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On the Mode of Interaction of β-Hydroxydecanoyl Thioester Dehydrase with Allenic Acid Derivatives*

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ABSTRACT: As a model system for the inhibition of β-hydroxydecanoyl thioester dehydrase by allenic compounds the reaction between 2,3-decadienoyl thioesters and histidine derivatives has been investigated. Refluxing *S*-ethyl 2,3-decadienoate with *N*-acetylhistidine in methanol for 3 hr affords an adduct (Ia) which has been characterized as a β,γ-olefinic enamine on the basis of ultraviolet and nuclear magnetic resonance spectroscopy, by analysis of ozonolysis products, and by mass spectrometry. Under the same conditions, the allenic thioester reacts with histidine methyl ester to form an α,β-olefinic enamine. Comparison of various derivatives of 2,3-decadienoic acid as dehydrase inhibitors established the following order of activities: thioester > oxygen ester > free acid > amide. It is also shown that an allene system conjugated with a carbonyl group is required for enzyme inhibition. The structural features in allenes necessary for enzyme inhibition and for adduct formation with histidine derivatives are compared.

The mode of action of β-hydroxydecanoyl thioester dehydrase (dehydrase) has been studied extensively in this laboratory (Bloch, 1969). This key enzyme in the generation of long-chain olefinic acids in *Escherichia coli* catalyzes a set of reversible reactions as shown below (Brock *et al.*, 1967).

$$CH_3(CH_2)_5CH_2CHOH\cdot CH_2\overset{O}{\overset{\|}{C}}R \rightleftharpoons CH_3(CH_2)_5CH_2CH{=}CH\cdot\overset{O}{\overset{\|}{C}}R \rightleftharpoons CH_3(CH_2)_5CH{=}CH\cdot CH_2\overset{O}{\overset{\|}{C}}R$$

The normal substrates of dehydrase are thioester derivatives of acyl carrier protein (ACP)[1] but simpler thioesters such as the *N*-acetylcysteamine (NAC)[1] derivatives are also transformed by the enzyme.

Several years ago a powerful and highly selective inhibitor of dehydrase was found in the form of 3-decynoyl-NAC (Brock *et al.*, 1967; and Helmkamp *et al.*, 1968). Unexpectedly this acetylenic substrate analog inhibited the enzyme irreversibly (Helmkamp *et al.*, 1968), inactivation resulting from a blockage of histidine residues at the active site (Helmkamp and Bloch, 1969). As was realized later, 3-decynoyl-NAC as such is not the inhibitory agent but is transformed by dehydrase into the allenic isomer which in turn combines irreversibly with enzyme protein (Endo *et al.*, 1970). Reaction

$$CH_3(CH_2)_5C{\equiv}C\cdot CH_2\overset{O}{\overset{\|}{C}}R \longrightarrow CH_3(CH_2)_5CH{=}C{=}CH\cdot\overset{O}{\overset{\|}{C}}R \quad (A)$$

$$CH_3(CH_2)_5CH{=}C{=}CH\cdot\overset{O}{\overset{\|}{C}}R + \text{enzyme} \longrightarrow \text{inactive enzyme} \quad (B)$$

A involves hydrogen abstraction at C_α and addition at C_γ of the fatty acyl chain in strict analogy to the isomerization of β,γ-decenoyl thioester to α,β-decenoyl thioester. Because the allenic product reacts with enzyme and inactivates it instantaneously, reaction A could not be demonstrated directly. However, the circumstantial evidence for an enzyme-catalyzed acetylene–allene isomerization is compelling. It includes the observation that enzyme inhibition by 2,2-dideuteriodecynoyl-NAC proceeds with a significant kinetic isotope effect (Endo *et al.*, 1970) which is of the same magnitude as that observed for removal of α-hydrogen in normal substrate–

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[1] Abbreviations used are: ACP, acyl carrier protein; NAC, *N*-acetylcysteamine.

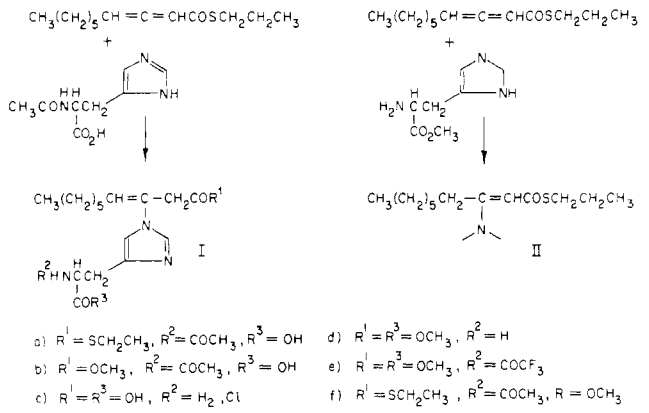


FIGURE 1: Structures of adducts formed between 2,3-decadienoyl ethyl thioester and histidine derivatives.

