Reaction of Cbz-Gly-Gly-Phe and ClCPL with both CPA-WT and CPA-Phe-248, as reflected in the k_{cat}/K_m vs. pH profile, is controlled by a base with $pK_{EH_2} = 6.2 \pm 0.3$ and by an acid with $pK_{EH} = 9.4 \pm 0.2$. These ionizations are similar to those observed for the hydrolysis of tripeptides[21] and cinnamate esters[4,23] as catalyzed by bovine CPA. Nitration of the bovine enzyme,[5] however, has been shown to alter significantly the shape of the pH dependence of the k_{cat}/K_m parameter for Cbz-Gly-Gly-Phe hydrolysis without changing the limiting value of this rate constant. This chemical modification is reasonably specific for Tyr-248[33] and causes the pK_{EH} for the basic limb in the pH profile to shift from a value of 9.0 for the native enzyme to 6.60 ± 0.17. The latter value correlates well with the spectroscopic pK_a assigned to the nitro-Tyr-248 residue,[5,26] and these findings have been frequently cited as evidence in support of the assignment of Tyr-248 as the ionizing group responsible for the inflection at pH 9 in the k_{cat}/K_m profile for peptide hydrolysis by the native enzyme.[2,5] In contrast, the decrease in esterase activity above pH 8.5 has been shown[4] not to be due to ionization of Tyr-248, since the basic limb of the pH-rate profile for ClCPL hydrolysis is not shifted subsequent to nitration of Tyr-248. Our results now demonstrate unequivocally that ionization of this residue cannot be responsible for the decrease in peptidase activity seen for CPA-WT above pH 8.5 either. Chemical modification of Tyr-248 apparently perturbs the system significantly, possibly by favoring the binding of the phenolate form of the modified Tyr-248 as a ligand to the active-site zinc ion.[34,35]

Elimination of the possibility that the ionizing group with pK_a at about 9 is Tyr-248 leaves open the question of the identity of the functional group responsible for this ionization. The most obvious candidates based on structural information are the zinc-water complex and an arginine residue involved in binding. We believe the assignment of this pK to the metal-bound water molecule is more probable. Chelation of arsanilazo-Tyr-248 to the active-site zinc ion of bovine CPA has been shown to be controlled by ionizations at pH 7.7 and 9.5.[25,27] The former correlates well with the spectroscopically determined value of the pK_a of the modified Tyr-248, while the latter has been argued[4] to be due to ionization of the zinc-bound water. It seems unlikely that the pK_a of an Arg residue would be lowered to the extent necessary to account for the observed ionization.

In conclusion, site-directed mutagenesis of Tyr-248 in CPA, in conjunction with measurements comparing the rates and pH dependencies for the catalytic action of the native and mutant enzymes, has demonstrated that the ionization state of the phenolic hydroxyl of the Tyr residue cannot be crucial for catalysis by CPA. This finding rules out the mechanistic proposals that have invoked Tyr-248 as an essential general acid or as a general base in the hydrolysis of amides and esters. Through related studies of other residues in the binding and catalytic sites of CPA, we hope to probe further the precise roles of functional groups in this hydrolytic enzyme.

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Registry No. CPA, 11075-17-5; ClCPL, 61556-61-4; L-Tyr, 60-18-4; L-Phe, 63-91-2; Cbz-Gly-Gly-Phe, 13171-93-2; Bz-Gly-OPhe, 3675-74-9.

(33) Muszynska, G.; Riordan, J. F. *Biochemistry* **1976**, *15*, 46-51.
(34) Riordan, J. F.; Muszynska, G. *Biochem. Biophys. Res. Commun.* **1974**, *57*, 447-451.
(35) Johansen, J. T.; Vallee, B. L. *Biochemistry* **1975**, *14*, 649-659.

A Thorough Study of the Stereochemical Consequences of the Hydration/Dehydration Reaction Catalyzed by β-Hydroxydecanoyl Thioester Dehydrase

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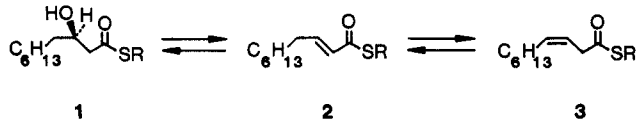
Abstract: β-Hydroxydecanoyl thioester dehydrase is the pivotal enzyme in the biosynthesis of unsaturated fatty acids under anaerobic conditions, catalyzing the equilibration of thioesters of (*R*)-3-hydroxydecanoic acid, (*E*)-2-decenoic acid, and (*Z*)-3-decenoic acid. Substrates chirally labeled with deuterium have been synthesized and incubated with dehydrase. Analysis of labeled products by ^2H NMR spectroscopy has shown that the *pro*-2*S* hydrogen is removed in the course of the dehydration reaction, which is therefore a syn elimination. In the complementary experiment, the *N*-acetylcysteamine thioesters of (*E*)-2-[2-^2H]decenoic acid and unlabeled (*E*)-2-decenoic acid were hydrated by dehydrase in ^1H_2O- and ^2H_2O-based buffers, respectively. Analysis of the resulting products by ^1H NMR spectroscopy demonstrated that the hydration is a synfacial process, with addition of the elements of water to the *si* face of the C-2/C-3 double bond.

β-Hydroxydecanoyl thioester dehydrase,[1] the crucial enzyme in the biosynthesis of unsaturated fatty acids under anaerobic conditions, catalyzes the equilibration of thioesters of (*R*)-3-hydroxydecanoic acid (**1**), (*E*)-2-decenoic acid (**2**), and (*Z*)-3-decenoic acid (**3**). In vivo, acyl carrier protein (ACP) thioesters are utilized, although other thiol moieties, including *N*-acetylcysteamine ($HSCH_2CH_2NHAc$; NAC), function acceptably in vitro.[2]



1 **2** **3**

Herein, we report the results of experiments that have defined the dehydrase-catalyzed hydration-dehydration as a syn addition-elimination, a finding that is mechanistically consistent with the stereochemical course of the allylic rearrangement, **2** to **3**, as reported previously.[3]

(1) Bloch, K. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic: New York, 1971; Vol. 5, pp 441-464.
(2) Brock, D. J. H.; Kass, L. R.; Bloch, K. *J. Biol. Chem.* **1967**, *242*, 4432-4440.

