β-Hydroxydecanoyl Thioester Dehydrase. Complete Characterization of the Fate of the "Suicide" Substrate 3-Decynoyl-NAC

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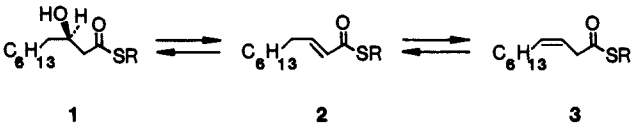
Abstract: β-Hydroxydecanoyl thioester dehydrase, the pivotal enzyme in the biosynthesis of unsaturated fatty acids under anaerobic conditions, catalyzes the interconversion of thioesters of (*R*)-3-hydroxydecanoic acid (**1**), (*E*)-2-decenoic acid (**2**), and (*Z*)-3-decenoic acid (**3**). Dehydrase is irreversibly inactivated by the *N*-acetylcysteamine thioester of 3-decynoic acid (3-decynoyl-NAC), via dehydrase-catalyzed conversion of the acetylenic thioester to 2,3-decadienoyl-NAC. This is the classic example of "suicide" or "mechanism-based" enzyme inactivation. NMR-based experiments have been carried out in order to define the mechanistic relationship between "normal" catalysis and suicide inactivation of dehydrase, by providing detailed structural information on the enzyme-bound inactivator moiety. 3-[2-^{13}C]Decynoyl-NAC was synthesized and incubated with homogeneous dehydrase. ^{13}C NMR spectroscopy at 100.6 MHz showed that when 2,3-decadienoyl-NAC is attacked by the active-site histidine, the product is (3-imidazolyl-3-decenoyl)-NAC. This adduct is slowly isomerized to (3-imidazolyl-2-decenoyl)-NAC. One molecule of inactivator is bound per subunit of the dimeric enzyme. Model histidine–allene adducts have been made and characterized. Comparisons of NMR data reveal that the double-bond configuration of the decenoyl moiety of the enzyme-bound inactivator is *E*. Analysis of these findings strongly suggests that the histidine residue that is alkylated by 2,3-decadienoyl-NAC is *the* active-site base. The structure of the product formed by inactivation of dehydrase by 3-decynoyl-NAC and the mechanism of the inactivation are readily explained in terms of the mechanisms of the normal dehydrase-catalyzed reactions as well as the stereochemical relationships between enzyme and substrates in those normal reactions.

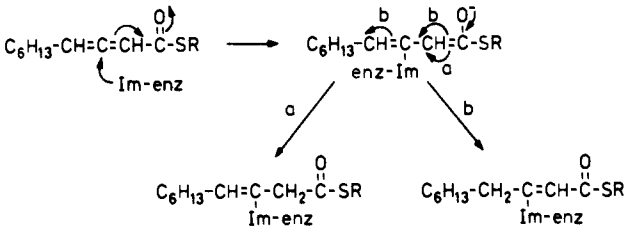
β-Hydroxydecanoyl thioester dehydrase,[5] the pivotal enzyme in the biosynthesis of unsaturated fatty acids under anaerobic conditions, catalyzes the interconversion of thioesters of (*R*)-3-hydroxydecanoic acid (**1**), (*E*)-2-decenoic acid (**2**), and (*Z*)-3-decenoic acid (**3**) (Scheme I).

While clearly important metabolically, dehydrase is perhaps better known for the fact that it is rapidly and completely inactivated by incubation with the *N*-acetylcysteamine (NAC) thioester of 3-decynoic acid.[6,7] This experimental observation constituted the original demonstration of "suicide" enzyme inactivation,[8] a phenomenon often referred to as "mechanism-based" enzyme inactivation. Unfortunately, the mechanistic bases for the action of mechanism-based inactivators have, until very recently,[9–13] always been inferred rather than proven unequivocally through carefully designed experiments. Such has been the case for the inactivation of dehydrase by 3-decynoyl-NAC. Over the past several years we have sought a better understanding of both the "normal" dehydrase-catalyzed reactions (equilibration of **1**, **2**, and **3**) and the inactivation of the enzyme by 3-decynoyl-NAC, in order to better define the mechanistic relationship between these processes.

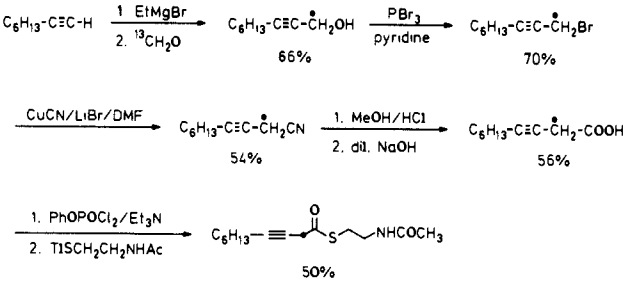
Scheme I. Reactions Catalyzed by β-Hydroxydecanoyl Thioester Dehydrase



Scheme II. Possible Partial Structures for the Dehydrase-Linked Inactivator



Scheme III. Synthesis of 3-[2-^{13}C]Decynoyl-NAC



Herein, we describe NMR-based experiments that provide a complete regio- and stereochemical picture of the fate of 3-decynoyl-NAC, following incubation with dehydrase.[9]

Results

Regiochemistry of the Enzyme-Bound Vinyl Imidazole. The initial step in characterizing the structure of the dehydrase–in-

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(5) Bloch, K. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic: New York, 1971; Vol. 5, pp 441–464.
(6) Brock, D. J. H.; Kass, L. R.; Bloch, K. *J. Biol. Chem.* **1967**, *242*, 4432–4440.
(7) Kass, L. R.; Bloch, K. *Proc. Natl. Acad. Sci. U.S.A.* **1967**, *58*, 1168–1173.
(8) Walsh, C. *Tetrahedron* **1982**, *38*, 871–909.
(9) Schwab, J. M.; Li, W.-b.; Ho, C.-K.; Townsend, C. A.; Salituro, G. M. *J. Am. Chem. Soc.* **1984**, *106*, 7293–7294.
(10) Badet, B.; Roise, D.; Walsh, C. T. *Biochemistry* **1984**, *23*, 5188–5194.
(11) Roise, D.; Soda, K.; Yagi, T.; Walsh, C. T. *Biochemistry* **1984**, *23*, 5195–5201.
(12) Gelb, M. H.; Abeles, R. H. *Biochemistry* **1984**, *23*, 6596–6604.
(13) Ringe, D.; Seaton, B. A.; Gelb, M. H.; Abeles, R. H. *Biochemistry* **1985**, *24*, 64–68.

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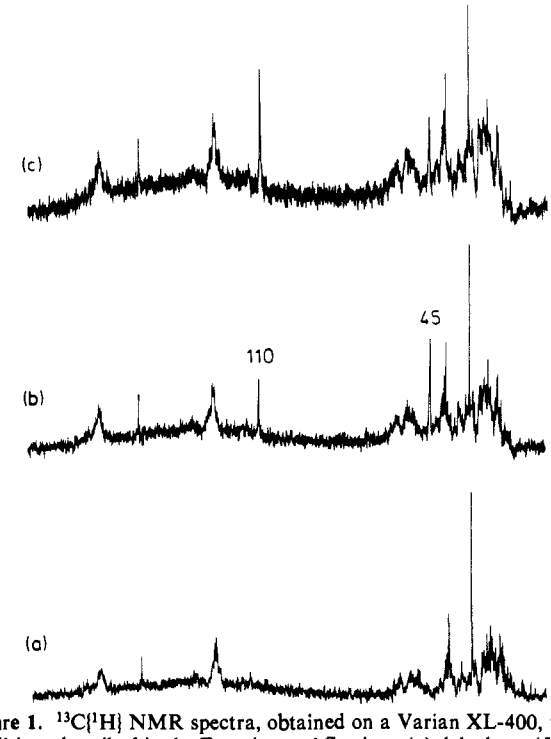


Figure 1. $^{13}C\{^1H\}$ NMR spectra, obtained on a Varian XL-400, under conditions described in the Experimental Section: (a) dehydrase (51 mg, 1.42 μmol), 75 000 scans; (b) dehydrase in (a), but after addition of 0.34 mg (1.26 μmol) of 3-[2-^{13}C]decynoyl-NAC; (c) dehydrase in (b), but after 2-week storage in a refrigerator at 4 °C.

activator adduct is to determine the position of the carbon–carbon double bond in the inactivator moiety. Specifically, attack of the active-site histidine at C-3 of the allenic thioester will lead to a thioester dienolate (or dienol) that could in principle be protonated either at C-2 or C-4 (Scheme II). Protonation at C-2 would give a nonconjugated vinyl imidazole thioester, while protonation at C-4 would afford the conjugated species. While a chemical degradation approach had been used previously to access this point,[14] the experiments were arduous, and the results were not unequivocal. A carbon-13 labeling experiment (using ^{13}C NMR for product analysis) was therefore designed.

