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Carbon-13 NMR Characterization of the Papain Adduct Formed by Peptidyl Acyloxy-, Aryloxy-, and Chloromethyl Ketone Irreversible Inhibitors[1]

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N-Benzyloxycarbonyl-L-phenylalanyl-glycine-derived mesitoyloxy- and pentafluorophenoxymethyl ketones, incorporating carbon-13 labels at the ketone carbonyl and α-keto methylene positions, were synthesized. These compounds act as quiescent affinity labels of cysteine proteinases. NMR studies have established that these compounds form an irreversible covalent adduct with papain, via release of mesitoic acid or pentafluorophenol. The same adduct was formed in each case, with ^{13}C NMR signals at 214.7 ppm ($COCH_2$) and 38.1 ppm ($COCH_2$). Identical spectra were observed for the adduct formed from the analogous ^{13}C-labeled chloromethyl ketones; the structure of this type of adduct has been previously established by X-ray studies. Our NMR data are consistent with this structure, and provide a sensitive measure of the hydrogen-bonding to the P_1 carbonyl. Disruption of this hydrogen-bonding by denaturation produced a ^{13}C NMR shift of 10 ppm to higher field in the carbonyl carbon, to a chemical shift in accordance with that of a model thiomethyl ketone.

INTRODUCTION

During the last 15 years, nuclear magnetic resonance spectroscopy (NMR)[3] has emerged as a powerful tool for the characterization of enzyme–substrate and enzyme–inhibitor complexes (*1, 2*). Papain (EC 3.4.22.2), a cysteine proteinase isolated from papaya fruit, has been the subject of a number of such investigations. Thus, the thiohemiacetal adducts formed by reversible inhibition of papain with peptidyl aldehydes have been investigated by ^{13}C NMR and found to be stereospecific (*3*). The reversible inhibition of papain by nitriles has also been studied; in this case, ^{13}C NMR techniques have provided strong evidence for the formation of a covalent thioimidate ester adduct (*4–6*). Recently, the adduct formed upon irreversible inhibition of papain by the epoxysuccinyl peptide Ep-475 (also known

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[3] Abbreviations used: NMR, nuclear magnetic resonance; Phe, phenylalanyl; Ala, alanyl; Gly, glycyl; Z, benzyloxycarbonyl; TMS, tetramethylsilane; TSP, sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4; ir, infrared; THF, tetrahydrofuran; DMSO, dimethyl sulfoxide; Ac, acetyl; MS, mass spectrum; DMF, dimethylformamide.

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as E-64c) was investigated by ^{13}C NMR (*7*). These studies established that the papain–inhibitor adduct results from a regioselective alkylation of the cysteine-25 thiol by the C-3 carbon of the epoxide.

Cathepsin B (EC 3.4.22.1) is a lysosomal cysteine proteinase which has been implicated in a number of conditions, such as inflammation (*8*), malignancy (*9*), bone resorption (*10*), and muscular dystrophy (*11*). A diverse variety of inhibitors have been developed for this enzyme (*12–16*). We recently communicated our design and investigation of peptidyl acyloxy- and aryloxymethyl ketones as potent irreversible inhibitors of cathepsin B (*17*). These compounds are "quiescent" affinity labels, in which an appropriate peptidyl group delivers a weakly reactive nucleofuge to the powerfully nucleophilic active site thiol group.

Papain serves as an excellent model for cathepsin B; the amino acid sequences of these enzymes show a very high degree of homology in the active site regions, and it has been concluded that the overall gross protein folding patterns and catalytic mechanisms are the same (*18, 19*). Our acyloxy- and aryloxymethyl ketone inhibitors are structurally analogous to chloromethyl ketones, which are well-known as cysteine proteinase inhibitors (*16, 20–22*). Indeed, the X-ray crystal structures of papain adducts derived from three Phe–Ala and Phe–Gly peptidyl chloromethyl ketones have been determined (*23*), and similar cysteine-25 thiomethyl ketone structures were obtained in each case. We have therefore prepared a series of ^{13}C-labeled Z-L-Phe–Gly acyloxymethyl, aryloxymethyl, and chloromethyl ketones and determined the NMR characteristics of the papain adducts derived from each inhibitor. As communicated previously (*17*), this study has established that a covalent cysteine-25 thiomethyl ketone adduct is obtained in each case. We now report the details of our NMR studies.

EXPERIMENTAL PROCEDURES

Synthesis

All reactions were conducted under a dry argon atmosphere, using anhydrous solvents except as stated otherwise. Proton NMR spectra were recorded with a Bruker WP80 at 80 MHz relative to TMS (or TSP for D_2O solutions) at 0 ppm, while IR spectra were obtained with a Perkin–Elmer Model 298 grating spectrophotometer. Optical rotations were measured on a Rudolph Research Autopol III automatic polarimeter. Mass spectra were recorded on Finnigan MAT CH7 and MAT 311A instruments. Elemental analyses were determined by Syntex Analytical Research.