0002-7863/86/1508-5304$01.50/0

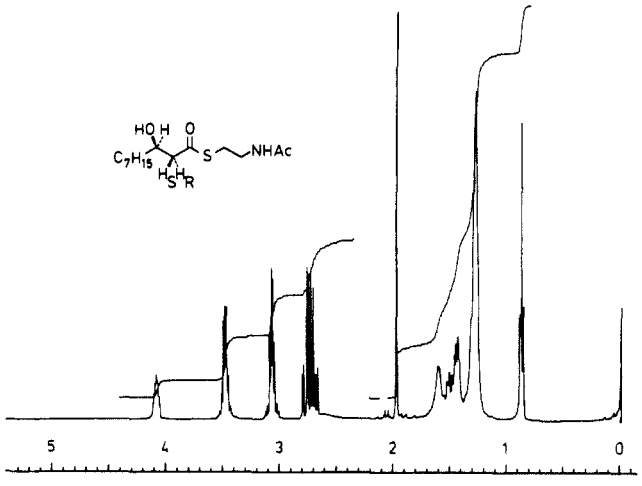


Figure 1. ^1H NMR spectrum (400 MHz) of (*R,S*)-(3-hydroxydecanoyl)-NAC.

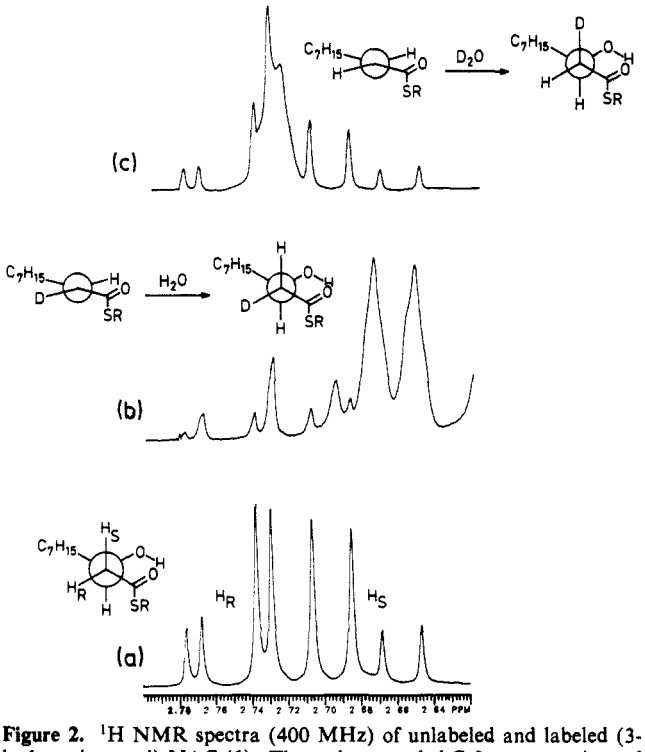


Figure 2. ^1H NMR spectra (400 MHz) of unlabeled and labeled (3-hydroxydecanoyl)-NAC (**1**). The scale-expanded C-2 proton regions of (a) unlabeled (*R,S*)-**1**, (b) (2*R*,3*R*)-**1**, from enzyme-catalyzed hydration of **2** in ^1H_2O, and (c) (2*S*,3*R*)-**1**, from enzyme-catalyzed hydration of unlabeled (*E*)-2-decanoyl-NAC (**2**) in ^2H_2O.

Results

Since only the *R* enantiomer of (3-hydroxydecanoyl)-NAC is a substrate for dehydrase,[1] it was necessary to determine only whether the *pro*-2*R* or *pro*-2*S* hydrogen was being labilized by the enzyme. This result was obtained for both the hydration and the dehydration reaction.

Hydration of (*E*)-2-Decenoyl-NAC. Figure 1 portrays the 400-MHz ^1H NMR spectrum of unlabeled (3-hydroxydecanoyl)-NAC. As seen in the expanded spectrum (Figure 2a), the C-2 proton signals clearly form the AB portion of an ABX system. Analysis shows that H_A and H_B resonate at 2.75 ppm (J_{AX} = 3.3 Hz) and 2.68 ppm (J_{BX} = 8.6 Hz), respectively, with J_{AB} = 15.4 Hz. Assuming that the preferred conformation of (3-hydroxydecanoyl)-NAC is governed by hydrogen bonding between the hydroxyl group and the carbonyl oxygen (with the heptyl sustituent in a quasi-equatorial orientation), absolute signal assignments can be made. When the vicinal couplings are considered,[4] the higher and lower field C-2 resonances can be assigned to the *pro*-2*S* and *pro*-2*R* protons, respectively. This forms the basis for analysis of (3-hydroxydecanoyl)-NAC chirally labeled with deuterium at C-2.

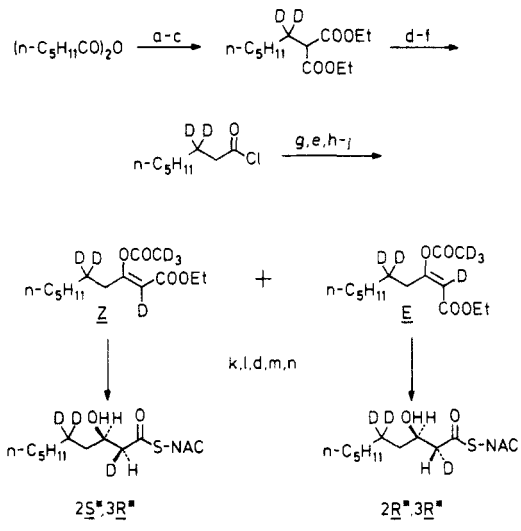
(*E*)-2-[2-^2H]Decenoyl-NAC was synthesized as described previously[3] and was incubated with a partially purified preparation of dehydrase. (3-Hydroxydecanoyl)-NAC thus obtained was examined by high-field NMR spectroscopy, as shown in Figure 2b. The most noticeable feature of this spectrum is the broadened doublet (J = 8.2 Hz) centered at 2.66 ppm. This resonance can be assigned to the *pro*-2*S* proton, the *pro*-2*R* position being occupied by a deuterium atom.