3-[2-^{13}C]Decynoic acid was synthesized (Scheme III) from [^{13}C]formaldehyde by a modification of the route used by Helmkamp[15] for the synthesis of 3-[1-^{14}C]decynoic acid. The acid was thioesterified by conversion to a mixed anhydride,[16] followed by treatment of the latter with the thallium salt of NAC.[17,18]

Homogeneous dehydrase was prepared from the cloned, overproducing mutant *Escherichia coli* DM51-A by straightforward methods, based largely on the prior work of Helmkamp[15] and Kass et al.[19] Since protein concentration determinations proved to be strongly dependent on the nature of the protein standard, gravimetric analyses were performed on aliquots of dehydrase. In this way, the quantities of protein used in subsequent NMR experiments could be accurately known.

A 100.6-MHz $^{13}C\{^1H\}$ NMR spectrum was obtained (Figure 1a) on 51 mg of dehydrase. By use of WALTZ-16 proton decoupling,[20] the sample temperature reached only 27 °C, and so no external cooling was required to prevent denaturation. In fact,

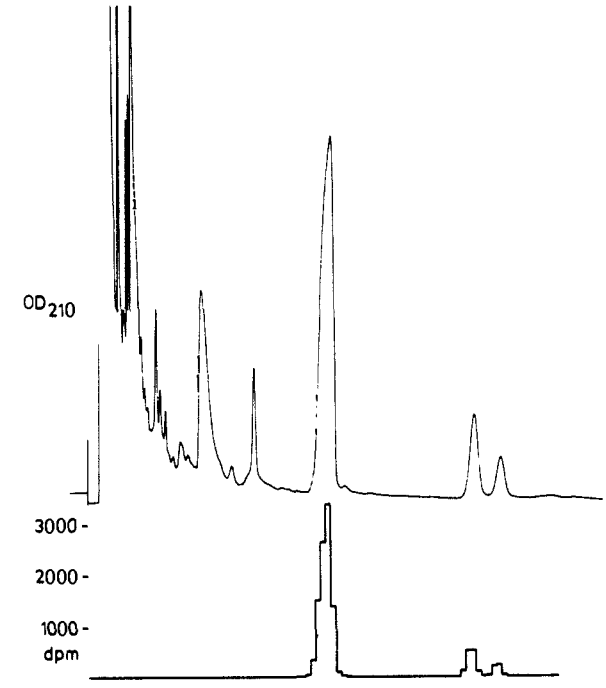
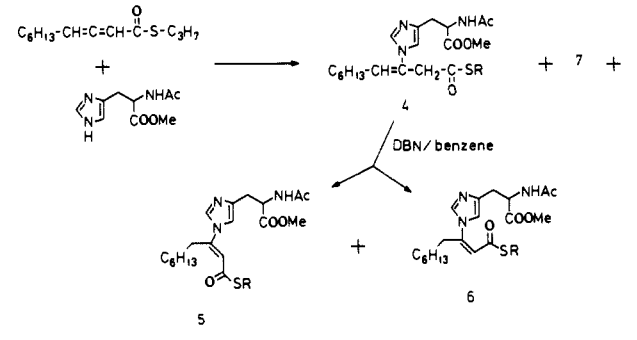


Figure 2. Reversed-phase HPLC profile of the hydrolysate from dehydrase inactivated with 3-[1-^{14}C]decynoyl-NAC. Both UV and radioactivity profiles are shown. The chromatographic conditions are described in the Experimental Section.

Scheme IV. Synthesis of Model Adducts



only 15% of the original enzyme activity was lost over the course of a 17-h data acquisition. A substoichiometric quantity of 3-[2-^{13}C]decynoyl-NAC was then added and a second spectrum obtained (Figure 1b). Two new resonances are evident, at 45.0 and 110.2 ppm, with a ratio of integrals of roughly 2:1. The line widths (36 and 28 Hz, respectively) indicate that these signals owe to enzyme-bound inactivator. (Dehydrase is a dimeric enzyme, with a subunit molecular weight of 18 000.) Significantly, it was found that aged, inactivated enzyme (2 weeks at 4 °C) gave the spectrum shown in Figure 1c, exhibiting a *reversal* of the ratio of integrals, such that the 110.2 ppm peak became predominant. This, plus the fact that early in data acquisition *only* the 45 ppm signal was seen (the 110 ppm signal was imperceptible), suggests that the species responsible for the 45 ppm peak is formed rapidly and that there is a slow, subsequent conversion of it to the species with the C-2 chemical shift of 110 ppm.

The assignment of structures to the moieties with the 45 and 110 ppm C-2 chemical shifts rests on comparisons to model compounds. As had been found by Morisaki and Bloch,[21] and as shown in Scheme IV, the nonconjugated vinyl imidazole thioester (tentatively assigned structure **4**; vide infra) is formed in the reaction between *N*-acetyl-L-histidine methyl ester and 2,3-decadienoic acid, *n*-propyl thioester (Morisaki had used the ethyl thioester). Carbon-2 of **4** resonates at 45.1 ppm. When

(14) Stein, J. P. Ph.D. Thesis, Harvard University, Cambridge, MA, 1970.
(15) Helmkamp, G. M., Jr. Ph.D. Thesis, Harvard University, Cambridge, MA, 1970.
(16) Liu, H.-J.; Sabesan, S. I. *Can. J. Chem.* **1980**, *58*, 2645–2648.
(17) Schwab, J. M.; Klassen, J. B. *J. Am. Chem. Soc.* **1984**, *106*, 7217–7227.
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(19) Kass, L. R.; Brock, D. J. H.; Bloch, K. *J. Biol. Chem.* **1967**, *242*, 4418–4431.
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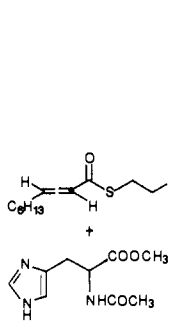
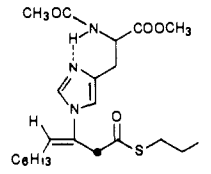
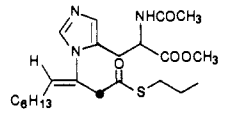
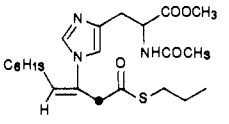
(21) Morisaki, M.; Bloch, K. *Biochemistry* **1972**, *11*, 309–314.

Table I. Spectral Characteristics of Adducts Formed between *N*-Acetyl-L-histidine Methyl Ester and 2,3-[2-^{13}C]Decadienethioic Acid, *S*-1-Propyl Ester

reactants	product	compd	amt recovered, mg	$^3J_{CH}$, Hz	C-2 δ
		**4**	25	7.0	45.1
		**8**	10	7.0	46.1
		**7**	1	4.9	51.3

treated with DBN in benzene, **4** suffers double-bond isomerization, producing two new diastereomeric compounds, **5** and **6**, with C-2 chemical shifts of 114.0 and 116.4 ppm. (The tentative configuration assignments rest on comparisons of proton chemical shifts of **5** and **6** to those of vinyl imidazole itself. These assignments are not crucial to the present argument.) The enzyme–inactivator adduct with the C-2 chemical shift of 45 ppm is accurately modeled by compound **4**. Compounds **5** and **6** are reasonable models for the slowly formed species with the C-2 chemical shift of 110.2 ppm.

Confirmation of the foregoing assignments came from comparisons of the products of hydrolysis of inactivated dehydrase with those formed by hydrolysis of model compounds **4–6**. Helmkamp[15] and Stein[14] had each inactivated dehydrase with 3-[1-^{14}C]decynoyl-NAC and had found two radiolabeled peaks in the effluent from ion-exchange chromatography of the hydrolysate of the resulting inactivated enzyme. Repetition of this experiment, but with the use of reversed-phase HPLC rather than ion-exchange chromatography at atmospheric pressure, has revealed that *three* radiolabeled compounds are formed (Figure 2). The major component has a retention time identical with that of the amino diacid produced by hydrolysis of model adduct **4**. Hydrolysis of *either* **5** or **6** gives an identical *mixture* of amino diacids, with retention times the same as those of the two slow-eluting components from hydrolysis of inactivated dehydrase. Since the conjugated adducts interconvert under the conditions used for acid hydrolysis, it is impossible to say whether the enzyme adduct with the 110 ppm C-2 chemical shift has the *E* or *Z* double-bond configuration or whether both structures are formed under the incubation conditions. (Interestingly, the nonconjugated amino diacid is *not* formed under the conditions that result in the interconversion of the conjugated adducts. Furthermore, hydrolysis of **4** proceeds cleanly and is not accompanied by detectable double-bond migration.)