N-Benzyloxycarbonyl-L-phenylalanyl-[1-^{13}C]glycine (Z-L-Phe-NHCH$_2$-^{13}COOH). Dicyclohexylcarbodiimide (50 mmol, 10.3 g) was added to a solution of Z-L-phenylalanine (Sigma, 50 mmol, 15.0 g) and *N*-hydroxysuccinimide (Aldrich, 50 mmol, 5.75 g) in 400 ml of THF at 0°C. The mixture was stirred at 0°C for 2 h, then at room temperature overnight. The mixture was cooled to 0°C and filtered and the filtrate was rotary evaporated. The residual oil was dissolved in ethyl acetate, washed with aqueous $NaHCO_3$ (2×) and brine, dried ($MgSO_4$), rotary

evaporated, and dried at high vacuum to provide 19.1 g (96%) of Z-L-phenylalanine *N*-hydroxysuccinimide ester as a white solid, mp 134–136°C (lit. (*24*) mp 136–137.5°C). Then, following the method of Itoh (*25*), triethylamine (20 mmol, 2.8 ml) was added to a mixture of [1-^{13}C]glycine (10 mmol, 761 mg, 99 atom% ^{13}C, Merck) in 100 ml of 1 : 1 dioxane–water. Solid carbon dioxide pieces were added with stirring until pH 8 was achieved. Z-L-phenylalanine *N*-hydroxysuccinimide ester (10 mmol, 3.96 g) was then added with stirring at room temperature. After 20 h, the mixture was acidified by gradual addition of 1 N HCl and then diluted with ethyl acetate. The organic phase was separated, washed with 1 N HCl (3×), water (2×), and brine, dried ($MgSO_4$), rotary evaporated, and dried at high vacuum to provide 3.07 g (86%) of Z-L-phenylalanyl-[1-^{13}C]glycine as a white solid, mp 153–154.5°C (lit. (*25*) mp 151–152°C for unlabeled Z-Phe–Gly-OH); ^{1}H NMR (DMSO-d_6) δ 8.3 (br t, NH), 7.5 (d, NH), 7.2 (app s, 10 H, 2× Ph), 4.9 (s, PhCH_2O), 4.3 (m, 1 H, CHCH$_2$Ph), 3.8 (app t (dd), 2 H, J_{HH} = 6 Hz, J_{CH} = 6 Hz, NHCH_2CO), 3.4 (br s, H_2O), 3.3–2.6 (m, 2 H, CHCH_2Ph).

N-Benzyloxycarbonyl-L-phenylalanyl-[1-^{13}C]glycine bromomethyl ketone (*Z-Phe-NHCH$_2$-^{13}CO-CH$_2$Br*) **(1a)**. Z-L-phenylalanyl-[1-^{13}C]glycine (1.0 g, 2.8 mmol) was dissolved in THF (15 ml) and cooled in an ice/acetone bath (−10°C). The stirred solution was treated with *N*-methylmorpholine (407 μl, 3.70 mmol) followed by isobutylchloroformate (463 μl, 3.57 mmol) over 5 min. The resulting suspension was stirred a further 15 min at −10°C, treated with diazomethane in ether (41 ml, approx. 0.3 M, prepared from Diazald (Aldrich) according to the supplier's directions) and warmed to room temperature over 4 h.

A 1 : 1 solution of AcOH and 48% HBr (6 ml) was added dropwise with stirring to the reaction mixture at 0°C. After stirring a further 15 min at room temperature, the reaction mixture was diluted with EtOAc. The aqueous layer was removed; the organic phase was washed with water (1×), brine (2×), and saturated $NaHCO_3$ (1×), dried (anhydrous Na_2SO_4), and evaporated to dryness. The residue was purified by column chromatography (silica gel, 50% EtOAc–hexane eluant) to yield **1a** as an off-white solid (840 mg, 69%), which was suitable for further reactions; ^{1}H NMR ($CDCl_3$) showed δ 4.3 (app t (dd), 2 H, J_{CH} = 5 Hz, J_{HH} = 5 Hz, NHCH_2CO), 3.9 (d, 2 H, J_{CH} = 4 Hz, COCH_2Br); electron impact MS, *m/z* 353 (0.3%, M$^+$ − HBr), 254 (6%, Z-NHCHCH$_2$Ph$^+$), 91 (100%, $C_7H_7^+$); exact mass calcd for $C_{19}{}^{13}CH_{20}N_2O_4$ (M$^+$ − HBr) 353.1457, measured 353.1452.

This material was otherwise identical to the similarly obtained "unlabeled" Z-L-Phe–Gly-CH$_2$Br: mp 96.5–97.5°C; $[\alpha]_D^{21}$ −0.8° (*c* = 1.19, $CHCl_3$); ir (KBr) 1740, 1690, 1640 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.5–7.0 (m, 10 H, 2× Ph), 6.5 (br t, NH), 5.3 (d, NH), 5.1 (s, PhCH_2O), 4.7–4.3 (app q (ddd), 1 H, CHCH$_2$Ph), 4.3 (d, 2 H, *J* = 5 Hz, NHCH_2CO), 3.9 (s, COCH_2Br), 3.1 (app d, 2 H, *J* = 7 Hz, CHCH_2Ph); anal. C,H,N (±0.2%).

