rium from Scheme I with an equation similar to eq 1;[5b] using the known values for K_2K_3 and K_4 we may estimate K_2 and K_3 to be $<10^{-11}$ and >1, respectively. The ionization pK_a for the amidine (K_2) is relatively low presumably owing to the powerfully electron-withdrawing ammonio group.

Assuming equilibrium of the species II–VI and rate-limiting proton-catalyzed hydrolysis of the free carbodiimide VI, we may estimate the acid plateau rate constant (pH 0–4) from proton attack on the trimethylammonio model ($k_H = 3 \times 10^2$ M^{-1} s^{-1}). The overall rate constant for reagent I becomes $k_HK_1K_2K_3/K_4$[5c] in the acid plateau region which gives 3×10^{-2} s^{-1} on substituting values for the equilibrium constants and k_H. The estimated value is much larger than that observed (10^{-3} s^{-1}) indicating that one of the steps K_1–K_4 is rate limiting for the carbodiimide mechanism. The decomposition rate constant for VI increases with decrease in pH and it is likely that K_3 becomes the rate-limiting step because this is pH independent and the other steps involve proton transfer to and from electronegative atoms. The overall rate constant for production of V is hydroxide ion dependent and the observation of the low, pH independent rate constant in the acid region is therefore consistent with a different mechanism, namely water attack on the ammonioamidinium dication (II).

Hydrolysis of I in the alkaline pH region follows the hydrolysis of the neutral model *N,N'*-di-*n*-propylcarbodiimide. Assuming that species II–VI are in equilibrium and using values of the equilibrium constants deduced above, we estimate that the fraction of reagent I present as V is 50% in the alkaline region; the other 50% is IV. Thus we should expect an observed rate constant some 50% less than that of the model at the corresponding pH. The close proximity of the data (Figure 1) confirms that the alkaline hydrolysis proceeds via the neutral carbodiimide V.

Attack of acetate (1 M) buffer on the trimethylammoniocarbodiimide at pH 5.4 has the rate constant 1.6×10^{-3} s^{-1}; at pH 5.4 the proportion of protonated carbodiimide VI is calculated to be 0.091 using the equilibrium constants determined above. The predicted rate constant for I with 1 M acetate is thus $0.091 \times 1.6 \times 10^{-3} = 1.46 \times 10^{-4}$ s^{-1}. This is considerably less than the observed value (see Figure 1) and the reaction is therefore considered to proceed via attack of acetate ion on the ammonioamidinium dication (II) as in Scheme II. The adduct from this reaction (VII) is probably decomposed by reaction with further acetate to yield acetic anhydride or by other nucleophiles.

The observation of buffer catalysis in decomposition of I in the region pH 3.5–7 is consistent with other observations of catalysis of amidinium ion hydrolysis.[6,7] We have shown that regular carbodiimides hydrolyse without buffer effects.[2b]

The data of DeWolfe[6] and Robinson and Jencks[7] suggest that the reactivity of II with water is consistent with amidinium ion hydrolysis.[8]

The water-soluble carbodiimide I and its analogues have been used in peptide synthesis[10] and in protein modification.[11] While the fate of the initial complex (analogous to VII) via direct attack by nucleophile or via anhydride is yet to be determined, this work indicates that initial reaction occurs at neutral and acid pH's via the ammonioamidinium dication and not via carbodiimide.

We point out here the possible synthetic utility in dehydration reactions of amidinium cations activated by powerful electron-withdrawing substituents.

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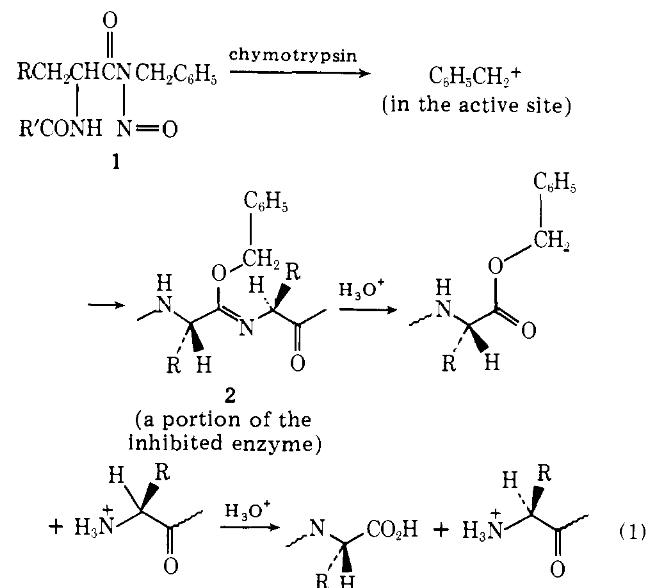
Labeling of Amide Linkages in Active Site Mapping: Carbonium Ion and Extended Photoaffinity Labeling Approaches

Sir:

Nitrosoamides of amino acids (**1**) are active site directed inhibitors[1] of the suicide type[2] for the enzyme α-chymotrypsin. Work with model systems now show that the carbonium ions generated by these reagents alkylate amide linkages along the backbone of the protein to give preferentially O-alkylation. The resulting imidate ester groups (**2**) are readily hydrolyzed at pH ~5 to give the amine and carboxylic acid fragments[3] (eq 1). Thus, each "hit" leads to a break in the chain at that point to give two unlabeled peptides.