Stoichiometry of the Inactivation Process. With homogeneous dehydrase it was possible to accurately determine the number of moles of inactivator bound per mole of dehydrase dimer by titration of enzyme with 3-[2-^{13}C]decynoyl-NAC and analysis by ^{13}C NMR spectroscopy.[9] Thus, addition of 1.39 μmol of labeled inactivator to 1.39 μmol (50 mg) of dehydrase (2.78 μmol of subunits) gave an adduct whose ^{13}C{^{1}H} spectrum was essentially identical with that shown in Figure 1b. After addition of a second 1.39-μmol aliquot of inactivator, the NMR spectrum was unchanged, except for an increase in the total peak areas of the 45 and 110 ppm signals (relative to constant protein) and a change (as before) in the proportions of the peak integrals relative to one another. As a control, a third equivalent of 3-[2-^{13}C]decynoyl-NAC was added. Two new features were readily evident. A large, narrow signal (line width 3 Hz) was seen at 27.5 ppm. This is the chemical shift of C-2 of 3-decynoic acid, which is apparently formed through the nonspecific, protein-catalyzed hydrolysis of 3-decynoyl-NAC.[22-28] In addition, there was a peak at 45.4 ppm with an intermediate line width (13 Hz, including 3 Hz line broadening). The similarity of this chemical shift to that of C-2 of **4** suggests that this new peak also stems from a nonconjugated vinyl imidazole thioester. The line width is consistent with adduct formation on the periphery of the enzyme.

Stereochemistry of the Initial Enzyme–Inhibitor Adduct. As mentioned above, reaction of *N*-acetyl-L-histidine methyl ester with the *n*-propyl thioester of 2,3-decadienoic acid leads to the formation of a substantial quantity of **4**. In addition, by careful chromatographic fractionation two isomeric adducts, **7** and **8**, can be isolated. The detailed structures of **4**, **7**, and **8** were revealed by a combination of NMR techniques.

It was clear from a cursory inspection of the ^{1}H NMR spectra that all three compounds are nonconjugated vinyl imidazole thioesters (cf. structure **4**, Scheme IV); accordingly, double-bond configurational isomers were sought. While configurations of trisubstituted olefins are often difficult to assign, allylic $^3J_{CH}$ values have proven to be useful indicators.[29] To facilitate the determination of the relevant coupling constants, the *n*-propyl thioester of 2,3-[2-^{13}C]decadienoic acid (made by base-catalyzed isomerization of the corresponding acetylenic thioester) was reacted with *N*-acetyl-L-histidine methyl ester, as previously. The C-4 proton signals of the three isomeric vinyl imidazole thioesters were relatively complex (even at 400 MHz) owing to proton–proton couplings, and so the $^3J_{CH}$ values could not be obtained cleanly. Nevertheless, the fully coupled ^{13}C NMR spectra readily provided the desired information. As shown in Table I, **4**, **7**, and **8** have $^3J_{CH}$ values of 7.0, 4.9, and 7.0 Hz, respectively, indicating that the configurations of the double bonds are *E*, *Z*, and *E*, respectively, as shown. These assignments are supported by two additional lines of reasoning. The chemical shifts of the C-4 protons of **4** and **7** are 5.79 and 5.68 ppm, respectively. These shieldings are consistent with those experienced by the cis and trans C-2 protons of vinyl imidazole itself, the chemical shifts of which are

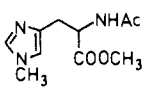
(22) Both lysozyme[23] and serum albumin[24-28] catalyze hydrolysis of esters in a nonspecific manner.

(23) Piskiewicz, D.; Bruice, T. C. *Biochemistry* **1968**, *7*, 3037–3047.
(24) Branchini, B. R.; Salituro, G. M.; Rosenstein, B. J.; Bruns, W. T. *Lancet* **1982**, *1*, 618–619.
(25) Branchini, B. R.; Salituro, G. M.; Rosenstein, B. J. *Pediatr. Res.* **1983**, *17*, 850–855.
(26) Bruno, J. J.; Ringold, H. J. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1969**, *28*, 840.
(27) Cassida, J. E.; Augustinsson, K.-B. *Biochim. Biophys. Acta* **1959**, *36*, 411–426.
(28) Kurono, Y.; Yamada, H.; Ikeda, K. *Chem. Pharm. Bull.* **1982**, *30*, 296–301.
(29) Marshall, J. L. *Carbon-Carbon and Carbon-Proton NMR Couplings*; Verlag Chemie Int.: Deerfield Beach, FL, 1983; pp 33–38.

5.27 and 4.88 ppm, respectively. Also, the C-2 chemical shifts of **4**, **7**, and **8** are 45.1, 51.2, and 46.1 ppm, respectively. The upfield shifts experienced by **4** and **8** can be rationalized by invoking the "cis effect",[30-32] involving the C-1/C-2 and hexyl substituents on the double bond.

The structural difference between **4** and **8** is readily detectable by examination of their 90-MHz ^{1}H NMR spectra. Slight variations in the coupling patterns of the protons attached to the α and β carbons of the amino acid moiety are evident, as well as in the chemical shifts of the imidazole protons. In particular, whereas the β protons of **4** exhibit a four-line pattern (the center fragment of the AB portion of an ABX system), the corresponding signal in the spectrum of **8** is a broadened doublet. This observation can be explained in terms of hydrogen-bonding interactions. In particular, it should be possible for the amide NH of **4** to hydrogen bond to the proximal imidazole ring nitrogen (N^3), whereas a comparable interaction is inaccessible in **8**. The result is that rotation of the alanyl moiety of **4** should be hindered, but there should be no such restriction on the motion of that portion of **8**.

The foregoing hypothesis was tested by synthesis of *N*-acetyl-N^1- and *N*-acetyl-N^3-methyl-L-histidine methyl esters (**9** and **10**) and



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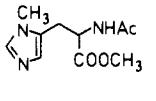

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inspection of their 90-MHz NMR spectra. Indeed, the β proton signals of **9** and **10** are identical with the signals of the corresponding protons of **4** and **8**, respectively.

It is worth noting that, at 400 MHz, the α and β protons of **4**, **7**, and **8** form ABX coupling systems, the AB portions of which comprise the expected eight-line patterns.

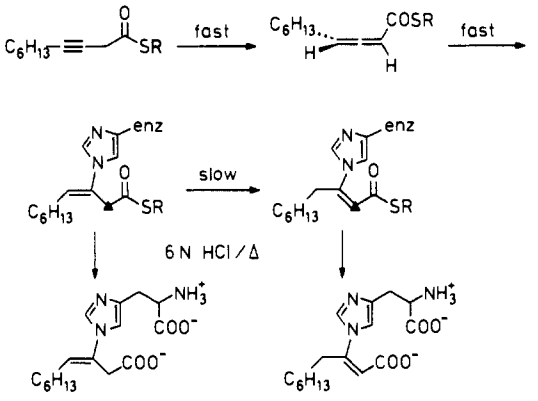
Of the three nonconjugated vinyl imidazole model adducts, two (**4** and **8**) have C-2 chemical shifts almost identical with that of the actual enzyme–inactivator adduct. These model compounds are the ones with *E* double-bond configurations. The chemical shift of C-2 of **7**, with the *Z* double-bond configuration, is downfield by over 6 ppm. On this basis *we conclude that the rapidly formed enzyme–inactivator adduct has the E double-bond configuration.*

Discussion

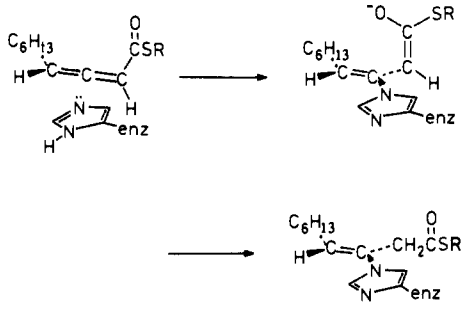
The inactivation of dehydrase by 3-decynoyl-NAC involves 2,3-decadienoyl-NAC as an intermediate. The allenic thioester, formed via dehydrase-mediated propargylic rearrangement of the acetylenic precursor, is very electrophilic and rapidly[33] alkylates a histidine residue[34] at the enzyme's active site. As mentioned above, in principle, either of two vinyl imidazole thioester regioisomers could be formed, depending on whether the implicit thioester dienolate is protonated at C-2 or at C-4. (See Scheme II.)

While this question of regiochemistry had previously been approached by the best methods available at the time, involving chemical degradation of the enzyme–inactivator adduct,[14] the data obtained were not entirely clear-cut. (The ultimate structure assignment was correct, however, as we have now found.) In recent years it has been possible to observe directly, by NMR spectroscopy, the formation of adducts between enzymes and substrates or inhibitors.[35,36] In this way, an accurate, detailed picture of

Scheme V. Interaction of 3-Decynoyl-NAC with Dehydrase and Chemistry of the Resulting Adduct



Scheme VI. Three-Dimensional Representation of Adduct Formation between 2,3-Decadienoyl-NAC and Dehydrase



dehydrase inactivation by 3-decynoyl-NAC has now been obtained.

