gested.[14a,16] Simple β-hydroxy*alkyl*silanes were found to be unreactive to these conditions.[14a,17] The work described here suggests that the fluoride ion induced reactions may proceed according to Scheme I, with fluoride ion acting as a base to generate alkoxide.

According to the mechanistic rationale of Scheme I, the base-induced protiodesilylation of a β-hydroxysilane might be viewed as a homo-Brook rearrangement (followed by hydrolysis of the resulting silyl ether). The Brook rearrangement,[18] the conversion of an α-hydroxysilane to a silyl ether with a catalytic amount of base (typically Na/K alloy or an amine), is normally very slow unless the carbon bearing the silicon is substituted with an anion-stabilizing group (e.g., phenyl).[19] We were therefore interested in determining whether protiodesilylations of simple unactivated α-hydroxysilanes could be accomplished under our conditions.

α-Hydroxysilane **17**[8] was prepared by addition of Me_3SiLi to 2-methylcyclohexanone (54% yield) (Scheme IV). The stereochemistry was initially assigned by assuming predominant attack of the silyl reagent trans to the methyl group. When **17** was treated with 5% KO-*t*-Bu in 19:1 Me_2SO:H_2O at room temperature, reaction was complete in 1 h, giving the protiodesilylation product, 2-methylcyclohexanol, in 72% yield. The stereochemistry of the product (97% cis) suggested that the protiodesilylation took place with predominant or complete retention of configuration. The Brook rearrangements of *α-phenyl*-α-hydroxysilanes under quite different conditions (Na/K alloy in ether, or with amines in various solvents) have been shown to take place with *inversion* of configuration at carbon.[18] Therefore an additional experiment was undertaken to confirm the stereochemistry in our reaction.

The isomeric α-hydroxysilane **19**[8a] was prepared from vinylsilane **18**[20] by treatment with BH_3·THF followed by H_2O_2/NaOH[21] (89% crude yield). When **19** was treated with 5% KO-*t*-Bu in 19:1 Me_2SO:H_2O (1 h), *trans*-2-methylcyclohexanol (>99% trans) was formed in 69% yield. These results indicate that these protiodesilylation reactions of α-hydroxysilanes, like those of the β-hydroxysilanes discussed above, take place with stereospecific retention of configuration at carbon.[22]

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Registry No. 1, 58541-11-0; **2**, 83511-14-2; **3**, 79705-13-8; **4**, 61580-73-2; **5**, 3429-76-3; **6**, 83511-15-3; **7**, 20584-41-2; **8**, 20584-43-4; **9**, 64997-08-6; **10**, 83511-16-4; **11**, 83511-17-5; **12**, 83511-18-6; **13**, 83511-19-7; **14**, 83511-20-0; **15**, 83511-21-1; **16**, 83511-22-2; **17**, 83511-23-3; **18**, 55860-92-9; **19**, 83511-24-4; Me_3SiLi, 18000-27-6; 1-octanol, 111-87-5; *trans*-2-methoxycyclohexanol, 7429-40-5; cyclohexanol, 108-93-0; 2-methylcyclohexanone, 583-60-8; *cis*-2-methylcyclohexanol, 7443-70-1; *trans*-2-methylcyclohexanol, 7443-52-9; 2-octanol, 123-96-6.

(16) A few examples of base-induced protiodesilylations (*without* fluoride ion) of β-hydroxyalkenylsilanes (Ruden, R. A., personal communication, and ref 14d) and β-hydroxy-α-alkoxysilanes (ref 3b, and footnote 18 therein) were known. Fluoride-induced protiodesilylations of epoxysilanes (Chan, T. H.; Lau, P. W. K.; Li, M. P. *Tetrahedron Lett.* **1976**, 2667-2670) and base-induced protiodesilylations of α-silyl esters having a β-OH group[2f] are known and have been found to take place with retention of stereochemistry at carbon; for these reactions, the β-hydroxyl group is presumably not necessary.

(17) In accord with these observations, we found that β-hydroxysilane **1** was inert to CsF in acetonitrile at 80 °C and that **1** and **4** were inert to CsF in Me_2SO at room temperature, reacting very slowly at 80 °C to give mixtures of products resulting from elimination and protiodesilylation.

