2.00 g). The two solutions were mixed and stirred thoroughly. The gel, with a composition $0.29\,Na_2O:0.30\,K_2O:1.0\,SiO_2:0.054\,Ce_2O_3:34.8\,H_2O$, was placed in an autoclave for eight days at 230°C. The crystalline product was filtered, washed with distilled water, and dried at ambient temperature. Bulk chemical analysis (ICP) yielded an Si/Ce/(Na + K) ratio of 8.6:1.0:2.3. The Si/Ce molar ratio of 8.6:1 is slightly larger than expected assuming a montregianite-type structure for which Si/Ce = 8:1. This is partially due to the presence of some amorphous siliceous phase. Indeed, the baseline of the ^{29}Si MAS NMR spectrum is slightly raised, although (due to the horizontal expansion) this is not clearly seen in Figure 3a. In addition, powder X-ray diffraction exhibits faint reflections from unknown impurities, while scanning electron microscopy (SEM) shows the presence of some poorly defined particles of very small size. The (Na + K)/Ce ratio of 2.3:1 is between 2 and 3, as expected for samples containing only Ce^{IV} or Ce^{III}, respectively. The K/Na ratio is 0.4:1.

Although small (40 μm at best) some of the AV-5 crystals seemed to be of good quality. It was, however, not possible to solve the structure by single-crystal methods. The sample was ground prior to data collection for Rietveld refinement. Powder XRD data were collected on a X'Pert MPD Philipps difractometer ($Cu_{K\alpha}$ radiation) with a curved graphite monochromator, constant slit, and a flat plate sample holder, in a Bragg-Brentano para-focusing optics configuration. Different grinding times were used and several data sets obtained. The unit cell parameters were first obtained with program TREOR90.[15] The Rietveld refinement was carried out with program Fullprof,[16] in the space group $P2_1/n$, range 5–100 2θ, using the atomic coordinates of montregianite and assuming full Ce for Y substitution. The background, peak shapes, and cell parameters were refined. The refinement reliability factors were relatively poor ($R_p = 0.138$, $R_{wp} = 0.177$), probably due to the presence of unknown impurities and to orientation effects.

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Is Hydrogen Tunneling Involved in AcylCoA Desaturase Reactions? The Case of a Δ^9 Desaturase That Transforms (*E*)-11-Tetradecenoic Acid into (*Z*,*E*)-9,11-Tetradecadienoic Acid**

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Introduction of double bonds into the unactivated aliphatic chains of fatty acids is one of the most remarkable transformations in living organisms. This reaction is catalyzed by specific desaturases, which are oxygen-dependent enzymes containing a non-heme diiron cluster at their active site.[1] The mechanism of enzymatic desaturation of fatty acids is a subject of currently active research. It has been proposed[2–5] that desaturation reactions begin with an initial hydrogen atom abstraction step by a hypervalent iron–oxo species to generate a very short lived intermediate, that is then transformed into the olefin via a one-electron oxidation/deprotonation or simple disproportion process. Large activation energies are required to cleave the stable C–H bonds in the substrates as the first step of desaturation. How the enzyme manages to perform such a difficult reaction is an interesting question and the contribution of hydrogen tunneling (H-tunneling) is an attractive possibility. It has been proposed[2, 3] that enzymatic desaturation and hydroxylation of unactivated aliphatic chains do share common mechanistic features and H-tunneling has been invoked in the oxidation of linoleic acid catalyzed by soybean lipoxygenase.[6] H-tunneling is defined as a phenomenon by which an atom transfers through a reaction barrier as a result of its wave-like properties.[7] Reports that show H-tunneling in enzymes are relatively recent.[7–9] This is an important event in enzyme catalysis, since it contributes to circumvent the thermodynamic difficulties associated with classical descriptions of C–H bond cleavage.

To investigate the putative effect of hydrogen tunneling in fatty acid desaturase reactions, we report herein on a mechanistic study on one of these catalysts, namely the Δ^9 desaturase of (*E*)-11-tetradecenoic acid,[10] using the moth *Spodoptera littoralis* as biological model.