An important aspect of these observations is that the normal peptides resulting from imidate hydrolysis can be analyzed by standard techniques. Thus, end-group analysis would lead to



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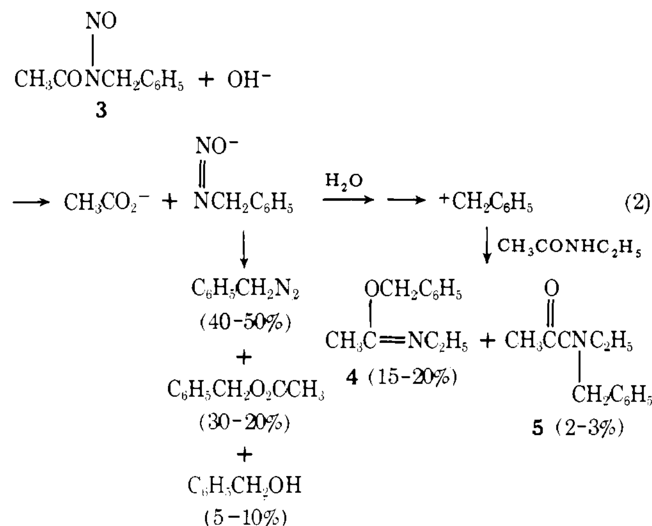
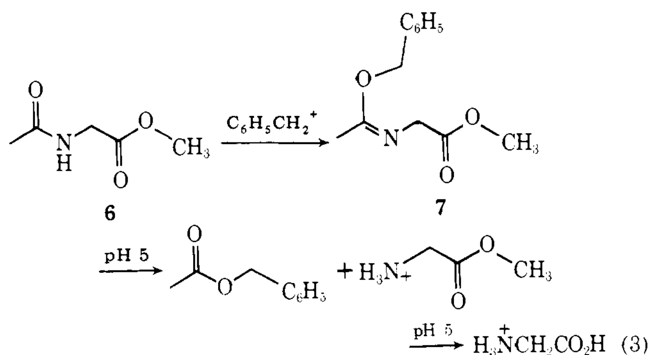
the identity of the amino acid labeled;[4] furthermore, if the sequence of the protein which was inhibited is known, sequencing[5] of the peptide for a small number of residues (three for α-chymotrypsin) should also identify the position of the labeled amino acid in the overall sequence of the protein. Since the carbonium-ion approach is applicable to hydrolases,[1] and probably to oxidases[1] and to enzymes capable of reacting with powerful alkylating agents such as oxonium ions (acetylcholine esterase),[6] this approach to labeling would appear to be of considerable utility. The utility is extended even further by the fact that the advantages cited above for the carbonium ion approach extend to photoaffinity labeling (vide infra).

Alkylation of Amides with Benzyl Carbonium Ions. (A) The addition of 12 M KOH (0.9 equiv) to a 1 M solution of *N*-benzyl-*N*-nitrosoacetamide (**3**) in *N*-ethylacetamide led to the immediate evolution of nitrogen and the appearance of the reddish orange color of phenyldiazomethane (products and yields in eq 2). The most significant observations are that an appreciable amount of the imidate ester (**4**) is formed and that



the ratio of **4**/**5** is ~7. Use of the strong base, OH^-, leads to the diversion of much of the diazotate to phenyldiazomethane;[7] this compound is not detected in the enzymic reaction at pH 7.9 (eq 1).

(B) A solution of 0.4 mmol of $Na^{+-}OCH_3$ in 0.2 mL of CH_3OH was added to 0.4 mmol of compound **3** dissolved in 4.4 mmol of methyl *N*-acetylglycinate (**6**). Phenyldiazomethane (40%), benzyl methyl ether (10%), benzyl acetate (3%), imidate ester **7** (10%), and unidentified products were formed (eq 3). Hydrolysis at pH 5 yielded methyl glycinate and



then glycine (5%).[8] In active site labeling the important yield would be that of labeled protein, and by using the K_{cat} approach[1,2] this could be made quantitative.

