a correlation is consistent with a thermodynamic origin for a kinetic α-effect in the gas phase and with analogous correlations of rates and equilibria for α-effect and normal nucleophiles for reactions in solution (see above). It cannot be emphasized too strongly that the α-effect represents unusual reactivity *relative to* the proton basicity of a nucleophile.[2-4]

Origin of the α-Effect. Greater basicity toward carbon or phosphorus than toward the proton for α-effect nucleophiles compared with normal nucleophiles can account for at least some of the observed kinetic α-effects, as described above.[22-28] There may be a differential stabilization that arises from the largely covalent character of bonds to carbon and phosphorus compared with the more ionic character of bonds to hydrogen. The heteroatom substituent of an α-effect base may cause a smaller decrease in the ability of the base to participate in a covalent bond for a given decrease in charge on the basic atom, compared with an electron-withdrawing substituent on a normal base.[55] This suggestion is supported by ab initio calculations that give a larger difference in Mulliken charge and a smaller difference in HOMO energy for hydrogen peroxide ion relative to methoxide ion than for formate ion relative to methoxide ion.[56,57] However, the α-effect compounds and normal bases: hydroxide, methoxide, hydrogen peroxide, and hypofluorite ions, follow a single correlation of Mulliken charge and HOMO energy, while formate ion deviates from this correlation. This suggests that it may be formate ion that is unusual, rather than the α-effect bases.

Electron-density difference maps from X-ray structural analysis show that formation of a covalent bond between two oxygen or nitrogen atoms results in a decrease in the electron density between the two atoms, while the electron density between carbon and oxygen or nitrogen atoms is increased upon bond formation.[58] This indicates that the electronic configuration of the α-effect bases is different from that of the normal bases. It is conceivable that this difference is related to the origins of the α-effect.

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(57) It has been suggested from these gas-phase calculations that the proton affinity of hydrogen peroxide ion is abnormal compared with hydroxide and methoxide ion because of an "abnormally large" decrease in Mulliken charge for the basic oxygen atom of hydrogen peroxide ion (ref 56). However, analysis of substituent effects for normal and α-effect bases in solution provides no indication of an abnormal proton affinity for the α-effect bases (ref 2 and Hall, H. K., Jr. *J. Am. Chem. Soc.* **1957**, *79*, 5441-5444). In addition, it has been argued that an abnormally low proton basicity does not account for the observed kinetic α-effects in solution (ref 2).
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Multiple Alkylation and Mapping of the Active Site of α-Chymotrypsin by Carbonium Ions Generated with Active-Site-Directed Enzyme-Activated Nitrosoamide Substrates

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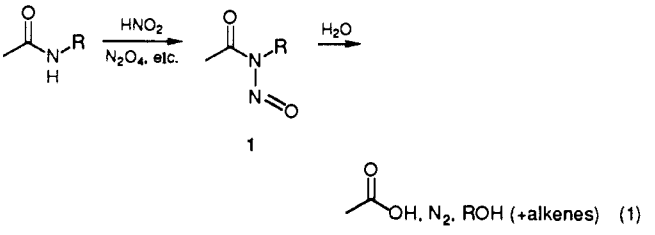
Abstract: The inhibition of α-chymotrypsin with ^{13}C-enriched alanine- and phenylalanine-based *N*-nitrosoamides, as active-site-directed and enzyme-activated inhibitors, results in the alkylation (benzylation) of side chains and also of the amide linkages of the protein backbone (both at O and N). ^{13}C NMR spectra of the denatured inhibited enzyme (in Gdn·HCl) indicate that alkylation has occurred at O, N, S, and C sites. ^{13}C NMR spectra of the amino acid mixtures from fully hydrolyzed inhibited enzymes show that the pattern of alkylation is strikingly different for inhibitions by the alanine- and phenylalanine-based inhibitors. In the case of the phenylalanine-based inhibitor, approximately equally intense signals are observed at 52.32, 51.31, 36.78, and 32.91 ppm, while with the alanine-based inhibitor, a major signal appears at 52.35 ppm, with minor signals appearing at 36.83 and 32.9 ppm. Chromatographic and NMR evidence is presented to indicate that the 52.32-52.35-ppm signal stems from *N*-benzylglycine. The chemical shift data suggest that the 51.31-ppm signal stems from *N*-benzylserine and the 36.78-36.83-ppm signal from *S*-benzylcysteine. Mechanisms are presented to account for the formation of those products.

N-Nitrosoamides,[1] lactams,[2] and sultams[3] are hydrolyzed far more rapidly than the corresponding amido precursors, and as "active" amides and peptides they can serve as substrates for hydrolytic enzymes (eq 1);[4] they are readily prepared by nitrosation



(1)

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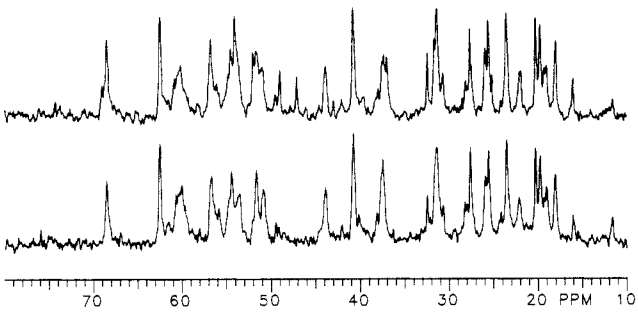
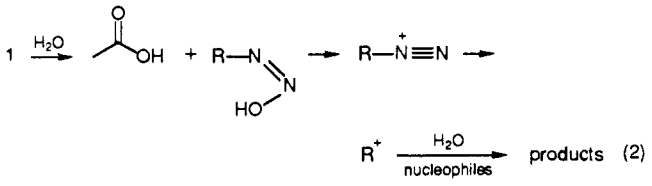


