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Inhibition of β-Hydroxydecanoyl Thioester Dehydrase by Some Allenic Acids and Their Thioesters[1]

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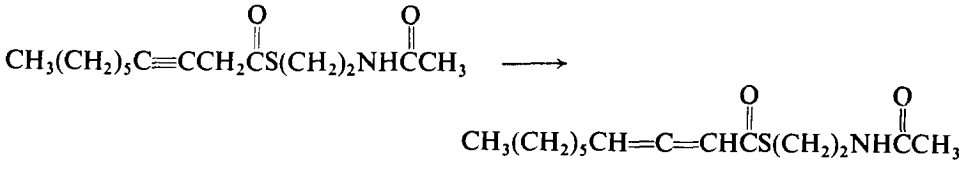
The allenic substrate analogue, 2,3-decadienoyl-*N*-acetylcysteamine, and free 2,3-decadienoic acid were previously shown to inhibit β-hydroxydecanoyl thioester dehydrase irreversibly. Racemic 2,3-decadienoic acid has now been resolved via its amphetamine salts. The dextrorotatory isomer is found to be twice as potent an inhibitor as the racemic acid, while the levorotatory allene affects enzyme activity only slightly.

A series of inhibitors varying in the length of the acyl chain (C_8–C_{14}) has been prepared. Enzyme inactivation occurs most rapidly with the C_{10}-congener of the acetylenic and allenic thioesters, while the C_{12} homologue is the most potent of the free allenic acid inhibitors.

INTRODUCTION

The *Escherichia coli* enzyme β-hydroxydecanoyl thioester dehydrase (dehydrase) catalyzes reactions leading to an equilibrium between D(−)-β-hydroxydecanoyl–NAC,[3] *trans*-2-decenoyl–NAC and *cis*-3-decenoyl-NAC and thus functions both as a reversible dehydrase and double-bond isomerase (*1*). The entire set of dehydrase-catalyzed reactions is potently and irreversibly inhibited by 3-decynoyl–NAC, the acetylenic analogue of one of the substrates (*2*). Essential structural features for inhibitory activity are a C_9, C_{10}, or C_{11} carbon chain, a β,γ-positioned triple bond, and a thioester function (*2*).

A recent study on the mode of action of this type of compound has revealed that 3-decynoyl–NAC is not an inhibitor *per se*, but that dehydrase recognizes it as a substrate and isomerizes it to the allenic 2,3-decadienoyl–NAC by a process analogous to the isomerization of *cis*-3-decenoyl– to *trans*-2-decenoyl–NAC.



The allenic isomer in turn inactivates the enzyme by binding covalently to a histidine residue at the active site (*3*). The ability of 3-decynoyl–NAC to be enzymatically

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[3] The abbreviation used is NAC = *N*-acetylcysteamine.

transformed explains why the structural requirements for it are as stringent as for the natural enzyme substrates. Granted that the acetylene–allene transformation is enzyme catalyzed and that it involves stereospecific abstraction and addition of hydrogen at C_α and C_γ respectively, the process will generate an optically active product. The present experiments were carried out to test this hypothesis and to ascertain whether the direct interaction between allene and dehydrase takes place in a stereospecific manner. Since free allenic acid also inactivates dehydrase, albeit at very much higher concentrations than the NAC derivative (*3*), 2,3-decadienoic acid was resolved into the two optically active forms. Only one of the isomers was active as an enzyme inhibitor. To study chain-length effects we have prepared various free allenic acids and their NAC-derivatives.