To determine the cryptoregiochemistry or initial oxidation site of the reaction, the same methodology previously validated for other desaturases[3–5, 11] was followed. This procedure is based on the calculation of intermolecular, primary kinetic isotope effects (KIE) in competitive experi-

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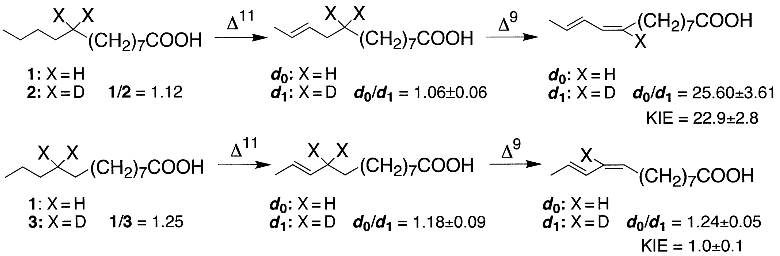
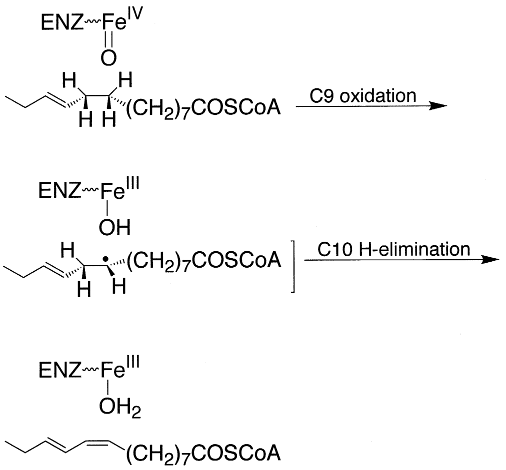


Scheme 1. Transformation of nonlabeled (**1**) and labeled (**2**, **3**) tridecanoic acids into nonlabeled (**d$_0$**) and labeled (**d$_1$**) (*E*)-11-tridecenoic acids and (*Z*,*E*)-9,11-tridecadienoic acids in the competitive experiments for KIE determination. Ratios between nonlabeled and labeled substrates in the administered mixture and ratios between nonlabeled and labeled metabolites are shown under the chemical structures. For the substrates, the ratio corresponds to a single determination with a $BF_3 \cdot MeOH$-derivatized sample of the used mixture. For the metabolites, ratios correspond to mean ± SD of seven or eight replicates. Δ^{11} means Δ^{11} desaturase and Δ^9 means Δ^9 desaturase. Product KIEs were calculated as described in the Experimental Section.



Scheme 2. Proposed mechanism for the (*Z*)-9 desaturation of (*E*)-11-tetradecanoic acid.

ments between a substrate and the same substrate dideuterated at either C9 or C10. In this case, tridecanoic acid (**1**), [9,9-D_2] tridecanoic acid (**2**), and [10,10-D_2] tridecanoic acid (**3**) were used as probes (Scheme 1). Although tridecanoic acid was not the actual enzyme substrate, the approach is valid because tridecanoic acid is transformed, after activation to the corresponding coenzyme A (CoA) ester, into (*E*)-11-tridecenoic acid[5] in the whole tissue assay. Furthermore, (*E*)-11 desaturation of tridecanoic acid **1** and of both the dideuterated probes, **2** and **3**, takes place at equal rates (Scheme 1). The base methanolyzed lipidic extracts, prepared after incubation of the moth pheromone sex glands with the probes, were analyzed by gas chromatography coupled to mass spectrometry under the selected ion monitoring mode (SIM-GC-MS). Integration of peaks corresponding to the nonlabeled and monodeuterated methyl (*Z*,*E*)-9,11-tridecadienoates formed from each mixture afforded the data required to determine the KIEs and, subsequently, the site of initial oxidation in the desaturation reaction.