Figure 1. ^{13}C NMR of **1a**-inhibited α-chymotrypsin (α-CT) (proton decoupled) in 6 M Gdn·HCl (20% D_2O), pH = 3 (upper curve) [no. of transients (NT) = 32 000] vs DFP-inhibited α-CT denatured in 6 M Gdn·HCl, pH = 3 (bottom) (NT = 64 000). A line broadening of 10 Hz was used in both cases. Sample temperature = 27 °C. Internal chemical shift standard = Gdn·HCl at 159.00 ppm; spectra were plotted with the signals at 40.74 ppm (Leu β + Lys ε signals from chymotrypsin) equal in intensity.

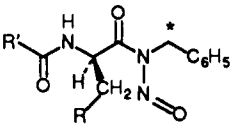
of the corresponding amides.[4,5] Subsequent to cleavage of these inhibitors at the carbonyl group, the leaving group rapidly forms alkyldiazonium ions and then "deaminatively formed" carbonium ions (eq 2). Such ions are extraordinarily reactive;[6] they have



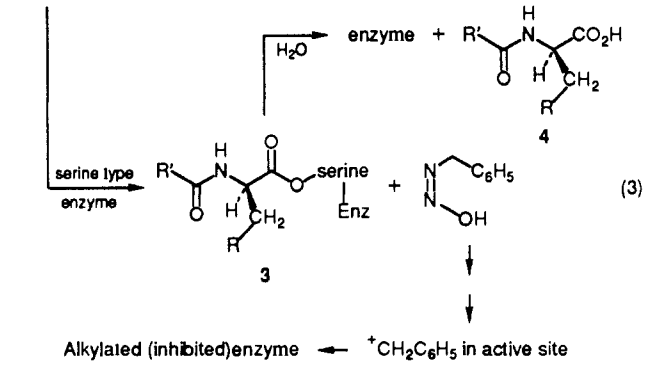
(2)

the capability of reacting (usually irreversibly) with all common organic functional groups except alkyl groups.[7] Suitably designed nitrosoamides have been shown to serve as irreversible inhibitors of α-chymotrypsin,[4] other proteases,[8] and an esterase;[9] related applications involving triazenes and sydnones as sources of deaminatively formed carbonium ions have been reported for *Escherichia coli* galactosidase[10] and cytochrome P-450[11] and suggested for monoamine oxidases.[12] Since these inhibitors require priming by the enzyme, they are "enzyme-activated inhibitors" (mechanism-based type),[13] and they can, generally, also be designed as active-site-directed inhibitors.[14]

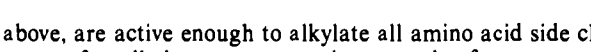
Inhibition of α-Chymotrypsin. We have examined in some detail the inhibition of α-chymotrypsin using *N*-nitroso-*N*-benzyl derivatives of alanine (**2a**) and phenylalanine (**2b**) as substrates (eq 3).[4] The carbonium ions generated in the active site, as mentioned



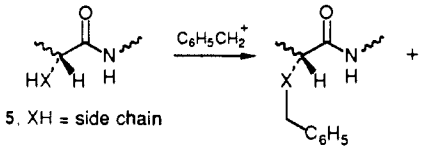
2a, R = H, R′ = 2-propyl
b, R = C_6H_5, R′ = 2-propyl
*, ^{14}C, ^{13}C

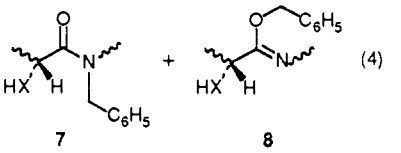


(3)



above, are active enough to alkylate all amino acid side chains except for alkyl groups; more importantly, from a mapping perspective, they also alkylate the amide linkages of the protein backbone (**5**) at both potential sites (O and N) (eq 4).[4d,f]





(4)

Through use of ^{14}C-labeled **2b**, we have shown that the inhibited enzyme contains one benzyl group per molecule;[2b] the location of the benzyl group differs, however, from one enzyme molecule to another. Further, we showed that the rate of inhibition was decreased by the competitive inhibitor *N*-acetyl-L-tryptophan.[2b] We now report on the inhibition of α-chymotrypsin with ^{13}C-labeled nitrosoamides **2a** and **2b** and the use of ^{13}C NMR spectroscopy to reveal the number and type of alkylation sites.

^{13}C NMR Spectra. α-Chymotrypsin was inhibited with ^{13}C-(90%) labeled **2a** (or **2b**), and the reaction mixtures were treated with DFP (diisopropyl fluorophosphate) to ensure the full inhibition of the enzyme (as a precaution against autolysis). ^{13}C NMR spectra (100 MHz) of these untreated reaction mixtures in pH 6.8 and 7.8 phosphate buffer were compared with spectra of α-chymotrypsin fully inhibited with DFP (the diisopropyl phosphate group is attached solely to the 3-oxygen of serine-195).[15] The difference spectra showed sharp peaks between 10 and 80 ppm attributed to small molecules such as benzyl alcohol, diisopropyl phosphate, DFP, and in the case of **2b** inhibitions, *N*-isobutyrylphenylalanine (**4b**) and benzyl *N*-isobutyrylphenylalaninate (the latter compound is a product of the thermal decomposition[1] of **2b** that is already present in small amounts in the samples of **2b** used); in addition, a number of small signals stemming from

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(7) When *N*-benzyl-*N*-nitrosoacetamide is treated with base in *N*-ethylacetamide as the solvent, benzylation occurs on both oxygen and nitrogen of the amide linkage. However, C-alkylation to yield *N*-ethyldihydrocinnamide or *N*-(3-phenylpropyl)acetamide was not detected by NMR spectroscopic examination of the product mixture.

