Irreversible Inhibition of Papain by Epoxysuccinyl Peptides. ^{13}C NMR Characterization of the Site of Alkylation

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E-64, ([*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]-amido(4-guanido)butane (**1**),[1] a natural product isolated from cultures of *Aspergillus japonicus* and synthesized by Hanada et al.,[2] is a potent, highly selective irreversible inhibitor of cysteine proteinases[3] that rapidly inhibits most cysteine proteinases without inhibiting other proteinases or alkylating free thiols in solution at physiological pH.[4] In addition to helping to identify new cysteine proteinases, E-64 exhibits interesting biological properties,[3,5] and related derivatives are undergoing preclinical evaluation.[6] Synthetic studies have established that the peptide portion of E-64 and related analogues, e.g., Ep-475 (**2**), determines the rate of onset of inhibition of cysteine proteinases.[4] A tritiated derivative was used to show that one molecule of E-64 reacts with one molecule of papain, and the disappearance of titratable enzyme thiol indicated that the epoxysuccinyl group alkylated the active site thiol group (Cys-25 in papain).[7] Two models for the interaction of the L-leucine side chain with the enzyme have been proposed[3,4,7] (Scheme I). However, direct observation of the alkylated cysteine has not been reported, nor has the electrophilic carbon in the epoxysuccinyl moiety been identified.

In order to determine the basis for the remarkable chemical selectivity of the epoxysuccinyl compounds for the cysteine proteinases, it is necessary first to identify the electrophilic atom in **2** that is attacked by the nucleophilic Cys-25 thiol. To do this, we have developed a new chiral synthesis of the *trans*-epoxysuccinyl group which is suitable for preparing the chiral, regiospecifically labeled ^{13}C derivative **2b**.[8] Reaction of **2b** with a cysteine proteinase in principle could lead to products derived from attack at either the C-3 or the C-2 carbon of **2b** (**3**, **4**; Scheme I). We report herein the results of ^{13}C NMR studies of the reaction of **2b** with papain that identify the site of attack as C-3 (**3**).

Ep-475 (**2b**) labeled at C-2 (99%) was synthesized as shown in Scheme II.[8] Model studies established that, in the absence

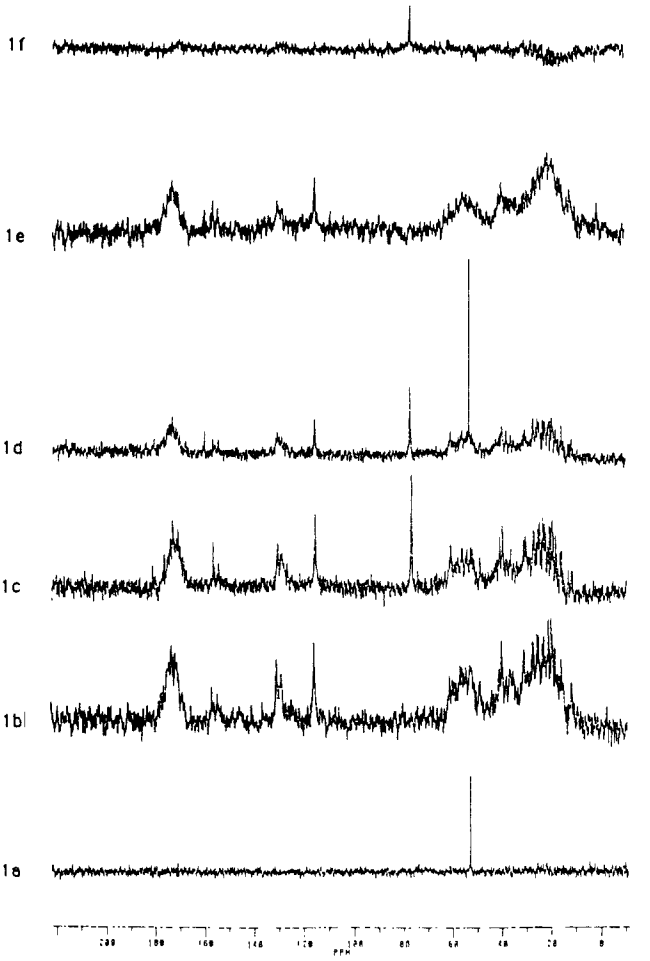
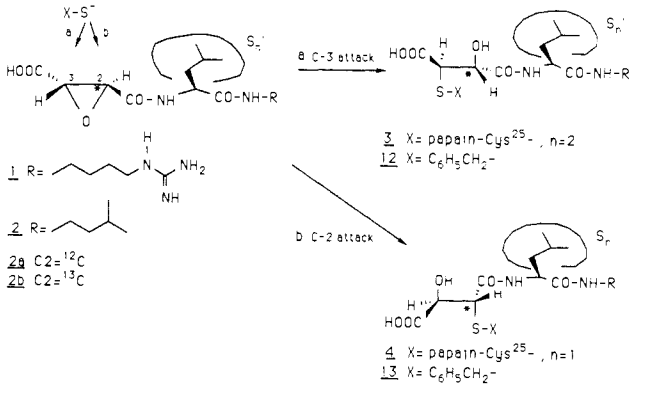


