Detection of a Tetrahedral Adduct in a Trypsin–Chloromethyl Ketone Specific Inhibitor Complex by ^{13}C NMR

J. Paul G. Malthouse,[1] Neil E. Mackenzie,[2]
Alan S. F. Boyd,[1] and A. Ian Scott*[2]

Department of Chemistry, University of Edinburgh Edinburgh EH9 3JJ, Scotland
Center for Biological NMR, Department of Chemistry Texas A&M University, College Station, Texas 77843
Received November 26, 1982

X-ray crystallographic studies at 1.9–2.6-Å resolution of inhibitor complexes of the serine proteases trypsin[3,4] and subtilisin BPN′[5] have so far failed to resolve the state of hybridization of the carbonyl group of the inhibitor.[6] Thus, although no significant distortion of carbonyl sp^2 hybridization of trypsin inhibitors has yet been revealed by NMR spectroscopy,[6,7] participiation of the serine hydroxyl group in covalent bond formation with the proximal carbonyl of inhibitors linked to the neighboring histidine residue is by no means excluded by the crystallographic evidence cited above. In order to resolve this question for the case of a trypsin–inhibitor complex, we have selected Z-lys–CMK (**1**),[8] which specifically alkylates N-3 of histidine-57.[9,10] As discussed below, clear evidence for a tetrahedral adduct is displayed by the ^{13}CNMR spectrum of this inhibitor complex with trypsin.

[2-^{13}C]Z-lys-CMK was synthesized[9,11] from [1-^{13}C]lysine (90 atom % enrichment). In aqueous solution (pH <7) resonances were detected (Figure 1a) at 204.7 and 95.4 ppm, which are assigned to the ketone (**1**) and its hydrate (**2**),[12] respectively (Scheme I). In 100% dimethyl sulfoxide a single resonance was detected at 200.6 ppm. Incubation of trypsin,[14] whose ^{13}C NMR spectrum at pH 3.2 is shown in Figure 1b, with Z-lys-CMK[17] produced no loss of activity at pH 3.2 and no perturbation of the spectrum of the chloromethyl ketone (Figure 1c). When the pH was adjusted to 6.9, enzyme activity was decreased and the Z-lys-CMK resonances at 95.4 and 204.7 ppm were replaced by a new signal at 98.0 ppm (Figure 1d). Addition of excess Z-lys-CMK resulted in the reappearance of the CMK signals at 95.4 and 204.7 ppm (Figure 1e), indicating that the resonance at 98.0 ppm does not arise from a rapid reversible exchange between bound and unbound Z-lys-CMK. Addition of a further aliquot of enzyme resulted in the loss of the resonances at 204.7 and 95.4 ppm with an increase in intensity of the resonance at 98.0 ppm (Figure 1f). Gel filtration of the labeled trypsin at this stage (Figure 1f) removed all low molecular weight material and confirmed that the signal at 98.0 ppm arises from covalently labeled trypsin (**3**, Figure 1g). As expected, addition of benzamidine (0.9 mM), a competitive inhibitor (K_I = 18.4 μM[18]) of trypsin, had no effect on the signal at 98.0 ppm. Incubation at pH 11.1 for 0.5 h resulted in some irreversible denaturation, since on restoration of the pH to 6.15 there was a decrease in the intensity of the resonance at 98.0 ppm while new signals appeared at 205.5 and 95.1 ppm (Figure 1h). Prolonged digestion (56 h) by adding active trypsin (0.13 mM) caused a further decrease in the resonance at