N-Benzyloxycarbonyl-L-phenylalanyl-glycine [^{13}C]bromomethyl ketone (*Z-Phe-NHCH$_2$CO-^{13}CH$_2$Br*) **(1b)**. In a similar manner, using ^{13}C-labeled diazomethane (6.1 ml, ca. 0.3 M in Et_2O, prepared from Aldrich [*N-methyl*-^{13}C]Diazald) and Z-L-Phe–Gly-OH (300 mg, 0.84 mmol), the desired product was obtained (190 mg, 52%); ^{1}H NMR ($CDCl_3$) showed δ 4.2 (d, 2 H, *J* = 5 Hz, NHCH_2CO), 3.8 (d, 2 H, J_{CH} = 152 Hz, COCH_2Br); electron impact MS, *m/z* 353 (0.2%, M$^+$ − HBr), 254

(2%, Z-NHCHCH$_2$Ph$^+$), 91 (100%, $C_7H_7^+$); exact mass calcd for $C_{19}{}^{13}CH_{20}N_2O_4$ (M^+ − HBr) 353.1457, measured 353.1448.

N-Benzyloxycarbonyl-L-phenylalanyl-[1-^{13}C]glycine chloromethyl ketone (*Z-Phe-NHCH$_2$-^{13}CO-CH$_2$Cl*) **(2a)**. By substituting the 1 : 1 solution of AcOH and 48% HBr in the above procedure with a 1 : 1 solution of AcOH and 37% HCl, was obtained the analogous chloromethyl ketone **2a** as a white powder, 49% yield, mp 96–98°C; ^{1}H NMR ($CDCl_3$) showed δ 4.2 (app t (dd), 2 H, J_{CH} = 5 Hz, J_{HH} = 5 Hz, NHCH_2CO), 4.1 (d, 2 H, J_{CH} = 4 Hz, COCH_2Cl); exact mass calcd for $C_{19}{}^{13}CH_{21}N_2O_4Cl$ 389.1223, measured, 389.1229.

This material was otherwise identical to similarly obtained unlabeled Z-L-Phe–Gly-CH$_2$Cl; mp 98–100°C; electron impact MS, *m/z* 388 (1%, M^+), 254 (27%, Z-NHCHCH$_2$Ph$^+$), 91 (100%, $C_7H_7^+$); exact mass calcd for $C_{20}H_{21}N_2O_4Cl$, 388.1190, measured 388.1194; anal. C,H,N (±0.2%).

N-Benzyloxycarbonyl-L-phenylalanyl-glycine [^{13}C]chloromethyl ketone (*Z-Phe-NHCH$_2$CO-^{13}CH$_2$Cl*) **(2b)**. In a similar manner, using ^{13}C-labeled diazomethane (ca. 0.3 M in Et_2O) and Z-L-Phe–Gly-OH, the desired product was obtained; ^{1}H NMR ($CDCl_3$) showed δ 4.1 (d, 2 H, J_{CH} = 150 Hz, COCH_2Cl).

N-Benzyloxycarbonyl-L-phenylalanyl-[1-^{13}C]glycine pentafluorophenoxymethyl ketone (*Z-Phe-NHCH$_2$-^{13}CO-CH$_2$OC$_6$F$_5$*) **(3a)**. Bromomethyl ketone **1a** (130 mg, 0.30 mmol) and pentafluorophenol (55 mg, 0.30 mmol) were dissolved in DMF (10 ml). The solution was treated with K_2CO_3 (41 mg, 0.30 mmol) and nBu_4NI (ca. 5 mg) and stirred at room temperature for 4 h. The mixture was diluted with EtOAc, and then washed with water (1×) and brine (4×), dried (anhydrous Na_2SO_4), and evaporated to give a solid residue. The product was purified by column chromatography (silica gel, 50% EtOAc–hexane eluant) followed by recrystallization (EtOAc) to give **3a** as a white solid (50 mg, 31%), mp 115–117°C; ^{1}H NMR ($CDCl_3$) showed δ 4.7 (d, 2 H, J_{CH} = 4 Hz, COCH_2O), 4.3 (app t (dd), 2 H, J_{CH} = 5 Hz, J_{HH} = 5 Hz, NHCH_2CO); exact mass calcd for $C_{25}{}^{13}CH_{21}N_2O_5F_5$ 537.1404, measured 537.1408.

This material was otherwise identical to similarly obtained unlabeled Z-L-Phe–Gly-CH$_2$OC$_6$F$_5$ (*17*); mp 110–115°C; $[\alpha]_D^{21}$ − 13.4° (acetone); ir (KBr) 1735, 1690, 1640 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.5–7.0 (m, 10 H, 2× Ph), 6.6 (br t, NH), 5.3 (br d, NH), 5.1 (s, PhCH_2O), 4.7 (s, 2 H, COCH_2O), 4.5 (app q (ddd), 1 H, app *J* = 8 Hz, C*H*CH$_2$Ph), 4.3 (d, 2 H, *J* = 5 Hz, NHCH_2CO), 3.1 (app d, 2 H, *J* = 7 Hz, CHCH_2Ph).