A very large isotope effect was observed for the carbon–hydrogen bond cleavage at C9 (KIE (25 °C) = 22.9 ± 2.8 [mean ± standard deviation], $n = 8$), but no isotope discrimination occurred in the H abstraction at C10 (KIE (25 °C) = 1.0 ± 0.1, $n = 7$). These results indicated that abstraction of C9–H is rate limiting in the desaturation reaction, in accordance to those previously reported for the (*Z*)-9 stearoyl CoA desaturase,[3] (*Z*)-12 oleoyl CoA desaturase[4] and Δ^{11} myristoyl CoA desaturase.[5] In all of these cases, large KIEs were found for the abstraction of the hydrogen atom located at the carbon atom nearer to the carboxyl group, whereas negligible isotope effects were obtained for the removal of the other hydrogen atom to form the double bond. In agreement with the model proposed by Buist et al.,[2, 3] the Δ^9 desaturation studied here occurs by an initial rate-limiting abstraction of C9–H[12] to give a carbon-centered radical intermediate, which affords the final olefin by fast elimination of C10–H[12] (Scheme 2). This result contrasts with what would have been expected from the chemical point of view: That cleavage of the allylic C10–H bond occurred more easily than that of the homoallylic C9–H. Proximity of C9 to the oxidizing iron-oxo species at the enzyme active site[2, 3] can account for initial oxidation of atom C9. On the other hand, formation of the homoallylic radical intermediate would avoid putative rearrangements that could occur from the allylic species,[13] that result from initial C10–H bond cleavage and it does, therefore, contribute to the regiospecificity of the enzymatic reaction.

The inflated KIE found for the Δ^9 desaturase studied here is not unprecedented in the literature. Large KIEs have been reported for several enzymes, especially those cleaving C–H bonds.[14–16] Although inflated isotope effects are suggestive of H-tunneling, the temperature dependence of the reaction rates and KIEs have to be determined and the results must be compared with the behavior expected for tunneling regimes in an Arrhenius plot.[17] Therefore, to confirm the contribution of H-tunneling to the Δ^9 desaturase of (*E*)-11-tetradecenoic acid, competitive experiments with tridecanoic acid and **2** were also performed in vitro at different temperatures. As shown in Figure 1, the resulting Arrhenius plots afforded almost parallel lines for the nonlabeled and the deuterated sub-

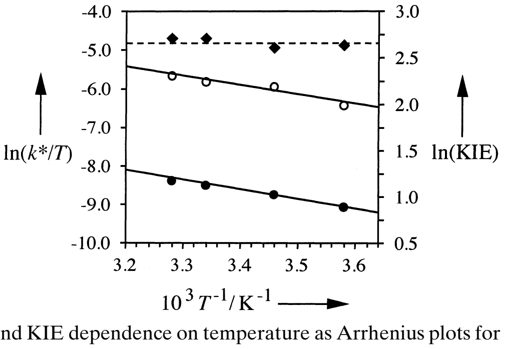


Figure 1. Rate and KIE dependence on temperature as Arrhenius plots for the Δ^9 desaturase of (*E*)-11-tetradecenoic acid. The measurements were performed in competitive experiments with tridecanoic acid and [9,9-D_2]tridecanoic acid as substrates. Data were collected under conditions of substrate saturation using a gland culture assay (see Experimental Section). Reaction rates (k^*) were defined as the relative amounts of nonlabeled (●) or labeled (○) diene with respect to the internal standard, methyl pentadecanoate. Each point was obtained from 6–8 replicates. Linear regression yields: $y = 1.58 - 2.22x$ (correlation coefficient $R^2 = 0.96$; nonlabeled diene) and $y = -0.60 - 2.37x$ ($R^2 = 0.98$; deuterated diene). Product KIEs (◆) were calculated as described in the Experimental Section.

strates, which indicates that the difference in activation enthalpies are near zero and that the KIEs are almost independent of temperature. Extrapolation of the data in Figure 1 to a zero reciprocal temperature leads to an isotope effect on the pre-exponential factor of the Arrhenius function, $A_\mathrm{H}/A_\mathrm{D} = 19.8 \pm 2.9$, which is markedly different from that expected for a classical transition-state barrier model ($A_\mathrm{H}/A_\mathrm{D} \approx 1$). These results are consistent with contribution of H-tunneling to the C9–H abstraction step.