(3*R*)-(3-[2-$^2H_1$]Hydroxydecanoyl)-NAC was recovered from the incubation of unlabeled (*E*)-2-decenoyl-NAC with dehydrase in ^2H_2O,[3,5] and its NMR spectrum is reproduced in Figure 2c. Aside from the peaks readily attributable to the C-2 protons of unlabeled (3-hydroxydecanoyl)-NAC, the most prominent resonance is the somewhat distorted doublet (J = 2.4 Hz) at 2.73 ppm. (The distortion apparently owes to the exact overlap of the lower field component of the doublet with the fourth line of the AB multiplet.) This signal stems from the *pro*-2*R* proton, and this time the *pro*-2*S* position is substituted with deuterium.

Clearly, these results are complementary and indicate that, in each case, protonation has occurred on the *si* face at C-2 of (*E*)-2-decenoyl-NAC. The coupling patterns observed are readily interpretable, as in each case the vicinal coupling is retained, while the geminal H–D coupling is indistinct. (Hydrogen–deuterium coupling constants are only 1/6.5 of the corresponding H–H values, i.e., 15.4/6.5 = 2.4 Hz.) The small discrepancies in the chemical shifts of the C-2 protons of the labeled vs. the unlabeled hydroxy thioester samples are readily explained in terms of the well-known isotopic shift.[6]

Dehydration of (3-Hydroxydecanoyl)-NAC. (2*R**,3*R**)- and (2*S**,3*R**)-(3-[2,5,5-$^2H_3$]hydroxydecanoyl)-NAC were made by the route shown in Scheme I, which is adapted from Rozzell's synthesis of chirally labeled 3-hydroxybutyrate.[7,8] A useful

Scheme I. Synthesis of (2*R**,3*R**)- and (2*S**,3*R**)-(3-[2,5,5-$^2H_3$]Hydroxydecanoyl)-NAC[a]



[a] Key: (a) $LiAlD_4$; (b) *p*-TsCl, pyridine; (c) $CH_2(CO_2Et)_2$, NaOEt; (d) OH^-; (e) H_3O^+; (f) $SOCl_2$; (g) Meldrum's acid, pyridine; (h) EtOH, heat; (i) D_2O, THF; (j) CD_3COCl, pyridine; (k) flash chromatography; (l) H_2, Rh/C (5%); (m) $(EtO)_2POCl$, Et_3N; (n) $TlSCH_2CH_2NHAc$.

(4) House, H. O.; Crumrine, D. S.; Teranishi, A. Y.; Olmstead, H. D. *J. Am. Chem. Soc.* **1973**, *95*, 3310–3324.
(5) Schwab, J. M.; Klassen, J. B. *J. Chem. Soc., Chem. Commun.* **1984**, 298–299.
(6) Abraham, R. J. *The Analysis of High Resolution NMR Spectra*; Elsevier: Amsterdam, 1971; pp 58–83.

(3) Schwab, J. M.; Klassen, J. B. *J. Am. Chem. Soc.* **1984**, *106*, 7217–7227.

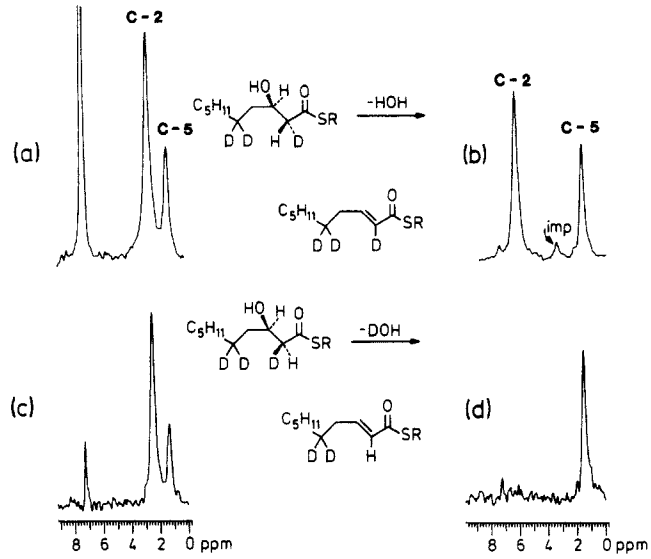


Figure 3. ^2H NMR spectra (13.71 MHz) of chirally labeled (3-hydroxydecanoyl)-NAC (**1**) samples and the dehydration product, (*E*)-2-decenoyl-NAC (**2**), derived from dehydrase-catalyzed dehydration of each substrate sample: (a) (2*R**,3*R**)-[2,5,5-$^2H_3$]-**1**; (b) [2,5,5-$^2H_3$]-**2**, derived from (2*R**,3*R**)-[2,5,5-$^2H_3$]-**1**; (c) (2*S**,3*R**)-[2,5,5-$^2H_3$]-**1**; (d) [5,5-$^2H_2$]-**2**, derived from (2*S**,3*R**)-[2,5,5-$^2H_3$]-**1**.

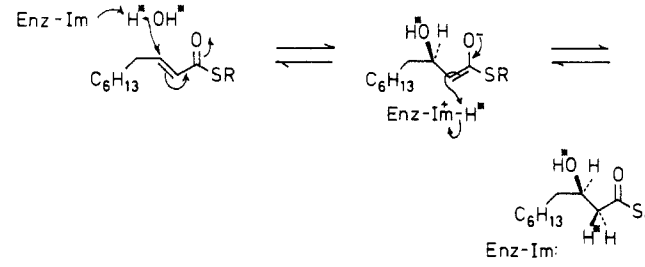
innovation, however, is the incorporation of a reference deuterium label at C-5, a position that is uninvolved in dehydrase-catalyzed reactions.

[3,3-$^2H_2$]Octanoic acid, made from 1-[2,2-$^2H_2$]hexyl tosylate via the malonic ester synthesis, was converted to the corresponding labeled acid chloride. The latter material was then diluted with a greater than threefold excess of unlabeled octanoyl chloride. The purpose of the dilution was to make sure that the label of interest [at C-2 of (3-hydroxydecanoyl)-NAC] would be present in substantial excess, relative to the reference label. Thus, loss or retention of label at C-2 would be especially easy to assess. Use[9] of the acid chloride for acylation of Meldrum's acid gave ethyl 3-oxo-[5,5-$^2H_2$]decanoate, which was subsequently labeled at C-2 by exchange with ^2H_2O.