N-Benzyloxycarbonyl-L-phenylalanyl-glycine pentafluorophenoxy-[^{13}C]methyl ketone (*Z-Phe-NHCH$_2$CO-^{13}CH$_2$OC$_6$F$_5$*) **(3b)**. In a similar manner, using bromomethyl ketone **1b** (0.16 mmol, 70 mg), was obtained the desired product (48 mg, 56%) as a white solid, mp 110–114°C; ^{1}H NMR ($CDCl_3$) showed δ 4.7 (d, 2 H, J_{CH} = 148 Hz, COCH_2O); electron impact MS, *m/z* 537 (0.3%, M^+), 386 (2%, M^+ − PhCH$_2$OCONH$_2$), 254 (6%, Z-NHCHCH$_2$Ph$^+$), 91 (100%, $C_7H_7^+$); exact mass calcd for $C_{25}{}^{13}CH_{21}N_2O_5F_5$ 537.1404, measured 537.1395.

N-Benzyloxycarbonyl-L-phenylalanyl-[1-^{13}C]glycine mesitoyloxy-methyl ketone (*Z-Phe-NHCH$_2$-^{13}CO-CH$_2$OCO-(2,4,6-Me$_3$)Ph*) **(4a)**. Anhydrous potassium fluoride (0.87 mmol, 51 mg) was added to a solution of bromomethyl ketone **1a** (0.30 mmol, 130 mg) in 15 ml of DMF. The mixture was stirred 3 min at room tempera-

ture, 2,4,6-trimethylbenzoic acid (mesitoic acid) (0.32 mmol, 53 mg) was added, and the mixture was stirred at room temperature overnight. The mixture was diluted with ethyl ether, washed with water (4×), saturated $NaHCO_3$ (2×), and brine, dried ($MgSO_4$), and evaporated. The residue was purified by column chromatography (silica gel, 20–50% EtOAc–hexane eluant) to give the product **4a** (93 mg, 60%) as a white solid, mp 118–119°C; ^1H NMR ($CDCl_3$) showed δ 4.9 (d, 2H, J_{CH} = 4 Hz, $COCH_2O$), 4.2 (app t (dd), 2H, J_{CH} = 5 Hz, J_{HH} = 5 Hz, $NHCH_2CO$); exact mass calcd for $C_{29}{}^{13}CH_{32}N_2O_6$ 517.2294, measured 517.2288.

This material was otherwise identical to similarly obtained unlabeled Z-L-Phe–Gly-CH_2OCO-(2,4,6-Me_3)Ph (*17*); mp 116–118°C; $[\alpha]_D^{21}$ −12.0° (*c* = 1.00, acetone); ir (KBr) 1725, 1690, 1660 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.5–7.0 (m, 10 H, 2× Ph), 6.9 (s, 2 H, Ar*H*), 6.6 (br t, NH), 5.3 (br d, NH), 5.1 (s, $PhCH_2O$), 4.9 (s, 2 H, $COCH_2O$), 4.5 (app q (ddd), 1 H, app *J* = 7 Hz, $CHCH_2Ph$), 4.2 (d, 2 H, *J* = 5 Hz, $NHCH_2CO$), 3.1 (app d, 2 H, *J* = 7 Hz, $CHCH_2Ph$), 2.3 (2 s, 9 H, $(CH_3)_3Ar$); electron impact MS, *m/z* 516 (0.4%, M^+), 365 (0.7%, $M^+ - PhCH_2O-CONH_2$), 254 (5%, Z-$NHCHCH_2Ph^+$), 91 (100%, $C_7H_7^+$); exact mass calcd for $C_{30}H_{32}N_2O_6$ 516.2260, measured 516.2260.

N-Benzyloxycarbonyl-L-phenylalanyl-glycine mesitoyloxy-[^{13}C]methyl ketone (*Z-Phe-NHCH$_2$CO-^{13}CH$_2$OCO-(2,4,6-Me$_3$)Ph*) **(4b)**. In a similar manner, using bromomethyl ketone **1b** (0.15 mmol, 65 mg), the desired product was obtained (53 mg, 68%) as a white solid, mp 111–114°C; ^1H NMR ($CDCl_3$) showed δ 4.8 (d, 2 H, J_{CH} = 147 Hz, $COCH_2O$).

N-Benzyloxycarbonyl-L-phenylalanyl-glycine ethylthiomethyl ketone (*Z-L-Phe-Gly-CH$_2$SEt*) **(5)**. A solution of (unlabeled) Z-L-Phe-Gly-CH_2Br (200 mg, 0.46 mmol) in DMF (15 ml) was treated with sodium ethyl thiolate (EtS^-Na^+, 41 mg, 0.49 mmol; prepared by treatment of NaH in Et_2O with EtSH, followed by filtration and drying at high vacuum). After the mixture was stirred overnight at room temperature, it was diluted with ether and washed with water (5×), aqueous $NaHCO_3$, and brine, dried ($MgSO_4$), and evaporated. The residue was purified by column chromatography (silica gel, 40% EtOAc–hexane eluant) to afford 94 mg (49%) of the product **5** as an off-white powder, mp 112–114°C; $[\alpha]_D^{21}$ −17.4° (*c* = 0.82, acetone); ir (KBr) 1720, 1695, 1645 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.5–7.0 (m, 10 H, 2× Ph), 6.4 (br t, NH), 5.2 (br d, NH), 5.1 (s, $PhCH_2O$), 4.5 (app q (ddd), 1 H, app *J* = 7 Hz, $CHCH_2Ph$), 4.3 (d, 2 H, *J* = 5 Hz, $NHCH_2CO$), 3.2 (s, 2 H, $COCH_2S$), 3.1 (app d, 2 H, *J* = 7 Hz, $CHCH_2Ph$), 2.4 (q, 2 H, *J* = 7 Hz, CH_2CH_3), 1.2 (t, 3 H, *J* = 7 Hz, CH_2CH_3); electron impact MS, *m/z* 414 (12%, M^+), 91 (100%, $C_7H_7^+$); exact mass calcd for $C_{22}H_{26}N_2O_4S$ 414.1613, measured 414.1610.