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(19) A few examples are known where no anion-stabilizing groups are present: Brook, A. G.; Warner, C. M.; McGriskin, M. E. *J. Am. Chem. Soc.* **1959**, *81*, 981-983. Brook, A. G.; Iachia, B. *Ibid.* **1961**, *83*, 827-831. See also: Manuel, G.; Mazerolles, P.; Gril, J. *J. Organomet. Chem.* **1976**, *122*, 335-343.

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(21) de Jesus, M.; Rosario, O.; Larson, G. L. *J. Organomet. Chem.* **1977**, *132*, 301-320.

(22) While this manuscript was in preparation, we learned that Wilson has carried out an aliphatic Brook rearrangement (by using KH in HMPA) which occurred with retention of configuration at carbon: Wilson, S. R.; Hague, M. S.; Misra, R. N. *J. Org. Chem.* **1982**, *47*, 747-748.

Cryoenzymology of Proteases: NMR Detection of a Productive Thioacyl Derivative of Papain at Subzero Temperature†

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It is generally accepted[3] that the hydrolysis of peptides and amides catalyzed by the thiol protease papain can be represented by a minimal three-step pathway[4] as in Scheme I. The reactions are controlled by a thiolate ion (cys-25) at the active site of papain in a sequence involving binding, acylation, and deacylation. Structural evidence for the thioacyl intermediate **1** is limited to electronic absorption data in which acylation of papain by *N*-cinnamoylimidazole gave rise to a UV spectrum red shifted by 20 nm relative to the model, (*S*)-*trans*-cinnamoylcysteine.[5] More direct evidence bearing on this point comes from the observation[6] of a species assigned to a dithioester structure with λ_{max} 313 nm (cf. dithioacetate, λ_{max} 305 nm) in the papain-catalyzed hydrolysis of methyl thionohippurate. As a result of the development in our laboratory of reliable protocols for the observation of covalently bound intermediates of enzymes and their substrates by ^{13}C NMR spectroscopy at subzero temperatures, we can now report on the direct observation of a productive thioacyl intermediate prepared from papain and [^{13}C=O]-*N*-benzoylimidazole by adapting the techniques of cryoenzymology[7] to a ^{13}C NMR experiment. To monitor the extent of benzoylation of papain and the rate of deacylation, we used the high reactivity of 2,2′-dipyridyl disulfide[8] toward the thiolate ion of cys-25 in papain at pH 3.8 to titrate free thiolate in aliquots of incubation mixtures corresponding to the time course NMR experiment, using 1-2 mM solutions of papain and a large excess (~20 mM) of substrate in formate buffer. After many trials the following conditions gave completely reproducible results in which a suitable concentration (~1 mM) and $t_{1/2}$ (>30 min) of the intermediate were achieved. Papain (1.7 mM) in formate buffer (0.1 M, pH 4.1) was mixed with 90% enriched [^{13}C=O]-*N*-benzoylimidazole[9,10] (23.6 mM) in 25% Me_2SO-d_6 at 0 °C then rapidly cooled to -6 °C. An aliquot of this solution was kept at -6 °C and active site thiol concentration measured throughout the NMR time course.

At 0 °C papain was 96% acylated (thiolate assay) while at -6 °C the half-life of deacylation is 96 min. The time course of the CMR experiment is shown in Figure 1 a-f. The broad (25 ± 5 Hz) resonance at 196.0 ppm is assigned to the thiobenzoate (**2**, Scheme II) of papain labeled at ^{13}C=O (cf. phenylthiobenzoate, δ 189.1;[11] *n*-butyl thioacetate, 194.1[12]). The rate of disappearance of the signal at 196.0 ppm (allowing for experimental error due

† Dedicated to the memory of the late Professor F. Šorm.

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(1) University of Edinburgh.

(2) Texas A&M University.