Formation of labeled enol acetates[10] was complicated by incomplete retention of label at C-2. Indeed, ^2H NMR spectra of the enol acetates indicated that a substantial amount of deuterium had been incorporated into the acetate methyl groups. To circumvent this difficulty, benzoyl chloride was substituted for acetyl chloride; however, it was found that, under the conditions required for hydrolysis of the benzoates, label was once again lost from decanoyl C-2. An expedient (albeit extravagant) solution was the use of [$^2H_3$]acetyl chloride for the enol ester synthesis.

The enol acetates were readily separated by column chromatography and their double-bond configurations revealed by proton NMR spectroscopy. The allylic C-4 protons of these compounds resonated at 2.81 and 2.26 ppm, and on the basis of the deshielding effect of a *cis*-alkoxycarbonyl group,[11,12] these were assigned the *E* and *Z* configurations, respectively. Mohrig et al. have used similar logic to assign configurations to enol benzoates of methyl acetoacetate.[13] In addition, the allylic $^4J_{H-H}$ values (0 Hz for the *E* isomer and a small but decidedly non-zero value for the *Z*) are consistent with the assignments.[14-16]

Scheme II



Chirally labeled 3-hydroxydecanoic acid samples were readily synthesized as described by Rozzell and Benner.[7,8] Thus, hydrogenation of each labeled enol acetate diastereomer gave a mixture of enantiomeric diesters, which were saponified with dilute methanolic NaOH. Proton NMR spectra indicated that the racemates were diastereomerically pure, with C-2/C-3 proton–proton coupling constants that were entirely consistent with the configurational assignments shown.

The published method for thioesterification of 3-hydroxydecanoic acid is reported to lead to exchange at C-2.[17] Although the yields were poor, NAC thioesters were made by the method of Masamune et al.[18] Proton NMR spectroscopy proved that the integrity of the label had been maintained.

Each racemate, (2*R**,3*R**)- and (2*S**,3*R**)-(3-[2,5,5-$^2H_3$]-hydroxydecanoyl)-NAC, was incubated with dehydrase as previously,[3] and (*E*)-2-decenoyl-NAC was isolated. ^2H NMR spectra of the substrates and the derived products are reproduced in Figure 3. The spectrum of each substrate sample shows a preponderance of deuterium at C-2, relative to the reference label at C-5. Very clearly, (*E*)-2-decenoyl-NAC derived from the (2*R*,3*R*) substrate has retained most of the label at C-2 (now a vinyl deuteron), while the unsaturated thioester from the (2*S*,3*R*) substrate bears no deuterium at C-2.

Discussion

Separate experiments have now shown that *the dehydration and hydration reactions catalyzed by β-hydroxydecanoyl thioester dehydrase from Escherichia coli are synfacial processes.* This result provides a unified view of the "normal" reactions catalyzed by dehydrase, as it had been previously shown[3] that the allylic rearrangement of (*E*)-2-decenoyl-NAC to (*Z*)-3-decenoyl-NAC proceeds suprafacially.

The present finding is also stereochemically consistent with what is known about the mechanism of the "suicide" inactivation of dehydrase.[2,19] The configuration of the allenic inactivator of dehydrase (2,3-decadienoyl-NAC) produced by the enzyme from the "suicide" substrate 3-decynoyl-NAC is *S*.[20] This outcome was predicted[3] on the basis of the assumption that the propargylic rearrangement is mediated by the same active-site base as is the normal allylic rearrangement.

It now appears that *all* of the dehydrase-catalyzed reactions utilize a unique active-site base, undoubtedly a histidine residue.[21,22] The role of this base in the allylic rearrangement has already been discussed.[3] In the hydration reaction (Scheme II), the histidine residue presumably functions as a general base, enhancing nucleophilic attack by water on C-3 of (*E*)-2-decenoyl-NAC. The resulting imidazolium ion supplies the water-derived proton to C-2 of the intermediate. The dehydration occurs, of course, in

(7) Rozzell, J. D. *Tetrahedron Lett.* **1982**, *23*, 1767–1770.
(8) Rozzell, J. D., Jr.; Benner, S. A. *J. Am. Chem. Soc.* **1984**, *106*, 4937–4941.
(9) Oikawa, Y.; Sugano, K.; Yonemitsu, O. *J. Org. Chem.* **1978**, *43*, 2087–2088.
(10) Casey, C. P.; Marten, D. F. *Tetrahedron Lett.* **1974**, 925–928.
(11) Jackman, L. M.; Wiley, R. H. *J. Chem. Soc.* **1960**, 2886–2890.
(12) House, H. O.; Kramar, V. *J. Org. Chem.* **1963**, *28*, 3362–3379.
(13) Mohrig, J. R.; Dabora, S. L.; Foster, T. F.; Schultz, S. C. *J. Org. Chem.* **1984**, *49*, 5179–5182.
(14) Whipple, E. B.; Goldstein, J. H.; Mandel, L. *J. Am. Chem. Soc.* **1960**, *82*, 3010–3013.
(15) Whipple, E. B.; Goldstein, J. H.; McClure, G. R. *J. Am. Chem. Soc.* **1960**, *82*, 3811–3813.
(16) Jackman, L. M.; Wiley, R. H. *J. Chem. Soc.* **1960**, 2881–2886.
(17) Rando, R. R.; Bloch, K. *J. Biol. Chem.* **1968**, *243*, 5627–5634.
(18) Masamune, S.; Kamuta, S.; Diakur, J.; Sugihara, Y.; Bates, G. S. *Can. J. Chem.* **1975**, *53*, 3693–3695.
(19) Kass, L. R.; Bloch, K. *Proc. Natl. Acad. Sci. U.S.A.* **1967**, *58*, 1168–1173.
(20) Schwab, J. M.; Lin, D. C. T.; He, C.-h.; Clardy, J. *Tetrahedron Lett.* **1984**, *25*, 4909–4912.
(21) Helmkamp, G. M., Jr.; Bloch, K. *J. Biol. Chem.* **1969**, *244*, 6014–6022.
(22) Schwab, J. M.; Li, W.-b.; Ho, C.-K.; Townsend, C. A.; Salituro, G. M. *J. Am. Chem. Soc.* **1984**, *106*, 7293–7294.

exactly the reverse manner, with histidine-mediated C-2 deprotonation followed by expulsion of hydroxide from substrate C-3. Carbon–oxygen bond cleavage is facilitated by donation of a proton from the protonated histidine.