(8) We have found that *N*-nitrosovalerolactam (and the more complex lactam covered in ref 2b) inhibits porcine elastase, subtilisin BPN′, human thrombin, and human plasmin, while *N*-nitroso-*N*-benzyl-6-aminocapramide hydrochloride inhibits thrombin but not plasmin (the latter observation was made by Dr. Thomas J. Ryan, New York Department of Health, Albany, NY).

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Table I. ^{13}C NMR: Chemical Shifts of Benzyl Methylene Moieties of Model Compounds

compounds	chemical shifts, ppm[a] pH(D) 1	pH(D) 13	other solvents
O-benzyl series			
O-Bzl-Ser	74.30[b]		
O-Bzl-Thr	73.06[b]		
O-Bzl-Tyr	71.45[b]		
benzyl phosphate, dicyclohexylamine salt	69.10[b]		
N-*t*-BOC-Asp β-benzyl ester			68.30[c]
N-*t*-BOC-Asp α-benzyl ester			68.25[c]
N-isobutyrylphenylalanine benzyl ester			68.32[c]
			67.22 ($CDCl_3$)
benzyl *N*-ethylacetimidate (*Z* form)			67.83 (CD_3OD)
			66.78 (CD_3CN)
			65.98 ($CDCl_3$)
N-isobutyrylalanine benzyl ester			67.01 ($CDCl_3$)
benzyl alcohol			65.17 (CD_3OD)
			64.76[c]
N-benzyl series			
N-Bzl-Pro	59.84[b]		
N-(2-aminoethyl)-N^{α}-acetyl-N^{α}-benzylglycinamide			54.89[d] (major, plus minor at 51.54)
$N3^{im}$-Bzl-His	54.00[b]		
N-Bzl-Cys	52.85[b]		
N-Bzl-Val	52.39[e]	53.16[f]	
N-Bzl-Gly	52.32[e]	53.62[f]	
	52.02[b]		
N-Bzl-Ile	52.27[e]	52.77[f]	
	52.17[b]		
N-Bzl-Asp	52.11[e]	52.76[f]	
N-Bzl-Thr	52.07[e]	53.02[f]	
N-Bzl-Lys	51.82[e]		
N-Bzl-Glu	51.45[b]	52.36[g]	
N-Bzl-Tyr	51.45[b]	52.18[g]	
N-Bzl-Phe	51.41[b]	52.21[g]	
N-Bzl-Met	51.40[b]	52.24[g]	
N-Bzl-Ser		51.32[e]	
N-Bzl-Leu	51.26[e]	52.34[f]	
N-Bzl-Ala	51.03[e]	52.68[f]	
benzylamine	44.60[b]		
N-benzyl-N^{α}-isobutyrylalaninamide			43.59[d]
N-nitroso-*N*-benzyl-N^{α}-isobutyrylalaninamide			42.95 ($CDCl_3$)
N-nitroso-*N*-benzyl-N^{α}-isobutyrylphenylalaninamide			42.34 ($CDCl_3$)
S-benzyl Series			
(3-amino-3-carboxypropyl)benzylmethylsulfonium chloride			46.97 (pH 3)
Bzl-S-S-Cys			42.86 (2 N DCl–D_2O)
Bzl-S-CH_3			38.30 ($CDCl_3$)
S-Bzl-Cys	36.77[e]		
S-benzylhomocysteine	36.11[b]		
	36.13[e]		
C-benzyl series			
1-phenylpropane			38.20 ($CDCl_3$)
3-Bzl-Tyr			36.40 (1 N DCl–D_2O)
2-benzyl-Δ^2-imidazoline	33.43[b]		

[a] For aqueous solvents, the α-carbon of Gly was used as an internal reference [41.44 ppm (at pH 1) and 46.33 ppm (at pH 13) from external TMS]. For organic solvents, TMS was used as the internal reference. The pH(D) 13 samples were obtained by titration of pH(D) 1 samples with 40% KOD in D_2O; pH (and "pD") were checked with a Beckman Model 4500 digital pH meter (no correction made for D). [b] Solvent = 0.1 N HCl (H_2O)–0.1 N DCl (D_2O), 90:10 (v/v). [c] 50 mM pH 6.8 potassium phosphate in 10% D_2O–90% H_2O. [d] Same as footnote *c* but pH 7.8. [e] Solvent = 0.1 N DCl (D_2O). [f] Titration of a sample in the solvent of footnote *e* with 40% KOD (D_2O) to pD = 13. [g] Titration of a sample in the solvent of footnote *b* with 40% KOD (D_2O) to pD = 13.

^{13}C benzyl groups covalently attached to the enzyme were observed.

The small molecules were removed by dialysis against 1 mM HCl; the ^{13}C NMR spectrum of the **2a**-inhibited enzyme in 1 mM HCl, compared to the spectrum of DFP-inhibited enzyme, now showed 10 broad signals between 37 and 71 ppm. Attempts to identify the sites of the alkyl groups were not pursued aggressively at these stages of the project, since the observed bands were broad, fully substituted amide models showed syn and anti isomers [Table I, *N*-(2-aminoethyl)-N^{α}-acetyl-N^{α}-benzylglycinamide, for example], the chemical shifts of basic compounds such as benzyl *N*-ethylacetimidate are sensitive to the pH, and many microenvironments leading to different chemical shifts exist in the protein molecule. It seems safe to conclude, however, that the signals seen in the 60–70-ppm region of the spectrum stem from various *O*-benzyl compounds such as ethers, esters, and imidate esters (Table I).