Enzyme Preparations and Assays

Papain (Sigma) was purified by a slight modification of the procedure described by Sluyterman and Wijdenes (*26*). Elution of the organomercurial agarose gel column (Affi-Gel 501 from Bio-Rad) was performed with sodium acetate buffer (pH 5.0, 10 mM), but organic solvents (dimethyl sulfoxide and butanol) were omitted. The enzyme was thereby obtained as mercury papain, and was concentrated as required with an Amicon ultrafiltration unit fitted with a PM-10 filter

(10,000 molecular weight cutoff). The enzyme was activated immediately before use by stirring with β-mercaptoethanol (5 mM) which was subsequently removed by passage of the solution through a PD-10 Sepharose column (Pharmacia). Thiol content was determined by titration with 5,5′-dithiobis-(2-nitrobenzoic acid) (*27*). Enzyme activity was measured spectrophotometrically at 412 nm (Perkin–Elmer Model 559A uv/visible spectrophotometer) at pH 6.0 with the substrate α-*N*-benzoyl-L-arginine-*p*-nitroanilide (*28*).

NMR Studies

NMR spectra were obtained with either (a) a Bruker WP-80 spectrometer equipped with a dual 5-mm carbon/proton probe at 80.1 MHz for ^{1}H, or a 5-mm fluorine probe at 75.4 MHz, or (b) a Bruker AM-500 spectrometer with a dual 5-mm probe at 125 MHz for ^{13}C. Carbon spectra (32 K data points) were generally acquired using a 30° pulse angle and an acquisition time of 0.52 s, with the probe temperature maintained at 27°C. Protons were composite pulse decoupled. The spectral width was 31,250 Hz, in quad detection. Chemical shifts were assigned relative to the internal standards acetone-d_6 at 29.8 ppm or DMSO-d_6 at 39.5 ppm for ^{13}C spectra. External trifluoroacetic acid was used as a reference and assigned at −78.9 ppm for ^{19}F spectra.

NMR spectra of inhibitors and model compounds were measured as DMSO-d_6 solutions. For the enzyme–inhibitor adducts, inhibitors were each dissolved in DMSO-d_6 or acetone-d_6, and then immediately added to the activated enzyme solution to provide final concentrations of 0.30 mM enzyme and 0.30 mM inhibitor for the carbonyl ^{13}C-labeled compounds, and 1.8 mM enzyme and 1.8 mM inhibitor for the methylene ^{13}C-labeled compounds. All enzyme solutions were 10 mM potassium phosphate buffer at pH 7.0. For the carbonyl ^{13}C-label experiments, DMSO-d_6 was present at 5% (v/v), and was used both as a lock signal and as an internal standard. For the methylene ^{13}C-label experiments, 5% (v/v) acetone-d_6 was used as the lock and internal standard, since DMSO-d_6 would have interfered with the chemical shift region of interest. Linewidths were measured at half peak height, and corrected for line broadening.

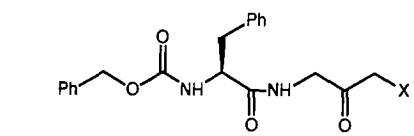
Following NMR measurement, each enzyme–inhibitor solution was passed through a PD-10 column (Pharmacia) using water as eluant to separate the protein from any low molecular weight species. Fractions containing protein were identified by uv absorbance measurements at 280 nm. ^{13}C NMR measurements were then repeated on the protein fraction. The small-molecule fractions were collected and lyophilized. In experiments with **3a**, the small-molecule fraction was dissolved in D_2O adjusted to pH 10 with K_2CO_3, and the ^{19}F NMR spectrum was measured. In experiments with **4a**, the small-molecule fraction was dissolved in D_2O and the ^{1}H NMR spectrum was recorded. NMR spectra were also obtained for authentic samples: (a) potassium mesitoate: ^{1}H NMR (D_2O) δ 6.97 (s, 2H), 4.76 (s, HDO), 2.29 (s, 9H); (b) pentafluorophenol: ^{19}F NMR (D_2O/K_2CO_3, pH 10) −167.8 (app dd, J = 10 and 20 Hz), −169.3 (app dd, J = 21 and 23 Hz), −182.7 (app tt, J = 10 and 21 Hz).

For the papain-adduct denaturation experiment, the papain–inhibitor adduct

from **4a** was prepared, and any small-molecule components were removed by Sepharose chromatography, as described above. The resulting aqueous solution of papain–inhibitor adduct (2 mM) was then gradually heated in increments to 75°C, with periodic measurement of the ^{13}C NMR spectrum. No significant change in the spectral pattern was observed; however, the NMR signals gradually decreased in intensity as the protein precipitated from solution. The heterogeneous sample (0.5 ml) was then stirred with aqueous 6 M guanidine hydrochloride solution (30 ml) for 72 h to effect partial resolubilization of the protein. The sample was centrifuged, and the supernatant was concentrated and washed with 6 M guanidine hydrochloride on an Amicon ultrafiltration device (as described above) to a final volume of 0.5 ml. The ^{13}C NMR spectrum of this solution (ca. 1 mM) was then recorded, using added D_2O as a lock signal. A strong signal for guanidine was observed at 159.2 ppm. Chemical shift assignments were based on a solution of 6 M guanidine hydrochloride containing 5% DMSO-d_6 at 39.5 ppm.