dehydrase interactions (Rando and Bloch, 1968). Secondly, synthetic 2,3-decadienoyl-NAC surpasses the inhibitory activity of 3-decynoyl-NAC by a factor of at least ten (Endo *et al.*, 1970) and it also attaches to a histidine residue at the active enzyme site. As for the chemistry of reaction B, it is to be noted that allenic acids and some of their derivatives are prone to attack by various nucleophiles (Adams and Ulich, 1920; Kurtz *et al.*, 1959). In the present instance the imidazole moiety of the "active" histidine would be the candidate attacking group for joining one of the allenic carbon atoms to the enzyme.

In order to facilitate the structure elucidation of the allene–enzyme adduct we have explored the reaction between simple histidine derivatives and 2,3-decadienoyl thioesters as a chemical model. These types of compounds react with surprising ease, affording isomeric enamines of the general structures I and II (Figure 1). The description of this model system and its relevance to the interaction of allenic thioesters with the active site of dehydrase are the subject of this paper. We have also examined the effects of modifying the carboxyl group of 2,3-decadienoic acid on enzyme inhibition and on the course of the model reaction.

Experimental Section

Syntheses. 2,3-DECADIENOIC ACID AND 2,3-DECADIENOYL-*N*-ACETYLCYSTEAMINE. These compounds were synthesized as previously described (Endo *et al.*, 1970).

4(5)-METHYLIMIDAZOLE was prepared by the method of Nozaki *et al.* (1957): mp 110–112°; nmr (D_2O), δ 2.3 (3 H, singlet), 7.1 (1 H, singlet), and 8.5 ppm (1 H, singlet).

HISTIDINE METHYL ESTER. L-Histidine methyl ester dihydrochloride (Chemical Corp.) was converted to the free base by treatment with 2 equiv of sodium methoxide in methanol (Fischer and Cone, 1908): nmr (D_2O), δ 3.0 (2 H, doublet, J = 6 Hz), 3.8 (3 H, singlet), 7.1 (1 H, singlet), and 7.8 ppm (1 H, singlet).

N-ACETYLHISTIDINE METHYL ESTER. L-*N*-Acetylhistidine monohydrate (Calbiochem) was treated with methanolic hydrochloric acid, followed by neutralization with sodium methoxide: nmr (D_2O), δ 2.0 (3 H, singlet), 3.1 (2 H, doublet, J = 7 Hz), 3.8 (3 H, singlet), 4.7 (1 H, multiplet), 7.1 (1 H, singlet), and 8.0 ppm (1 H, singlet).

2,3-DECADIENOYL ETHYL THIOESTER. 3-Decynoic acid (1 g) was dissolved in 10 ml of oxalyl chloride and the solution heated at 40–50° for 30 min. The excess of oxalyl chloride was removed by a nitrogen stream. To the acid chloride was added, with ice cooling, 10 ml of anhydrous benzene containing ethanethiol (1.5 ml) and pyridine (0.7 ml). The solution was allowed to stand in an ice bath for 15 min and the crude product chromatographed on silicic acid (Unisil, Clarkson Chemical Co.) with *n*-hexane–ether (100:1, v/v) to give a pale yellow oil of 3-decynoyl ethyl thioester (718 mg, 60% of theory): ir, ν_{max} 6.1 μ; uv, λ_{max} 232 nm (ϵ 3600); nmr (CCl_4), δ 2.9 (2 H, quartet, J = 8 Hz) and 3.3 ppm (2 H, triplet, J = 2 Hz).

To the acetylenic ethyl thioester (570 mg) dissolved in 50 ml of anhydrous ethanol was added 0.1 ml of 1 M sodium ethoxide solution in ethanol. After standing at room temperature for 5 min, the mixture was diluted with water and extracted with methylene chloride. The crude product was purified by column chromatography on Unisil. Elution with *n*-hexane–ether (100:1) afforded 400 mg (70%) of the allenic ethyl thioester: ir, λ_{max} 6.0 and 5.1 μ; uv, λ_{max} 264 nm (ϵ 3800); nmr ($CDCl_3$), δ 5.7 ppm (2 H, multiplet).

2,3-DECADIENOYL METHYL ESTER. To 2,3-decadienoic acid (100 mg) in 1.0 ml of ether, a solution of diazomethane in ether was added dropwise until a pale yellow color persisted (an excess of the reagent should be avoided). The solvent was evaporated and the residue was filtered through a Unisil column with *n*-hexane–ether (100:1) to give the methyl ester (70 mg): ir, λ_{max} 5.8 and 5.1 μ; uv, λ_{max} 225 nm (ϵ 1700); nmr ($CDCl_3$), δ 3.8 (3 H, singlet) and 5.6 ppm (2 H, multiplet).