On the basis of ^{13}C NMR studies of inactivated enzyme, we have shown that dehydrase acts upon 3-decynoyl-NAC so as to quickly produce an (*E*)-3-(N^{im}-histidinyl)-3-decenoyl thioester adduct (cf. structure **4**) at the active site. This species is slowly converted to the 2-decenoyl thioester congener. (See Scheme V.)

These findings are readily rationalized on mechanistic grounds and constitute strong evidence in support of the contention that suicide inactivation of dehydrase is, indeed, mechanism-based.

As mentioned above, and as illustrated in Scheme VI, attack at C-3 of 2,3-decadienoyl-NAC by the active-site histidine presumably leads initially to a thioester dienolate (or dienol). Significantly, the two double bonds do not form a single π system but are orthogonal to one another. As a result, this intermediate could be protonated at C-4 *only* after a 90° rotation about the C-2/C-3 single bond. There is, however, no analogous stereoelectronic barrier to protonation at C-2. If one assumes that substrates (and inactivators) undergo minimal motion at enzymes' active sites,[37,38] then it is most reasonable that the thioester dienolate would be protonated at C-2, giving the nonconjugated vinyl imidazole thioester.

The stereochemical outcome is significant, as well. We had previously shown that the inhibitory allene has the *S* configuration[39] and that the active-site histidine (the "single base") effects removal (or supply) of the *pro*-2*S* proton.[17,40] Accordingly, C-3 of (*S*)-2,3-decadienoyl-NAC is attacked from a point located over the plane of the paper (as it is portrayed in Scheme VI) and orthogonal to the plane of the C-2/C-3 π system. This necessarily

(30) Dorman, D. E.; Jautelat, M.; Roberts, J. D. *J. Org. Chem.* **1971**, *36*, 2757–2766.
(31) Gaudemer, A. In *Stereochemistry: Fundamentals and Methods*; Kagan, H. B., Ed.; Georg Thieme Verlag: Stuttgart, 1977; pp 44–136.
(32) Perlin, A. S. In *Carbon-13 in Organic Chemistry*; Buncel, E., Lee, C. C., Eds.; Elsevier: Amsterdam, 1977; pp 171–235.
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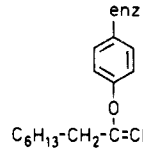
means that the *n*-hexyl and histidinyl substituents on the C-3/C-4 double bond are situated trans to one another. That is, it is *predicted* that the configuration of this double bond will be *E*. This is indeed the outcome.

There is an important corollary interpretation of the vinyl imidazole double-bond configuration. Clearly, the histidine that is alkylated approaches the allenic thioester from the same perspective as does the active-site base in catalyzing reactions involving the normal dehydrase substrates.[17,40] It is unlikely that this observation is coincidental; rather, it constitutes strong evidence that the alkylated histidine is *the* active-site base. Such a finding is especially significant since the use of affinity labels does not *always* provide results that can be interpreted unambiguously. A case in point is the modification of His-57 (rather than the active-site nucleophile, Ser-195) of chymotrypsin by (*p*-tolylsulfonyl)-L-phenylalanyl chloromethyl ketone (TPCK).[41]

An original goal of our studies on dehydrase was to determine the number of active-site acid/base groups. This question was raised by Helmkamp's studies,[15,34] which had clearly implicated an active-site histidine and had provided rather equivocal evidence for tyrosine involvement, as well. Helmkamp's[15] and Stein's[14] findings that two radiolabeled components are found in the hydrolysate of enzyme inactivated with 3-[1-^{14}C]decynoyl-NAC suggest that one might be a histidine adduct and the other a tyrosine adduct. That scenario could be rationalized mechanistically in the following way. If the initial histidine adduct (cf. structure **4**) suffers double-bond rearrangement (cf. **5** and **6**), then the product is a vinylogous thiolocarbamate. The amino moiety of the latter is an imidazole ring, a particularly facile leaving group. It is not unreasonable to postulate conjugate attack by the hydroxyl group of the putative active-site tyrosine, with expulsion of the histidine residue.

Even in the absence of our data involving comparison of the dehydrase hydrolysate to the products from hydrolysis of the model histidine adducts, the implausibility of the foregoing scenario was evident. The tyrosine adduct **11** rationalized above is an aryl enol



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ether. Under acid hydrolysis conditions, such a compound would readily undergo hydrolysis to the corresponding β-keto acid (which would decarboxylate), plus free tyrosine. Both Helmkamp[15] and Stein[14] used 3-decynoyl-NAC labeled with ^{14}C at C-1. All of the radioactivity from adduct **11** would be lost as $^{14}CO_2$, and none would be found in the effluent from ion-exchange chromatography. Accordingly, only histidine adducts are expected to be detectable.

Experimental Section

Unless otherwise indicated, all solvents and reagents were of reagent grade and were used as received from commercial sources. D_2O and $CDCl_3$ were purchased from Aldrich, and [^{13}C]paraformaldehyde and Na^{14}CN came from Merck Sharp and Dohme and Amersham, respectively. Tetrahydrofuran was distilled from $LiAlH_4$ and degassed with nitrogen prior to use. DMF and pyridine were stirred over KOH overnight, then distilled from BaO, and stored over 3A molecular sieves. Lithium bromide was heated at 160 °C for 1 h in vacuo before use. Triethylamine was distilled from CaH_2. Except as noted, organic extracts were dried over anhydrous magnesium sulfate, filtered, and concentrated via rotary evaporation, using a Cole-Parmer aspirator pump.

Proton NMR spectra were obtained on either a Varian EM-360A or a JEOL FX-90Q, with $CDCl_3$ as solvent, in 5-mm tubes. Chemical shifts are given in parts per million downfield from tetramethylsilane (Me_4Si) added as an internal standard. Proton-decoupled carbon-13 NMR spectra were obtained on a JEOL FX-90Q or a Varian XL-400, using $CDCl_3$ or acetone as an internal standard.

Analytical gas chromatography was accomplished on Packard 428 and Varian 2100 instruments, with 6 ft × 2 mm (i.d.) columns packed with 7.5% Carbowax 20M on Chromosorb W AW-DMCS. Infrared spectra were recorded on a Perkin-Elmer 298 spectrophotometer, with solutes dissolved in $CHCl_3$.

Thin-layer chromatograms were run on glass-supported silica gel plates purchased from Analtech. Silica gel 60 (230–400 mesh) for flash chromatography[42] was purchased from E. Merck.

All mobile-phase components used for HPLC were "HPLC grade". Analytical and preparative HPLC separation of enzyme substrates was carried out on a system that included an Altex 110A pump, an ISCO 1840 absorbance monitor, and a Rheodyne 7125 injector. A 10-μm Lichrosorb Si-60 column (250 × 4.6 mm) packed by Chromanetics was used.

Analytical reversed-phase HPLC separation of amino acids and amino acid derivatives was performed on a system that included an Altex 110A pump, a Kratos 757 absorbance monitor, a Rheodyne 7125 injector.

Radioactivity was assayed on a Beckman LS 1801 liquid scintillation counter. The fluor for aqueous samples was supplied by Beckman, while Scintiverse (Fisher) was used for organic-soluble samples. A Berthold LB276 TLC scanner was used for determining the radioisotopic purity of 3-[1-^{14}C]decynoyl-NAC.

E. coli DM51A was routinely grown in 2-L flasks in a New Brunswick G-25R incubator shaker. A large-scale (300-L) incubation was also conducted. Deionized water was used, and all chemicals used for enzyme purification or analysis were of enzyme grade or the purest grade available. An ISCO UA-5 detector was used for monitoring all enzyme column chromatography steps. Enzyme kinetics studies and protein assays were performed on a Gilford 252-Beckman DU UV–vis spectrophotometer. Centrifugation of enzyme solutions was performed in a Servall RC-2 centrifuge equipped with a GSA or an SS-34 rotor. Polyacrylamide gel electrophoresis was carried out on Buchler Polyanalyst (tube gels) and Hoeffer slab gel apparati. An Amicon Model 8MC microultrafiltration system was employed for concentration of enzyme solutions. Dehydrase was freeze-dried on a Virtis lyophilizer.

