multiple additions of MDA units to the initial MDA-base adduct.[15] The ability of MDA to act as an electrophile and a nucleophile is responsible for this oligomerization.

The present report raises the number of structurally distinct adducts that MDA forms with nucleosides to five.[6,9] Four of them result from the reaction of both carbonyl equivalents of the molecule with the nucleic acid component, which is consistent with the structure-activity relations for the induction of frame-shift mutations.[4,16] The effect of each of these unique adducts on DNA replication and their importance in MDA mutagenesis is under investigation.

Acknowledgment. This work was supported by a research grant from the National Cancer Institute (CA 22206). L.J.M. is a recipient of a Faculty Research Award from the American Cancer Society (FRA 243). We are grateful to Norman LeBel for helpful discussions.

Supplementary Material Available: Chemical shifts and assignments for guanosine adduct **2** in $^2H_6$-Me_2SO and in ^2H_2O and guanine adduct **2** in ^2H_2O, ^2H_2O-proton decoupled, and ^2H_2O-fully coupled (3 pages). Ordering information is given on any current masthead page.

(15) We cannot exclude the possibility that MDA dimerizes before reacting with the nucleosides.
(16) The four adducts are **1**, **2**, and the cyclopropylidene-containing adducts to adenosine and cytosine.[6]

Reversible Covalent Inhibition of Papain by a Peptide Nitrile. ^{13}C NMR Evidence for a Thioimidate Ester Adduct

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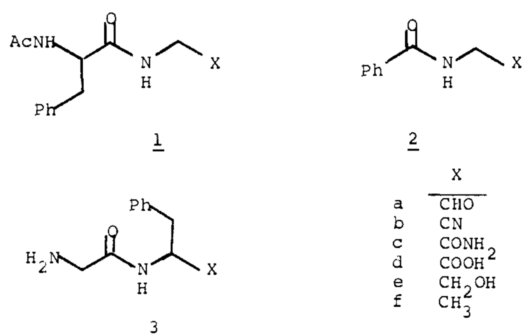
Received July 22, 1985

The powerful but reversible inhibition of cysteine and serine proteases by peptide aldehydes was first demonstrated independently by Westerick and Wolfenden working with papain[2] and by Thompson working with elastase.[3] The extraordinary potency of these inhibitors was attributed to their forming a covalent tetrahedral adduct with the enzyme via its active site nucleophile; these adducts were thought to resemble transition states or intermediates involved in catalysis, except for being incapable of breaking down to form products. Indirect support for this hypothesis was provided by secondary[4] and solvent[5] deuterium isotope effects on enzyme-inhibitor binding constants, ^1H NMR cross-saturation experiments,[6] and ^{19}F NMR studies.[7] Recently a tetrahedral covalent adduct of **1a** with papain has been observed directly by ^{13}C NMR.[8]

Lewis and Wolfenden reported that nitrile **1b** was also a powerful competitive inhibitor of papain (K_i = 0.000 73 mM) and that it was not hydrolyzed by papain.[4] They proposed that "nitriles may also bind covalently to the active site of papain". Nitrile **2b** also inhibits papain (K_i = 0.38 mM)[9,10] but is not a substrate.[10]



Although **2b** is not as potent as **2a** (K_i = 0.025 mM),[2] it is much more potent than the related compounds **2c-f** (K_i = 10-1000 mM).[2,4] Recently we prepared nitrile **3b** and showed it to be a powerful but reversible competitive inhibitor of another cysteine protease DPP-I;[11] for comparison the K_i of amide **3c** (6.2 mM) is over 5000 times greater than that for nitrile **3b** (0.0011 mM).[12] Compound **3b** also protects DPP-I from irreversible inhibition by an affinity labeling reagent specific for this enzyme.[12]

These observations raise the interesting prospect that peptide nitriles may be a *general* class of *reversible covalent* inhibitors for cysteine proteases,[13] interacting with the active site thiol by the mechanism shown in Scheme I. To test this hypothesis we undertook an NMR study of the interaction of [*nitrile*-^{13}C]-**1b**[14] with papain.[17] The results of this study are shown in Figure 1. Traces (a) and (b) show partial ^{13}C NMR spectra of [^{13}C]-**1b** and papain, respectively. When a 50 mol % excess of [^{13}C]-**1b** was added to papain a major new resonance appeared at 182.08 ppm (spectrum c). The chemical shift of the new resonance is entirely consistent with the proposed thioimidate ester linkage shown in Scheme I, since it falls between the usual ranges for thioamide carbons (200-210 ppm) and amide and peptide carbons (160-170 ppm).[22] The inhibition of papain by **1b** is readily reversible by dialysis.[12] To show that the new peak at 182.08 ppm

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(14) For the synthesis of [^{13}C]-**1b**, $Na^{13}CN$ (1 g, 99% ^{13}C, Stohler/KOR) was condensed[15] with NH_4Cl and CH_2O to form the trimer of "methyleneaminoacetonitrile", which was recovered by extraction into CH_2Cl_2 (44% yield after recrystallization from EtOH). Vigorous shaking with ethanolic HCl (1.17 M),[16] followed by evaporation to dryness and recrystallization from EtOH, gave 0.62 g (71%) of $H_2NCH_2{}^{13}CN{\cdot}HCl$. The latter was coupled to Ac-L-Phe in THF by using *N*-methylmorpholine and *i*-BuOCOCl, giving 0.76 g (46%) of **1b** after recrystallization from EtOH/hexane (1:1).