2,3-DECADIENOYL-*N*-ACETYLETHYLENEDIAMIDE. 3-Decynoyl chloride was prepared from 900 mg of 3-decynoic acid as described above. To the stirred and chilled (−5°) solution of acid chloride in 20 ml of methylene chloride was added 600 mg of monacetylethylenediamine, which had been prepared by reacting ethylenediamine with ethyl acetate at 110°, 48 hr, followed by distillation: bp 132–133° (4 mm) (Hill and Aspinal, 1939). After stirring at room temperature for 3 hr the reaction mixture was washed with 2 N HCl and saturated $NaHCO_3$ solution and dried over magnesium sulfate. Evaporation of solvent afforded a pale yellow amorphous product (447 mg). The crude amide (350 mg) was chromatographed on Unisil with methylene chloride–methanol (100:3) to give 3-decynoyl-*N*-acetylethylenediamide (120 mg): nmr ($CDCl_3$), δ 2.0 (3 H, singlet), 3.2 (2 H, triplet, J = 2 Hz), 3.5 (4 H, diffused triplet), 6.8 (1 H, broad), and 7.5 ppm (4 H, broad).

The acetylenic amide (100 mg) was treated with 35 ml of 57 mM sodium ethoxide solution in ethanol at room temperature for 7 min. The reaction mixture was poured into a NaCl solution, extracted with ether, washed with NaCl, and dried over Na_2SO_4. The crude product was chromatographed twice on Unisil with methylene chloride–methanol (100:1) to give the allenic amide (30 mg): mp 123–124°, ir, λ_{max} 5.1 and 6.0 μ; uv, λ_{max} 220 nm (ϵ 7300); nmr, δ 2.0 (3 H, singlet), 3.4 (4 H, diffused triplet), 5.6 (2 H, multiplet), and 6.7 to 7.2 ppm (2 H, broad).

ETHYL 3,4-DECADIENOATE. This was synthesized by the method of Crandall and Tindell (1970). A mixture of 1-octyn-3-ol (Aldrich) (3.78 g), triethyl orthoacetate (30 g), and propionic acid (100 mg) was heated at 140–150° for 2.5 hr with removal of ethanol by distillation. The excess ortho ester was evaporated *in vacuo* to give a pale yellow residue (6.5 g). A 350-mg aliquot of the residue was chromatographed on Unisil with hexane–ether (100:1) to give the β-allenic ethyl ester (230 mg): ir, λ_{max} 5.1 and 5.8 μ; nmr ($CDCl_3$), δ 3.0 (2 H, multiplet), 4.2 (2 H, quartet, J = 7 Hz), and 5.2 ppm (2 H, multiplet).

ETHYL 3,4-UNDECADIENOATE was synthesized from 1-nonyn-3-ol by the procedure described for ethyl 3,4-decadienoate in

52% yield: ir, λ_{max} 5.1 and 5.8 μ; nmr ($CDCl_3$), δ 3.0 (2 H, multiplet), 4.2 (2 H, quartet, J = 7 Hz), and 5.3 ppm (2 H, multiplet). The nonynol was prepared from heptanal and LiC≡CH·$NH_2CH_2CH_2NH_2$ (Foote Mineral Co.) in tetrahydrofuran.

The intensity of the characteristic infrared absorption at 5.1 μ served as a criterion of purity for the various allenic derivatives.

Reactions of Allenic Acid Thioesters with Histidine Derivatives. In all runs the molar ratio of allene to histidine derivative was 1:2.

2,3-DECADIENOYL ETHYL THIOESTER[2] AND *N*-ACETYLHISTIDINE. A mixture of 2,3-decadienoyl ethyl thioester (600 mg) and *N*-acetylhistidine monohydrate (1.2 g) was refluxed in 10 ml of methanol for 3 hr, and the products were partitioned between sodium chloride solution and chloroform. The fraction soluble in the organic phase (1.3 g) was chromatographed on silicic acid (20 g) with methylene chloride–methanol (15:1) as the eluting solvent. The isolated adduct (810 mg, 70% of theory) was resolved into two spots on silica gel tlc, probably the cis and trans isomers. The major isomer (Ia) has the following spectra: ir, λ_{max} 2.9 and 6.0 μ; uv, λ_{max} 232 nm (ε 8100); nmr ($CDCl_3$), δ 2.0 (3 H, singlet), 2.9 (2 H, quartet, J = 7 Hz), 3.3 (2 H, broad doublet, J = 5 Hz), 3.7 (2 H, singlet), 4.7 (1 H, multiplet), 5.9 (1 H, triplet, J = 7 Hz), 6.9 (1 H, singlet), and 7.8 ppm (1 H, singlet).

