Active-Site-Directed Alkylation of Chymotrypsin by Reagents Utilizing Various Departing Groups

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Affinity-labeling reagents are useful for the inactivation of proteases in vivo but are apparently limited in application by the possibility of side reactions. In addition, increased specificity would be desirable. Substrate-derived chloromethyl ketones, one class of protease inhibitors, have been examined with the hope that replacement of the departing group halogen by other substituents might lead to improved inhibitor characteristics. Analogues of Z-Phe-Ch_2X were synthesized in which X is a sulfonate or carboxylate substituent and examined as inactivators of chymotrypsin. The sulfonate esters were found to be more reactive than the previously studied halogen derivatives.

Serine proteases are readily inactivated by amino acid derivatives including peptides which satisfy the specificity of the protease but contain a chloromethyl ketone group instead of a free carboxylic acid[1] or hydrolyzable derivative. The initial observations were reported by Schoellman and Shaw with the bromomethyl and chloromethyl ketone derived from tosylphenylalanine which alkylates the active center histidine in chymotrypsin.[2] Trypsin and related enzymes[3,4] and elastase[5,6] form two major additional specificity classes of serine proteinases to which this affinity-labeling approach has been applied. Although introduced as a means of studying protein structure, the possibility was realized that agents of this type might be of therapeutic value. Some important indications of in vivo activity have been obtained in situations in which a protease is known to be involved or is implicated. For example, the chloromethyl ketone Tos-Lys-CH_2Cl (also known as TLCK), an inactivator of trypsin,[3] also inactivates certain enzymes of related specificity, such as acrosin, a trypsin-like protease attached to sperm and essential for fertilization.[7] This inhibitor prevents conception in animals[8] presumably due to inactivation of acrosin. Since Tos-Lys-CH_2Cl is a potential alkylating agent, reaction in vivo at many sites, for example at thiol groups on proteins, is possible and the observed in vivo result may be due to such side reactions.

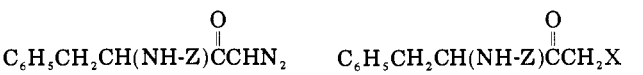
Observations made in a different area of physiological interest also relate to the usefulness of this class of protease inactivators as diagnostic tools and potential therapeutic agents. Tos-Phe-CH_2Cl and Tos-Lys-CH_2Cl have been found to inhibit the tumorigenesis produced in mouse skin by application of dimethylbenzanthracene followed by croton oil promotion.[9] A similar result has been obtained with the nontoxic antibiotic leupeptin.[10] In these experiments an increase in tissue proteolytic activity is known to occur. Both Tos-Lys-CH_2Cl and leupeptin are inhibitors of trypsin-like enzymes. Tos-Phe-CH_2Cl, on the other hand, inactivates chymotrypsin, not trypsin. However, all three reagents inhibit a thiol protease, cathepsin B, which has been implicated in the pathological changes accompanying carcinogenesis.[11]

A different protease, a cellular plasminogen activator, has been shown to be secreted in increased amounts by malignantly transformed cells of varied origin.[12] It is a serine protease of trypsin-like specificity[13] and has been assigned an important role in endowing cells with properties associated with malignancy.[14] Preliminary observations indicate that this plasminogen activator is susceptible[15] to certain lysyl peptide chloromethyl ketones.

In these and analogous situations protease inhibitors devoid of side reactions and endowed with narrower enzyme specificity would be of considerable value in attempting to deduce the role of a given protease in a pathological process.[16] It may be that a sequence of proteolytic events is involved as in zymogen activation or that several independent proteases act at various stages of the pathological process.

One possibility for obtaining diminished side reactions with affinity-labeling alkylating agents may lie in the utilization of departing groups other than chloride or bromide. Studies were carried out with the carbobenzyloxyphenylalanylmethyl ketone series, Z-Phe-CH_2X (Z = benzyloxycarbonyl), as inhibitors of chymotrypsin. The chloro and bromo derivatives have been shown[17] to be very effective affinity labels for this protease. It was considered advisable to examine, in particular, the properties of derivatives in which the departing group, X^-, might be difficultly displaceable in the expectation that an agent with fewer side reactions would result than may be the case with Z-Phe-CH_2Cl. The derivatives reported in this initial study are sulfonate and carboxylate esters.