Finally, it is worth noting that there are numerous examples of both syn[23-30] and anti[31-40] enzyme-catalyzed dehydration/hydration reactions. The evolutionary and mechanistic factors[8,41] that have led to this dichotomy remain unknown.

Experimental Section

A description of the general experimental conditions has been published.[3] For the present studies, ^2H and routine ^1H NMR spectra were run on a JEOL FX-90Q, and high-field ^1H spectra were obtained on a Varian XL-400.

1-[1,1-$^2H_2$]Hexanol. Hexanoic anhydride (4.99 g, 23.3 mmol) in 25 mL of Et_2O was treated with $LiAlH_4$ (1.47 g, 35 mmol). Following overnight reflux and a standard workup, 4.62 g (95%) of labeled hexanol was obtained. The product was shown by GC to be 98% pure. ^1H NMR ($CDCl_3$): δ 0.89 (t, J = 6 Hz, 3 H, CH_3), 1.2–1.7 (m, 8 H, CH_2), 2.6–2.8 (br s, 1 H, OH). ^2H NMR (CCl_4): δ 3.4 (s, CD_2OH). IR: 3601, 3451, 2911, 2846, 2180, 2080, 1605, 1461, 1376, 1286, 1186, 1146, 1116, 1073, 946, 891 cm^{-1}.

1-[1,1-$^2H_2$]Hexyl *p*-Toluenesulfonate. 1-[1,1-$^2H_2$]Hexanol (4.62 g, 44.3 mmol) was reacted with *p*-toluenesulfonyl chloride (13.2 g, 69.3 mmol) in pyridine (50 mL). Workup provided 11.1 g (43 mmol, 97%) of product as a yellowish oil. ^1H NMR: δ 0.84 (t, J = 5.5 Hz, 3 H, CH_3), 1.1–1.5 (m, 6 H, CH_2), 1.5–1.7 (m, 2 H, CH_2CD_2), 2.44 (s, 3 H, $ArCH_3$), 7.3–7.8 (AB q, J = 8 Hz, 4 H, ArH). ^2H NMR (CCl_4): δ 3.85 (s, CD_2OTs). IR: 2911, 2846, 1601, 1463, 1356, 1168, 1091, 948 cm^{-1}.

1-[1,1-$^2H_2$]Hexylmalonic Acid, Diethyl Ester. 1-[1,1-$^2H_2$]Hexyl *p*-toluenesulfonate (11.0 g, 42.3 mmol) was dissolved in 30 mL of absolute EtOH and added dropwise to a stirred solution made from 1.08 g of sodium (47 mmol) and 7.2 g (45 mmol) of diethyl malonate in 75 mL of absolute EtOH. The reaction mixture was heated at reflux for 12 h, and after it had cooled, the EtOH was removed at reduced pressure. The residue was taken up in dilute HCl and extracted several times with Et_2O. The organics were washed with brine, dried, filtered, and concentrated, giving 10.0 g (92%) of product. ^1H NMR ($CDCl_3$): δ 0.88 (t, J = 5.5 Hz, 3 H, CH_3), 1.1–1.4 (m, 14 H, CH_2 and OCH_2CH_3), 3.3 [s, 1 H, $CD_2CH(COOEt)_2$], 4.2 (q, J = 7 Hz, 4 H, OCH_2CH_3). ^2H NMR (CCl_4): δ 1.72 [s, $CD_2CH(COOEt)_2$]. IR: 3023, 2966, 2931, 2866, 1755, 1735, 1471, 1376, 1329, 1305, 1158, 1118, 1098, 1031 cm^{-1}.

[3,3-$^2H_2$]Octanoic Acid. The foregoing labeled diester (10.0 g, 39.1 mmol) was saponified and decarboxylated by heating at reflux first with 50% aqueous KOH and then with 16 N H_2SO_4. Workup gave 5.09 g (89%) of a clear liquid, the GC of which showed a single major peak, with a retention time identical with that of authentic, unlabeled octanoic acid. ^1H NMR ($CDCl_3$): δ 0.88 (t, J = 5.5 Hz, 3 H, CH_3), 1.0–1.5 (m, 8 H, CH_2), 2.34 (s, 2 H, CD_2CH_2COO), 11.46 (s, 1 H, COOH). ^2H NMR (CCl_4): δ 1.58 (s, CD_2CH_2COOH). IR: 3011, 2931, 2921, 2851, 1716, 1465, 1411, 1371, 1250, 1030 cm^{-1}.

Ethyl 3-Oxo-[5,5-$^2H_2$]decanoate. [3,3-$^2H_2$]Octanoic acid (5.09 g, 34.8 mmol) was converted to the acid chloride by heating at reflux for 5 h in $SOCl_2$. Removal of excess reagent in vacuo gave 5.70 g of product that was used without further purification. The labeled acid chloride, along with 18.8 g of unlabeled octanoyl chloride, was added to an ice-cold solution of 2,2-dimethyl-1,3-dioxane-4,6-dione (Meldrum's acid) (22.1 g, 153.3 mmol) in CH_2Cl_2 (100 mL) and pyridine (25 mL). The deep red solution was allowed to warm to room temperature and stirred for 3 h and then partitioned between dilute HCl and CH_2Cl_2. Repeated extraction with CH_2Cl_2 gave an organic phase that was washed with water, dried, filtered, and concentrated. The dark red, viscous liquid residue was heated at reflux with 100 mL of absolute EtOH for 6 h, after which the volatiles were removed in vacuo. The residue, a dark red liquid, was purified by flash chromatography (6 in. × 50 mm; 1:16, EtOAc/petroleum ether), giving 30.1 g (95%) of the keto ester as a clear, colorless liquid. ^1H NMR (CCl_4): δ 0.86 (t, J = 5.5 Hz, 3 H, CH_3), 1.1–1.4 (m, 11 H, CH_2 and CH_3CH_2O), 1.4–1.7 (m, ca. 1.5 H, CH_2CH_2CO), 2.5 (t, J = 7 Hz, 2 H, CH_2CH_2CO), 3.4 (s, 2 H, $COCH_2COO$), 4.18 (q, J = 7 Hz, 2 H, CH_3CH_2O). ^2H NMR (CCl_4): δ 1.5 (s, CD_2CH_2CO). IR: 3010, 2916, 2848, 1737, 1716, 1465, 1406, 1368, 1301, 1211, 1091, 1016 cm^{-1}.