2,3-DECADIENOYL METHYL ESTER AND *N*-ACETYLHISTIDINE. Methyl 2,3-decadienoate (43 mg) and *N*-acetylhistidine monohydrate (100 mg) were refluxed in 1.0 ml of methanol for 3 hr. The fraction extractable by methylene chloride (52 mg) was chromatographed on silicic acid to afford 36 mg (40%) of an adduct (Ib), whose nmr ($CDCl_3$) showed δ 2.0 (3 H, singlet), 3.2 (2 H, broad doublet, J = 5 Hz), 3.5 (2 H, singlet), 3.7 (3 H, singlet), 4.6 (1 H, multiplet), 5.8 (1 H, triplet, J = 7 Hz), 6.9 (1 H, singlet), and 7.8 ppm (1 H, singlet).

2,3-DECADIENOIC ACID AND *N*-ACETYLHISTIDINE. 2,3-Decadienoic acid (240 mg) and *N*-acetylhistidine monohydrate (575 mg) were refluxed in 6 ml of methanol for 3 hr. The chloroform-soluble fraction (240 mg) was fractionated by column chromatography on silicic acid. Apart from a nonpolar substance (95 mg) whose R_F coincided with that of the starting allenic acid, 100 mg of a polar product was obtained. The nmr spectrum of this material lacked any peaks assignable to imidazole or *N*-acetyl groups.

BASE TREATMENT OF ADDUCT Ia. Adduct Ia (85 mg) was refluxed in 10 ml of 10% NaOH solution for 2.5 hr. After acidification, the reaction mixture was extracted with ether. Only 2 mg of a brown oil was obtained. An nmr spectrum of the acidified aqueous phase showed that the β,γ-enamine structure was still intact. Also, when *S*-ethyl 2,3-decadienoate and *N*-acetylhistidine were refluxed in methanol in the presence of MeONa, the uv spectrum of the reaction mixture showed a maximum at 232 nm but not at 319 nm. Therefore, base-catalyzed isomerization of the β,γ-olefin to the α,β structure does not seem to occur.

ACID TREATMENT OF THE ADDUCT. Adduct Ia (120 mg) was suspended in 4 ml of 6 N HCl and heated in a sealed tube at 100° for 24 hr. After washing with methylene chloride, the aqueous phase was evaporated *in vacuo* to give 78 mg of an amorphous white solid (Ic): nmr (D_2O), δ 3.5 (2 H, doublet, J = 6 Hz), 3.8 (2 H, singlet), 4.5 (1 H, multiplet), 6.3 (1 H, triplet, J = 7 Hz), 7.8 (1 H, doublet, J = 1 Hz), and 9.0 ppm (1 H, doublet, J = 1 Hz).

Ozonolysis of Adduct Id

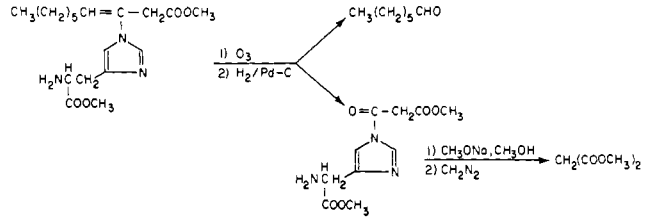


FIGURE 2: Ozonolysis of adduct Id.

N-TRIFLUOROACETYL DIMETHYL ESTER DERIVATIVE (Ie). Substance Ic (40 mg) obtained by acid hydrolysis of adduct Ia was refluxed for 2 hr in 10 ml of a 1.3 N solution of HCl in methanol. The reaction mixture was evaporated, the residue redissolved in 5 ml of water and the chilled solution brought to pH 8 with $NaHCO_3$. Extraction with chloroform and evaporation of solvent gave 13 mg of oil, whose nmr was consistent with the dimethyl ester structure (Id). About 0.5 mg of dimethyl ester was treated with 0.2 ml of trifluoroacetic anhydride in 1.8 ml of anhydrous methylene chloride at 100° for 1 hr in a screw-capped test tube. Gas–liquid chromatography of the acylated product (Ie) was carried out on a Perkin-Elmer 900 instrument equipped with a flame ionization detector. The column (6.0 ft × 0.08 in.), was packed with 3% OV-1 on Gaschrom Q (100–200 mesh) and the temperature programmed (3°/min) from 200° to 250°. The sample was detected as a single peak at 13.5 min. Mass spectrometry of Ie was carried out on a AEI, MS-9 double-focussing instrument. Besides the molecular ion peak at *m/e* 447, prominent peaks were observed at *m/e* 416, 388 (base peak), 335, 334, 299, 275, 263, 248, and 206.