Chemistry. Sulfonic esters were accessible from Z-phenylalanine diazomethyl ketone (1), which is also the precursor for Z-Phe-CH_2Cl[18] and Z-Phe-CH_2Br,[19] by the action of the appropriate sulfuric acid under anhydrous conditions. Both the *p*-toluenesulfonyl ester (2, X = H_3C-C_6H_4-SO_3^-) and the methanesulfonyl ester (2, X = H_3C-SO_3^-) were thus prepared.



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Carboxylic esters could not be obtained by this method since carboxylic acids are not, in general, strong enough acids to catalyze the decomposition of diazomethyl ketones. A procedure found to be suitable for this purpose was the nucleophilic displacement of chloride from Z-Phe-CH_2Cl (2, X = Cl^-) with the appropriate carboxylic acid anion under aprotic conditions. A tetrabutylammonium salt of the latter was prepared by neutralization of the carboxylic acid with exactly 1 equiv of tetrabutylammonium hydroxide followed by thorough drying. These dried salts were found to be very soluble in solvents such as acetone and acetonitrile and the displacement reaction with ZPCK could be carried out at room temperature in the presence of an excess of the salt. The acetate [2, X = $H_3CC(=O)^-$], as well as the *N*-acetylglycine and *N*-acetylglycylglycine esters, was prepared by this method.

In addition, it was found that the reaction of ZPCK with tetrabutylammonium trifluoroacetate in acetone containing at least 1 equiv of water gives a good yield of the α-hydroxymethyl ketone.

Results and Discussion

The derivatives of Z-Phe-CH_2– had a limited solubility in water which restricted the concentration range that could be examined in their evaluation as inactivators of chymotrypsin. Some organic solvent was generally included in the incubation mixture. With very reactive inhibitors, this offered no problem. In fact, in the case of

Table I. Rate of Inactivation of Chymotrypsin at pH 6.8 by Z-Phe-CH_2X

X	Temp, °C	[I], M	[E], M	$t_{1/2}$, min[a]	K_2,[b] M^{-1} s^{-1}
OTos	25	2×10^{-7}	3×10^{-8}	7.8	7400
OMs	25	9×10^{-7}	3×10^{-8}	7	1800
		4.5×10^{-7}	3×10^{-8}	14.5	
		2.25×10^{-7}	3×10^{-8}	28.5	
		1.12×10^{-7}	3×10^{-8}	69	
Br	25				790[c]
Cl	25				69[c]
OAc	37	4.6×10^{-5}	1.8×10^{-7}	>400	0
		1.35×10^{-4}	3×10^{-8}	>57	
OH	37	1.44×10^{-3}	5×10^{-8}	>500	

[a] Time for loss of 50% of initial activity; apparent first-order kinetics for this process were observed. [b] $K_2 = K_1/[I]$. [c] Cf. ref 17.

the sulfonic esters, the rates of inactivation of α-chymotrypsin were so rapid that incubations had to be performed at high dilutions of enzyme and inhibitor to diminish the rate to a measurable level. A sample of chymotrypsin inactivated by the toluenesulfonyl ester was found, on amino acid analysis, to contain one less histidine residue than chymotrypsin, an indication that this reagent acts in the same way as the chloro derivative.[17] These esters represent a new series of potent chymotrypsin inhibitors.

In the case of the carboxylic esters and the hydroxy compound, the rates of reaction with the enzyme, if, in fact, they were different from zero, were slower than could be detected by our methods even with long incubation times and higher incubation temperatures. Thus, it is apparent that, in this series of inhibitors and inhibitor analogues, variation of the susceptibility of the leaving group to nucleophilic attack by, in this case, the histidine-57 nitrogen produces a wide range of reactivities. This ability to vary the activity of the functionality responsible for chemical modification of the protease active site is one of potential importance to the design of inhibitors with increased specificity and reduced general reactivity. The latter property may remove reactivity to glutathione, for example.[20]

In the case of the acetylglycylglycine ester, it was established that the inhibitor was complexing with chymotrypsin. A K_i of 9.5×10^{-4} was determined. Thus the failure to detect inactivation was not due to failure of this step to take place. The conditions that were used and the results obtained are summarized in Table I.