2-[1-^{13}C]Nonyn-1-ol.[43] To a dry 500-mL three-necked flask equipped with a condenser, a magnetic stirbar, and a rubber septum were placed 15 mL of anhydrous ether and 1.66 mL (4.99 mmol) of EtMgBr. To this was added, dropwise and under a blanket of nitrogen, 0.605 g (5.49 mmol) of 1-octyne. The reaction mixture was stirred and heated at reflux for 2 h and then cooled to room temperature. Gaseous $^{13}CH_2O$, generated by heating 0.141 g (4.53 mmol) of [^{13}C]paraformaldehyde at 180–190 °C, was bubbled into the Grignard reagent solution. When the introduction of formaldehyde was complete, 10 mL of anhydrous ether was added, and the reaction mixture was stirred at room temperature overnight. The mixture was then poured into 20 mL of 10% aqueous NH_4Cl and extracted several times with ether. The combined extracts were dried, filtered, and concentrated. The crude product was purified by flash chromatography (6 in. × 30 mm; 85:15, petroleum ether/EtOAc), providing 0.389 g (66%) of the labeled propargylic alcohol as a colorless oil. ^{1}H NMR: δ 0.89 (t, J = 6 Hz, 3 H, CH_3), 1.05–1.50 (m, 8 H, CH_2), 2.0–2.5 (m, 2 H, $CH_2CH_2C{\equiv}$), 4.25 (dt, $^1J_{CH}$ = 166 Hz, $^5J_{HH}$ = 3 Hz, 2 H, $^{13}CH_2OH$). ^{13}C NMR: δ 51.43. IR: 3610, 3435, 2925, 2859, 2282, 2220, 1468, 1380, 1190, 1135, 1005, 620 cm^{-1}.

1-Bromo-2-[1-^{13}C]nonyne. 2-[1-^{13}C]Nonyn-1-ol was converted to the corresponding bromide by the procedure of Newman and Wotiz.[43] To 15 mL of ether were added 0.380 g (2.70 mmol) of 2-[1-^{13}C]nonyn-1-ol and 10 μL of pyridine, under nitrogen. The reaction mixture was heated to reflux temperature, and 110 μL (1.17 mmol) of PBr_3 was added dropwise, so as to maintain a gentle reflux. The reaction mixture was stirred continuously for an additional 3 h. GC analysis showed that the starting material had been consumed. The mixture was cooled to room temperature, poured into ice, and extracted with ether several times. The combined ether extracts were washed with saturated aqueous $NaHCO_3$ and then brine, dried, filtered, and concentrated, giving 0.396 g of an oily product (70%), 96% pure, by GC. ^{1}H NMR: δ 0.89 (t, J = 6 Hz, 3 H, CH_3), 1.05–1.60 (m, 8 H, CH_2), 2.0–2.5 (m, 2 H, $CH_2CH_2C{\equiv}$), 3.93 (dt, $^1J_{CH}$ = 176 Hz, $^5J_{HH}$ = 3 Hz, 2 H, ${\equiv}C^{13}CH_2Br$). ^{13}C NMR: δ 15.77. IR: 2930, 2860, 2304, 2235, 1468, 1430, 1380, 1330, 1190, 1135, 610 cm^{-1}.

1-Cyano-2-[1-^{13}C]nonyne. The title compound was prepared by the procedure described by Brandsma.[44] To a stirred solution of 380 mg (1.88 mmol) of 1-bromo-2-[1-^{13}C]nonyne in 7 mL of DMF (in a dry, two-necked flask equipped with a condenser and a thermometer) was

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added 236 mg (2.63 mmol) of dry CuCN, followed by 59 mg (0.68 mmol) of LiBr, under nitrogen. The suspension was heated at 80 °C for 15 min and then at 100 °C for 30 min. An aliquot was removed, and after workup, GC analysis showed that no starting material was left. The brown reaction mixture was allowed to cool to 40 °C, poured into a vigorously stirred solution containing 7 g of NH_4Cl and 35 mL of 4 N HCl, and extracted with five 20-mL aliquots of ether/petroleum ether (1:1). The organic extracts were washed with saturated aqueous NH_4Cl, then dried, filtered, and concentrated, leaving a brown oil that was further purified by flash chromatography (6 in. × 20 mm; 95:5, petroleum ether/EtOAc). Removal of the solvent from the pooled fractions afforded 152 mg of product, as a pale yellow oil (54%). ^1H NMR: δ 0.89 (t, J = 6 Hz, 3 H, CH_3), 1.05–1.60 (m, 8 H, CH_2), 2.0–2.33 (m, 2 H, $CH_2CH_2C{\equiv}$), 3.31 (dt, $^1J_{CH}$ = 155 Hz, $^1J_{HH}$ = 3 Hz, 2 H, $\equiv C^{13}CH_2CN$). ^{13}C NMR: δ 9.26. IR: 2920, 2860, 2300, 2258, 2240, 1466, 1410, 1315, 1190, 1125, 915, 620 cm^{-1}.

Methyl 3-[2-^{13}C]Decynoate. The title compound was synthesized by Helmkamp's method.[15] The foregoing labeled nitrile (140 mg, 0.93 mmol) was added to a solution of 1.5 mL of MeOH and 0.167 mL of deionized H_2O. The resulting solution was stirred, and HCl gas was bubbled through for 10 min. The mixture was then cooled to room temperature, and 4 mL of H_2O was added. GC analysis showed that the starting nitrile had been completely converted to the ester. The reaction mixture was extracted with four 5-mL aliquots of petroleum ether. These extracts were dried, filtered, and concentrated, providing 140 mg of the ester as a colorless oil. ^1H NMR: δ 0.89 (t, J = 6 Hz, 3 H, CH_3), 1.05–1.70 (m, 8 H, CH_2), 2.0–2.35 (m, 2 H, $CH_2CH_2C{\equiv}$), 3.26 (dt, $^1J_{CH}$ = 148 Hz, $^5J_{HH}$ = 3 Hz, 2 H, $\equiv C^{13}CH_2COOMe$), 3.74 (s, 3 H, OCH_3). ^{13}C NMR: δ 25.86.

3-[2-^{13}C]Decynoic Acid. To the foregoing labeled ester was added 24 mL of 0.05 N NaOH in 50% aqueous MeOH. The resulting suspension was stirred under nitrogen for 24 h at room temperature. After it had clarified, the reaction mixture was washed twice with 15-mL aliquots of petroleum ether, acidified to pH 2.0 with 4 N HCl, and extracted with 4 × 15 mL of petroleum ether. The organic extracts were dried, filtered, and concentrated, leaving 89 mg of 3-[2-^{13}C]decynoic acid (56%, from the nitrile). ^1H NMR: δ 0.89 (t, J = 6 Hz, 3 H, CH_3), 1.12–1.74 (m, 8 H, CH_2), 2.0–2.42 (m, 2 H, $CH_2CH_2C{\equiv}$), 3.33 (dt, $^1J_{CH}$ = 148 Hz, $^5J_{HH}$ = 3 Hz, 2 H, $\equiv C^{13}CH_2COOH$), 9.3–10.2 (br s, 1 H, COOH). ^{13}C NMR ($CDCl_3$): δ 25.9. ^{13}C NMR ($^1H_2O/^2H_2O$): δ 27.5. IR: 3095, 2925, 2855, 2240, 1735, 1698, 1435, 1420, 1400, 1282, 1225, 1190, 1130, 935, 620 cm^{-1}.

3-[2-^{13}C]Decynethioic Acid, S-[2-(Acetylamino)ethyl] Ester (3-[2-^{13}C]Decynoyl-NAC). The mixed anhydride of 3-[2-^{13}C]decynoic acid (89 mg, 0.53 mmol) was prepared by the procedure of Liu and Sabesan,[16] using an equimolar quantity of phenyl dichlorophosphate. The filtered anhydride solution was diluted with THF to a total volume of 20 mL and cooled to 0 °C. Thallium–NAC suspension (0.53 mmol)[17] was added dropwise. When the color of the reaction mixture had turned to white, the precipitate was removed by filtration through Celite, and the filtrate was concentrated. The residue was extracted thoroughly with ether, and the combined extracts were concentrated, leaving a crude yellowish oil, which was purified by flash chromatography (6 in. × 20 mm; 86:14, CH_2Cl_2/acetone), affording 71 mg (50%) of the thioester as a slightly yellow crystalline solid. ^1H NMR: δ 0.89 (t, J = 6 Hz, 3 H, CH_3), 1.12–1.75 (m, 8 H, CH_2), 1.97 (s, 3 H, $COCH_3$), 2.0–2.40 (m, 2 H, $CH_2CH_2C{\equiv}$), 3.04 (t, J = 7 Hz, 2 H, CH_2S), 3.42 (dt, $^1J_{CH}$ = 149 Hz, $^5J_{HH}$ = 3 Hz, 2 H, $\equiv C^{13}CH_2CO$), 3.45 (dt, J = 7, 7 Hz, 2 H, CH_2NH), 5.73–6.20 (br m, 1 H, NH). ^{13}C NMR ($^1H_2O/^2H_2O$): δ 33.6. IR: 3460, 2930, 2860, 2230, 1765, 1520, 1470, 1375, 1265, 1190, 1130, 1070, 995, 615 cm^{-1}.