OZONOLYSIS OF THE DIMETHYL ESTER DERIVATIVE OF THE ADDUCT (FIGURE 2). The dimethyl ester (Id) (80 mg) was dissolved in 7 ml of methylene chloride. The solution was cooled in Dry Ice–acetone and ozonization was carried out for 5 min when the solution turned blue. After the excess of ozone had evaporated, the ozonide was decomposed by catalytic hydrogenation on 5% palladium/charcoal (20 mg) for 1 hr. Heptanal was identified by gas chromatography on 3% OV-1 at 65°. After removal of solvent,[3] the crude ozonolysis products were dissolved in 1 ml of methanol, 25 μl of 0.5 M sodium methoxide solution was added, and the solution was kept at room temperature for 3 hr. After treatment with diazomethane–ether, the products were analyzed by gas chromatography on a column of 3% OV-1 column at 65°. Dimethyl malonate was identified as the major product. Dimethyl oxalate could not be detected.

2,3-DECADIENOYL ETHYL THIOESTER AND 4(5)-METHYLIMIDAZOLE. A mixture of allenic thioester (60 mg) and 4(5)-methylimidazole (50 mg) was refluxed in 0.9 ml of methanol for 3 hr. Partition between water and chloroform gave 60 mg of chloroform-soluble material. The nmr spectrum of this fraction gave no indication for the presence of an imidazole moiety.

[2] Originally we used the *N*-acetylcysteamine thioester of 2,3-decadienoic acid in the model system. We replaced it by the more accessible *S*-ethyl thioester which proved as effective as the NAC derivative both as an enzyme inhibitor and as a reactant in the model system.

[3] At this stage it is essential to remove excess heptanal by evaporation to avoid condensation of the aldehyde with the subsequently formed malonate.

TABLE I: Comparison of C_{10}-Allenic Acid Derivatives as Dehydrase Inhibitors.[a]

Inhibitor	Concn Required for 50% Inhibn (M)
$CH_3(CH_2)_5CH{=}C{=}CHCOSCH_2CH_2NHCOCH_3$	5.3×10^{-8}
$CH_3(CH_2)_5CH{=}C{=}CHCOSCH_2CH_3$	7.1×10^{-8}
$CH_3(CH_2)_5CH{=}C{=}CHCOOCH_3$	4.2×10^{-7}
$CH_3(CH_2)_5CH{=}C{=}CHCOOH$[b]	1.4×10^{-5}
$CH_3(CH_2)_5CH{=}C{=}CHCONHCH_2CH_2NHCOCH_3$	$>1 \times 10^{-4}$

[a] Enzyme (4.5×10^{-8} M) was preincubated in 0.2 ml of phosphate buffer, pH 6.0, $I = 0.05$, with various amounts of inhibitors for 10 min at room temperature. Then, 0.8 ml of *cis*-3-decenoyl-*N*-acetylcysteamine (2.4×10^{-4} M) solution in the same buffer was added and the change in absorbance at 263 nm was recorded. The concentration required for 50% inhibition was estimated graphically. [b] At pH 6 the acid is present mainly as the anion. As has previously been shown, the acid form is the active inhibitory species (Morisaki and Bloch, 1971).

TABLE II: Effects of Allenic Oxygen Esters on Dehydrase Activity.[a]

Inhibitor	Concn (M)	ΔA_{263} (nm/min)	Control (%)
None		0.040	100
$CH_3(CH_2)_5CH{=}C{=}CHCOOH$	1.4×10^{-5}		75[b]
$CH_3(CH_2)_5CH{=}C{=}CHCOOCH_3$	5×10^{-7}	0.013	32
$CH_3(CH_2)_7CH{=}C{=}CHCOOCH_3$	5×10^{-7}	0.001	3
$CH_3(CH_2)_4CH{=}C{=}CHCH_2COOC_2H_5$	5×10^{-5}	0.037	93
$CH_3(CH_2)_5CH{=}C{=}CHCH_2COOC_2H_5$	5×10^{-5}	0.040	100

[a] Dehydrase (4.5×10^{-8} M) was first incubated in 0.2 ml of phosphate buffer, pH 7.0, $I = 0.01$, with inhibitors at room temperature for 10 min. Subsequently, 0.8 ml of *cis*-3-decenoyl-*N*-acetylcysteamine solution (2.4×10^{-4} M) in phosphate buffer, pH 6.0, $I = 0.05$, was added and the increase of optical density at 263 nm was followed with a Gilford spectrophotometer. [b] Calculated from the data in Table I.