Reaction of Benzyl Carbonium Ions with α-Chymotrypsin. α-Chymotrypsin inactivated by ^{14}C-labeled nitrosoamide **1** ($R = C_6H_5$; $R' = CH(CH_3)_2$) and containing one benzyl group total per enzyme molecule was hydrolyzed in 6 N HCl to give a mixture of amino acids which had retained ~1/3 of the benzyl groups; ~2/3 of the labels were labile and were removed in the volatile fraction. Model studies suggest that the stable labeled amino acids contain benzyl groups attached to N, S, and C atoms, and that the labile labeled amino acids are largely imidate esters accompanied by *O*-benzyl derivatives of tyrosine, etc.

In dialysis of the inhibited enzyme against 10^{-3} M hydrochloric acid, the full mole of benzyl groups per mole of enzyme was retained. However, dialysis at pH 3 in the presence of 8 M urea, which leads to denaturation of the enzyme, led to the hydrolysis of ~1/2 of the benzyl groups. Evidence that chain cleavage accompanied the loss of benzyl groups was provided by chromatography of this material (after reduction of the cystines and alkylation of the thiol groups) on Sephadex G-50, superfine; in addition to protein eluting in the void volume (intact and alkylated B and C chains of chymotrypsin), several bands for peptides were resolved in later fractions. Enzyme inhibited with diisopropylfluorophosphate produced only normal B and C chains appearing in the void volume.

An Extension of Photoaffinity Labeling. In this approach substrates are modified to yield, upon irradiation, carbenes and nitrenes, highly active intermediates capable of reacting with functional groups on the enzyme.[9] In practice, the yields of recovered amino acids derivatized through the functional groups have been quite low.[10] We could find no reference in the literature to the possibility that carbenes should, as electrophilic species, react with the amide linkage; in fact, they do.

When a solution of 0.1 mmol of phenyldiazomethane in 6 mmol of *N*-ethylacetamide was irradiated at 20 °C, a 51% yield of imidate **4** was obtained; none of the isomer **5** was detected. Further, the irradiation of a solution of 1 mmol of ethyl diazoacetate in 6 mmol of methyl *N*-acetylglycinate (**6**), followed by hydrolysis at pH 5, yielded glycine[8] (~2% based on the diazo ester). The yields of imidate in this study were minimal because of the loss of most of the carbenes to give azines, ethyl glycolate, and ethoxyketene; the "azine" diversion of the reactive intermediate is minimized in protein work by the low concentrations of reagents normally used and by the "encapsulation" of the reactive species by the active site.

These studies show that the advantages outlined above for the imidate aspects of the carbonium ion approach (amide labeling in the active site, chain cleavage, identification of amino acids which had been *O*-benzylated), extend to photoaffinity labeling.[11]

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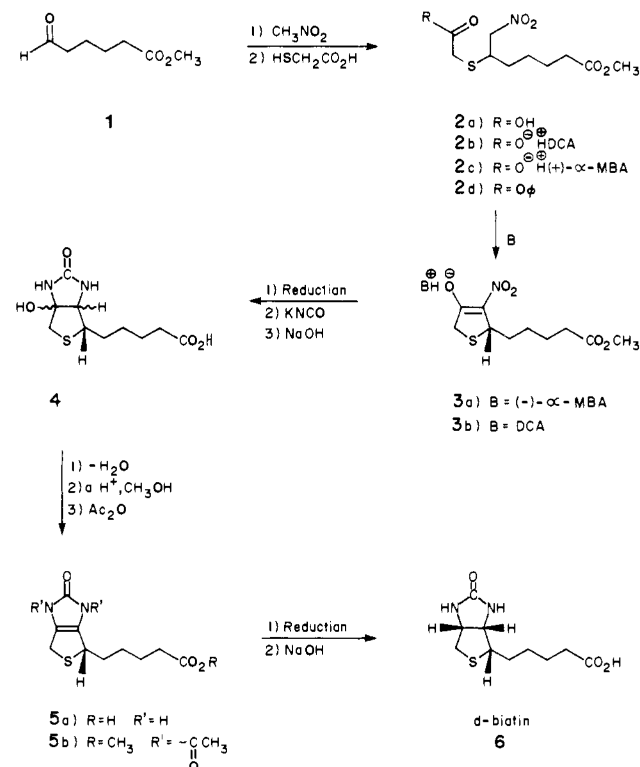
Synthesis of *d*-Biotin via Dehydrobiotin

Sir:

The synthesis[1] of *d*-biotin (**6**) reported here avoids the problem of diastereomer formation by maintaining two of the three chiral carbons in a prochiral form until a late stage. Its success also depends on several other critical features: (1) a new carbon–carbon bond-forming reaction leading to an α-nitro ketone; (2) easy resolution of α-nitro ketone **3** or its precursor acid **2a**; and (3) a virtually stereospecific, economic catalytic hydrogenation of a derivative of dehydrobiotin **5b**.