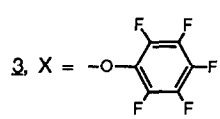
RESULTS AND DISCUSSION

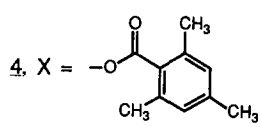
^{13}C-labeled inhibitors were synthesized by utilizing either [1-^{13}C]glycine or [^{13}C]diazomethane via established procedures (*29, 30*) to obtain *N*-benzyloxycarbonyl-L-phenylalanyl-glycine bromomethyl and chloromethyl ketones **1a,b** and **2a,b,** respectively. Displacement reactions on the bromomethyl ketones (**1a,b**) then afforded the corresponding pentafluorophenoxymethyl (**3a,b**) and mesitoyloxymethyl (**4a,b**) ketones (*17*).

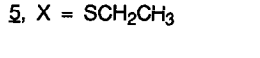


1, X = Br

2, X = Cl







a: labeled as ^{13}CO-CH_2-X

b: labeled as CO-$^{13}CH_2$-X

Carbonyl ^{13}C-Label Experiments

The ^{13}C NMR characteristics of compounds **2a**, **3a**, and **4a** were first examined. In DMSO-d_6, each of these compounds shows a single resonance at 198–200 ppm (Table 1) for the ^{13}C-labeled carbonyl. Due to low solubility, spectra could not be obtained in fully aqueous solution; however, in 80% DMSO-d_6/H_2O measurements

TABLE 1

^{13}C NMR Chemical Shift Characteristics for Inhibitors **2–4**, Their Papain–Inhibitor Adducts, and Model Derivative **5**

Sample	Chemical shift (ppm)[a]	
	$^{13}C{=}O$	$^{13}CH_2$
2, Z-Phe–Gly-CH_2Cl	198.5	47.4
3, Z-Phe–Gly-$CH_2OC_6F_5$	201.0	75.7
4, Z-Phe–Gly-$CH_2OCO(2,4,6\text{-}Me_3)Ph$	200.2	66.9
5, Z-Phe–Gly-CH_2SEt	202.1	37.3
	202.0[b]	37.3[b]
	200.2[c]	38.0[c]
Papain–inhibitor adduct (from **2**, **3**, or **4**)	214.7[d]	38.1[d]
Papain–inhibitor adduct, denatured (from **4a**)	204.9[e]	

[a] In DMSO-d_6, except as noted.
[b] In acetone-d_6.
[c] In $CDCl_3$.
[d] In 10 mM potassium phosphate, pH 7, 5% (v/v) DMSO-d_6 or acetone-d_6 (see under Experimental Procedure).
[e] In 6 M guanidine hydrochloride.

were possible, and for **4a** resonances were observed at 200.8 and 93.5 ppm (ca. 8 : 1 ratio by peak height), attributed to the ketone and hydrate (*gem*-diol), respectively. These observations are in accord with those found for *N*-tosyl-[$^{13}C{=}O$]phenylalanine chloromethyl ketone (203.6 and 96.6 ppm; in 50% DMSO-d_6/H_2O) (*31*) and *N*-benzyloxycarbonyl-[$^{13}C{=}O$]lysine chloromethyl ketone (204.7 and 95.4 ppm; in aqueous solution, pH 3–7) (*32*). Similarly, ketone and hydrate signals were observed in 80% DMSO-d_6/H_2O for compound **2a** at 199.3 and 94.3 ppm, and for **3a** at 201.7 and 94.0 ppm.

Solutions of active papain (10 mM phosphate buffer, pH 7.0), were treated with equimolar amounts of compounds **2a**, **3a**, and **4a** (each dissolved in DMSO-d_6). After several (10–20) minutes, the enzyme activity was measured and complete inhibition was established. ^{13}C NMR measurements were performed, and a single resonance was observed at 214.7 ppm for each enzyme–inhibitor solution. A linewidth of 5 Hz was observed, in good agreement with that expected (ca. 3 Hz) for a carbonyl carbon (*1*). The remaining portion of each spectrum was virtually identical to that obtained for untreated papain.

Upon removal of low molecular weight components by gel filtration, NMR measurements were repeated, and the spectra were found to retain the resonance at 214.7 ppm and to be virtually unchanged. In addition, enzyme activity measurements confirmed that complete inhibition was persistent. The "small-molecule" column fractions were collected and lyophilized. The ^{19}F NMR spectrum (in D_2O/K_2CO_3, pH 10) of the small-molecule sample originating from the pentafluorophe-

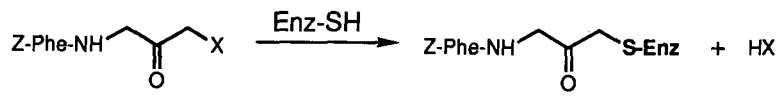


FIG. 1. Reaction of affinity labels **2**, **3**, or **4**, with papain to form a thiomethylketone papain–inhibitor adduct.

noxy inhibitor **3a** gave a spectrum which was identical to that of authentic pentafluorophenol, while the ^1H NMR spectrum (in D_2O) of the small-molecule fraction derived from the mesitoyloxy inhibitor **4a** was identical to that of authentic potassium mesitoate. No other proton resonances were observed in this spectrum, confirming the attachment of the peptide fragment to the protein.