EXPERIMENTAL METHODS

Resolution of 2,3-decadienoic acid. (+)-Amphetamine (405 mg) was added to a solution of 504 mg racemic 2,3-decadienoic acid (*3*) in 3 ml of ether, and the mixture was allowed to stand in a refrigerator overnight. Evaporation of solvent at room temperature gave the diastereomeric salts, which were fractionally crystallized nine times from *n*-pentane/ether or ether/methanol. The ninth crystallizate, mp 97–98° and $[\alpha]_D$ +114°, was decomposed by shaking with a mixture of 4% HCl and ether. The ether-soluble fraction was washed first with 4% HCl and then with saturated NaCl solution. Drying over Na_2SO_4 and evaporation of solvent afforded (+)-2,3-decadienoic acid (11 mg), $[\alpha]_D$ + 145°.

Similarly (−)-amphetamine gave the salt, mp 97–98° and $[\alpha]_D$ −111° and then (−)-2,3-decadienoic acid, $[\alpha]_D$ −151°.

Allenic acids. 2,3-Octa-, 2,3-deca-, and 2,3-dodecadienoic acids were prepared by the method previously described for the C_{10}-congener (*3*). 2,3-Tetradecadienoic acid was synthesized as follows: 3-Butyn-1-ol tetrahydropyranyl ether (8.2 mmole) prepared from the corresponding alcohol by reaction with 1 equiv of dihydropyran in methylene-chloride solution containing a trace of *p*-toluenesulfonic acid was converted into the lithium salt by treatment with 8.2 mmole of butyllithium in *n*-pentane-tetrahydrofuran at −65°. To this solution 8.2 mmole of 1-decanol *p*-toluenesulfonate was added, and the mixture was heated to 50° for 24 hr. The crude product was subjected to acid-catalyzed methanolysis, and was purified by column chromatography on silicic acid (Unisil) to yield 0.21 g (12%) of 3-tetradecyn-1-ol. The alcohol was oxidized by Jones' reagent to 3-tetradecynoic acid, mp 55–56° in 44% yield, and the acid was isomerized to 2,3-tetradecadienoic acid, mp 46–47° by treatment with 18% potassium carbonate, yield 20%.

Each acid had the characteristic infrared-absorption band at 5.10 μ, attributable to an allene. The methyl esters of the allenic acids gave single peaks on GLC analysis on 10% DEGS, with the following retention times, relative to those of the corresponding methyl esters of 3-alkynoic acids (1.0):

	C_8	C_{10}	C_{12}	C_{14}
Column temperature	138°	140°	161°	169°
Relative retention time	0.76	0.77	0.78	0.83

Allenic thioesters. 2,3-Octa-, 2,3-deca-, and 2,3-dodecadienoyl–NAC derivatives were prepared by base treatment of the corresponding 3-alkynoyl–NACs as described

earlier (*3*). The purity of each sample was checked and confirmed by thin-layer chromatography on silica gel and spectroscopically (uv absorption at 263 nm and ir absorption at 5.10 μ).

Enzyme assay. Enzyme activity was assayed by monitoring the initial velocity of the isomerization of *cis*-3-decenoyl–NAC to *trans*-2-decenoyl–NAC at 263 nm with a Gilford model 240 recording spectrophotometer (*4*). Inhibitors to be tested were preincubated with enzyme (sp act 900 (*4*)) in the appropriate buffer for varying intervals (for details see figure legends). At zero time the substrate *cis*-3-decenoyl–NAC was added, and the increase in optical density at 263 nm was recorded. The allenic thioesters also absorb at 263 nm but since their concentration is only 1/3000 that of substrate, this contribution is negligible. The potency of an inhibitor is judged from the fraction of dehydrase activity remaining as compared to controls run for the same length of time in the absence of inhibitor. The partially purified dehydrase used in the present experiments was kindly provided by Dr. G. Helmkamp and *cis*-3-decenoyl–NAC by Mr. J. Stein.