2,3-DECADIENOYL ETHYL THIOESTER AND HISTIDINE METHYL ESTER. A mixture of allenic thioester (68 mg) and histidine methyl ester (108 mg) was refluxed in 2.5 ml of methanol for 3 hr. The chloroform-soluble fraction (111 mg) was chromatographed on a silicic acid column. On elution with methylene chloride–methanol (80:1), 50 mg (40%) of adduct (II) was isolated: ir, λ_{max} 2.7, 5.7, and 6.2 μ; uv, λ_{max} 319 nm (ϵ 25,000); nmr ($CDCl_3$), δ 2.9 (2 H, quartet, $J = 7$ Hz), 3.1 (2 H, doublet, $J = 6$ Hz), 3.8 (3 H, singlet), 4.5 (1 H, multiplet), 5.0 (1 H, singlet), 6.9 (1 H, singlet), and 7.6 ppm (1 H, singlet).

REACTION OF 2,3-DECADIENOYL ETHYL THIOESTER WITH *N*-ACETYLHISTIDINE METHYL ESTER. A mixture of allenic thioester (70 mg) and *N*-acetylhistidine methyl ester (140 mg) was refluxed in 1.4 ml of methanol for 3 hr. The chloroform-soluble fraction (126 mg) showed uv maxima at 232 and 319 nm (ϵ 6400 and 1000, respectively). Column chromatography on silicic acid gave 46 mg (33%) of the major product (If) whose nmr ($CDCl_3$) showed δ 2.0 (3 H, singlet), 2.9 (2 H, quartet, $J = 7$ Hz), 3.1 (2 H, doublet, $J = 6$ Hz), 3.8 (3 H, singlet), 4.9 (1 H, multiplet), 5.9 (1 H, triplet, $J = 7$ Hz), 6.9 (1 H, singlet), and 7.6 ppm (1 H, doublet, $J = 1$ Hz).

Enzyme Assays. Enzyme activity was assayed as previously described (Kass *et al.*, 1967) (for details, see Tables I and II). Partially purified β-hydroxydecanoyl thioester dehydrase was kindly provided by Dr. G. Helmkamp. The substrate, *cis*-3-decenoyl-*N*-acetylcysteamine, was prepared as described (Brock *et al.*, 1967).

Results and Discussion

Model Reactions for Interaction between Dehydrase and Allenic Inhibition. Both 3-decynoyl-NAC and 2,3-decadienoyl-NAC combine stoichiometrically with dehydrase and inactivate it by modifying histidine residues at the active enzyme site (Helmkamp *et al.*, 1968; Helmkamp and Bloch, 1969; Endo *et al.*, 1970). The actual dehydrase inhibitor is the allenic molecule which arises from the acetylenic thioester by an enzyme-catalyzed isomerization (Endo *et al.*, 1970). As for the chemistry of the enzyme–inhibitor adduct, Helmkamp had found (Helmkamp and Bloch, 1969; Helmkamp, 1970) that dehydrase treated with [1-^{14}C]decynoyl-NAC yields on hydrolysis with 6 N HCl 1 to 2 histidines less than native enzyme and also a radioactive product which is soluble in water but not in organic solvents. From these findings it was concluded that the adduct contained the intact C_{10} chain of the inhibitor and that this was connected by an acid-stable linkage to a basic or amphoteric molecule.

For the reasons given in the introduction, we have carried out the described model reactions between allenes and simple histidine derivatives to obtain information that might be applicable to the structure of the enzyme–inhibitor complex. These experiments have furnished us with two types of adducts which may be useful for this purpose but which in any event demonstrate facile interactions between allenic acid esters and the histidine derivatives.

Under the chosen conditions (refluxing for 3 hr in meth-

anol), *S*-ethyl 2,3-decadienoate[2] (1 mole) and *N*-acetylhistidine (2 moles) afforded in 70% yield a compound to which we assign the enamine structure Ia[4] on the basis of spectroscopic data, chemical identification of ozonolysis products, and mass spectrometry. The ultraviolet spectrum of adduct Ia showed a maximum at 232 nm typical for thioesters. However, the extinction coefficient (ε 8100) was much higher than is usual for simple thioesters (ε 4000) probably due to overlap with the enamine moiety. The nmr signal at 5.9 ppm (1 H, triplet, J = 7 Hz) indicates an olefinic proton adjacent to a methylene group and the two broad singlet signals at 6.9 and 7.8 ppm can be ascribed to an intact imidazole nucleus.

If, as these spectral data indicate, adduct Ia is a β,γ-olefinic enamine, hydrolysis should proceed readily and afford β-ketodecanoic or its decarboxylation product nonan-2-one. Surprisingly, however, treatment with 6 N HCl or 10% NaOH at reflux temperature failed to yield any material that was extractable by organic solvents. Moreover, the unchanged nmr spectrum of the aqueous phase of acid-hydrolyzed Ia showed that the enamine moiety had remained intact. Whatever the explanation for the extreme and atypical acid stability of this enamine,[5] it should be noted, as already pointed out, that in the enzyme–inhibitor adduct the bond linking the fatty acid chain to histidine also survives drastic hydrolysis (Helmkamp, 1970).