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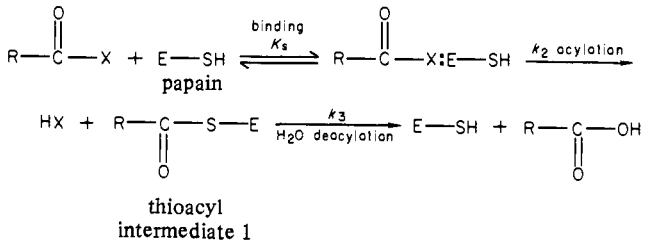
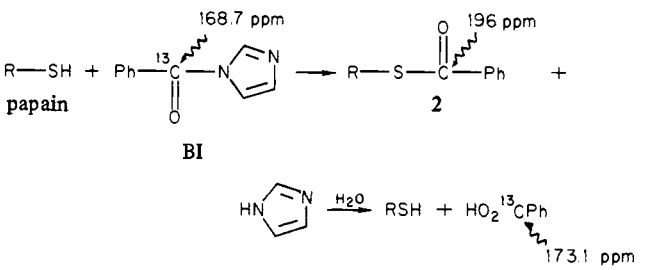
Scheme I



Scheme II



to line broadening) corresponds well with $t_{1/2}$, measured independently by titration with 2,2-dipyridyl disulfide. Moreover, the thiobenzoate signal at 196.0 ppm was not observed in the control hydrolysis experiment in the *absence* of papain at pH 4.1. Apart from the resonances due to papain at natural abundance, in which Arg C-6 (158 ppm) and Tyr C-7 (156 ppm)[13] are clearly discerned, together with broad carbonyl resonances between 170 and 180 ppm, a signal at 165.2 ppm (A in Figure 1a,b) was also detected in the absence of both formate and papain and is ascribed to the carbonyl resonance of benzoic anhydride, which has been shown to hydrolyze to benzoic acid at pH 4.1. It should be noted that the remaining triplet centered at 170.1 ppm (pH 4.1) (Figure 1a–f) is due to partially decoupled formate and is shifted to 169.3 ppm at pH 3.8 (Figure 1g).

After 17 h at −6 °C, the reaction mixture used in the above experiments was diluted (×4), low molecular weight material was removed by gel filtration, and the sample was concentrated by ultrafiltration, whereupon titration with 2,2′-dipyridyl disulfide showed no loss of fast-reacting thiol at pH 3.8. The ^{13}C NMR spectrum of this sample exhibited a broad resonance at 168–170 ppm, which resolved clearly into two peaks at 168.7 and 169.1 ppm by subtraction of the ^{13}C NMR spectrum of papain. By analogy with model compounds and studies on trypsin,[14] the main sites of this nonspecific benzoylation are assigned to the amino and phenolic side chains of the 10 lysine and 19 tyrosine residues of papain.

From the above data we conclude that (a) papain reacts with benzoylimidazole to form a thioacyl intermediate unambiguously detected by ^{13}C NMR spectroscopy at −6 °C, (b) the rate of decay of this intermediate is equal to the rate of regeneration of the active center thiolate ion of papain as measured by titration with 2,2′-dipyridyl disulfide, and (c) *nonspecific* benzoylation of lysine and tyrosine residues of papain can be observed by virtue of the appearance of resonances at 168–170 ppm.

These results show that it is possible to characterize a labile covalent enzyme–substrate intermediate under well-defined cryoenzymological conditions by ^{13}C NMR spectroscopy and to observe its transformation to product. Previous studies[15] have provided ^{13}C NMR evidence for acetyl chymotrypsin stabilized at pH 5.1 at room temperature. The experiments described herein show that it is now possible to observe at subzero temperatures enzyme–substrate intermediates that would escape detection above 0 °C or at higher ratios of substrate to enzyme. Further refinement of the technique to evolve parameters for the observation of acyl and tetrahedral intermediates[16,17] of thiol and serine proteases is in progress.