RESULTS AND DISCUSSION

Stereospecificity. The time course of enzyme inactivation by equal concentrations of (+)-, (−)- or racemic-2,3-decadienoic is shown in Fig. 1. Dehydrase activity is diminished most rapidly by the (+)-allenic acid, more slowly by the racemic mixture,

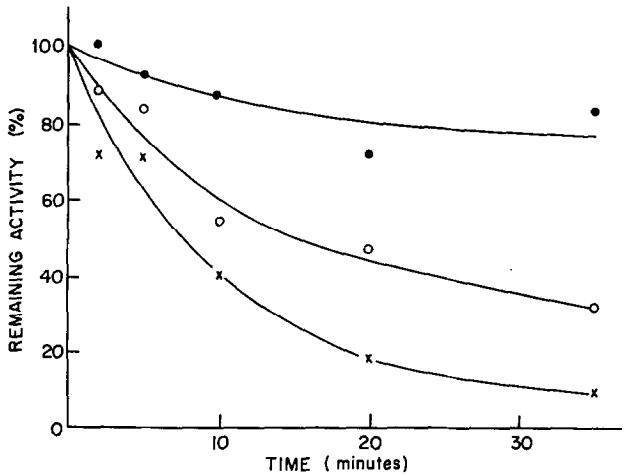

FIG. 1. Comparison of racemic and optically active 2,3-decadienoic acids as dehydrase inhibitors. Dehydrase (4.5×10^{-8} *M*) was preincubated with 5×10^{-5} *M* racemic (o–o–o)-, dextro (×–×–×)-, or levo (●–●–●)-allenic acids in 0.2 ml of succinate buffer pH 5.0, $I = 0.01$ at room temperature. At various times, 0.8 ml of *cis*-3-decenoyl–NAC (2.4×10^{-4} *M*) in phosphate buffer, pH 6.0, $I = 0.05$, was added and the initial velocity of product formation was recorded. The degree of inactivation (remaining activity, %) was based on comparison with the rate of a similar reaction carried out in the absence of inhibitor.

and only very slightly by (−)-allenic acid. These results clearly establish the stereoselective nature of the interaction between 2,3-decadienoic acid and dehydrase, the (+)-isomer being the active species. Steric preference rather than absolute stereospecificity in the allene–enzyme reaction is indicated by the slight but significant inhibitory activity of the levorotatory acid. However, in the absence of proof that the

resolved acids are optically pure, it is not possible to decide whether inhibition by the (−) acid is intrinsic or due to contamination with (+)-isomer. In any event there is no *a priori* reason why the chemical modification of an enzyme—in contrast to an enzyme-catalyzed reaction—should exhibit absolute stereospecificity.

It seemed reasonable to assume that enzyme inhibition by 2,3-decadienoyl-NAC would display the same stereoselectivity that had been observed with the free allenic acids. Unfortunately, this point could not be tested because we were unable so far to prepare the thioesters of the resolved allenic acids.

Enzyme reactions, as a rule, proceed stereospecifically and, therefore, the dehydrase catalyzed isomerization of 3-decynoyl–NAC is likely to proceed stereospecifically to afford the inhibitory, optically active 2,3-decadienoyl–NAC. This supposition cannot be readily verified for the following reason. In the interaction between dehydrase and 3-decynoyl–NAC the enzyme-catalyzed isomerization is the slow, rate-limiting step. The product, 2,3-decadienoyl–NAC, does not accumulate but immediately combines with the protein in an irreversible, chemical reaction.

It may be noted that the present data provide the first experimental evidence for the ability of an enzyme to distinguish isomeric allenes.

Chain-length specificity. In a previous study a strict parallelism between the relative activities of dehydrase substrates and inhibitors as a function of carbon chain-length was observed (*2*). This phenomenon—remarkable because of the noncompetitive nature of the inhibition—could be reasonably explained by the finding that 3-decynoyl–NAC is not an inhibitor *per se* but a very active dehydrase substrate (*3*). The enzyme transforms the substrate analogue into the isomeric allene which is the true inhibitor. Since the reaction between allene and protein is chemical, the question arose whether for this process the length of the carbon chain and other structural features are equally critical. We have already reported on one of these aspects, the dispensability of the thioester for inhibition by the allenes (*3*). 2,3-Decadienoic acid inactivates dehydrase whereas 3-decynoic acid does not.