To gain sharper signals, the inhibited enzyme was denatured with 6 M guanidine hydrochloride (Gdn·HCl). The spectrum of the **2a**-inhibited enzyme now showed approximately eight new major signals (25–69 ppm) relative to ^{13}C spectra of DFP-inhibited enzyme (natural abundance) under the same conditions (Figure 1). The chemical shifts of these eight signals indicate that they stem from benzyl CH_2 groups attached to O, N, S, and C sites (76–64, 60–51, 47–36, and ~36 ppm, respectively). The remaining common signals in the two spectra can, in most cases, be assigned to carbon atoms of the amino acids making up the protein backbone of α-chymotrypsin.[16]

Hydrolysis of the **2a**-inhibited enzyme (6 N HCl, 110 °C, 22 h in the presence of phenol) led to a mixture of native amino acids and N-, S-, and C-benzylated amino acids (all *O*-benzyl groups

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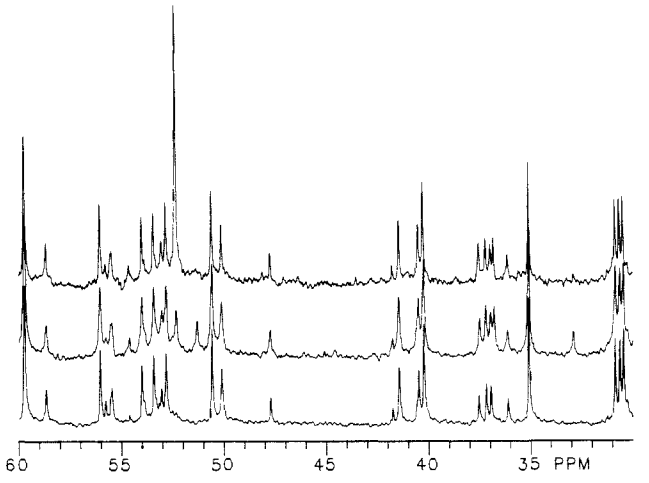


Figure 2. 100-MHz ^{13}C NMR (proton decoupled) of 6 N HCl hydrolysates: **1a**-inhibited α-CT (upper) (NT = 40 000), **1b**-inhibited α-CT (middle) (NT = 30 000), and α-CT (bottom) (NT = 50 000). Solvent = 0.1 M DCl in D_2O, and sample temperature = 20 °C. Internal standard = Gly α-carbon at 41.44 ppm; the spectra were plotted with equal intensities of this signal; a line broadening of 5 Hz was used.

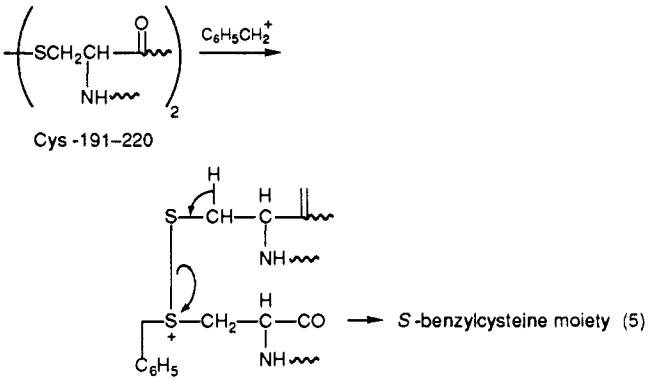
are hydrolyzed under the conditions used; the phenol was added to scavenge benzyl chloride formed from those groups and thus to avoid alkylation by that compound). The ^{13}C NMR spectrum of this mixture (Figure 2) compared to that of an analogous product from native enzyme showed one major new signal for the CH_2 of a benzyl group at 52.35 ± 0.03 ppm and two minor signals at 36.83 and 32.9 ppm. The ^{13}C NMR spectrum of analogous hydrolysis products from the **2b**-inhibited enzyme, relative to native enzyme, showed four approximately equal signals for benzylated amino acids at 52.32, 51.31, 36.78, and 32.91 ppm (Figure 2).

Patterns of Alkylation. A comparison of the NMR spectra in Figure 2 shows that the alkylation patterns resulting from use of the alanine- (**2a**) and phenylalanine- (**2b**) based inhibitors—with formation of the same "leaving group" and alkylating agent—are strikingly different. Thus, the alkylation pattern observed in the aromatic binding region of the active site is revealed to be a sensitive function of structural differences of the acyl portions of the inhibitors, which exert their influence at some distance from this aromatic binding region,[17] and while severed from the carbonium ion alkylating agent. The high reactivity of deaminatively formed carbonium ions ensures that alkylation of the protein will occur near the release point of the ion. In stark contrast, the key species formed in all other "mechanism-based" inhibitions are relatively unreactive; such species could diffuse considerable distances until a good nucleophilic center such as sulfur or the histidine nitrogen, for example, is encountered.[13] Thus, the nitrosoamide approach to enzyme inhibition is capable of providing a type of map of the active site reflecting the positions of amino acid residues with respect to the release point of the carbonium ion. In principle, this method could detect residues that approach near, or enter, the active site only during the catalytic step. X-ray crystallographic techniques would not detect such residues. Also, enzymes in the crystalline state can exhibit altered reactivity, a result presumably of altered structure. Thus, both α- and γ-chymotrypsin react with various chloromethyl ketones in solution, but in the crystalline state, only the γ form reacts.[17c]