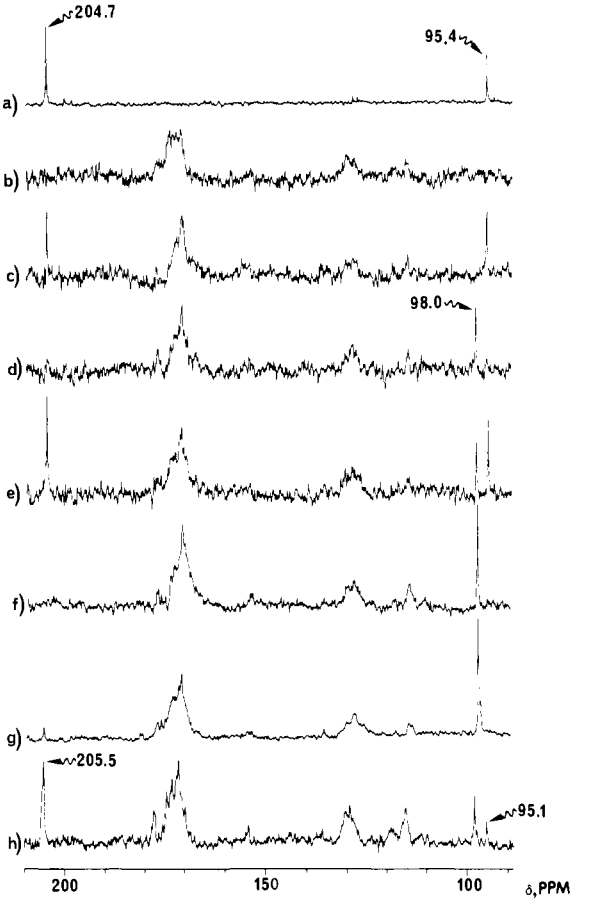
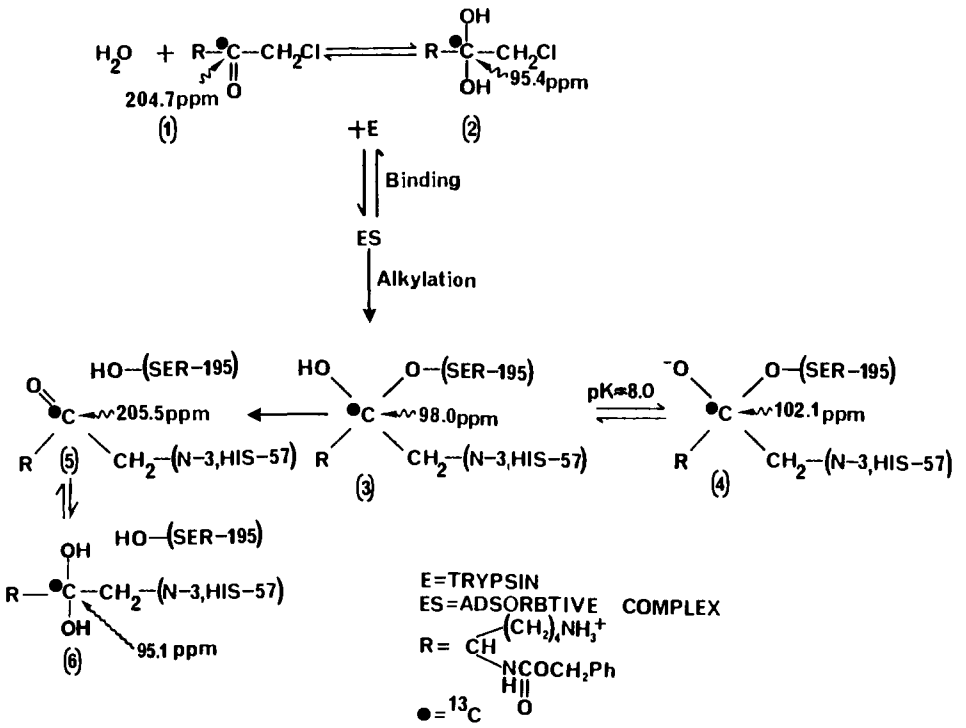


Figure 1. (a) Z-lys-CMK 47.6 mM (by weight), 1 mM HCl, D_2O 16.7% v/v, volume 0.6 mL, no. of accumulations = 1660, pH 3.1. (b–e) 20 mM sodium phosphate; 12.5% v/v D_2O; 10000 accumulations, trypsin 0.28 mM (concentration of fully active enzyme), volume 8 mL. Z-lys-CMK concentrations, pH, and enzyme activities[14] toward α-*N*-benzoyl-DL-arginine *p*-nitroanilide were as follows: (b) 0.00 mM, pH 3.2, 100%; (c) 0.38 mM, pH 3.2, 100%; (d) 0.37 mM, pH 6.9, 0.06%; (e) 0.74 mM, pH 6.9, 0.06%. (f) Trypsin 0.44 mM, 15 mM sodium phosphate buffer, D_2O 12.5% v/v, Z-lys-CMK 0.59 mM, activity 8.5%, volume 10.2 mL, no. of accumulations = 10000, pH 6.9. (g) After gel filtration and concentration by ultrafiltration: trypsin 0.48 mM, 20 mM sodium cacodylate buffer, D_2O 12.5% v/v, activity 2.1%, volume 8 mL, no. of accumulations = 200000, pH 6.1. (h) After incubating for 0.5 at pH 11.1: trypsin 0.41 mM, 17 mM sodium cacodylate buffer, D_2O 12.5% v/v, volume 9.4 mL, no. of accumulations = 64000, pH 6.1.