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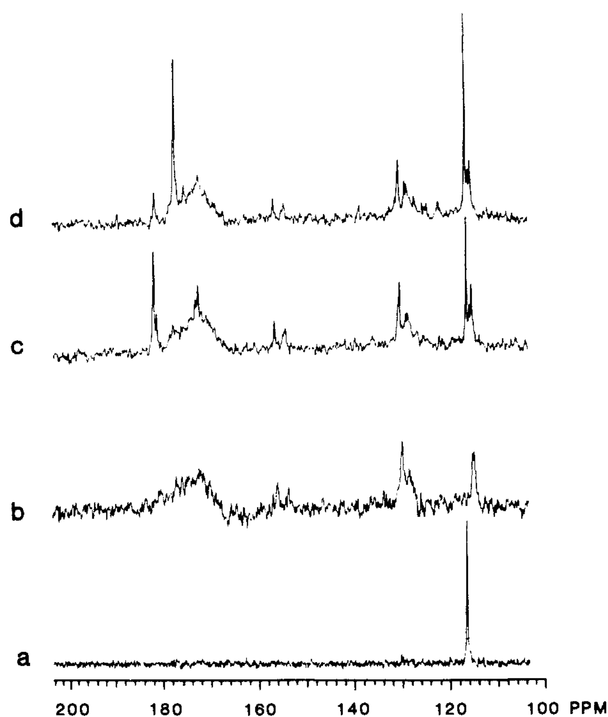

Figure 1. ^{13}C NMR spectra. Samples were prepared in 10-mm NMR tubes in 50 mM phosphate buffer, pH 6.20, containing 1% MeOH and 50% D_2O. The pH of all samples was checked before and after spectral acquisition and did not change. All spectra were recorded at 15 °C by using a Varian XL-300 spectrometer. Broad-band decoupling was performed in all experiments using the Waltz-16 program provided by Varian (1.0-W power). A pulse width of 5.0 μs and a pulse delay of 0.0 s were used with an acquisition time of 0.750 s. Methanol was used as the internal reference (49.00 ppm). The concentrations of papain given are based on active site titration.[19,20] (a) 1.0 mM Ac-L-PheGly^{13}CN (800 transients). (b) 1.0 mM papain (43 000 transients). After recording this spectrum Ac-L-PheGly^{13}CN was added (1.5 mM) and the spectrum shown in (c) was recorded (50 000 transients). The sample was next acidified with 1% v/v glacial acetic acid (pH 4.05), 2,2′-dipyridyl disulfide (6 mM) was added, and the spectrum shown in (d) was recorded (39 800 transients; 179 ppm = HOAc).

Scheme I

derives from the *reversible* addition of the active site sulfhydryl of papain to the ^{13}C nitrile carbon, the sample used to record spectrum (c) was acidified and treated with the thiol reagent 2,2′-dipyridyl disulfide to trap free E–SH covalently.[8,19] This resulted in the rapid (i.e., within 10 min) and complete disappearance of the 182.08 ppm peak with concomitant growth in intensity of the peak due to free inhibitor (spectrum d).

The above experiments clearly indicate that **1b** and papain interact via reversible formation of a covalent adduct involving the nitrile carbon. Since the nitrile carbon in **1b** corresponds directly with the carbonyl carbon of typical papain substrates (e.g. **1c**), the adduct formed is most likely the thioimidate depicted in Scheme I. This is completely consistent with the known chemical reactivity of nitriles and thioimidate esters. It is surprising, however, that the thioimidate adduct fails to yield hydrolysis products, since it is a close analogue of the acyl enzyme intermediate formed during turnover of normal substrates by papain.

Acknowledgment. Financial support for this research was provided by the University of Kansas, NIH Predoctoral Training Grant GM-07775, and a grant from the NIH Shared Instrumentation Program (RR-413). We also appreciate helpful suggestions from Dr. Grover Everett and the assistance of Dr. Charles Decedue in the purification of papain.

Characterization of [Dimethyl *N*,*N*′-ethylenebis(L-cysteinato)(2–)-*S*,*S*′]copper(II), $Cu(SCH_2CH(CO_2CH_3)NHCH_2–)_2$, a Stable Cu(II)–Aliphatic Dithiolate

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We describe here the first stable Cu(II) complex (**1**) that incorporates "biological" S(cys) ligation. Except for Cu(tet *b*)-$SCH_2CH_2CO_2$ (**2**),[1] stable Cu(II) aliphatic thiolates have been limited to type 1 proteins[2] and possibly the Cu_A site in cytochrome *c* oxidase.[3-7] From X-ray absorption edge,[8,9] EXAFS,[10] EPR, and ENDOR studies, Chan et al.[3] suggested that the Cu_A site is a pseudotetrahedral $CuN_2(his)S_2(cys)$ unit with considerable Cu(I)–thiyl radical[11] or unusually covalent Cu(II)–thiolate character.[5] The large covalency of Cu(II)–thiolate bonding recently has been evaluated.[12]

A solution of *N*,*N*′-ethylenebis(L-cysteine)[13] (3 g) in 100 mL of dry methanol (saturated with HCl(g) at 268 K) was heated to 318 K for 10 h. The resulting diester dihydrochloride was isolated (2.9 g) after the solution was reduced to 50 mL and cooled to 298 K. A suspension of the dihydrochloride in dry ether was treated with $NH_3(g)$ for 0.5 h, NH_4Cl was removed, and the solvent evaporated to yield the free ester. The title complex deposited as aggregated red-brown plates (≈90% yield) from an argon-purged solution of 0.2 mM of ester and 0.2 mM of Cu(tet *a*)·$2ClO_4$[14] in 10 mL of DMF/MeOH/H_2O (5:1:1). This ligand-exchange reaction depends on the Cu(II) starting material.[16]

Due to the importance of the structure, a data set was collected on the only apparently single plate even though twinning was

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