3-[1-^{14}C]Decynoyl-NAC. The ^{14}C-labeled thioester was made by the same procedures used for the synthesis of 3-[2-^{13}C]decynoyl-NAC. $Cu^{14}CN$, made from $Na^{14}CN$, by the method of Reid and Weaver[45] (as described by Murray and Williams[46]), was reacted with 1-bromo-2-nonyne, providing 1-[^{14}C]cyano-2-nonyne in 50% yield. From 40 mg (107 μCi) of the latter was obtained 20 mg (26 μCi) of 3-[1-^{14}C]decynoyl-NAC (28% chemical and 24% radiochemical yields, based on the nitrile).

Purification of β-Hydroxydecanoyl Thioester Dehydrase. *E. coli* DM51A was grown in 0.1 M KPO_4 (pH 7.5) containing 10 g of casein hydrolysate, 5 g of yeast extract, 5 g of glycerol, 25 mg of thymine, and 200 mg of ampicillin/L of medium. (A concentrated solution of thymine and ampicillin was filter sterilized and added separately.) A 100-mL starter culture was inoculated with 10 mL of a stock suspension of bacteria stored in 50% glycerol at −80 °C and then incubated at 37 °C, with vigorous agitation. After 17 h of growth, this culture was added to 1000 mL of culture medium in a 2-L Erlenmeyer flask, and incubation was continued at 37 °C for an additional 17 h. The cells were harvested by centrifugation (9000g) and washed with 20 mM Tris-HCl containing 50 mM $MgCl_2$ (pH 8.0). They were frozen in liquid nitrogen and stored at −80 °C.

All enzyme purification steps were carried out at 0–4 °C. Frozen cells (40 g) were suspended in 20 mM Tris-HCl containing 50 mM $MgCl_2$, pH 8.0 (6 mL of buffer/g of cells), homogenized in a Waring Blendor for 2 min, and filtered through cheesecloth. The cells were then disrupted in a French pressure cell (SLM-Aminco) at 16 000 psi, following which the mixture was centrifuged at 25000g for 30 min. The supernatant was made 4 M in NaCl, and then 40 mg of Dextran T500 (Pharmacia) and 60 mg of poly(ethylene glycol) 6000 (PEG-6000; Sigma)/mL were added.[47] After the solutes had dissolved, the solution was spun at 1500g for 10 min. The combined upper phase was reextracted with an equal volume of fresh bottom phase to remove most of the nucleic acids, following which solid $(NH_4)_2SO_4$ was added to the upper phase to the point of saturation (0.13 g/mL). After being stirred for 40 min, the mixture was centrifuged at 10000g for 10 min. Following removal of the pelleted PEG-6000, the supernatant was dialyzed exhaustively against 1 mM KPO_4, pH 7.0. The dialysate was centrifuged at 10000g for 20 min, and the supernatant was then loaded onto a DEAE-cellulose (Whatman DE-52) column (30 × 2.5 cm). The column was eluted with a linear gradient comprising 300 mL each of 1 mM KPO_4 (pH 7.4) and 0.1 KCl in the same buffer. The flow rate was adjusted to 1.0 mL/min, and 7-mL fractions were collected. Dehydrase isomerization activity was found in fractions 55–85, and fractions 57–78 were pooled, based on the UV absorbance profile of the effluent. The pooled fractions were taken to 90% of saturation with $(NH_4)_2SO_4$, and the precipitated protein was collected by centrifugation at 25000g for 20 min. After dissolution in 15 mL of 10 mM KPO_4 (pH 7.0), the enzyme was dialyzed against the same buffer. Dialyzed dehydrase was loaded onto a hydroxylapatite-C column (8 × 2.5 cm), which was eluted with a linear gradient comprising 250 mL each of 10 mM KPO_4 and 200 mM KPO_4, pH 7.0. Fractions of 4.5 mL were collected at a rate of 0.35 mL/min. Dehydrase activity was found in fractions 37–60. The elution was continuously monitored at 254 nm, and on the basis of the protein peak shape, fractions 43–56 were pooled. This solution was taken to 90% of saturation with $(NH_4)_2SO_4$, and protein was collected by centrifugation of the resulting suspension. The pellet was redissolved in 10 mL of 10 mM KPO_4, pH 7.0, and after dialysis against the same buffer, the enzyme solution was concentrated by ultrafiltration. The concentrated solution of dehydrase was frozen in liquid nitrogen and stored at −80 °C until needed.

Enzyme purity was determined by polyacrylamide gel electrophoresis under both native[19] and denaturing[48] conditions, and protein was stained with Coomassie Brilliant Blue R-250. No impurities could be detected in highly purified dehydrase.

Protein was assayed routinely by the Bio-Rad method;[49] however, the results were heavily dependent on which protein standard was employed. An accurate benchmark was obtained by performing gravimetric analysis on samples of dehydrase (vide infra).

Enzyme activity was determined by the isomerase assay of Kass and Brock.[50] One unit of isomerase activity corresponds to the formation of 1.0 nmol of (*E*)-2-decenoyl-NAC [from (*Z*)-3-decenoyl-NAC]/min.

The specific activity of highly purified dehydrase was 8700 units/mg.

Gravimetric Analysis of Dehydrase. Blakeley and Zerner's method[51] was used for gravimetric analysis of dehydrase. In analyses of 3.1 and 6.0 mg of protein, extinction coefficients of 1.347 and 1.288 $(g/L)^{-1}$ cm^{-1} (280 nm) were obtained (cf. lit.[15] value 0.88, at 278 nm). The applicability of the method and the skill of the experimenter were verified by determining the extinction coefficient of bovine serum albumin (0.663; cf. lit.[54] value 0.660).

Incubation of 3-[2-^{13}C]Decynoyl-NAC with Dehydrase. A carefully measured quantity of homogeneous dehydrase in 10 mM KPO_4 (pH 7.0), ca. 50 mg in each experiment, was added to a 10-mm NMR tube along with 150 μL of D_2O. EDTA was added (final concentration 1.0 μM), and the total volume was adjusted to 3.0 mL with 10 mM KPO_4, pH 7.0. Several microliters of an acetone solution of 3-[2-^{13}C]decynoyl-NAC was added, the tube was lowered into the probe of a Varian XL-400, and data acquisition was begun. $^{13}C\{^1H\}$ NMR spectra were obtained at 100.6 MHz, using WALTZ-16 decoupling, and 32K data points were collected over 200 ppm, giving an acquisition time of 0.8 s and digital resolution of 1.22 Hz. A 90° pulse (24 μs) was employed, with no pulse delay. For each spectrum, 75 000 transients were collected. Spectra were processed

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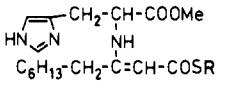
typically with 3.0 Hz of line broadening.

3-[2-^{13}C]Decynethioic Acid, *S*-1-Propyl Ester. Oxalyl chloride (1.5 mL) and 3-[2-^{13}C]decynoic acid (162 mg, 0.96 mmol) were combined and heated at reflux for 20 min. After the reaction mixture had cooled to room temperature, the excess oxalyl chloride was removed with a gentle stream of nitrogen, and to the residue were added 2 mL of benzene and 110 μL of pyridine (1.36 mmol). The resulting solution was cooled in an ice bath, and 300 μL (3.3 mmol) of 1-propanethiol was added. The reaction mixture was stirred at 0 °C for 20 min and at room temperature for another 5 min and then poured into 10 mL of H_2O. The aqueous phase was extracted with 3 × 20 mL of Et_2O, and the combined organic extracts were washed first with 1 N HCl and then with water (4 × 20 mL), dried, filtered, and concentrated. The crude product was purified by flash chromatography (6 in. × 20 mm; 99:1, petroleum ether/Et_2O), giving 108 mg of the thioester (50%). ^{1}H NMR: 0.95 (t, J = 7 Hz, 6 Hz, CH_3 and CH_3), 1.1–1.8 (m, 10 H, CH_2), 2.1–2.3 (m, 2 H, $CH_2CH_2C{\equiv}$), 2.88 (t, J = 7 Hz, 2 H, CH_2S), 3.39 (dt, $^1J_{CH}$ = 133 Hz, $^5J_{HH}$ = 3 Hz, 2 H, $^{13}CH_2CO$).

2,3-[2-^{13}C]Decadienethioic Acid, *S*-1-Propyl Ester.[52] To 108 mg (0.48 mmol) of the ^{13}C-labeled acetylenic thioester in 9 mL of EtOH was added 19.7 μL of a 1 M solution of NaOEt in EtOH. This solution was stirred for 7 min, diluted with H_2O, and extracted with 4 × 30 mL of CH_2Cl_2. The organic extracts were dried, filtered, and concentrated, giving 99 mg (92%) of the allenic thioester. ^{1}H NMR: δ 0.97 (t, J = 7 Hz, 6 H, CH_3 and CH_3), 1.1–1.8 (m, 10 H, CH_2), 2.0–2.3 (m, 2 H, CH_2CH=), 2.87 (t, J = 7 Hz, 2 H, CH_2S), 5.71 (dt, J = 7, 7 Hz, 1 H, CH_2CH=), 5.84 (ddt, $^1J_{CH}$ = 174 H, $^4J_{HH}$ = 3 Hz, $^5J_{HH}$ = 3 Hz, 1 H, =^{13}CHCO).