(1) University of Edinburgh.
(2) Texas A&M University.
(3) Ruhlmann, A.; Kukla, D.; Schwager, P.; Bartels, K.; Huber, R. *J. Mol. Biol.* **1973**, *77*, 417–436. Sweet, R. M.; Wright, H. T.; Janin, J.; Chothia, C. H.; Blow, D. M. *Biochemistry* **1974**, *13*, 4212–4228.
(4) Huber, R.; Kukla, D.; Bode, W.; Schwager, P.; Bartels, K.; Deisenhofer, J.; Steigemann, W. *J. Mol. Biol.* **1976**, *89*, 73–101. Huber, R.; Bode, W.; Kukla, D.; Kohl, U.; Ryan, C. A. *Biophys. Struct. Mech.* **1975**, *1*, 189–201.
(5) Poulos, T. L.; Alden, R. A.; Freer, S. T.; Birktoft, J. J.; Kraut, J. *J. Biol. Chem.* **1976**, *251*, 1097–1103.
(6) Steitz, T. A.; Shulman, R. G. *Ann. Rev. Biophys. Bioeng.* **1982**, *11*, 419–444.
(7) Hunkapiller, M. W.; Forgac, M. D.; Yu, E. H.; Richards, J. H. *Biochem. Biophys. Res. Commun.* **1979**, *87*, 25–31. Baillargeon, M. W.; Laskowski, M.; Neves, D. E.; Porubcan, M. A.; Santini, R. E.; Markley, J. L. *Biochemistry* **1980**, *19*, 5703–5710. Richards, R.; Tschesche, H.; Wuthrich, K. *Ibid.* **1980**, *19*, 5711–5715.
(8) Abbreviation: Z-lys-CMK: 1-chloro-3-(carbobenzyloxyamino)-7-aminoheptan-2-one.
(9) Coggins, J. R.; Kray, W.; Shaw, E. *Biochem. J.* **1974**, *138*, 579–585.
(10) Shaw, E.; Springhorn, S. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 391–397.
(11) Bezas, B.; Zervas, L. *J. Am. Chem. Soc.* **1961**, *83*, 719–723.
(12) The ^{13}C NMR spectrum of Z-lys-CMK was pH independent over the pH range 3.0–7.0. Between pH 7 and 9 the signal at 95.4 ppm decreased in intensity as expected for a hydrated carbonyl group.[13] At pH values >9.0 both signals at 204.7 and 95.4 ppm were not detected. Restoration to neutrality and prolonged scanning showed that this was due to the irreversible breakdown/reactions of Z-lys-CMK, at least four new species being formed. The resonance at 95.4 ppm is therefore assigned to the hydrated form of Z-lys-CMK. Reaction of Z-lys-CMK with *N*-acetylcysteine (Gamcsik, Malthouse, Mackenzie, Boyd, and Scott, unpublished results) resulted in the disappearance of the signals at 95.4 and 204.7 ppm and the appearance of a new signal at 207.8 ppm. This downfield shift is expected for an alkylated thiol group.
(13) Hellström, N.; Almqvist, S.-O. *J. Chem. Soc. B* **1970**, 1396–1400.
(14) Concentrations of fully active trypsin were determined by titration with *p*-nitrophenyl-*p*′-guanidobenzoate.[15] The activity of trypsin with or without Z-lys-CMK was determined by removing aliquots and assaying with α-*N*-benzoyl-DL-arginine *p*-nitroanilide at pH 7.0 in 0.1 M phosphate as previously reported.[16]
(15) Chase, T.; Shaw, E. *Biochem. Biophys. Res. Commun.* **1967**, *29*, 508–514.
(16) Malthouse, J. P. G.; Brocklehurst, K. *Biochem. J.* **1976**, *159*, 221–234.
(17) Trypsin activity toward α-*N*-benzoyl-DL-arginine *p*-nitroanilide decreased linearly with Z-lys-CMK concentration. Z-lys-CMK concentration could therefore be determined by assuming stoichiometric inhibition.
(18) Mares-Guia, M.; Shaw, E. *J. Biol. Chem.* **1965**, *240*, 1579–1585.

0002-7863/83/1505-1685$01.50/0

Scheme I



98.0 ppm and an additional resonance at 205.1 ppm appeared (not shown).