Comparison of dehydrase inhibition by 3-alkynoic-, and allenic-thioesters as a function of chain length shows very similar dependencies, at least qualitatively (Fig. 2A and B). Thus after a 5-min exposure of enzyme to the acetylenic thioesters, the inhibitions were, with C_8, 10%; C_{12}, 20%, and C_{10}, 50%. The corresponding figures for the allenic thioesters were C_8, 10%; C_{12}, 25%, and C_{10}, 65%.

The evidence is, therefore, strong that allenic inhibitors occupy the same unique site in the enzyme as the substrate and that their dimensions are of critical importance. Not only the reactivity of the allenic moiety but also the close structural resemblance to substrate must be responsible for the effectiveness of this active-site reagent.

From the data in Fig. 2 it can be seen that the allenic thioesters are far more effective inhibitors than the corresponding acetylenes. At 5×10^{-8} *M* they diminish enzyme activity at least as rapidly as the 3-alkynoyl thioesters at 10 times higher concentrations (5×10^{-7} *M*). The reasons for these rate differences have already been given. The acetylenic thioesters must first be enzymatically isomerized and this process is slow compared to the direct chemical interaction of allene and enzyme.

On the presumption that 2,3-decadienoyl–NAC is a highly specific reagent which combines with a limited number of enzyme sites, we have determined the stoichiometry of the dehydrase-inhibitor reaction. In such titration experiments 8×10^{-8} *M* (±)-2,3-decadienoyl–NAC was found to cause 100% inhibition of a 4.5×10^{-8} *M* solution of enzyme. Since only one of the stereoisomers reacts, we conclude that no more than 1 mole of (+)-2,3-decadienoyl–NAC binds per mole of dehydrase and that this is sufficient for complete enzyme inactivation.

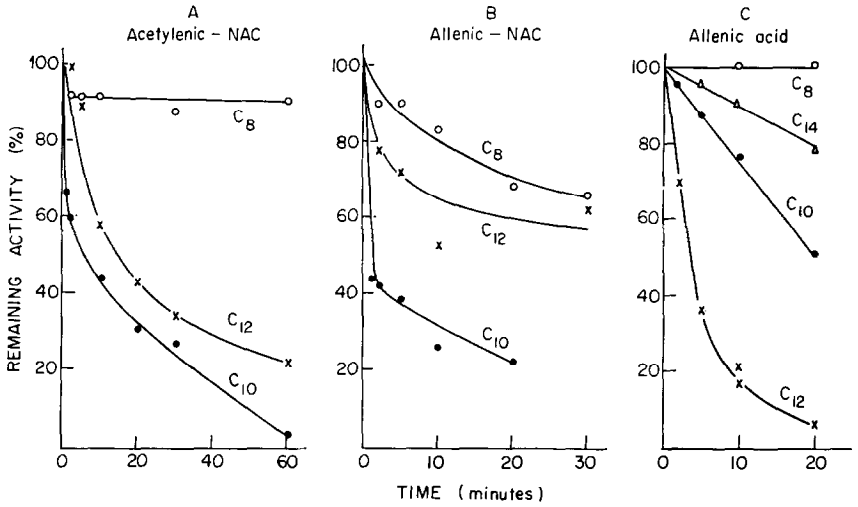

FIG. 2. Chain-length specificities of acetylenic and allenic dehydrase inhibitors. Acetylenic–NAC (5×10^{-7} *M*) or allenic–NAC (5×10^{-8} *M*) was preincubated with dehydrase (4.5×10^{-8} *M*) in 0.5 ml of phosphate buffer, pH 6.0, $I = 0.05$ at room temperature. At various times, 0.5 ml of *cis*-3-decenoyl–NAC (1.5×10^{-4} *M*) in the same buffer was added, and the initial velocity of product formation was recorded. Allenic acids (1.0×10^{-5} *M*) were preincubated with enzyme in 0.2 ml of succinate buffer, pH 5.0, $I = 0.01$ at room temperature. At various times, 0.8 ml of substrate (*cis*-3-decenoyl–NAC, 2.4×10^{-4} *M*) in phosphate buffer, pH 6.0, $I = 0.05$, was added and the initial velocity of product formation recorded.