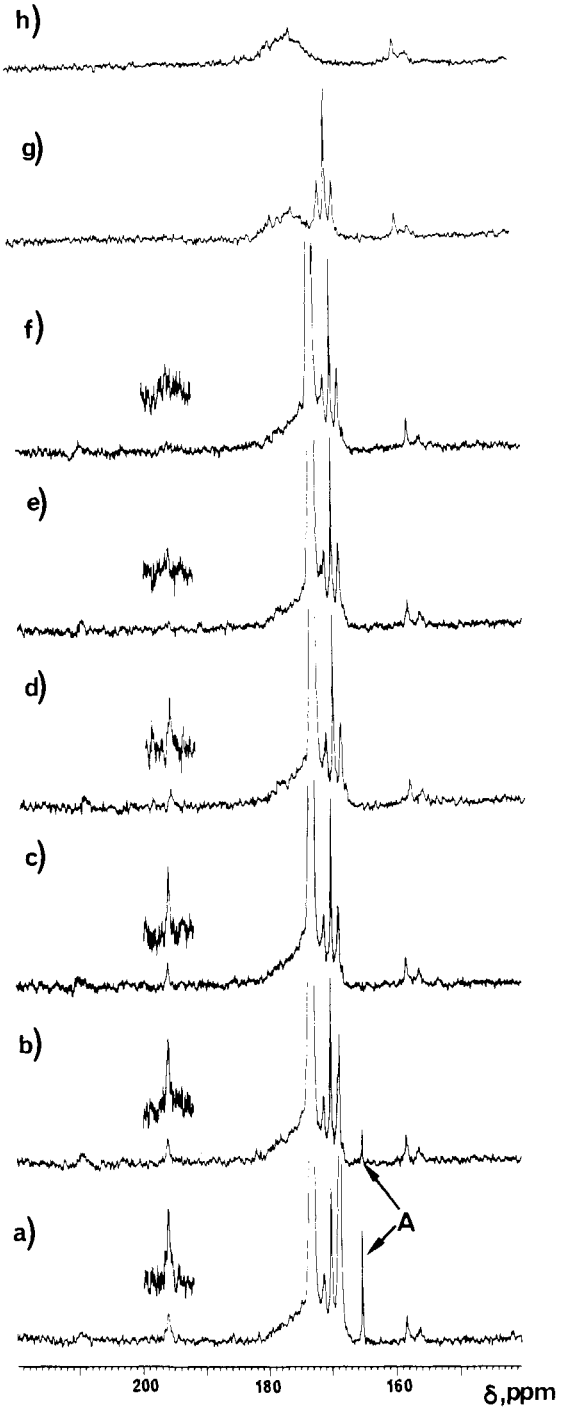


Figure 1. (a–f) 1.7 mM papain (72% active enzyme), 5.4 mM potassium chloride, 25% v/v Me_2SO, 0.1 M sodium formate buffer (pH 4.1), 23.6 mM benzoylimidazole. [^{13}C=O]Benzoylimidazole was added at 0 °C after 1.5 min, the reaction mixture cooled to −6 °C, and the NMR data acquisition commenced 6 min after the reaction was initiated. Spectra a–f represent 10000 accumulations recorded sequentially starting at 6, 51, 96, 141, 186, and 231 min after adding benzoylimidazole. The insert at 200–192 ppm is a 3-fold vertical expansion of this spectral range. (g) 2.03 mM papain (72% active enzyme), 6.5 mM potassium chloride, 25% v/v Me_2SO, 0.1 mM sodium formate buffer (pH 3.8). (h) 2.3 mM papain (72% active enzyme), 7.5 mM potassium chloride, 25% v/v Me_2SO (pH = 6.4). Papain was purified by salt precipitation and covalent chromatography[18,19] to ≥95% activity. Concentration and treatment with aqueous Me_2SO reduced activity to 72%.

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A Critical Examination of Transient Assignments in the Laser Flash Photolysis of 9-Diazofluorene[1]

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Laser photolysis of 9-diazofluorene (DAF; 1×10^{-3} M) in acetonitrile yields a transient species with λ_{max} 470 nm, which decays with first-order kinetics and has a lifetime of 27 ns at room temperature.[2,3] The decay of this transient leads to two new absorption bands at 400 and 500 nm.[2,3] In their original study of this system, Zupancic and Schuster assigned the absorption at 470 nm to singlet fluorenylidene, ^{1}Fl, while those at 400 and 500 nm were assigned to the triplet carbene, ^{3}Fl.[2] Further experiments based on these assignments led to the unusual conclusion that singlet fluorenylidene added nonstereospecifically to olefins.[4]

Experiments carried out in this laboratory[3] showed that the original assignment of the 500-nm absorption to ^{3}Fl was incorrect and that it was in fact due to the 9-fluorenyl radical. On further investigation, we have found that the original assignments[2] of the other bands also require revision. We conclude that the absorption at 470 nm was due to ^{3}Fl while that at 400 nm was due to an ylide formed in the reaction of fluorenylidene with acetonitrile. Singlet fluorenylidene was not detected, presumably because its lifetime was shorter than 10 ns.