It is clear that native trypsin stabilizes[19] the tetrahedral adduct (**3**;[20] 98.0 ppm) as only nucleophilic attack by the hydroxyl group of serine-195 and covalent bond formation would cause the upfield shift of >100 ppm. A shift of this magnitude cannot be rationalized by a simple perturbation of the carbonyl trigonal hybridization. It is unlikely that tetrahedral geometry results from aqueous hydration of the enzyme-bound label for the following reasons. First, such a species should persist on denaturation, whereas new signals at 205.5 and 95.1 ppm (Figure 1h), observed on denaturation, provide clear evidence for the free ketone (**5**) and its hydrate (**6**). Secondly, with increasing pH the peak at 98.0 ppm was gradually shifted downfield ($pK_a \approx 8.0$) to a limiting value of 102.1 ppm, suggesting that there is stabilization of the *ionized* tetrahedral intermediate (**4**) by hydrogen bonding to the backbone NH groups of serine-195 and glycine-193 (Scheme I). No evidence of a carbonyl resonance (~205 ppm) was found over the pH range 3-11 prior to denaturation. The un-ionized tetrahedral intermediate (**3**) may also be stabilized by hydrogen bonding to one of the backbone carbonyl groups and/or electrostatic interaction with the protonated imidazole of histidine-57.[22] At pH 3.2 there is no evidence (Figure 1c) for the formation of a reversible tetrahedral adduct prior to alkylation of histidine-57, but this does not exclude the possibility of prior addition at pH >3.2.

Recently it has been claimed that a tetrahedral intermediate (99 ppm) has been detected by ^{13}C NMR with pepsin and a ketone inhibitor.[23] This result appears to be similar to those reported herein and taken together with the previous ^{13}C NMR studies indicate that the NMR method is an excellent probe for the diagnosis of carbon hybridization in complexes of inhibited proteases. The stage is now set for detailed studies on the enzyme–substrate complexes where tetrahedral intermediates can, in principle, be intercepted at subzero temperature and clearly recognized by ^{13}C NMR spectroscopy.

Acknowledgment. We thank the Science & Engineering Research Council (U.K.) for generous support of this work and for the provision of a Bruker WB 300-MHz spectrometer.

Registry No. CMK, 52780-79-7; trypsin, 9002-07-7.

(19) On the basis of peak heights (Figure 1g) at least 90% of the labeled carbon is sp^3 hybridized.
(20) Analogous chemical shifts are found in the anomeric carbon of many sugars.[21]
(21) Rosenthal, S. N.; Fendler, J. H. *Adv. Phys. Org. Chem.* **1976**, *13*, 279-424.
(22) Kossiakoff, A. A.; Spencer, S. A. *Biochemistry* **1981**, *20*, 6462-6474.
(23) Rich, D. H.; Bernatowicz, M. S.; Schmidt, P. G. *J. Am. Chem. Soc.* **1982**, *104*, 3535-3536.

Stereospecific Synthesis of Racemic Daunosamine. Diastereofacial Selectivity in a Nitrone Cycloaddition[1]

Philip DeShong* and Joseph M. Leginus

Department of Chemistry
The Pennsylvania State University
University Park, Pennsylvania 16802

Received October 25, 1982

There are several recent reports of stereoselective cycloadditions involving nitrones and nitrile oxides. Uskoković,[2] Belzecki,[3] and Vasella[4] have reported that nitrones carrying a chiral substituent on nitrogen (R^1 in **1**) displayed high diastereoselectivity in cycloadditions with a variety of achiral dipolarophiles (Scheme I). Koizumi[5] and Kozikowski[6] have found that the reaction of chiral dipolarophiles with nitrones and nitrile oxides, respectively, leads to the diastereoselective formation of cycloadducts. Since the N,O bonds of these cycloadducts are readily cleaved to produce acyclic molecules, the diastereoselectivity displayed in the cycloadditions serves as a means of controlling acyclic stereochemistry.[7] We

(1) Presented in part at the 183rd National Meeting of the American Chemical Society in Las Vegas, NV, Ap 1982.
(2) Wovkulich, P. M.; Uskoković, M. R. *J. Am. Chem. Soc.* **1981**, *103*, 3956 and references cited therein.
(3) Belzecki, C.; Panfil, C. *J. Org. Chem.* **1979**, *44*, 1212.
(4) Vasella, A.; Voeffray, R. *Helv. Chim. Acta* **1982**, *65*, 1134 and references cited therein.
(5) Koizumi, T.; Hirai, H.; Yoshii, E. *J. Org. Chem.* **1982**, *47*, 4005.
(6) Kozikowski, A. P.; Chen. Y. Y. *Tetrahedron Lett.* **1982**, *23*, 2081. Kozikowski, A. P.; Ghosh, A. K. *J. Am. Chem. Soc.* **1982**, *104*, 5788.

0002-7863/83/1505-1686$01.50/0