The bulk of the molecule is rapidly assembled by sequential treatment of adipaldehydic acid methyl ester[2] (**1**) in dimeth-



ylformamide with nitromethane (1.4 equiv), magnesium sulfate (1.6 equiv), piperidine (1.1 equiv), and thioglycolic acid (1.0 equiv) at 3 °C. Acidification with 2 N sulfuric acid and extraction with toluene gives 6-carboxymethylthio-7-nitroheptanoic acid methyl ester (**2a**) as an oil, characterized as its dicyclohexylamine (DCA) salt, **2b**, mp 100–105 °C,[3] in 90% yield. A salt of the *S* enantiomer of **2a**, appropriate to the synthesis of *d*-biotin, is obtained by its treatment with (+)-α-methylbenzylamine ((+)-α-MBA) in ethyl acetate followed by dilution with ether. Two recrystallizations from ethyl acetate give **2c** in 30% yield (from *dl*-**2a**) with >97% ee,[4] mp 88–89 °C, $[\alpha]^{25}_{365}$ 155.0° (*c* 1.55, $CHCl_3$).

The final carbon–carbon bond is formed by first converting **2c** to enantiomerically pure **2b**.[5] Treatment of **2b** with phenol, thionyl chloride, and a catalytic amount of pyridine overnight at room temperature yields the oily phenyl ester **2d** in 95% crude yield. Mixing of this oil with (−)-α-methylbenzylamine in ethyl acetate at 3 °C gives crude 5-(2,5-dihydro-4-hydroxy-3-nitrothien-2-yl)pentanoic acid methyl ester (−)-α-MBA salt (**3a**),[6,10] which is purified by acidification followed by reprecipitation with (−)-α-MBA to give **3a** in 75% yield, mp 138–139 °C, $[\alpha]^{25}_{589}$ −252° (*c* 0.595, 95% ethanol, >97% ee[11]). Alternatively, the racemic nitro ketone obtained analogously (characterized as *dl*-**3b**, mp 157–159 °C dec, UV max 348 nm (ϵ 15 800)) may be resolved by treatment of an ether solution with (−)-α-MBA (0.5 equiv) to give crude **3a** in 40% yield (based on *dl*-**3b**).

Construction of the remaining heterocyclic ring commences by the low-pressure hydrogenation of the nitro group over 10% Pd/C in ~8:1 mixture of acetic acid and 3 N hydrochloric acid. The reaction is complete in 3 h at room temperature. The uncharacterized amino ketone hydrochloride is reacted with aqueous potassium cyanate at pH 5–6. Treatment with sodium hydroxide and then acidification leads to hexahydro-6a-hydroxy-2-oxo-1*H*-thieno[3,4-*d*]imidazole-4-pentanoic acid (**4**) (*dl*-**4**, mp 192–200 °C). Dehydration of **4** takes place in acetic acid by continuous stripping at 55 °C, yielding 2,3,4,6-tetrahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazole-4-pentanoic acid (**5a**)[12] (mp 210–214 °C, $[\alpha]^{25}_{589}$ −76.5° (*c* 1.00, 0.1 N NaOH)). Acid-catalyzed reaction with refluxing methanol for 8 h adds methanol to the double bond and produces a mixture of methoxybiotin methyl esters. After neutralization with sodium bicarbonate and removal of methanol, the mixture is treated with acetic anhydride for 6 h at 110 °C. Filtration of an ether solution of the crude product through silica gel and recrystallization from 2-propanol gives enantiomerically pure 1,3-diacetyl-2,3,4,6-tetrahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazole-4-pentanoic acid methyl ester (**5b**)[13] (mp 89 °C, $[\alpha]^{25}_{389}$ −113° (*c* 1.00, CH_2Cl_2)) in 42% yield from **3a**.

In striking contrast to many other sulfur-containing compounds including dehydrobiotin (**5a**), **5b** is an excellent substrate for catalytic hydrogenation.[14] The reduction is carried out in a 5–8% solution in acetic anhydride at 550 psi of hydrogen pressure, 85 °C, 6 h, and a 10% loading of 5% Pd/C to give, after crystallization from 2-propanol, enantiomerically pure *N,N*-diacetylbiotin methyl ester (mp 71–71.5 °C, $[\alpha]^{25}_{589}$ −66.8° (*c* 1.00, CH_2Cl_2)). The palladium catalyst can be recycled a number of times. Basic hydrolysis of this substance in aqueous methanol and recrystallization from water give pure *d*-biotin (mp 228–228.5 °C, $[\alpha]^{25}_{289}$ +91.3° (*c* 1.00, 0.1 N NaOH)) in 85% yield (from (**5b**)). The yield of *d*-biotin can be made quantitative by using more catalyst and hydrogen pressures of >3000 psi.

The above reduction justifies our planar strategy and completes a synthesis which is ~90% stereoselective. The overall yield is 7.2% optically pure *d*-biotin from adipic acid half-aldehyde.

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