Reaction of *N*-Acetyl-L-histidine Methyl Ester with 2,3-[2-^{13}C]Decadienethioic Acid, *S*-1-Propyl Ester.[21] Purification and Characterization of Products. *N*-Acetyl-L-histidine methyl ester (274 mg, 1.3 mmol; made by esterification of commercial *N*-acetyl-L-histidine) and the ^{13}C-labeled allenic thioester (99 mg, 0.44 mmol) were dissolved in 2 mL of THF, and the resulting suspension was heated at reflux for 3 h. (The failure of the reactants to go into solution completely suggests that the *N*-acetyl-L-histidine methyl ester was contaminated with histidine methyl ester hydrochloride. This is supported by the fact that a substantial quantity of enamine adduct **12** was formed here.) The reaction mixture was cooled



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to room temperature and concentrated in vacuo, and the residue was fractionated by flash chromatography (94:6, CH_2Cl_2/MeOH). In addition to starting material, a mixture of products (134 mg) was obtained, which was again applied to a flash column. Two fractions were obtained: 79 mg of a mixture of **4**, **7**, and **12** and 10 mg of **8**. The mixture was further purified by semipreparative HPLC (10 × 250 mm silica gel column, CH_3CN solvent, 4.6 mL/min, effluent monitored at 215 nm), providing 25 mg of **4**, 1 mg of **7**, and 28 mg of **12**.

For **4**. ^{1}H NMR: δ 0.8–1.05 (m, 6 H, CH_3 and CH_3), 1.2–1.7 (m, 10 H, CH_2), 2.06 (s, 3 H, $NHCOCH_3$), 2.1–2.4 (m, 2 H, CH_2CH=), 2.87 (t, J = 7 Hz, 2 H, CH_2S), 3.05 [m (AB portion of an ABX system), 2 H, $CH_2CH(NHAc)COOMe$], 3.65 (br d, $^1J_{CH}$ = 130 Hz, 2 H, $^{13}CH_2COS$), 3.70 (s, 3 H, $COOCH_3$), 4.80 [ddd (X portion of an ABX system) J = 8, 5, 5 Hz, 1 H, $CH_2CH(NHAc)COOMe$], 5.78 (dt, $^3J_{HH}$ = 7 Hz, $^3J_{CH}$ = 7 Hz, 1 H, CH=), 6.83 (s, 1 H, imidazole CH), 7.12 (br d, J = 8 Hz, 1 H, NH), 7.51 (s, 1 H, imidazole CH). ^{13}C NMR ($CDCl_3$): δ 45.0 ($^3J_{CH}$ = 7.0 Hz, when run without any proton decoupling).

For **7**. ^{1}H NMR: δ 0.94 (t, J = 7 Hz, 6 H, CH_3 and CH_3), 1.1–1.7 (m, 10 H, CH_2), 1.93 (m, 2 H, CH_2CH=) 2.04 (s, 3 H, $NHCOCH_3$), 2.84 (t, J = 7 Hz, 2 H, CH_2S), 3.06 [m (AB portion of an ABX system), 2 H, $CH_2CH(NHAc)COOMe$], 3.49 (d, $^3J_{CH}$ = 131 Hz, 2 H, $^{13}CH_2COS$), 3.68 (s, 3 H, $COOCH_3$), 4.82 [ddd (X portion of an ABX system), J = 8, 5, 5 Hz, $CH(NHAc)COOMe$], 5.67 (m, 1 H, CH=), 6.68 (s, 1 H, imidazole CH), 7.10 (br d, J = 8 Hz, 1 H, NH), 7.36 (s, 1 H, imidazole CH). ^{13}C NMR ($CDCl_3$): δ 51.2 ($^3J_{CH}$ = 4.9 Hz, when run without any proton decoupling).

For **8**. ^{1}H NMR: δ 0.8–1.05 (m, 6 H, CH_3 and CH_3), 1.1–1.7 (m, 10 H, CH_2), 1.98 (s, 3 H, $NHCOCH_3$), 2.1–2.3 (m, 2 H, CH_2CH=), 2.84 (t, J = 7 Hz, 2 H, CH_2S), 3.04 [apparent d, J = 7 Hz, 2 H, $CH_2CH(NHAc)COOMe$], 3.63 (d, J = 130 Hz, 2 H, $^{13}CH_2COS$), 3.73 (s, 3 H, $COOCH_3$), 4.65–4.95 [m, 1 H, $CH(NHAc)COOMe$], 5.65–5.95 [m (t, J = 8 Hz, in unlabeled **8**), 1 H, CH=], 6.14 (d, J = 8 Hz, 1 H, NH), 6.78 (s, 1 H, imidazole CH), 7.44 (s, 1 H, imidazole CH). ^{13}C NMR ($CDCl_3$): δ 46.1 ($^3J_{CH}$ = 7.0 Hz, when run without any proton decoupling).

For **12**. ^{1}H NMR (unlabeled material): δ 0.7–1.1 (m, 6 H, CH_3 and CH_3), 1.1–1.4 (m, 10 H, CH_2), 1.4–1.75 (m, 2 H, CH_2CH_2S), 1.9–2.1 (m, 2 H, CH_2CIm=), 2.82 (t, J = 7 Hz, CH_2S), 3.10 [br d, J = 6 Hz, $CH_2CH(NH)COOMe$], 3.70 (s, 3 H, $COOCH_3$), 4.35–4.65 [m, 1 H, $CH(NH)COOMe$], 4.98 (s, 1 H, =CHCOS), 6.87 (s, 1 H, imidazole CH), 7.53 (s, 1 H, imidazole CH), 9.60 [d, J = 10 Hz, $CH(NH)$-$COOMe$], 11.07 (br s, 1 H, imidazole NH).

A similar reaction between 226 mg of unlabeled allenic thioester and 420 mg of freshly prepared *N*-acetyl-L-histidine methyl ester gave (after flash chromatography) 245 mg of **4** plus 52 mg of **8**. The ^{1}H NMR spectrum of **4** indicated that the sample was contaminated with a small amount of **7**. Compound **12** was not produced.

Isomerization of the Adduct 4 Formed between *N*-Acetyl-L-histidine Methyl Ester and 2,3-Decadienoic Acid, *S*-1-Propyl Ester. Compound **4**, 500 mg, was dissolved in 5 mL of benzene, and 150 mg of 1,5-diazabicyclo[4.3.0]non-5-ene (DBN) was added. The reaction mixture was stirred at room temperature for 5 h, after which time volatiles were removed in vacuo. Following preliminary purification by flash chromatography, the product was subjected to semipreparative HPLC (10 × 250 mm silica gel column, CH_3CN solvent, 4.5 mL/min, effluent monitored at 215 nm). Four fractions were obtained: 130 mg of **5**, 14 mg of **6**, 26 mg of **7**, and 46 mg of **4** (recovered starting material).

For **5**. ^{1}H NMR: δ 0.75–1.1 (m, 6 H, CH_3 and CH_3), 1.1–1.8 (m, 12 H, CH_2), 2.03 (s, 3 H, $NHCOCH_3$), 2.8–3.2 [m, 6 H, CH_2S, CH_2-C=, and $CH_2CH(NHAc)COOMe$], 3.71 (s, 3 H, $COOCH_3$), 4.86 [ddd, J = 8, 5, 5 Hz, $CH(NHAc)COOMe$], 6.16 (s, 1 H, =CHCOS), 7.00 (s, 1 H, imidazole CH), 7.09 (br d, J = 8 Hz, NH), 7.76 (s, 1 H, imidazole CH). ^{13}C NMR: δ 113.95 (C-2).

For **6**. ^{1}H NMR: δ 0.7–1.05 (m, 6 H, CH_3 and CH_3), 1.1–1.7 (m, 12 H, CH_2), 2.05 (s, 3 H, $NHCOCH_3$), 2.1–2.5 (m, 2 H, CH_2C=), 2.86 (t, J = 7 Hz, 2 H, CH_2S), 3.0–3.15 [m, 2 H, $CH_2CH(NHAc)COOMe$], 3.71 (s, 3 H, $COOCH_3$), 4.86 [ddd, J = 8, 5, 5 Hz, 1 H, $CH(NHAc)$-$COOMe$], 5.98 [s (showing slight t character), 1 H, =CHCOS], 6.79 (s, 1 H, imidazole CH), 7.03 (br d, J = 8 Hz, 1 H, NH), 7.54 (s, 1 H, imidazole CH). ^{13}C NMR: δ 116.43 (C-2).