After acid hydrolysis of Ia to the carboxylic acid (Ic), the *N*-trifluoroacetyl dimethyl ester (Ie) was prepared and found to be homogeneous by glc analysis. Its mass spectrum showing a molecular ion peak at *m/e* 447 is also in accord with structure Ie ($C_{20}H_{28}O_5N_3F_3$). Finally the olefinic bond of Ia was located chemically by degradation as shown in Figure 2. Ozonolysis of the dimethyl ester (Id) gave heptanal which was identified by glc. An *N*-acylimidazole was the expected second ozonolysis product. This should contain a labile C–N bond and indeed treatment of the crude ozonolysis mixture with sodium methoxide and diazomethane yielded dimethyl malonate which was identified by glc.

The nature of the ester moiety in the reacting allene profoundly influences the extent of adduct formation. While the yield of adduct was 70% with the *S*-ethyl thioester, methyl 2,3-decadienoate afforded only 40% of product under the same conditions. Even more strikingly, 2,3-decadienoic acid failed to give adduct in any detectable amount. With the free allenic acid, polymerization appeared to take place. The relative reactivity of the various allenic derivatives in the model system and as enzyme inhibitors will be discussed below.

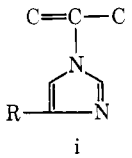
Variation of Imidazole Component. Under the conditions established for facile adduct formation between *S*-ethyl 2,3-decadienoate and *N*-acetylhistidine, 4(5)-methylimidazole failed to react. Only starting materials were recovered. On the other hand, histidine methyl ester readily reacted with the allenic thioester affording a new adduct (II) in 40% yield. Spectroscopically (II) is clearly distinct from the *N*-acetylhistidine-derived enamine Ia. Unlike Ia, II absorbs strongly at 319 nm suggesting an enamine moiety in conjugation with thioester. Moreover, the nmr signal at 5.0 ppm (1 H, singlet) shows the presence of a vinylic proton at C_α of an α,β unsaturated thioester confirming the enamine structure II. This adduct also contains the imidazole moiety as shown by the nmr signals at 6.9 (1 H, singlet) and 7.6 ppm (1 H, singlet). Further characterization of the α,β enoic adduct is needed. In particular our spectroscopic data do not reveal whether in II an imidazole nitrogen or the α-amino nitrogen of histidine is linked to the fatty acyl chain.

N-Acetylhistidine methyl ester was the final nucleophile tested for adduct formation with *S*-ethyl 2,3-decadienoate. It afforded a mixture displaying uv absorption maxima at both 232 and 319 nm indicative of both β,γ- (Type I) and α,β- (Type II) enoic adducts. From the uv intensity at these two wavelengths and the respective extinction coefficients, the ratio of the two isomers in the mixture was estimated to be 30:1. The structure of the more abundant adduct (Type I) was confirmed by nmr.

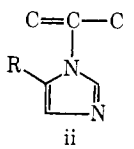
Structural Requirement for Adduct Formation. From the structural evidence presented it seems clear that one of the imidazole nitrogens of histidine can serve as the nucleophile in the reaction with allenic thioester. Yet 4(5)-methylimidazole fails to undergo this type of addition and, therefore, the amino acid portion of histidine must be critically involved. In particular, our data illustrate the prominent part played by the α-NH_2 group, free *vs.* acylated, in determining the course of the addition reaction. *N*-Acetylhistidine and *N*-acetylhistidine methyl ester both afford the β,γ-enoic enamine, the former exclusively, and the latter predominantly. On the other hand, when the α-NH_2 group is free as in histidine methyl ester the addition reaction affords the stabler α,β-olefinic product. This change of direction may be due to the more basic character of histidine methyl ester as compared to the *N*-acetyl derivative. It is known that α,β adducts are formed from allene carboxylic acid esters on treatment with amines (Eglinton *et al.*, 1954). In accord with this interpretation *N*-acetylhistidine methyl ester yields significant if small amounts of α,β adduct in addition to β,γ isomer whereas the more acidic *N*-acetylhistidine does not. As previously mentioned, adduct Ia retains the β,γ-olefinic structure on treatment with alkali. It is, therefore, unlikely that II arises by alkali-catalyzed isomerization of the β,γ-olefinic enamine.