Photoaffinity labeling[18] also possesses the advantages cited above; however, in the version of the latter approach in which an enzyme–substrate derivative is irradiated, low yields of alkylated sites can result. In the present approach, using an excess of inhibitor, the enzyme continues to turn over substrate until it becomes fully alkylated, and being an enzyme-activated process utilizing highly reactive ions, the resulting alkylation should be confined to the active site. Consistent with this view is the observation that in the inhibition by **2a** and **2b** the majority of the alkyl groups are located on the C-chain of α-chymotrypsin[4f] (with the exception of His-57 and Asp-102, the active site is constructed of residues from the C-chain).[19]

N- and S-Benzylated Amino Acids. A combination of HPLC and ^{13}C NMR spectroscopy was used to identify the major alkylated amino acid obtained from 6 N HCl hydrolysis of the **2a**-inhibited α-chymotrypsin.[20] The compound responsible for the large signal in the ^{13}C NMR spectrum of the amino acid mixture from the alanine- (**2a**) based inhibition (52.35 ppm) (and also for the 52.32-ppm signal from the **2b** inhibition) was identified as *N*-benzylglycine in the following way. From the chemical shifts observed with model benzyl alkylated amino acids (Table I), it was shown that the 52.35-ppm signal could stem only from *N*-benzylvaline (52.39), *N*-benzylglycine (52.32 ppm), *N*-benzylisoleucine (52.27 ppm), *N*-benzylaspartic acid (52.11 ppm), or *N*-benzylthreonine (52.07 ppm). The amino acid mixture was separated arbitrarily into seven fractions by HPLC on a Nova Pak C-18 column. ^{13}C NMR spectra of each fraction located the 52.35-ppm peak in fraction 2, limiting now the choices to *N*-benzylglycine (R_f 2.4 min) and *N*-benzylthreonine (R_f 2.5 min) [eliminating *N*-benzylisoleucine (R_f 6.3 min, fraction 6), *N*-benzylvaline (R_f 4.6 min, fraction 4), and *N*-benzylaspartic acid (R_f 1.8 min, fraction 1)]. ^{13}C NMR spectra at pD 13 pinpointed the unknown as being *N*-benzylglycine, since its CH_2 resonance at 53.62 ppm matched that of the unknown compound at 53.60 ppm, which differed significantly from the value for *N*-benzylthreonine (53.02 ppm). Confirmation of the assignment was made by derivatization of the amino acids in fraction 2 (above) with phenyl isothiocyanate[21] and HPLC separation of the products. A peak was found at R_f 9.2 min for the unknown, which corresponds to the measured R_f of the *N*-benzylglycine derivative (9.2 min); no peak was found near the R_f value of the *N*-benzylthreonine derivative (8.6 min).

The remaining ^{13}C NMR signals are considerably weaker than the 52.32-ppm peak for *N*-benzylglycine just discussed, and only comparisons with chemical shift values for model compounds (Table I) are possible at this point. On this basis, the 51.31-ppm signal probably stems from *N*-benzylserine and the 36.78–36.83-ppm signal from *S*-benzylcysteine (eq 5).



The alkylation reaction of eq 5 was not achieved with benzyl cations obtained from the reaction of 6 N HCl with benzyl alcohol; the only products obtained were recovered alcohol, benzyl chloride, and the 2- and 4-(chloromethyl)diphenylmethanes. The same

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(20) The ε of benzyl groups is ~200. If more material were available, UV absorption in more concentrated solutions or longer path-length cells could be used to detect the alkylated amino acids. In the present case, we found ^{13}C NMR spectroscopy to be a more sensitive method. The latter method was a highly specific one in which fewer artifactual signals were found than in the detection of phenyl isothiocyanate derivatives by HPLC.

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reaction carried out in the presence of phenol yielded only the 2- and 4-hydroxydiphenylmethanes. The latter result shows that phenol is an efficient scavenger of benzyl cations under the reaction conditions used for the hydrolysis of proteins.

Conclusions. We had earlier reported that in the inhibition by **2b** one of the "hits" occurred on the carbonyl group of an amide to form an imidate ester; this alkylation labilizes the amide bond and permits chain cleavage at that point (eq 4).[4f] Analysis of the peptides led to the identification of serine-214 as the amino acid under attack; further, we showed that tryptophan was modified during the inhibition. One of the three portions of the protein C-chain making up the active site of chymotrypsin has the sequence -Ser(214)-Trp(215)-Gly(216)-Ser(217)- in the aromatic binding region.[17] An examination of a molecular model of γ-chymotrypsin[22] led to the following predictions for reactions of the benzyl cation released in the active site: Ser-214 (carbonyl O), Trp-215 (amide N), Gly-216 (N atom), Ser-217 (N atom), and Cys(191)-Cys(220) (S atoms) (eq 5).[23] The benzyl cation in the inhibition by **2b** appears to have been released near this stretch of the peptide chain. Work is in progress to verify this hypothesis through standard techniques of protein chemistry: enzymatic cleavage of the protein chains followed by separation and analysis of the peptides.

The present study appears to be the first in which inhibition of an enzyme results from alkylation, or other alteration, at multiple locations in the active site.[24] ^{13}C NMR spectroscopy was useful in this study in providing a rapid method for the observation of the alkylation patterns; it has the potential of revealing quickly how variables such as pH, temperature, salt concentration, and inhibitor structure might alter the alkylation map.