Ethyl 3-Oxo-[2,2,5,5-$^2H_4$]decanoate. Keto ester labeled at C-5 was stirred with a solution of 20 mL of ^2H_2O in 60 mL of THF for a period of 8 h, after which the THF was removed in vacuo, and the aqueous suspension was extracted with CH_2Cl_2. The solvent was removed, and the residue was treated three more times in this manner, until virtually complete exchange of the C-2 protons had been effected. All of the foregoing manipulations were performed in a glovebag, under N_2, in order to exclude atmospheric ^1H_2O. ^1H NMR ($CDCl_3$): δ 0.86 (t, J = 5.5 Hz, 3 H, CH_3), 1.1–1.4 (m, 11 H, CH_2 and CH_3CH_2O), 1.4–1.7 (m, ca. 1.5 H, CH_2CH_2CO), 2.5 (t, J = 7 Hz, 2 H, CH_2CH_2CO), 3.4 (s, 0.08 H, residual $COCH_2COO$), 4.18 (q, J = 7 Hz, 2 H, CH_3CH_2O).

(*E*)- and (*Z*)-3-[$^2H_3$]Acetoxy-2-[2,5,5-$^2H_3$]decenoic Acid, Ethyl Ester. [$^2H_3$]Acetyl chloride (18.3 g, 224 mmol) was added dropwise to a stirred, ice-cold solution of the labeled keto ester (30.0 g, 138.9 mmol) in 100 mL of dry pyridine. The reaction mixture, which soon solidified, was placed in a refrigerator for 18 h, after which time it was extracted five times with Et_2O. The combined Et_2O extracts were washed with dilute HCl and then with water before being dried, filtered, and concentrated. The residual enol acetate mixture, 25 g of an orange-red liquid, was subjected to flash chromatography (11 in. × 50 mm; 1:20, EtOAc/petroleum ether). The faster and slower enol acetate fractions corresponded to the *E* and *Z* isomers, respectively (as shown by ^1H NMR spectroscopy), and the recoveries were 9.5 g (26%) of *E* and 11.1 g (31%) of *Z*.

***E* Isomer.** ^1H NMR ($CDCl_3$) δ 0.88 (t, J = 5.5 Hz, 3 H, CH_3), 1.15–1.77 (m, ca. 13 H, CH_2 and CH_3CH_2O), 2.81 (t, J = 7.3 Hz, 2 H, CH_2CH_2CO=), 4.18 (q, J = 7 Hz, 2 H, CH_3CH_2O); ^2H NMR (CCl_4) δ 1.45 (s, 0.28 D, CD_2CH_2CO=), 2.1 (s, 3.0 D, CD_3CO), 5.59 (0.81 D, =CDCOO). IR: 2921, 2839, 1763, 1716, 1651, 1466, 1368, 1200, 1108, 1056, 896 cm^{-1}.

***Z* Isomer.** ^1H NMR ($CDCl_3$): δ 0.88 (t, J = 5.5 Hz, 3 H, CH_3), 1.1–1.7 (m, ca. 13 H, CH_2 and CH_3CH_2O), 2.26 (t, J = 7.3 Hz, 2 H, CH_2CH_2CO=), 4.13 (q, J = 7 Hz, 2 H, CH_3CH_2O). ^2H NMR (CCl_4): δ 1.45 (s, 0.28 D, CD_2CH_2CO=), 2.1 (s, 3.0 D, CD_3CO), 5.5 (s, 0.79 D, =CDCOO). IR: 2901, 2839, 1761, 1711, 1651, 1461, 1366, 1190, 1111, 1044, 898 cm^{-1}.

(2*S,3*R**)-3-[2,5,5-$^2H_3$]Hydroxydecanoic Acid** The labeled *Z* enol acetate was converted to (2*S**,3*R**)-3-[2-$^2H_1$]hydroxydecanoic acid by the method of Rozzell.[7,8] The enol acetate (4.92 g, 18.9 mmol) was hydrogenated in 750 mL of THF, with 2.25 g of 5% Rh/C as catalyst. The reaction mixture was stirred for 8 h, after which H_2 uptake ceased. It was then filtered through celite and the THF removed under reduced pressure, giving 4.48 g (90.5%) of the diester as a clear, colorless liquid. This diester was purified by flash chromatography (9 in. × 50 mm; 1:15, EtOAc/petroleum ether). ^1H NMR: δ 0.88 (t, J = 5.5 Hz, 3 H, CH_3), 1.1–1.45 (m, 13 H, CH_2 and OCH_2CH_3), 1.6 (d, J = 5.6 Hz, 2 H, CD_2CH_2CHOAc), 2.52 (d, J = 2.6 Hz, 1 H, CHDCOOEt), 4.1 (q, J = 7 Hz, 2 H, OCH_2CH_3), 5.21 (dt, J = 5.6, 5.6 Hz, 1 H, CHOAc). ^2H NMR (CCl_4): δ 1.25 (s, 0.33 D, CD_2CH_2CHOAc), 1.9 (s, 3.0 D, $CHOCOCD_3$), 2.3 (s, 0.86 D, CHDCOOEt). IR: 2901, 2834, 1723, 1456, 1361, 1200, 1066, 1011, 891 cm^{-1}.

The diester (574 mg, 2.21 mmol) was added to a solution of 870 mg (21.8 mmol) of NaOH in 870 mL of MeOH, and the resulting solution was stirred at room temperature for 5 h. Acidification and concentration gave a white solid, which was triturated with THF. The solvent was removed, and the residual solid was crystallized from EtOAc/pentane,

