with the alkyne group in bond formation at the cationic carbon to which the leaving group is initially attached.

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A New Enzyme Containing a Synthetically Formed Active Site. Thiol-Subtilisin[1]

Sir:

We wish to report the synthesis of a new, active enzyme containing the backbone of subtilisin,[2] but containing a thiol group rather than a hydroxyl group as the essential nucleophile of the active site. We call this synthetic enzyme thiol-subtilisin.

The alteration of the active site of an enzyme may give mechanistically meaningful results. To this end, we have investigated the alteration of the active site of subtilisin in order to convert it from a "serine enzyme" to a "cysteine enzyme." Subtilisin contains a serine hydroxyl group at its active site; this group serves as a nucleophile during enzymatic catalysis, with the intermediate formation of an acyl-enzyme in which the acyl group is attached to this serine hydroxyl group. Recent investigations on small serine peptides showed the possibility of changing a serine to a cysteine residue without racemization: the O-tosyl derivative of a serine peptide was treated with either thiolacetate or thiolbenzoate ion in various organic solvents, yielding the thiol ester of the cysteine residue. The thiol ester could easily be decomposed to the mercaptan and acid.[3,4] By using this synthetic procedure subtilisin has been changed to a synthetic enzyme bearing a thiol group.

The transformation of subtilisin to thiol-subtilisin was carried out in three steps according to eq 1. The first step involves the almost instantaneous stoichiometric reaction of phenylmethanesulfonyl fluoride-^{14}C

$$\text{subtilisin-OH} \xrightarrow[-\text{HF}]{+\text{C}_6\text{H}_5\text{CH}_2\text{SO}_2\text{F}} \text{subtilisin-OSO}_2\text{CH}_2\text{C}_6\text{H}_5 \xrightarrow[-\text{C}_6\text{H}_5\text{CH}_2\text{SO}_3^-]{+\text{CH}_3\overset{\overset{\text{O}}{\|}}{\text{C}}\text{S}^-} \text{subtilisin-S}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{CH}_3 \xrightarrow[-\text{CH}_3\text{CO}_2^-]{+\text{H}_2\text{O}} \text{subtilisin-SH} \quad (1)$$

(1.2×10^{-3} *M*) with subtilisin (1×10^{-3} *M*) at pH 7.0, 0.1 *M* phosphate buffer. The resultant phenylmethanesulfonyl-subtilisin contained 0.9 equiv of carbon-14 per mole of enzyme,[5] and its enzymatic activity, tested with *p*-nitrophenyl acetate, was less than 1% of the original activity. The second step of the transformation involves the displacement of the phenylmethanesulfonyl group by thiolacetate ion in an SN2 displacement reaction.[6] The reaction was carried out in 0.7 *M* thiolacetate ion at pH 5.25 and 25° for 48 hr. During this period, more than 99% of the phenylmethanesulfonyl groups were removed from the enzyme as monitored by the loss of protein-bound carbon-14. The third step of the transformation involves the spontaneous hydrolysis of the acetyl group from the thiol ester, presumably assisted by the enzyme.

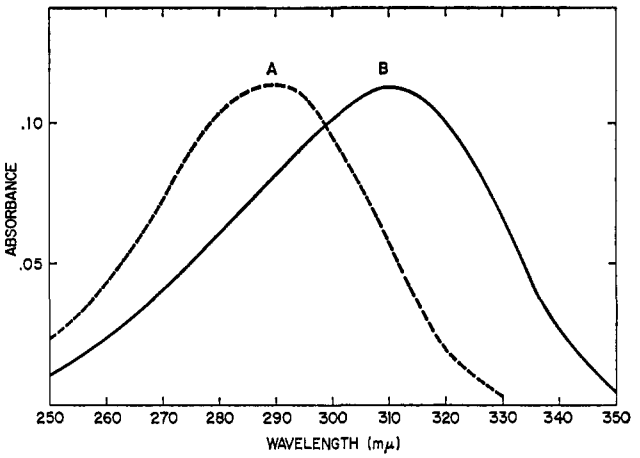

Figure 1. The difference spectra of *trans*-cinnamoyl-subtilisin *vs.* subtilisin (A) and *trans*-cinnamoyl-thiol-subtilisin *vs.* thiol-subtilisin (B) at pH 5, 0.1 *M* acetate buffer. *N-trans*-Cinnamoylimidazole (5.3×10^{-6} *M*) was treated with excess enzyme (9.2×10^{-6} *M*) to transform completely the cinnamoyl group to cinnamoyl-enzyme.