Experimental Section

Materials and Methods. α-Chymotrypsin (α-CT) (Type I-S) and diisopropyl fluorophosphate (DFP) were obtained from Sigma Chemical Co. Deuterium oxide (99.8% atom D), 37 wt % DCl (99% atom D) in D_2O, 40 wt% KOD (98+% atom D) in D_2O, benzoyl-L-tyrosine ethyl ester (BTEE), and guanidine hydrochloride (Gdn·HCl) were obtained from Aldrich Chemical Co. Constant-boiling 6 N HCl was purchased from Pierce Chemical Co. All other chemicals were of the highest purity available. Phosphate buffer (0.05 M, pH 6.8 and 7.8) was prepared with double-distilled and deionized water.

All the spectra were acquired on a Varian Associates XL-400 NMR spectrometer (updated to VWR-400) operating at 100.563-MHz resonance frequency for ^{13}C. Waltz-16 decoupling was used to decouple the protons from carbon resonances. The decoupling efficiency was optimized for each run by executing the "Gamma H2 test" (Varian Associates XL-400 software program) to set the decoupler modulation frequency, decoupler offset, and decoupler power. The same type of NMR tube containing a 3% solution of dioxane in the same solvent that was used for the sample under investigation was used to optimize the decoupler parameters. The carbon of Gdn-HCl (159.0 ppm at pH 3 from external TMS) and the glycine α-carbon (41.44 ppm at pH 1 and 46.33 ppm at pH 13 from external TMS) were used as internal references in the ^{13}C runs of denatured and hydrolyzed enzymes, respectively. For proton NMR in D_2O, sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (TSP) was used as an internal reference.

HPLC analyses and separation were performed on a Waters Associates HPLC system with a Nova Pak C-18 reverse-phase column, with some samples undergoing phenyl isothiocyanate (PITC) derivatization for labeling.[25] Both labeled and unlabeled samples were monitored with UV detection at 254 nm. The enzymatic activity of α-CT samples was determined by the method of Hummel.[26] The pH(D) was measured with a Beckman Model 4500 digital pH meter with no correction for deuterium.

Benzylated Amino Acids. *O*-Benzyl-Ser and -Thr, *N*-benzyl-His, -Phe, -Pro, -Thr, -Tyr, and -Val, and *S*-benzylhomocysteine were obtained from Vega-Fox Biochemical Co., *O*-benzyltyrosine, *S*-benzylcysteine, and $N3^{im}$-benzylhistidine were obtained from Aldrich Chemical Co., and *N*-benzylisoleucine was obtained from Accurate Chemical and Scientific Co. *N*-Benzyl-Ala, -Glu, -Leu, -Lys, and -Ser were prepared as described by Quitt et al.[27] and *N*-benzyl-Cys and -Met as described by Kanao and Sakayori.[28] *N*-Benzyl-Asp was prepared as described by Frankel et al.[29] and *N*-benzyl-Gly as described by Baker et al.[30] *S*-(Benzylthio)cysteine was prepared by the method of Nogami et al.,[31] and the *S*-benzylsulfonium salt of methionine was prepared by the method of van Bergen et al.[32] Finally, 3-benzyltyrosine was prepared by the method of Iselin.[33]

Benzyl *N*-Ethylacetimidate. This compound was prepared by the methods of Pilotti et al.[34] and Bredereck et al.[35] The stable form is believed to be the *Z* isomer.[36]

The syntheses of the ^{13}C-enriched inhibitors **1** and **2** followed the procedures of White et al.[4] except in the synthesis of methyl alaninate hydrochloride.

Methyl Alaninate Hydrochloride. Alanine (3.35 g, 37.6 mmol) was suspended in 40 mL of methanol and the mixture was cooled to 0 °C. Hydrogen chloride was introduced until all the alanine had dissolved (~7 g of HCl gas had been absorbed at this point). The clear solution was heated to reflux for 4 h. The solvent was removed by vacuum distillation to yield a yellowish solid, which was washed on a Hirsch funnel (under vacuum) with 0.5 mL of methanol and then with 0.5 mL of ether to remove the yellow impurity. A white solid (4.35 g, 31.2 mmol, 83%) was obtained and was used in the next step (isobutyrylation) without further purification: NMR (D_2O) δ 4.13 (q, 1 H, J = 7 Hz), 3.85 (s, 3 H), and 1.56 (d, 3 H, J = 7 Hz).

Inhibition of α-Chymotrypsin. The enzyme (100 mg, 0.004 mmol) was dissolved in 13.5 mL of 50 mM pH 7.8 phosphate buffer with gentle magnetic stirring at 22 °C (in a few runs, pH 6.8 buffer was used). After the enzyme had dissolved, two 5-μL aliquots were taken and added to two 1-mL volumes of pH 3 HCl. A 10-fold molar excess (0.04 mmol) of inhibitor **1** (14.2 mg) or **2** (11.1 mg) in 1.5 mL of acetonitrile (HPLC grade) was added over a period of approximately 5–8 min with gentle magnetic stirring to the enzyme solution. After 20 min, the yellow color of the inhibitor had disappeared and the solution became cloudy. Another two aliquots were taken (5 μL each) and injected into two 1-mL volumes of pH 3 HCl solution, respectively. A 10-fold molar excess (0.04 mmol) of DFP in CH_3CN (0.2 mL of 0.2 M) was added dropwise. Ten minutes after the addition was complete, a third batch of aliquots was taken. All three batches of aliquots were assayed for α-chymotrypsin activity via the BTEE method of Hummel.[26]

The inhibition solution was gravity-filtered through Whatman No. 1 paper to yield a clear solution. The filtrate was transferred to dialysis tubing with a molecular cutoff of 3500 and dialyzed in pH 3 HCl solution for 24 h at 4 °C with three bath changes (500 mL every 8 h). The

(22) Courtesy of Dr. David R. Davies of the National Institute of Arthritis, Metabolism, and Digestive Diseases. α- and γ-chymotrypsin have the same amino acid sequence and very similar electron-density maps at 2.7 Å.[17c]

(23) Other possibilities were Met-192 (N), Gly-193 (N), Cys-220 (N), and Met-192 (S).