It remains to be seen which of the isomeric adducts is the more faithful model for the reaction between allenic thioester and the histidine residue at the active enzyme site. Of course, in the enzyme protein the carboxyl and amino groups of histidine are not free but are engaged in the form of peptide bonds. For this reason the model reaction with *N*-acetylhistidine may more truly reflect the course of allene addition to the enzyme. On the other hand, the presence of a free or ester carboxyl group in *N*-acetylhistidine has only a minor effect; in both

[4] We have no evidence favoring structure i over structure ii for these adducts but consider the less hindered i more probable.

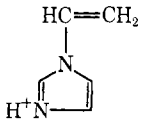


i

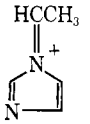


ii

[5] Professor T. A. Spencer has informed us that *N*-vinylimidazole (iii)



iii

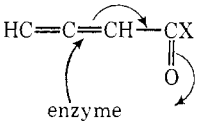


iv

is similarly stable. Heating this compound in 5.0 N HCl at reflux for 21 hr or keeping it at room temperature for 3 days in 1.5 M HCl produced no detectable free imidazole (Hupe *et al.*, 1972, and B. F. Sweeney and D. J. Hupe, unpublished). They suggest that the formation of the imidazolium ion iv may be energetically unfavorable, rendering this type of compound unusually stable to hydrolysis.

instances the β,γ-olefinic adduct is the predominant condensation product.

Enzyme Inhibition by Allenic Acid Derivatives. Some of the structural features necessary for dehydrase inhibition by allenic acids and their derivatives had been previously reported (Morisaki and Bloch, 1971). These included a 2,3-diene system, a limited range for the length of the carbon chain (C_8–C_{14}), and the appropriate stereochemistry of the allenic system (dextrorotatory acid). In contrast to the corresponding β,γ-alkynoic compounds which must be thioesters in order to be active as enzyme inhibitors (Brock *et al.*, 1967 and Helmkamp *et al.*, 1968), free allenic acids as well as their thioesters were found to inactivate dehydrase, especially at low pH (Endo *et al.*, 1970; Morisaki and Bloch, 1971). The rationale given for these differences was that the acetylenes are enzyme substrates and as such must be thioesters whereas the allenic isomers are active site reagents requiring less assistance by the carbonyl function for reaction with the enzyme. Nevertheless, the reactivity of the allenic acids is profoundly enhanced by esterification. In Table I are listed the concentrations of allenic acid and various esters required for 50% inhibition of dehydrase during a 10-min incubation period. These experiments were carried out at pH 6 because at this pH the various allenes have maximal or near maximal activity (Morisaki and Bloch, 1971). Under the chosen conditions, the concentration of 2,3-decadienoic acid necessary for 50% enzyme inhibition was about 300 times greater than for the corresponding *N*-acetylcysteamine or *S*-ethyl thioesters. Methyl 2,3-decadienoate was a much more potent inhibitor than the free acid but definitely inferior (by a factor of 8–10) to the two thioesters tested. Especially notable is the lack of enzyme inhibition by the allenic acid amide derivative. The inhibitory potencies shown in Table I (thioester > oxygen ester > free acid > amide) clearly follow an order which is inversely related to the relative contribution of the resonance form $-C(O^-)=X^+$ in substituted carboxyl groups which is greatest in amides and least in thioesters (Lienhard and Wang, 1968). The pH dependence of inhibition by free allenic acid (Morisaki and Bloch, 1971) can also be explained on this basis. For the interaction of enzyme with allenic inhibitor we may, therefore, visualize an attack by the nucleophilic imidazole group of histidine on the central allenic carbon in a Michael-type reaction



The data of Table II extend previous information on structural features which are important for dehydrase inhibition by allenes (Morisaki and Bloch, 1971). Confirming the results obtained with homologous 2,3-dienoic acids, the C_{12} methyl ester is a significantly more potent enzyme inhibitor than the corresponding C_{10} ester. Secondly, the two 3,4-dienoic esters tested had virtually no effect on enzyme activity even at 100 times higher concentrations. It is to be noted that the C_{11} 3,4-dienoic ester has the same allenic carbon chain as the very active C_{10} 2,3-dienoate but is nevertheless inert as an inhibitor. Clearly, the reactivity of an isolated diene system is insufficient; it must be enhanced by an adjacent carbonyl function. This result, in conjunction with the conclusions reached above from a comparison of the inhibitory potencies of various 2,3-decadienoate derivatives, leads to a further definition of the structural requirements for inhibition. A conjugated allenic carbonyl moiety is essential and the degree of reactivity is a function of carbonyl polarization.

While only a relatively small variety of allenic acid derivatives have so far been compared and tested in the chemical as well as in the enzyme reaction, they do show parallel reactivities in the two systems, at least under arbitrarily chosen conditions. Current structural studies on the enzyme–inhibitor complex should reveal whether there are any further analogies in the mechanistic behavior of allenes as reactants in the model system and as active site reagents for dehydrase.

Acknowledgment

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