***N*-Acetyl-N^3-methyl-L-histidine Methyl Ester (10). (A) From N^3-Methyl-L-histidine.** To 100 mg (0.59 mmol) of N^3-methyl-L-histidine (Sigma) was added 0.4 mL of H_2O and 102 mg (1.00 mmol) of Ac_2O. The reaction mixture was stirred at room temperature for 20 min, and then volatiles were removed under high vacuum, leaving 115 mg of crude N-acetylated product. The latter material was suspended in 4 mL of MeOH, and HCl gas was bubbled into the mixture for 10 min, during which time solution was effected. The reaction mixture was cooled to room temperature and concentrated in vacuo. The residue was treated with saturated $NaHCO_3$, and the resulting mixture was extracted several times with CH_2Cl_2. The CH_2Cl_2 extracts were dried, filtered, and concentrated, leaving methyl ester **10** as an amorphous solid.

(B) From Methylation of *N*-Acetyl-L-histidine Methyl Ester.[53] *N*-Acetyl-L-histidine methyl ester (210 mg, 1.00 mmol; made by esterification of commercial *N*-acetyl-L-histidine) was combined with 6 mL of MeI, and the reaction mixture was stirred at room temperature for 24 h. The mixture was then filtered, the filtrate was concentrated to dryness, and the residue was taken up in $CHCl_3$, filtered, and concentrated once again. The components of the product mixture were separated by flash chromatography (9:1, CH_2Cl_2/MeOH), providing 65 mg of **10**, along with 72 mg of **9**.

For **10**. ^{1}H NMR: δ 2.00 (s, 3 H, NHAc), 3.11 (apparent d, J = 6 Hz, 2 H, CH_2CH), 3.57 (s, 3 H, NCH_3), 3.75 (s, 3 H, $COOCH_3$), 4.7–4.95 [m, 1 H, $CH(NHAc)COOMe$], 6.45 (br d, J = 8 Hz, 1 H, NH), 6.75 (s, 1 H, imidazole CH), 7.38 (s, 1 H, imidazole CH).

***N*-Acetyl-N^1-methyl-L-histidine Methyl Ester (9).** The title compound was isolated from the reaction of MeI with *N*-acetyl-L-histidine methyl ester, as described above. ^{1}H NMR: δ 2.03 (s, 3 H, NHAc), 3.02 [m (AB portion of an ABX system), 2 H, CH_2CH], 3.63 (s, 3 H, NCH_3), 3.69 (s, 3 H, $COOCH_3$), 4.78 [ddd (X portion of an ABX system), J = 8, 5, 5 Hz, 1 H, $CH(NHAc)COOMe$], 6.65 (s, 1 H, imidazole CH), 7.1–7.4 (br m, 1 H, NH), 7.33 (s, 1 H, imidazole CH).

Incubation of 3-[1-^{14}C]Decynoyl-NAC with Dehydrase. To 10 mg (2.78 μmol) of homogeneous dehydrase in 1.43 mL of KPO_4 (pH 7.0) was added 5.6 μmol of 3-[1-^{14}C]decynoyl-NAC in 5.6 μL of acetone. After stirring at room temperature for 12 h, the reaction mixture was acidified to pH 2.0 with 4 N HCl and extracted with several 2-mL aliquots of ether. The remaining aqueous solution was dialyzed exhaustively against 0.2 M NH_4HCO_3, and the dialysate was lyophilized.

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Hydrolysis of Dehydrase Inactivated with 3-[1-^{14}C]Decynoyl-NAC. Lyophilized, inactivated dehydrase (4.3×10^5 dpm) was dissolved in 16 mL of 6 NHCl. After stirring for 3 h at room temperature, the suspension was transferred to a heavy-walled reaction tube, which was sealed under vacuum. The sample was heated at 110 °C for 20 h and then cooled to room temperature. After removal of HCl and H_2O, the sample (total recovered radioactivity, 4.12×10^5 dpm) was analyzed by HPLC: 33:67, MeOH/0.5% Et_3NHPO_4, pH 3.1; 250 × 4.6 mm Rainin Microsorb C-18 column; eluted at 1.0 mL/min, with effluent monitored at 210 nm (see Figure 2).

Hydrolysis of Model Adducts 4-6. Compounds **4-6** were hydrolyzed in the same way as were dehydrase and dehydrase inactivated with 3-decynoyl-NAC. The HPLC conditions were identical, as well.

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Proton Inventory of the Second Step of Ribonuclease Catalysis

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Abstract: The specificity ratio $k_{cat}/K_m = k_E$ has been measured for the bovine pancreatic ribonuclease A catalyzed hydrolysis of cytidine cyclic 2',3'-monophosphate at 25 °C, pH 7.47, and equivalent in H_2O–D_2O solutions containing a mole fraction n of D_2O. A graph of the partial kinetic solvent iostope effects $k_{E,n}/k_{E,0}$ vs. n is bowl shaped. The isotope effect data fit the equation $k_{E,n}/k_{E,0} = (1 - n + 0.57n)^2$. The transition state may involve two protons, each with an isotopic fractionation factor of 0.57. These results support previous studies that propose general-acid–general-base catalysis by the imidazole rings of histidine residues 12 and 119 as an essential feature of ribonuclease action.

The second step of ribonuclease (RNase) catalysis involves the hydrolysis of nucleoside cyclic 2',3'-phosphate esters to 3'-phosphates.[1] This reaction is believed to be catalyzed by donation of a proton to the 2' ester group and abstraction of a proton from water.[1,2] Although the chemical and physical evidence is complex,[1,2] the imidazole groups of histidine residues (12 and 119) are generally considered to be the proton donor and proton acceptor (Figure 1). We now report that a proton inventory[3,4] study of the bovine RNase A catalyzed hydrolysis of cytidine cyclic 2',3'-monophosphate (cCMP) supports this hypothesis. Our results indicate that, at about pH 7.5, two protons undergo a change of state upon activation of the dissociated enzyme and substrate to a single rate-controlling transition state.

Results

Partial kinetic solvent isotope effects (KSIEs) acquired for eight H_2O–D_2O mixtures on four occasions are given in Table I. The $k_{E,n}$ and $k_{E,0}$ are, respectively, the values of the specificity ratio k_{cat}/K_m in protium oxide containing a mole fraction n of deuterium oxide and in protium oxide. A graph of $k_{E,n}/k_{E,0}$ vs. n bulges downward, as shown in Figure 2. The dependence of the KSIEs on n was used with the Gross–Butler equation[3] (eq 1) to evaluate

Table I. Solvent Deuterium Isotope Effects on the Ribonuclease A Catalyzed Hydrolysis of Cytidine Cyclic 2',3'-Monophosphate[a]

n	$k_{E,n}/k_{E,0}$	average
0.117	0.927, 0.879, 0.932, 0.907	0.912 ± 0.024
0.234	0.801, 0.816, 0.793, 0.778	0.797 ± 0.016
0.352	0.688, 0.676, 0.737, 0.741	0.721 ± 0.024
0.469	0.627, 0.632, 0.645, 0.630	0.634 ± 0.008
0.586	0.536, 0.552, 0.562, 0.563	0.553 ± 0.013
0.703	0.513, 0.485, 0.500, 0.487	0.496 ± 0.013
0.820	0.447, 0.422, 0.438, 0.426	0.433 ± 0.011
0.935	0.364, 0.346, 0.354, 0.359	0.356 ± 0.008

[a] 25 °C; pH 7.47 in H_2O and its equivalent[3b,c] in H_2O–D_2O mixtures (0.10 M Tris buffer); pseudo-first-order conditions ($[E_0] \ll [S_0] \ll K_m$) throughout; average values of KSIEs are the mean ± 1 standard deviation of the KSIEs obtained at the same n in four separate proton inventories; average error in $k_{E,n}/k_{E,0}$ is 2.4%.

$$k_{E,n}/k_{E,0} = \frac{\overset{TS}{\prod}(1 - n + \phi^T_i n)}{\overset{TS}{\prod}(1 - n + \phi^R_j n)} \quad (1)$$

$$k_{E,n}/k_{E,0} = \overset{TS}{\prod}(1 - n + \phi^T_i n) \quad (2)$$

$$k_{E,n}/k_{E,0} = (1 - n + \phi^T n)^2 \quad (3)$$

the number of protons involved upon activation of the enzyme and substrate to the rate-controlling transition state or transition states. The ϕ^T_i and ϕ^R_j, respectively, represent the isotopic fractionation factors of the ith hydrogenic site of the transition state and jth hydrogenic site of the reactants. When $n = 1$, the overall KSIE is the product of all ϕ^T over the product of all ϕ^R. A reasonable initial assumption is that ϕ^R of the ionizable side-chain groups

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