Table I shows the results of three different analytical procedures used to characterize subtilisin before and after the treatment described above. (1) Spectrophotometric titration of thiol groups using *p*-chloromercuribenzoate ion[7] indicates the appearance of 0.7–0.8 equiv of thiol group/mole in the modified enzyme. (2) Amino acid analysis of the hydrolysate of the performic acid oxidized proteins using a Spinco amino acid analyzer shows that the thiol group of the synthetic enzyme exists in a cysteine residue and, moreover, that the amount of cysteine per mole is comparable to the amount of thiol groups per mole found in (1). (3) The wavelength of maximum absorption of an O-cinnamoyl compound is significantly lower than that of an S-cinnamoyl compound.[8] The difference spectra of O-cinnamoyl-subtilisin *vs.* subtilisin and S-cinnamoyl-subtilisin *vs.* thiol-subtilisin show the same relationship, as seen in Figure 1. Furthermore, in the S-cinnamoyl-

Table I. Comparison of Subtilisin and Thiol-Subtilisin

Determination	Subtilisin	Thiol-subtilisin
Thiol groups/molecule using titration with *p*-chloromercuribenzoate ion	<1.0	0.80
Cysteic acid/molecule from amino acid analyses of the hydrolysate from the performic acid oxidized protein	0.096	0.78
λ_{max} of (cinnamoyl-enzyme) *vs.* (enzyme)	289	~310

(1) This research was supported by grants from the National Institutes of Health.
(2) Bacterial Proteinase Novo, Novo Pharmaceutical Co., Copenhagen, Denmark.
(3) I. Photaki and V. Bradakos, *J. Am. Chem. Soc.*, 87, 3489 (1965).
(4) C. Zioudrou, M. Wilchek, and A. Patchornik, *Biochemistry*, 4, 1811 (1965).
(5) Based on a molecular weight of the enzyme of 27,600 and $A^{1\%}_{278} = 11.7$: H. Matsubara, C. B. Casper, D. M. Brown, and E. L. Smith, *J. Biol. Chem.*, 240, 1125 (1965).
(6) The alkanesulfonyl group is an excellent leaving group in such a reaction; the *p*-toluenesulfonyl group used for small peptides[3,4] was replaced by the phenylmethanesulfonyl group here because the first step of the transformation was much more rapid with the latter group.
(7) P. D. Boyer, *J. Am. Chem. Soc.*, 76, 4331 (1954).
(8) M. L. Bender and F. J. Kézdy, *Ann. Rev. Biochem.*, 34, 49 (1965).

enzyme, the absorption maximum of the O-cinnamoyl group is essentially nonexistent.

The thiol-subtilisin synthesized here is an active enzyme, catalyzing the hydrolysis of N-*trans*-cinnamoylimidazole, *p*-nitrophenyl acetate, and *p*-nitrophenyl N-benzyloxycarbonylglycinate, one of the more specific substrates of subtilisin. These catalyses are due to the synthetic thiol-subtilisin since: (1) the intermediate cinnamoyl-enzyme in the hydrolysis of N-*trans*-cinnamoylimidazole has the spectral characteristics of a thiol enzyme (see above); (2) the *p*-nitrophenyl acetate hydrolysis follows Michaelis–Menten kinetics; (3) the hydrolyses of *p*-nitrophenyl acetate and *p*-nitrophenyl N-benzyloxycarbonylglycinate are inhibited to the extent of 95 and 70%,[9] respectively, by *p*-chloromercuribenzoate, whereas the subtilisin catalyses of these hydrolyses are not; (4) the rate of deacylation of cinnamoyl-thiol-subtilisin and the k_{cat} of the *p*-nitrophenyl acetate hydrolysis are dependent on a basic group of *ca.* pK_a 7.5.[10]

The kinetic properties of thiol-subtilisin are now being investigated in two ways: (1) comparison with subtilisin; this comparison involves two enzymes of common basic group but different nucleophilic entity; (2) comparison with papain; this comparison involves two enzymes with the same nucleophilic entity but different basic group.

(9) This 70% inhibition means that a small amount of another enzymatic activity highly active toward the glycine substrate is still present in the thiol-subtilisin preparation. Theoretically ~10% of subtilisin could still be present since titration of the subtilisin with phenylmethanesulfonyl fluoride to complete inactivity required 0.9 equiv of inhibitor while only 0.8 equiv of thiol group was found in thiol-subtilisin. However, the extraneous enzymatic activity unaffected by *p*-chloromercuribenzoate is also unaffected by phenylmethanesulfonyl fluoride and thus cannot be regenerated subtilisin.

(10) K. E. Neet and D. E. Koshland, Jr., personal communication, have prepared a thiol-subtilisin. Both they and we have found no significant activity toward aromatic amino acid alkyl ester substrates (ethyl N-acetyl-L-tyrosinate and methyl N-acetyl-L-tryptophanate, respectively) (their suggestion). This apparent inactivity could be due to either a specificity of thiol-subtilisin for powerful acylating agents or because the rate of deacylation of acyl-thiol-subtilisins is much slower than that of acyl-subtilisins, as we have in fact found.