These results provide convincing evidence that a single molar equivalent of inhibitor (**2a**, **3a**, or **4a**) is sufficient for complete inhibition of enzyme activity, that the inhibition is irreversible, and that the peptidyl portion of the inhibitor becomes covalently attached to the enzyme while the group X is released (demonstrated for **3a** and **4a**). Also, a single enzyme–inhibitor adduct is formed, which is identical for all three inhibitors. As the adducts derived from Phe–Ala and Phe–Gly chloromethyl ketones have each been established by X-ray crystal studies to have a cysteine-25-derived thiomethyl ketone structure (*23*), it is clear that the adduct formed by **2a**, **3a**, or **4a** has an analogous structure (Fig. 1).

As a model of the papain–inhibitor adduct, the ethylthiomethyl ketone **5** was prepared.[4] The ^{13}C resonance observed for the adduct is ca. 14 ppm downfield from the corresponding signal for **5** in three different solvents (Table 1). However, such deshielding is consistent with extensive bond polarization in the carbonyl group, which could be accounted for by strong hydrogen bonding to the carbonyl oxygen in the active site. Indeed, a key feature of the papain adduct X-ray structures (*23*) is the close proximity of the carbonyl oxygen of the P_1 residue to two potential hydrogen-bond donors, the backbone NH of cysteine-25 and the NH_2 of glutamine-19 (Fig. 2). NMR chemical shift data provide valuable information regarding bond polarization and bond order (*33–35*), and, as an example, the carbonyl carbon resonance of acetone has been found to experience downfield shifts (relative to that of neat acetone) of 2.3 ppm in chloroform, 9.1 ppm in water, 14.1 ppm in trifluoroacetic acid, and 37.4 ppm in sulfuric acid (*36, 37*). These shifts are an effective measure of the extent of hydrogen-bonding to (or protonation of) the carbonyl oxygen, and it has been expressed that such NMR data provide a more sensitive measure of bond-order than X-ray crystal structures (*38*). If complete protonation is considered to cause a shift of 20–30 ppm to higher frequency, then our observed shift of 14 ppm suggests a reduction in the carbonyl bond order from 2 to 1.5 (ca. 50% protonation of the P_1 carbonyl oxygen) (*38*).

To further characterize the ketone carbonyl of this papain adduct, it was of interest to examine the ^{13}C NMR signal of this species with the key (potential)

[4] Compound **5** is not an effective inhibitor of the cysteine proteinase cathepsin B; L. J. Copp, unpublished results.

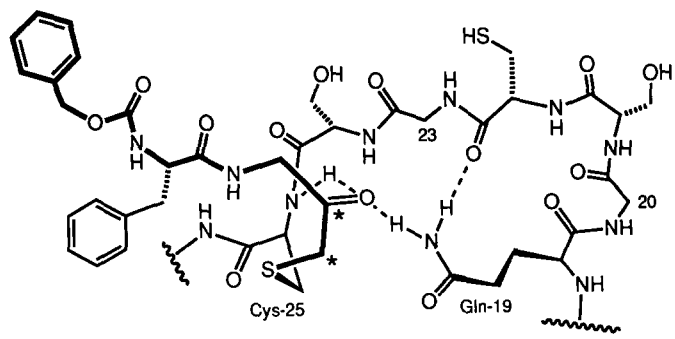


FIG. 2. Papain–inhibitor adduct structure; asterisks indicate the positions of the ^{13}C labels. The drawing is derived from the reported X-ray crystal structures of papain–chloromethyl ketone adducts (*23*).

hydrogen bonds disrupted. A sample of the papain adduct from inhibitor **4a** was therefore subjected to thermal unfolding, based on the conditions established for the unfolding of native papain (*39*). Unfortunately, poor solubility of the denatured papain adduct under these conditions precluded the observation of any new ^{13}C NMR signals. Resolubilization was achieved by stirring the mixture with aqueous 6 M guanidine hydrochloride, to give a protein sample which exhibited a spectrum similar to that obtained before denaturation, but with a new strong resonance at 204.9 ppm in place of the adduct carbonyl signal normally observed at 214.7 ppm. This observation, involving a shift of 10 ppm to higher field upon denaturation, suggests that the strong polarization of the carbonyl noted in the papain–inhibitor adduct is disrupted by unfolding of the protein. The chemical shift of this new resonance is now in strong agreement with that of the ketone carbonyl for model compound **5** (Table 1).

Methylene ^{13}C-Label Experiments

As the methylene carbon chemical shift should be less susceptible to specific active-site "environmental" effects, the analogous methylene ^{13}C-labeled inhibitors **2b**, **3b**, and **4b** were examined to confirm the structural identity of the papain adduct. As well, shifts of ≤ 1 ppm are observed in sp^3 carbons upon protonation of adjacent carbonyls (*34*).