Whether H-tunneling is a general event in desaturase reactions is now raised as an interesting question. In a recent review, Scrutton et al.[17] warned against the assumption that a measured KIE < 7 is indicative of classical hydrogen-atom transfer and emphasized that extensive tunneling could also occur in cases with low KIE values. Therefore, although KIEs in the range of 5.5–7.0 have been reported for other fatty acid desaturases thus far studied, it is possible that H-tunneling is a common mechanism of hydrogen-atom transfer in these oxidation reactions. Therefore, temperature dependence of the reaction rate and KIE should be determined in other desaturase enzymes to confirm that H-tunneling occurs. Further work along this line is in progress in our laboratory.

Experimental Section

The probes (9,9-$^2H_2$) tridecanoic acid (**2**) and (10,10-$^2H_2$) tridecanoic acid (**3**) were prepared following reported procedures.[5] The deuterium contents of the labeled substrates were determined by GC-MS analysis of their respective methyl esters and were found to be as follows: **2**: 89.0% 2 D, 8.7% 1 D, and 1.1% 0 D; **3**: 88.2.% 2 D, 8.7% 1 D, and 3.1% 0 D.

Cryptoregiochemistry was determined following the previously reported procedure.[5]

In vitro temperature dependence of reaction rates and KIE values were determined using a gland culture procedure. These experiments were carried out using round bottom, 96-well plates. To each well, a 5 μL drop of incubation medium was added and the plates were placed in an incubator at the desired temperature (6, 15, 23 and 30 °C). The incubation medium consisted of Grace's saline (135 μL) and a 1:1 mixture of tridecanoic acid and **2** (10 mg mL^{-1} each) in a dimethyl sulfoxide solution (15 μL). One-day-old virgin *S. littoralis* females, reared as reported elsewhere,[10] were briefly anesthetized on ice and the pheromone glands were excised, carefully cleaned, and immersed into a drop of the incubation medium at the given temperatures. Plates were sealed with adherent plastic covers and the incubation proceeded for 3 h. After this time, pheromone glands were collected and soaked in chloroform/methanol (2/1) at 25 °C for 1 h. The lipidic extracts thus obtained were base methanolyzed as described elsewhere[10] to obtain the fatty acid methyl esters. Methyl pentadecanoate was added as internal standard for quantification.

The extracts were analyzed by GC-MS at 70 eV on a Fisons gas chromatograph (8000 series) coupled to a Fisons MD-800 mass selective detector. The system was equipped with a nonpolar Hewlett Packard HP-1 capillary column (30 m × 0.20 mm i.d.) using the following temperature program: After an initial delay of 2 min at 120 °C, the temperature increased from 120 °C to 180 °C at 5 K min^{-1} and then to 260 °C at 2 K min^{-1}. Analyses were carried out under selected ion monitoring (SIM) mode. Selected ions were 223, 224, 225, 226, 227, 228, 229, 230, and 231. The dwell was set at 0.02 and mass span at 0.5.

KIEs were calculated from the ratios of product formed from unlabeled substrate and that from the deuterated analogs, and were based on the abundance of the respective molecular ions of the various isotopomers of methyl (*Z,E*) tridecadienoate ($\mathbf{d_0}$ 224, $\mathbf{d_1}$ 225). Isotope effects were corrected for the exact proportion of unlabeled and labeled substrates administered, which was determined by GC-MS analysis of a $BF_3 \cdot MeOH$ derivatized sample of the applied mixture. Corrections were also made for incomplete deuterium incorporation in the substrates and for the natural abundance of carbon and oxygen isotopes in the ions monitored. These latter values were obtained from the GC-MS chromatograms of gland extracts incubated with the individual substrates.

Reaction rates (k^*) were defined as the relative amounts of nonlabeled or labeled diene formed with respect to the internal standard.

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Molecular Recognition with Introverted Functionality**

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Both chemical and physical forces play roles in molecular recognition. Functional group complementarity influences selectivity and affinity in chemical recognition, and these interactions can be arranged using synthetic receptors with open, cleftlike structures. For receptors that completely

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