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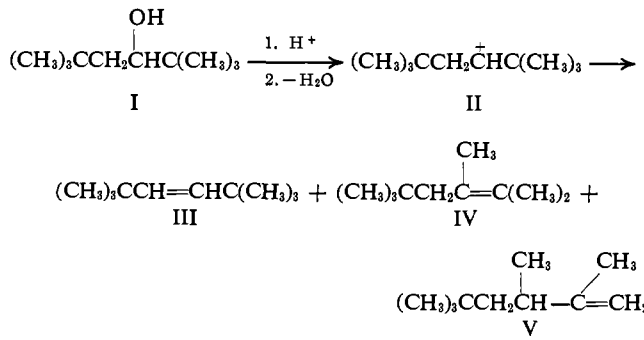
Dehydration, without Rearrangement, of a Neopentyl-Type Secondary Alcohol

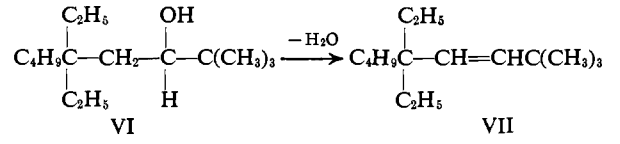
Sir:

The dehydration of a secondary alcohol, such as 2,2,5,5-tetramethyl-3-hexanol (I), has been shown to produce a mixture of olefins (III–V) which results from the intermediate carbonium ion (II) by the well-known Whitmore or Wagner–Meerwein type of rearrangement.[1,2]

We wish to report what appears to be the first example of the dehydration of an alcohol, similar to I, which affords only a single olefin uncontaminated with skeletal rearrangement products.

When 5,5-diethyl-2,2-dimethyl-3-nonanol (VI), bp 93–94° (1.4 mm), $n^{25}D$ 1.4528 (*Anal.* Calcd for $C_{15}H_{32}O$: C, 78.87; H, 14.12; Found: C, 79.17; H, 13.98), was heated with potassium hydrogen sulfate at 170–180° for 20 hr, there was obtained 5,5-diethyl-2,2-dimethyl-3-nonene (VII), bp 120–121° (40 mm), $n^{25}D$ 1.4394, in 81% yield. *Anal.* Calcd for $C_{15}H_{30}$: C, 85.63; H, 14.37. Found: C, 85.70; H, 14.40.





Analysis of the dehydration product by vapor phase chromatography, 10-ft Carbowax (15%) on Chromosorb W column at 170°, indicated the presence of only one olefin. The infrared spectrum of VII shows absorptions at 3040 and 970 cm^{-1} characteristic of a *trans*-olefin. The nmr spectrum has an AB absorption pattern centered at τ 4.8, with a coupling constant of 16 cps, representative of a *trans* structure. Although assignments cannot be made for all of the methyl and methylene absorptions of VII, they essentially are superimposable on those of 3,3-diethylheptanonitrile which had been converted to alcohol VI by treatment with *t*-butyllithium, followed by hydrolysis and reduction of the intermediate ketone with lithium aluminum hydride. The only additional absorption is a singlet at τ 9 which is attributed to the *t*-butyl group.

In order to substantiate the structure of VII further, 3-deuteroxy-5,5-diethyl-2,2-dimethylnonane-3,4,4-d_3, bp 125–126° (9 mm), $n^{25}D$ 1.4512, was prepared from the corresponding ketone by deuteration with deuterium oxide followed by reduction with lithium aluminum deuteride and treatment with deuterium oxide and base. *Anal.* Calcd for $C_{15}H_{28}D_4O$: C, 77.51; H + D, 15.61. Found: C, 77.41; H + D, 15.48. Removal of deuterium oxide from this deuteroxy compound by heating with potassium hydrogen sulfate gave 5,5-diethyl-2,2-dimethyl-3-nonene-3,4-d_2 in 74% yield, bp 133–134° (80 mm), $n^{25}D$ 1.4365. *Anal.* Calcd for $C_{15}H_{28}D_2$: C, 84.81; H + D, 15.19. Found: C, 84.61; H + D, 14.97. The nmr spectrum of the latter is devoid of vinyl proton absorption, but is identical otherwise with that of the olefin VII.

Treatment of I with potassium hydrogen sulfate under the conditions used for VI led to a mixture of three isomeric olefins as shown by gas chromatographic analysis.

Molecular models of II and the corresponding carbonium ion from VI indicate that crowding is far more serious in the latter. In addition, the hindrance appears to be still greater in the expected carbonium ions which might arise from the rearrangement of intermediates from VI. This suggests that steric factors

(1) F. C. Whitmore and P. L. Meunier, *J. Am. Chem. Soc.*, **55**, 3721 (1933).

(2) F. L. Howard, T. W. Mears, A. Fookson, and P. Pomerantz, *ibid.*, **68**, 2121 (1946).