The large majority of ^{13}C NMR studies of enzyme reactions has utilized *carbonyl* ^{13}C-enriched derivatives (*2*). At a given field strength, both longitudinal relaxation times (T_1) and linewidths ($1/\pi T_2$) are directly proportional to the rotational correlation time, which for papain, with a molecular weight of 23,350, is calculated to be 30 ns (*1*). For carbons with directly bonded protons, dipolar relaxation predominates even at high field strengths. Since this dipolar relaxation is inversely proportional to the sixth power of the carbon–proton separation, the linewidths of carbons with directly bonded protons (for example, methylene carbons) are expected to be significantly larger than those carbons with no attached protons (for example,

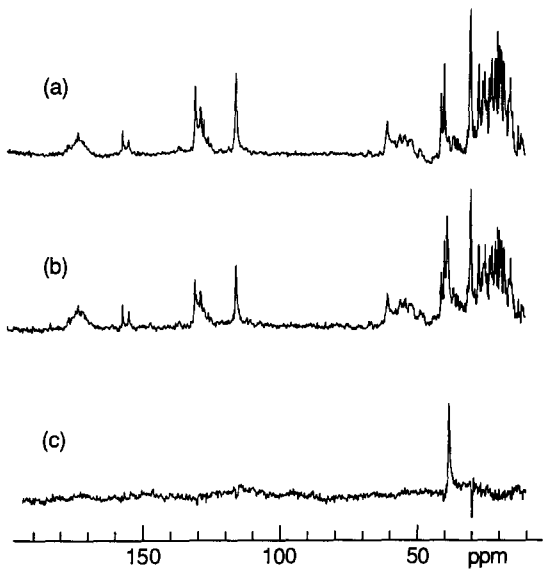


FIG. 3. ^{13}C NMR spectra (125.8 MHz) of the papain adduct with inhibitor **4**. The spectra were obtained with full proton decoupling, 45° pulse, 0.56-s acquisition time, 292,000 scans, 10-Hz line broadening, and 32K data points. (a) Sample containing 1.8 mM papain treated with 1.8 mM unlabeled **4**, in 10 mM potassium phosphate, pH 7, containing 5% (v/v) acetone-d_6. (b) Sample as in (a), except using 1.8 mM **4b** (i.e., labeled as CO-$^{13}CH_2$-). (c) Subtraction of spectrum (a) from (b), showing the ^{13}C-label signal at 38.1 ppm; the negative signal at 29.8 ppm is acetone-d_6.

carbonyl carbons). In addition, the aliphatic region of the protein ^{13}C spectrum is crowded, increasing the difficulty in observing spectral changes. Consequently, difference techniques were used to obtain a satisfactory spectrum of the adduct.

For the mesitoyloxy compound **4b**, the NMR spectrum of papain inactivated with unlabeled **4** was obtained in a manner similar to that described above, and this spectrum was subtracted from that obtained from papain inactivated with **4b** ([^{13}C]methylene labeled). The difference spectrum then cleanly exhibited a single resonance at 38.1 ppm, as illustrated in Fig. 3. The observed linewidth of 65 ± 5 Hz is in accord with that expected (calculated linewidth = 84 Hz) for a carbon with two directly bonded protons, and a molecular weight of 23,350 (*1*).[5] Enzyme–inhibitor solutions prepared from **2b** and **3b** provided virtually identical results. The chemical shift (38.1 ppm) of this adduct is in excellent agreement with the model thiomethyl ketone derivative **5**, which shows a corresponding resonance at 37–38 ppm (Table 1).

[5] Efforts were made to reduce the dipolar relaxation, and thereby improve the signal linewidth and intensity of the [^{13}C]methylene signal, by replacing the attached protons with deuterons (*1*). For an effectively "immobile" macromolecule (MW 280,000), relatively narrow CD_2 linewidths have been observed even without deuterium decoupling (*40*). However, our experiments with deuterium-labeled **4b** (labeled as CD_2CO-$^{13}CD_2$; obtained by exchange in MeOH-d_4/cat. aq. K_2CO_3) in D_2O did not provide adduct spectra with improved signal linewidths (in the absence of deuterium decoupling).

Conclusions

Peptidyl acyloxy- and aryloxymethyl ketones have been designed and developed as quiescent affinity labels of cysteine proteinases (*17*). The NMR studies of papain described in this report have established that the inactivation event occurs with concomitant release of pentafluorophenol from inhibitor **3**, and mesitoic acid from **4**. By the use of both carbonyl and methylene ^{13}C-labeled inhibitors, the covalent papain adduct formed by either type of (quiescent) inhibitor has been characterized as identical to that obtained from the analogous chloromethyl ketone (reactive) affinity label **2**. Papain chloromethyl ketone adducts have been previously established as cysteine-25 thiomethyl ketones by X-ray structure determinations (*23*). Consistent with these structures, our NMR data indicate alkylthio-substitution on the α-keto methylene, and strong hydrogen bonding to the P_1 carbonyl oxygen. Unfolding of the adduct structure by denaturation produces a ^{13}C NMR shift of 10 ppm to higher field for the carbonyl carbon, in accordance with loss of strong hydrogen-bonding to the carbonyl group.

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