Figure 1. ^{13}C NMR spectra of labeled Ep-475 and papain at 125.75 MHz (^{13}C). PW = 2.5 μs (30 °C). Chemical shifts are relative to TMS. All spectra were obtained in mixtures of H_2O/D_2O (90/10). Concentration of Ep-475, concentration of papain, number of scans: (a) 2.5 mM, 0 mM, 500; (b) 0 mM, 1.7 mM, 40000; (c) 1.7 mM, 1.7 mM, 70000; (d) 2.2 mM, 1.7 mM, 50000; (e) 1.7 mM (unlabeled Ep-475 (**2a**)), 1.7 mM, 50000; (f) subtraction of 1c and 1e.

Scheme I



of enzyme, mercaptans attack both carbons of epoxide **2b** in solution at high pH. Reaction of epoxide **2b** (C-2, 52.5 ppm; Figure 1a) with benzyl mercaptan anion ($C_6H_5CH_2SH/NaOH$, pH 10; Scheme I) gave two products that correspond to attack at C-3 or C-2, respectively, in a ratio of 4:1. The C-2 carbon in **12** resonates near 73 ppm while the corresponding carbon in **13** resonates near 53 ppm.

The site of attack of papain on epoxide **2b** was determined by the following ^{13}C NMR experiment. In separate samples, a solution of papain (Sigma, Type III) in phosphate buffer at pH 6.8 was activated by addition of dithiothreitol (DTT). Activated papain (^{13}C spectrum, Figure 1b) was inactivated with Ep-475, the papain-Ep-475 complex was dialyzed to remove excess inhibitor

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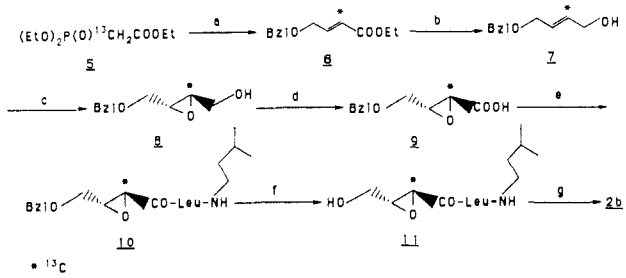
Scheme II. Synthesis of [^{13}C-2]-Ep-475 (**2b**)



(a) $BzlOCH_2CHO$, NaH, 1,2-dimethoxyethane, 25 °C, 30 min; 80% yield. (b) Diisobutylaluminum hydride, C_6H_6, 25 °C, 4 h, 84%. (c) Titanium tetraisopropoxide, (-)-diethyl tartrate, *tert*-butyl hydroperoxide,[10] CH_2Cl_2, -23 °C, 20 h, 88%. (d) O_2/Pt black, AcOEt/aq. $NaHCO_3$ (1:1),[11] 25 °C, 5 days 82%. (e) L-Leu-NH[$CH_2CH_2CH(CH_3)_2$],[2b] 1,3-dicyclohexylcarbodiimide, 1-hydroxybenzotriazole, 0 °C, 1 h; then 25 °C 7 h, 58%. (f) H_2/Pearlman's catalyst, EtOH, 25 °C, 6.5 h, 82%. (g) O_2/Pt black, AcOEt/aq. $NaHCO_3$ (1:1),[11] 25 °C, 4 days; then Dowex 50W (H^+ form), 77%.