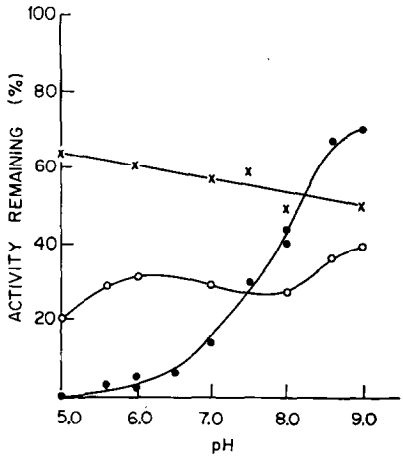

FIG. 3. Effect of pH on inhibition of dehydrase by 2,3-decadienoyl–NAC (o–o–o), 3-decynoyl–NAC (×–×–×), and 2,3-decadienoic acid (●–●–●). Allenic (5×10^{-8} *M*) and acetylenic (1×10^{-6} *M*) thioesters were dissolved in 0.5 ml of various buffers, $I = 0.01$, and 7.5 μg of partially purified dehydrase (4.5×10^{-8} *M*) was added at zero time. After preincubation at room temperature for 5 min, 0.5 ml of *cis*-3-decenoyl–NAC (1.5×10^{-4} *M*) in phosphate buffer, pH 6.0, $I = 0.05$, was added and the initial velocity of product formation was recorded. The allenic acid inhibitors (9.1×10^{-5} *M*) were incubated with 5 μg of enzyme in 0.1 ml of various buffers, $I = 0.01$, at room temperature for 30 min and then 1 ml of substrate (3.5×10^{-4} *M*) in Tris–HCl buffer, pH 8.2, $I = 0.05$, was added. The buffer systems used for the various pH ranges were NaOH–succinic acid, potassium phosphate, and Tris–HCl.

Examining the chain-length specificity of the free allenic acids we observed a deviation from the pattern given by the thioester derivatives of either alkynoic or allenic acids. Enzyme was more rapidly inactivated by the C_{12} acid than by the C_{10} congener (Fig. 2C). We have no explanation for this inversion of chain-length specificity. The possibility that 2,3-dodecadienoic acid, in addition to being an active-site reagent, inhibits the enzyme by detergent action (micelle formation) appears unlikely since 3-dodecynoic, *cis*-3-dodecenoic, and dodecanoic acids did not inhibit dehydrase significantly.[4]

pH Dependence

In order to determine dehydrase inactivation as a function of pH, the C_{10} inhibitors 2,3-decadienoic acid, 2,3-decadienoyl–NAC, and 3-decynoyl–NAC were incubated with enzyme in buffers ($I = 0.01$) in the absence of substrate. Substrate was then added and the remaining enzyme activity assayed either in Tris–HCl or phosphate buffer, $I = 0.05$ (Fig. 3). Under the chosen conditions (30 min preincubation) inhibition by free allenic acid was strongly pH dependent, with a maximum effect at pH 5 and no significant inhibition at pH 9. The undissociated allenic acid is, therefore, the active inhibitory species. The anion appears unable to combine with enzyme possibly due to charge repulsion. By contrast, the effect of pH on inhibition by alkynoic or allenic thioesters is small. A slight increase in the inhibitory activity of 3-decynoyl–NAC occurs with rising pH but this may be due to chemical isomerization of the alkyne to the more reactive allenic thioester.

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[4] M. Morisaki, unpublished.