Laser flash photolysis (337.1 nm, ~8 ns, up to 10 mJ, 300 K) of DAF in a variety of solvents showed that the buildup of the 400-nm band concurrent with the decay at 470 nm was only observed with nitrile solvents. It was therefore highly unlikely that the 400-nm band was due to ^{3}Fl. In hexafluorobenzene, for example, we measured a lifetime of 95 ns for the 470-nm band. The transient was quenched rapidly when cis-4-methyl-2-pentene was added, and the rate constant was found to be 2.1×10^8 M^{-1} s^{-1}. This result can be combined with a report by Jones and Rettig[5] to show that the 470-nm transient was in fact *triplet* fluorenylidene, rather than the singlet carbene as was originally reported.[2]

Jones and Rettig showed that when DAF was photodecomposed in hexafluorobenzene and *cis*-4-methyl-2-pentene mixtures containing from 5 to 100 mol % olefin, the degree of stereoselectivity in the cyclopropane products[6] was concentration dependent. Our laser experiments show that their lowest concentration was sufficient to quench over 90% of the 470-nm species. There would then be no reason for the ratio of any two products of reaction (such as the cyclopropanes) to be concentration dependent, *regardless of whether singlet addition is stereospecific or not.* A reasonable explanation for these data is that the 470-nm transient is not the singlet but instead the triplet carbene. The reason for the changes in stereoselectivity with olefin concentration should be attributed to the involvement of the shorter lived singlet carbene, which becomes trappable only a high olefin concentrations. Since the singlet carbene is trappable, yet undetectable by nonosecond techniques, its lifetime must be between 0.05 and 5 ns.

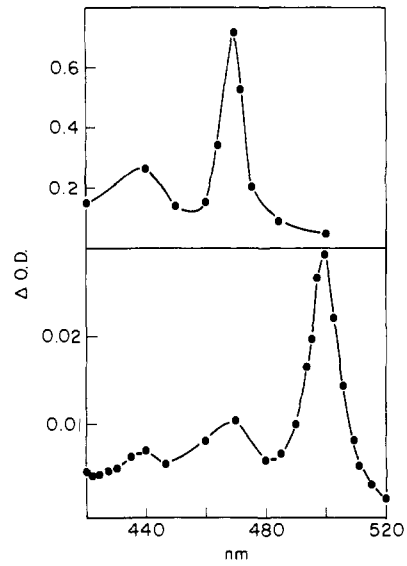

Figure 1. Transient spectra obtained during the laser flash irradiation of 10^{-3} M DAF in a 1:1 isopentane:diethyl ether glass at 77 K (top), and spectrum of the 9-fluorenyl radical obtained by attack of *tert*-butoxyl radicals (from peroxide photodecomposition) on fluorene at 300 K (bottom).

In support of this assignment, we have found that laser flash photolysis of DAF (1×10^{-3} M) in an isopentane:diethyl ether glass at 77 K led to the transient absorption at 470 nm, which had a lifetime of ca. 80 μs.[7] The band at 470 nm consisted of two components separated by ~1360 cm^{-1} (Figure 1). The form and separation of the maxima were virtually identical with those observed for the 9-fluorenyl and 9-chlorofluorenyl radicals.[3] This supports the assignment of the 470-nm absorption band to triplet fluorenylidene since diarylcarbenes and their corresponding radicals have similar electronic configurations and are therefore likely to have similar absorption spectra.[8]

Much of the case for the original, incorrect, assignment[2] of the band at 400 nm to ^{3}Fl was based on a comparison of that absorption with carbene absorption spectra in matrices reported by Closs. However, in his work[9] Closs only described details of the spectrum due to diphenylmethylene; that due to ^{3}Fl has not been reported. We believe that the 400-nm buildup, observed in nitrile solvents (acetonitrile, acetonitrile-d_3, pivalonitrile, and benzonitrile), must be due to reaction of fluorenylidene with nitriles,[10] presumably leading to an ylide, by analogy with reactions involving ketones.[12]

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