and DTT, and the ^{13}C NMR spectrum of the complex in H_2O was taken. The ^{13}C NMR spectrum of the complex displays a new broad signal (line width = 35 Hz) at 76.5 ppm (Figure 1c). When an excess of inhibitor **2b** was added to the reaction mixture and then the NMR spectrum retaken, only the resonance at 52.5 ppm for C-2 of the epoxide increased (Figure 1d) which established that the new resonance at 76.5 did not arise from artifacts. The chemical shift of the new carbon resonance at 76.5 ppm (Figure 1c) shows that the new signal which is formed from the reaction of **2b** with papain corresponds to a secondary alcohol at C-2. Moreover, no peak near 50 ppm for the alternative alcohol product which would be formed from attack of C-2 in **2b** could be detected. A higher resolution difference spectrum, obtained from samples prepared by activating papain separately in the presence of labeled (**2b**) and unlabeled (**2a**) Ep-475 (Figure 1e), clearly showed the presence only of the 76.5-ppm resonance (Figure 1f). This latter experiment gave a better difference spectrum because activated papain at the high concentrations needed for the NMR spectra undergoes autolysis in the absence of inhibitors. These data establish that the active site thiol group in papain cleanly attacks the C-3 carbon of the epoxysuccinyl group in Ep-475 (**12**).

Our approach complements ^{13}C NMR techniques that have been used to characterize other enzyme-inhibitor interactions[13-20] and enzyme catalytic mechanisms,[21,22] and has led to the discovery that the product formed results from attack of the Cys-25 thiol group in papain on the C-3 carbon of the epoxide **2b** (Scheme I, a). This highly regioselective attack, coupled with the rapid inactivation only of cysteine proteinases by epoxysuccinic acid derivatives (apparent second-order rate constants approach 10^6 M^{-1} s^{-1}),[4] suggests that the reaction of the Cys-25 sulfhydryl group with the epoxide moiety in E-64 and related derivatives is an enzyme-catalyzed process that occurs in the active site of cysteine proteinases. Now that the attachment site is known, molecular modeling studies of E-64 derivatives bound in the active site of cysteine proteinases can be initiated in order to determine both the enzyme subsites that E-64 derivatives bind to prior to the alkylation reaction and the relationship between the catalytic and inhibitor mechanisms.

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Formation of Nitridoiron(V) Porphyrins Detected by Resonance Raman Spectroscopy

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Formation of ferrylporphyrins is regarded as the most crucial step in the reaction cycle of cytochrome P-450.[1] A recent finding that liver microsomal cytochrome P-450-LM3,4 catalyzes functionalized nitrogen atom transfer[2] intra- as well as intermolecularly suggests that a multiple FeN bond similar to the ferryl FeO bond is involved in the reaction cycle. Nitridoiron porphyrins (formally written as N≡Fe(por)) may be regarded as models of such intermediate species. It is highly important therefore to prepare such compounds and characterize them by spectroscopic methods and to determine whether such nitridoiron porphyrins are formulated as Fe(V) porphyrins or Fe(IV) porphyrin π-cation radicals. Furthermore, it should be noted that only a few examples of iron(V) compounds are known thus far.[3]

Stable nitridomanganese(V) porphyrins[4,5] and nitridochromium(V) porphyrins[6] have been prepared by chemical oxidation as well as by photolysis[7,8] and their ν(M≡N) (ν is the stretching frequency) could be identified spectroscopically. Recently, Tsubaki et al.[8] observed ν(Mn≡N) of nitridomanganese(V) substituted myoglobin and horseradish peroxidase at 1010 and 1003 cm^{-1}, respectively, by resonance Raman (RR) spectroscopy. However, no reports are available on nitridoiron porphyrins. In this communication we report the formation of N≡Fe(TPP) (TPP = tetraphenylporphine) by laser photolysis of the azido complex and its RR characterization for the first time.

Azido(tetraphenylporphinato)iron, N_3Fe(TPP), and its ^{54}Fe, $^{15}N_3$, and $N_2{}^{15}N$ analogues were prepared by the method of Adams et al.[9] TPP, ^{54}Fe, and ^{15}N containing azides were purchased from Midcentury, Oak Ridge Laboratory, and Stohler Chemicals, respectively. Methylene chloride solutions of these azido complexes were evaporated on the surface of a cold tip and the films thus obtained were cooled to ≈30 K by a CTI Model 20/70 cryocooler. RR spectra were measured on a Spex Model 1403 double monochromator equipped with a Spex DM1B computer. The 514.5-nm line from a Spectra-Physics Model 2025 Ar^+ laser was used for photolysis and for RR excitation. Laser power levels up to 70 mW were applied to the samples through a cylindrical lens.

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