(23) Sedgwick, B.; Morris, C.; French, S. J. *J. Chem. Soc., Chem. Commun.* **1978**, 193–194.
(24) Messner, B.; Eggerer, H.; Cornforth, J. W.; Mallaby, R. *Eur. J. Biochem.* **1975**, *53*, 255–264.
(25) Anke, H.; Diekmann, H. *FEBS Lett.* **1971**, *17*, 115–117.
(26) Anke, H.; Diekmann, H. *Arch. Microbiol.* **1974**, *95*, 213–225.
(27) Scharf, K. H.; Zenk, M. H.; Onderka, D. K.; Carroll, M.; Floss, H. G. *J. Chem. Soc., Chem. Commun.* **1971**, 765–766.
(28) Avigad, G.; Englard, S. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1969**, *28*, 345.
(29) Hanson, K. R.; Rose, I. A. *Proc. Natl. Acad. Sci. U.S.A.* **1963**, *50*, 981–988.
(30) Aberhart, D. J.; Tann, C.-H. *J. Chem. Soc., Perkin Trans. 1* **1979**, 939–942.
(31) Englard, S. *J. Biol. Chem.* **1960**, *235*, 1510–1516.
(32) Englard, S.; Britten, J. S.; Listowsky, I. *J. Biol. Chem.* **1967**, *242*, 2255–2259.
(33) von der Mühll, P. A.; Settimj, G.; Weber, H.; Arigoni, D. *Chimia* **1965**, *19*, 595–596.
(34) von der Mühll, P. A. Ph.D. Thesis No. 4214, Eidgenössische Technische Hochschule, Zürich, 1968.
(35) Weber, H. Ph.D. Thesis No. 3591, Eidgenössische Technische Hochschule, Zürich, 1965.
(36) Arigoni, D.; Eliel, E. L. In *Topics in Stereochemistry*; Eliel, E. L., Allinger, N. L., Eds.; Wiley: New York, 1969; Vol. 4, pp 127–243.
(37) Cohn, M.; Pearson, J. E.; O'Connell, E. L.; Rose, I. A. *J. Am. Chem. Soc.* **1970**, *92*, 4095–4098.
(38) Cole, F. E.; Kalyanpur, M. G.; Stevens, C. M. *Biochemistry* **1973**, *12*, 3346–3350.
(39) Schroepfer, G. J., Jr. *J. Am. Chem. Soc.* **1965**, *87*, 1411–1412.
(40) Schroepfer, G. J., Jr. *J. Biol. Chem.* **1966**, *241*, 5441–5447.
(41) Rose, I. A.; Hanson, K. R. In *Applications of Biochemical Systems in Organic Chemistry*; Jones, J. B., Sih, C. J., Perlman, D., Eds.; Wiley: New York, 1976; pp 507–553.

giving 221 mg of the labeled hydroxy acid, mp 56–57 °C (lit.[42] mp 53–54 °C). ^{1}H NMR: δ 0.88 (t, J = 5.3 Hz, 3 H, CH_3), 1.0–1.6 (m, 12 H, CH_2), 2.51 (d, J = 1.7 Hz, 1 H, CHOHC*H*DCOOH), 3.9–4.15 (m, 1 H, C*H*OH), 5.2–6.0 (br s, 2 H, OH and COOH). ^{2}H NMR ($CHCl_3$): δ 1.38 (s, 0.48 D, CD_2CH_2CHOH), 2.51 (s, 1.0 D, CDHCOOH). IR: 3578, 3486, 3011, 2901, 2831, 1699, 1455, 1396, 1286, 1176 cm^{-1}.

(2*R,3*R**)-3-[2,5,5-$^2H_3$]Hydroxydecanoic Acid.** In the same manner as described above, 4.92 g (18.9 mmol) of the *E* enol acetate was hydrogenated, giving 4.41 g (89%) of the diester. ^{1}H NMR ($CDCl_3$): δ 0.88 (t, J = 5.5 Hz, 3 H, CH_3), 1.1–1.5 (m, 13 H, CH_2 and OCH_2CH_3), 1.6 (m, 2 H, CD_2CH_2CHOAc), 2.55 (d, J = 7.3 Hz, 1 H, C*H*DCOOEt), 4.14 (q, J = 7 Hz, 2 H, OCH_2CH_3), 5.22 (dt, J = 6, 6 Hz, 1 H, CHOAc). ^{2}H NMR (CCl_4): δ 1.25 (s, 0.31 D, CD_2CH_2CHOAc), 1.9 (s, 3.0 D, $CHOCOCD_3$), 2.3 (s, 0.83 D, CHDCOOEt). IR: 2901, 2828, 1723, 1456, 1361, 1200, 1061, 1008, 896 cm^{-1}.

The diester (500 mg, 1.9 mmol) was saponified as above, giving 184 mg of hydroxy acid, mp 55.5–57 °C (lit.[42] mp 53–54 °C). ^{1}H NMR ($CDCl_3$): δ 0.87 (t, J = 5.4 Hz, 3 H, CH_3), 1.0–1.7 (m, 12 H, CH_2), 2.44 (d, J = 8.5 Hz, 1 H, CHDCOO), 3.8–4.2 (m, 1 H, CHOH), 5.5–6.3 (br s, 2 H, OH and COOH). ^{2}H NMR ($CHCl_3$): δ 1.38 (s, 0.47 D, CD_2CH_2CHOH), 2.5 (s, 1.0 D, CHDCOOH). IR: 3571, 3478, 3021, 2896, 2829, 1698, 1451, 1391, 1220 cm^{-1}.

(2*S,3*R**)-3-[2,5,5-$^2H_3$]Hydroxydecanethioic Acid *S*-2-(Acetylamino)ethyl Ester [(3-Hydroxydecanoyl)-NAC].** The thioester was made in the manner described previously[3] except that diethyl phosphorochloridate[18] was used for activation of the carboxylic acid. From 200 mg (1.06 mmol) of (2*S**,3*R**)-3-[2,5,5-$^2H_3$]hydroxydecanoic acid was obtained (following flash chromatography) 39 mg of the NAC thioester. ^{1}H NMR ($CDCl_3$): δ 0.88 (t, J = 5.3 Hz, 3 H, CH_3), 1.0–1.7 (m, 12 H, CH_2), 1.95 (s, 3 H, CH_3CO), 2.6–2.9 [m, 2 H (simplifying to d, J = 2 Hz, 1 H following addition of D_2O), OH and CHDCOSR], 3.03 (t, J = 6 Hz, 2 H, CH_2CH_2S), 3.45 (dt, J = 6, 6 Hz, 2 H, CH_2NH), 3.9–4.2 (m, 1 H, C*H*OH), 5.8–6.2 (m, 1 H, NH). ^{2}H NMR (CCl_4): δ 1.35 (s, 0.43 D, CD_2CH_2CHOH), 2.7 (s, 1.0 D, CHDCOSR). IR: 3681, 3596, 3451, 2921, 2851, 1776, 1511, 1466, 1406, 1371, 1200, 1121, 1033 cm^{-1}.