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Table II. HPLC Elution Times of N- and S-Benzylated Amino Acids and Related Compounds[a]

compound	elution time, min	compound	elution time, min
N-Bzl-Asp	1.8	*N*-Bzl-Val	4.6
N-Bzl-Glu	2.0	*S*-Bzl-Cys	5.8
N-Bzl-Ser	2.4	*N*-Bzl-Met	6.2
N-Bzl-Gly	2.4	*N*-Bzl-Ile	6.3
N-Bzl-Thr	2.5	*N*-Bzl-Tyr	6.4
N-Bzl-Ala	2.6	$N3^{im}$-Bzl-His	6.5
N-Bzl-Pro	3.8	*N*-Bzl-Leu	6.8
Bzl-S-S-Cys	4.3[b]	*S*-Bzl-homocysteine	7.0
Bzl-S-S-Bzl	4.3		

[a] The eluant consisted of two solutions: solution A, 11.5 g of NaOAc, 1 mL of $N(Et)_3$ and 200 μL of EDTA (1 g/LH_2O) in 1 L of water adjusted to pH 5.7 with acetic acid; solution B, CH_3CN–H_2O [3:2 (v/v)]. The eluant composition (the flow rate was 1 mL/min) varied from 90% A at t = 0 min to 49% A at t = 10 min (Waters solvent programmer curve 5 was used). The flow rate was 1 mL/min and UV detection was employed at 254 nm. [b] *S*-(Benzylthio)cysteine appeared to disproportionate on the column, for a peak with an elution time equal to benzyl disulfide (4.30 min) was observed in all HPLC runs of Bzl-S-S-Cys.

dialyzed enzyme was lyophilized at 10^{-3} Torr to give 70–80 mg of inhibited enzyme.

For the inhibition with DFP only, a 10-fold molar excess of DFP was added dropwise to the enzyme; further treatment involved the procedures described above.

For ^{13}C NMR runs, the inhibition of ~15 mg of the enzyme was usually sufficient.

Hydrolysis of Inhibited Enzyme. The inhibited, dialyzed, and lyophilized enzyme (15–40-mg run) was hydrolyzed in 6 N HCl (1 mL/10 mg of enzyme) at 115 °C for 22 h. For the run using phenol as a carbonium ion scavenger, about 40 mg of phenol was used per run. After hydrolysis, the solution was extracted with chloroform and the aqueous phase was lyophilized.

^{13}C NMR Spectra of Inhibited Enzyme. The inhibition with **2** or DFP was carried out in pH 6.8 phosphate buffer containing 30% D_2O. The resulting mixture was transferred to an NMR tube and subjected to ^{13}C NMR spectrometry immediately. Acquisition parameters were a pulse width (pw) of 17.5 μs [pw(90°) = 25.0 μs] and an acquisition time of 0.54 s with no delay. The sample temperature was 25.0 ± 1.0 °C; 60 000–90 000 transients were usually acquired. A line broadening of 15 Hz was used in the Fourier transformation.

^{13}C NMR Spectra of Dialyzed Inhibited Enzyme. **2a**- or DFP-inhibited α-CT (40–50 mg) (after dialysis; see above) was dissolved in 3.0 mL of 1 mM HCl + 1 mM DCl [70:30 (v/v)]. The acquisition parameters were those listed for the nondialyzed samples.

^{13}C NMR Spectra of Inhibited, Dialyzed, and Denatured Enzyme. **2a**- or DFP-inhibited α-CT (75 mg) was denatured in 6 M Gdn·HCl with 20% D_2O as lock solvent, pH = 3; 10-mm OD NMR tubes were used. Acquisition parameters were the same as listed above. The sample temperature was 27.0 ± 1.0 °C, and 30 000–60 000 transients were usually acquired. A line broadening of 10 Hz was employed in the Fourier transformation.

^{13}C NMR Spectra of Inhibited and Hydrolyzed Enzyme. The hydrolysate (15–40 mg per run) was dissolved in 0.5–0.7 mL of 0.1 N DCl in D_2O for ^{13}C NMR recording; a 5-mm OD NMR tube was used. Some hydrolyzed samples were titrated with 40% KOD in D_2O to PD = 13, and spectra were then measured. The acquisition time was 0.75 s with no delay, and about 15 000–60 000 transients were usually acquired. Sample temperatures were 20.0 ± 1.0 °C. A line broadening of 5 Hz was used in the Fourier transformation.

HPLC Separation and Analysis. The hydrolysate solution after ^{13}C NMR recording was injected directly into the HPLC system[25] for separation. The eluant composition is described in the footnotes to Table II. Seven fractions were obtained: 1, 0–1.9; 2, 1.9–3.0; 3, 3.0–4.0; 4, 4.0–5.2; 5, 5.2–5.7; 6, 5.7–6.8; and 7, 6.8–9.9 min. The model benzylated amino acids were then analyzed under identical conditions (Table II).