It is apparent that the relative effectiveness of the derivatives of Z-Phe-CH_2– to inactivate chymotrypsin is comparable to the ease of nucleophilic displacement of the departing group. Presumably the departing groups in this limited series were all readily accommodated at the active center of chymotrypsin so that the geometry of the reagent–chymotrypsin complex was more or less constant with respect to the access of the imidazole ring of histidine-57 to the methylene carbon of the reagent at which displacement occurs. Conceivably this would not be true in certain instances and the expected reactivity of a given derivative might not be observed. The displaced groups extend into the region of the enzyme active center occupied during proteolytic action by a substrate amino acid residue; i.e., this region provides a departing group subsite. We have recently observed that a group of trypsin-like enzymes apparently have different spatial capacities in this region of their active centers, a difference that can be taken advantage of to obtain reagents selective for a given enzyme among closely related ones.[21] The methods described in the present work may be applied to exploit these differences with protease affinity-labeling reagents acting by alkylation, particularly among the numerous trypsin-like enzymes where the need for selectivity of action is great.

Experimental Section

Inhibitor Evaluation. The various Z-Phe-CH_2X derivatives were incubated with α-chymotrypsin at pH 6.8 in 0.1 M phosphate buffer. Acetonitrile or dimethyl sulfoxide up to 20% final concentration was added to increase inhibitor solubility. Other conditions are described in Table I. Residual enzyme activity was determined by diluting an aliquot of the incubation mixture with buffer (20% Me_2SO) and assaying with Z-Tyr-ONP.[22] Log plots of residual activity vs. time were linear through 90% inactivation except in those few cases where the inhibitor concentration was only slightly in excess of the enzyme concentration and linearity obtained only in the initial 40–50% drop. The rates of inactivation are the differences between the observed rates with inhibitor and those of a control containing only enzyme.

Synthesis of Reagents. Melting points were determined with a Fisher-John apparatus. NMR spectra obtained with a Varian T-60 spectrometer were consistent with the assigned structures. In the examination of NMR spectra of the substituted methyl ketone derivatives, the chemical shifts of the methylene protons in Z-Phe-CH_2X analogues in $CDCl_3$ were observed to be: substituent (parts per million from tetramethylsilane) Cl (4.02), OH (4.17), OAc (4.68), OTs (4.53), and OMs (4.92).

L-1-Diazo-3-(*N*-carbobenzyloxy)amino-4-phenyl-2-butanone (*N*-Z-Phe-CHN_2). This material has been alluded to[18,23] and has been characterized,[24] but the following procedure is superior.

N-Carbobenzyloxy-L-phenylalanine (10 g, 33.4 mmol) was dissolved in 30 ml of THF at −10 °C and treated with 4.6 ml of *N*-methylmorpholine and 6.0 ml of isobutyl chloroformate. After stirring 12 min at −10 °C, the reaction was diluted with 160 ml of diethyl ether and filtered rapidly into a dropping funnel. The filtrate, under dry nitrogen, was added dropwise to a cold (0 °C) 0.3 M solution of diazomethane in ether (150 ml). After addition was complete, the solution, under nitrogen, was allowed to warm to room temperature over a 2-h period. It was then washed twice with water and once each with 1 M aqueous sodium bicarbonate and saturated aqueous sodium chloride and then dried over anhydrous sodium sulfate. After filtering and concentrating, the residue was taken up in a small volume of ether and diluted with petroleum ether until slightly turbid. This was allowed to stand in the cold (0 °C) for 12 h, at which time the crop of crystals was collected and dried, yielding 8.26 g (25.5 mmol, 76.4%), mp 85–87 °C (lit.[24] mp 84–85°). A second recrystallization from the same solvent system did not change the observed melting point.

L-1-Toluenesulfonyloxy-3-(*N*-carbobenzyloxy)amino-4-phenyl-2-butanone (*N*-Z-Phe-CH_2OTos). A solution of 494 mg (1.5 mmol) of *N*-Z-Phe-CHN_2 in 20 ml of dry acetonitrile was treated with 3.5 ml of a 0.43 M solution of toluenesulfonic acid (anhydrous) in acetonitrile. After 1 min the reaction was diluted with 50 ml of water and extracted twice with 60-ml portions of ethyl acetate. The combined ethyl acetate extracts were washed with water and with saturated aqueous sodium chloride solution and were dried over anhydrous magnesium sulfate. The solvent was then evaporated and the residue was recrystallized from chloroform and petroleum ether: yield 500 mg (1.07 mmol, 71%); mp 86–89 °C. Anal. ($C_{25}H_{25}NO_6S$) C, H, N.

