmitate substrate would adopt a transoid (*Z*,*Z*)-diene producing conformation (**i**, Figure 6A), in which the hydrogen atoms lost from C11/C12 and C13/C14 have opposite configurations (**ii**,**iii**, Figure 6A). Because the Δ^{11} desaturation occurs with removal of the two *pro*-(*R*) hydrogen atoms from C11 and C12,[18] then the two hydrogen atoms lost from C13 and C14 must have a *pro*-(*S*) configuration.

In the enyne case (Figure 6B), rotation of the hexadecenoate substrate (**ii** → **iv**, Figure 6B) is required to locate the two sp^2 hydrogens in the appropriate spatial arrangement for elimination, after which the resulting acetylene conformer that best fits into the enzyme active site for Δ^{13} desaturation is that having the two *pro*-(*R*) C13−H and C14−H facing forward (**v**, Figure 6B). This conformer is similar to that initially adopted by palmitate (**i**, Figure 6A). In the acetylene case, the presence of the triple bond shortens the distance between C13 and the carboxylate end and makes C13 accessible to the oxidizing site.

Conclusions

In summary, we have extended the chemoenzymatic strategy previously reported to obtain enantiomerically enriched deu-

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terated palmitic acids with a tetradeuterium tag and use them as probes to decipher the stereochemistry of the Δ^{13} desaturation involved in the sex pheromone biosynthetic pathway of the processionary moth. The key step in the synthesis of probes is the kinetic lipase-catalyzed resolution of racemic mixtures of secondary propargylic alcohols. The presence of the acetylenic triple bond not only simplifies the absolute configuration determination of the resolved alcohols but also improves the lipase-mediated resolution of the alcohols assayed. Using the pentadeuterated palmitic acids thus prepared, we have demonstrated that the stereochemical course of the (*Z*)-13 desaturation is substrate-dependent, as the removal of hydrogen atoms in 11-hexadecynoate and (*Z*)-11-hexadecenoate are *pro*-(*R*)- and *pro*-(*S*)-specific *syn*-dehydrogenation processes, respectively.

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Supporting Information Available: General experimental section and copies of ^{1}H and ^{13}C NMR and DEPT spectra for compounds **2**−**4** and **6**−**12**. NMR spectra of (*R*)-MPA derivatives of compounds **5** and **7** used to determine the absolute configuration. This material is available free of charge via the Internet at http://pubs.acs.org.

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