(2*R,3*R**)-3-[2,5,5-$^2H_3$]Hydroxydecanethioic Acid *S*-2-(Acetylamino)ethyl Ester.** The (2*R**,3*R**) compound was made in the same way as was the diastereomeric thioester just described. From 200 mg (1.06 mmol) of the acid was obtained 30 mg of pure thioester. ^{1}H NMR ($CDCl_3$): δ 0.88 (t, J = 5.3 Hz, 3 H, CH_3), 1.1–1.6 (m, 12 H, CH_2), 1.97 (s, 3 H, CH_3CO), 2.51–2.91 [m, 2 H (simplifying to d, J = 8 Hz, 1 H following addition of D_2O), OH and CHDCOSR], 3.06 (t, J = 6 Hz, 2 H, CH_2S), 3.4 (dt, 6, 6 Hz, 2 H, CH_2NH), 3.9–4.2 (m, 1 H, CHOH), 5.7–6.0 (m, 1 H, NH). ^{2}H NMR (CCl_4): δ 1.35 (s, 0.47 D, CD_2CH_2CHOH), 2.7 (s, 1.0 D, CHDCOSR). IR: 3681, 3591, 3446, 2916, 2848, 1671, 1506, 1463, 1401, 1369, 1220, 1121, 1031 cm^{-1}.

Incubation of Labeled Thioester Substrates with Dehydrase. Dehydrase was obtained, purified, and incubated with labeled substrates by the procedures described previously,[3] with only small deviations. In each incubation, 20 mg of labeled hydroxy thioester was incubated with 77 mL of partially purified dehydrase (1.00 g of protein,[43] 87 units/mg of protein) in 500 mL of buffer, at 30 °C. From the (2*R**,3*R**) and (2*S**,3*R**) substrates were isolated 0.8 and 3 mg of pure (*E*)-2-decenoyl-NAC, respectively, following flash chromatography.

Labeled (*E*)-2-Decenoyl-NAC from (2*S,3*R**) Substrate.** ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 6 Hz, 3 H, CH_3), 1.1–1.4 (m, 10 H, CH_2), 1.87 (s, 3 H, CH_3CO), 2.05–2.35 (m, 2 H, CD_2CH_2CH=), 3.08 (t, J = 6 Hz, 2 H, CH_2S), 3.48 (dt, J = 6, 6 Hz, 2 H, CH_2NH), 5.7–6.0 (m, 1 H, NH), 6.05 (dd, $J_{vicinal}$ = 15, $J_{allylic}$ = 1 Hz, 1 H, CH=C*H*COSR), 6.94 (dt, J = 15, 7 Hz, 1 H, CH_2CH=CH). ^{2}H NMR (CCl_4; see Figure 3d): δ 1.45 (s, CD_2CH_2CH=). IR: 3446, 2916, 2846, 1666, 1631, 1509, 1461, 1368, 1210, 1026, 963 cm^{-1}.

Labeled (*E*)-2-Decenoyl-NAC from (2*R,3*R**) Substrate.** ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 6 Hz, 3 H, CH_3), 1.1–1.4 (m, 10 H, CH_2), 1.87 (s, 3 H, CH_3CO), 2.05–2.35 (m, 2 H, CD_2CH_2CH=), 3.1 (t, J = 6 Hz, 2 H, CH_2S), 3.48 (dt, J = 6, 6 Hz, 2 H, CH_2NH), 5.7–6.0 (m, 1 H, NH), 6.1 (dd, $J_{vicinal}$ = 15, $J_{allylic}$ = 1 Hz, ca. 0.2 H, CH=C*H*COSR), 6.94 (m, 1 H, CH_2CH=CD). ^{2}H NMR (CCl_4; see Figure 3b): δ 1.45 (s, 0.52 D, CD_2CH_2CH=), 6.01 (s, 1.0 D, =CDCOSR). IR: 3444, 2916, 2848, 1666, 1619, 1506, 1466, 1371, 1221, 1141, 1121, 991, 871 cm^{-1}.

Incubation of Unlabeled (*E*)-2-Decenoyl-NAC with Dehydrase in ^2H_2O Buffer. The details of this experiment have already been published.[3] (2*S*,3*R*)-(3-Hydroxydecanoyl)-NAC, ca. 50 mg, was isolated by flash chromatography. ^{1}H NMR (400 MHz; see Figure 2c): δ 0.84 (t, J = 7 Hz, 3 H, CH_3), 1.19–1.36 (m, 10 H, CH_2), 1.4–1.54 (m, 3 H, CH_2C-HOH and OH), 1.94 (s, 3 H, $COCH_3$), 2.73 (br d, J = 2.4 Hz, 1 H, CHDCOS), 3.01 (m, 2 H, CH_2S), 3.41 (m, 2 H, CH_2NH), 4.02 (m, 1 H, C*H*OH), 6.26 (m, 1 H, NH).

Incubation of (*E*)-2-[2-^{2}H]Decenoyl-NAC with Dehydrase in ^1H_2O Buffer. The published[3] procedure was used, with the following modifications: Dehydrase (5 mL, 13.1 mg/mL, 88 units/mg) in 50 mL of 10 mM KPO_4, pH 7.0, was incubated at 29–30 °C for 5 min with 12 mg of labeled substrate. (2*R*,3*R*)-(3-[2-$^2H_1$]Hydroxydecanoyl)-NAC, 5.6 mg, was recovered, following flash chromatography. ^{1}H NMR (400 MHz; see Figure 2b): δ 0.88 (t, J = 7 Hz, 3 H, CH_3), 1.2–1.4 (m, 10 H, CH_2), 1.4–1.6 (m, 2 H, CH_2CHOH), 1.97 (s, 3 H, $COCH_3$), 2.61 (d, J = 4 Hz, 1 H, OH), 2.66 (br d, J = 8.9 Hz, 1 H, CHDCOS), 3.05 (m, 2 H, CH_2S), 3.45 (m, 2 H, CH_2NH), 4.05 (m, 1 H, C*H*OH), 5.79 (m, 1 H, NH).

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(42) Thaler, H.; Geist, G. *Biochem. Z.* **1939**, *302*, 369–383.

(43) Bulletin 1069, Bio-Rad Laboratories, Richmond, CA, 1979.