The seven fractions were taken to dryness and each was dissolved in 0.1 N DCl–D_2O and titrated with 2 N DCl to pD = 1; ^{13}C NMR spectra were then run. The NMR parameters were the same as described for the hydrolysate before HPLC separation. The first fraction showed most of the background amino acids (Asp, Glu, Ser, Gly, His, Arg, Thr, Ala, Pro, Val, Ile, Leu, and Lys) and the HPLC eluent components sodium acetate (21.94, 178.02 ppm) and triethylamine (9.80, 48.12 ppm). Fraction 2 showed a peak at 52.36 ppm that was very close to that of the model compound *N*-Bzl-Gly (52.32 ppm); it also showed Leu (52.84, α; 40.27, β; 23.05, γ; and 22.41, δ), Tyr (55.53, α), Phe (36.88, β), and Arg (25.42, γ). Fraction 6 had one peak at 36.86 ppm, believed to be from the benzyl methylene carbon of *S*-Bzl-Cys (model *S*-Bzl-Cys: 36.77 ppm). Both fractions 2 and 6 showed sodium acetate and triethylamine signals in addition to the peaks mentioned above. Fraction 3 showed nothing but sodium acetate and triethylamine signals, while fractions 4, 5, and 7 showed only the sodium acetate (presumably triethylamine was pumped out during the drying process).

Fractions 1, 2, and 6 (the latter two containing ^{13}C enrichment) were also derivatized individually with PITC and then further analyzed on the same column by using a new gradient program: from t = 0 to 0.10 min, 90% A and 10% B; from t = 0.10 to 10.0 min, the eluant changed from 90% A and 10% B to 0% A and 100% B (solvent programmer curve 6). Certain N-benzylated amino acids, used as standards, were subjected to the same procedure. For derivatized fraction 1, Asp (2.33 min), Glu (2.62), Ser (4.32), Gly (4.79), His (5.37), Arg (6.03), Thr (6.27), Ala (6.45), Pro (6.65), Tyr (7.72), Val (8.07), Ile (8.80), Leu (8.86), and Lys (9.65) were observed. For derivatized fraction 2, a peak at 9.20 min was seen, which was the same as the elution time of the derivatized model compound *N*-Bzl-Gly (9.20 min) injected immediately after the unknown sample. The elution times of some other N-benzylated and derivatized amino acids were *N*-Bzl-Thr, 8.56 min; *N*-Bzl-Val, 9.95 min; *N*-Bzl-Ser, 10.23 min; and *N*-Bzl-Ile, 10.55 min. It was found that if the same sample was injected twice within very short intervals, i.e., within 1 or 2 h, the experimental error was usually in the range of 0.01 min. However, if the same sample was injected on different days, the experimental error could be as large as ±0.15 min. For example, nine runs of *N*-Bzl-Gly on different days gave elution times ranging from 9.08 to 9.38 min. For parallel injections close together, however, there was one peak from fraction 2 that was the same as the peak of model *N*-Bzl-Gly (i.e., for the last three runs, fraction 2 showed a peak at 9.20 min, the same value as measured for *N*-Bzl-Gly). For fraction 6 it was found that the model *S*-Bzl-Cys did not yield any reasonable peak after PITC derivatization, presumably because of its poor solubility in the derivatization solvent [ethanol–H_2O–triethylamine–PITC, 7:1:1:1 (v/v)].

Reaction of Cystine and Benzyl Alcohol in 6 N HCl. Cystine (23.6 mg, 0.0983 mmol) mixed with 10.4 μL of α-^{13}C-labeled (90%) benzyl alcohol (0.102 mmol) was dissolved in 2 mL of 6 N HCl, and the mixture was degassed. The mixture was kept at 115 °C for 22 h. After being cooled to room temperature, the solution was extracted with $CDCl_3$. The $CDCl_3$ solution was dried over anhydrous Na_2SO_4 and the ^1H NMR spectrum was recorded. The spectrum showed that at least four compounds were present: benzyl-α-^{13}C alcohol [4.69, d, J = 142 Hz (lit.[37] 4.69)], benzyl-α-^{13}C chloride [4.54, d, J = 151 Hz (lit.[37] 4.50)], 4-(chloromethyl-^{13}C)diphenylmethane-^{13}C [3.97, d, J = 127 Hz [lit.[37] 3.99 (chloromethyl)] and 4.68, d, J = 151 Hz [lit.[37] 4.50 (CH_2)]], and 2-(chloromethyl-^{13}C)diphenylmethane-^{13}C (3.93, d, J = 127 Hz, and 4.54, d, J = 151 Hz). The spectrum showed aromatic signals around δ 7, as expected.

Reaction of Cystine and Benzyl Alcohol in 6 N HCl in the Presence of Excess Phenol. The general procedure was the same as described above, except that 40.2 mg of phenol (0.428 mmol) was used along with the mixture of cystine (23.4 mg, 0.0975 mmol) and benzyl-α-^{13}C alcohol (10.4 μL, 0.102 mmol). The ^1H NMR spectrum only showed two products: 4-hydroxydiphenylmethane-^{13}C [3.89, d, J = 127 Hz (lit.[37] 3.89)] and 2-hydroxydiphenylmethane-^{13}C [3.96, d, J = 127 Hz; (lit.[37] 3.96)]. The formation of these compounds indicates that the phenol was a good scavenger for carbonium ions produced during the hydrolysis. The ^{13}C NMR spectra of the aqueous phases in both cases (with or without phenol) gave only cystine signals.

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