L-1-Methanesulfonyloxy-3-(*N*-carbobenzyloxy)amino-4-phenyl-2-butanone (*N*-Z-Phe-CH_2OMs). A solution of 248 mg (0.77 mmol) of Z-Phe-CHN_2 in 100 ml of diethyl ether was treated with a solution of 0.50 ml of methanesulfonic acid in 25 ml of diethyl ether at 0 °C for 10 min. The reaction was then extracted with 20 ml of dilute aqueous sodium bicarbonate, 20 ml of water and 20 ml of saturated aqueous sodium bicarbonate, 20 ml of water, and 20 ml of saturated sodium chloride. The organic phase was then dried over anhydrous sodium sulfate and concentrated. The residue was recrystallized from ether and petroleum ether: mp 95–96 °C; 220 mg (73%).

L-1-Hydroxy-3-(*N*-carbobenzyloxy)amino-4-phenyl-2-butanone (*N*-Z-Phe-CH_2OH). *N*-Z-Phe-CH_2Cl (2 g, 6.03 mmol) was dissolved in 70 ml of a 0.176 M solution of tetrabutylammonium trifluoroacetate in acetone (with 1% water) and the

reaction mixture was refluxed for 48 h. It was then concentrated, diluted with 100 ml of water, and extracted with three 50-ml portions of ethyl acetate. The combined extracts were dried over anhydrous sodium sulfate and concentrated. The residue was chromatographed on silica gel (3:1 benzene–ethyl acetate) and two fractions were collected. The first, unreacted Z-Phe-CH_2Cl weighed 300 mg. The second fraction crystallized on concentration and was recrystallized from diethyl ether and petroleum ether. The product, mp 76.5–77.5 °C, weighed 850 mg (45% yield). Anal. ($C_{18}H_{19}NO_4$) C, H, N.

L-1-Acetoxy-3-(*N*-carbobenzyloxy)amino-4-phenyl-2-butanone (*N*-Z-Phe-CH_2OAc). A solution of 1.49 g (4.5 mmol) of Z-Phe-CH_2Cl in 50 ml of acetone was added to a solution of 50 ml of 0.125 M tetrabutylammonium acetate in acetone. The reaction was stirred for 3 h at room temperature. After concentrating and diluting with 100 ml of ethyl acetate, the solution was washed four times with 100-ml portions of water and once with 100 ml of saturated aqueous sodium chloride. The solution was then dried over anhydrous sodium sulfate and concentrated. The residue, an orange oil, crystallized on standing. The crystals were washed with a small volume of ether and then dissolved in chloroform and recrystallized with pentane. The purified material, mp 103–104 °C, weighed 950 mg (2.6 mmol, 50%). Anal. ($C_{20}H_{21}O_5N$) C, H, N. The acetate was also obtainable from the alcohol. Z-Phe-CH_2OH (20 mg, 63.8 μmol) was dissolved in 5 ml of pyridine and treated with 5 ml of acetic anhydride. The resulting solution was stirred at room temperature for 12 h, then 5 ml of water was added, and the whole mixture was concentrated leaving a white powder as residue. This was dissolved in ethyl acetate, washed well with water, dried over anhydrous magnesium sulfate, and concentrated. The residue, a buff-colored powder, weighed 15.6 mg (68.8% yield).

Z-Phe-CH_2OH *N*-Acetylglycylglycine Ester. To a solution of 12 g (28.9 mmol) of tetrabutylammonium acetylglycylglycinate in 100 ml of dry acetonitrile was added 500 mg (1.5 mmol) of Z-Phe-CH_2Cl in acetonitrile. After stirring for 24 h at room temperature the solution was concentrated, diluted with water, and extracted several times with ethyl acetate. The organic extract was dried over anhydrous sodium sulfate and concentrated. The residue was chromatographed on a column of 10 g of silica gel. After a preliminary fraction was eluted with ethyl acetate, the product was eluted with isopropyl alcohol yielding a crystalline residue, 50 mg, of the desired ester. It was shown by TLC in ethyl acetate to be free of Z-Phe-CH_2Cl. Anal. ($C_{24}H_{27}O_7N_3$) C, H, N.

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