of product which contained trace amounts of impurities from side fractions. HPLC analysis of several product batches showed the presence of 0.8–1.5% of the D-histidine containing tripeptide. Further characterization data are given in Tables III and IV.

<Glu-His-Pip-OMe (2). Route B. To a solution of <Glu-His-OMe[30] (2.1 g) in 40 mL of MeOH was added at 10 °C with stirring 25 mL of 95% hydrazine. The solution was allowed to warm to 20 °C, and the solvent was removed in vacuo. EtOH was added, and the precipitated solid was filtered and washed with EtOH to give 1.4 g of <Glu-His-hydrazide: R_f 0.25 (60:30:10 CMW), 0.33 (60:30:4:6 CMWA). A suspension of 1.5 g (5.37 mmol) of <Glu-His-hydrazide in 40 mL of DMF was acidified at −25 °C with 4.08 mL of 5.25 N HCl in THF (21.4 mmol) and treated with portions of isoamyl nitrite (total 0.77 mL) until a positive test on starch–KI paper was obtained. After 40 min at −25 °C, 965 mg (5.37 mmol) of methyl pipecolate hydrochloride was added, and the solution was neutralized to pH 7.2 (moist pH paper, range 6–8) with 3.5 mL of NEt_3 and stored at 5 °C for 96 h and at 25 °C for 24 h. NEt_3 was added periodically to maintain pH 7.2. Examination of the reaction mixture by TLC showed the presence of 25% of racemized histidine tripeptide: R_f 0.49 (80:20:2 CMA). The reaction mixture was filtered, and the filtrate was evaporated to a residual gum (2.8 g) which was purified by chromatography on 600 g of silica gel (Baker AR, 80–200 mesh), packed in 90:10:1 CMW. Elution with the same solvent yielded 788 mg of product 2 containing <2% of the D-His diastereoisomer as determined by TLC and the Manning–Moore procedure.[16] Product fractions containing varying amounts of D-histidine containing diastereoisomer were combined to give an additional 800 mg.

Acknowledgment. We gratefully acknowledge the help of Dr. William Randall for obtaining CD spectra, Susan Bergstrand for technical assistance, and Dr. David Cochran for helpful discussions concerning NMR spectra.

(30) Boler, J.; Chang, J.; Enzmann, F.; Folkers, K. *J. Med. Chem.* 1971, *14*, 475–476.

Inactivation of Trypsin-like Proteases by Depsipeptides of *p*-Guanidinobenzoic Acid†

Vishwas S. Ganu and Elliott Shaw*

Department of Biology, Brookhaven National Laboratory, Upton, New York 11973. *Received October 27, 1980*

A number of esters of *p*-guanidinobenzoic acid have been synthesized which contain a glycolyl peptide as the departing group. In the case of several enzymes such as trypsin and plasma kallikrein, depsipeptides were obtained which were considerably more reactive than the ethyl ester in inactivation of the protease by acyl-enzyme formation; the depsipeptide possessing $-CH_2CO$-Phe-NH_2 as a leaving group displayed the highest reactivity. They were less effective in the case of urokinase, plasmin, and urinary kallikrein. Boar acrosin was very susceptible to inactivation by both ethyl and peptidyl esters. Depsipeptides possessing a longer peptide chain and a secondary carbon as a leaving group showed lower activities. The results demonstrate the productive use of the departing group region of protease active centers to obtain selectivity.

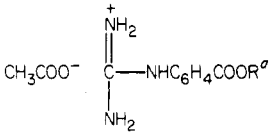
Serine proteases of trypsin-like specificity are involved in a variety of important physiological processes such as digestion, blood clotting, fertilization, biosynthesis of polypeptide hormones, and several other functions.[1] It has been suggested that affinity labels (active-site-directed agents) capable of inactivating one of these enzymes without affecting others may generate new chemotherapeutic agents.[2] Previous reports from our laboratory on affinity labeling of trypsin-like enzymes have indicated progress in design, synthesis, and effectiveness of affinity labels which can discriminate among such enzymes.[2-6] These affinity labels fall into two main classes, esters and chloro ketones, both of which inactivate by forming a covalent bond in the active center of the target enzyme. The esters act as affinity labels by forming a stable acyl-enzyme, for example, as in the hydrolysis of ethyl or nitrophenyl *p*-guanidinobenzoate. The chloromethyl ketones, on the other hand, form an enzyme complex in which a histidine side chain becomes irreversibly alkylated. Studies on the active center of enzymes such as chymotrypsin, thrombin, and elastase (all serine proteases) indicate these enzymes have an extended active site which binds at least five to six amino acid residues.[7-9] These studies also reveal that for improved substrate behavior with an enzyme, proper interactions of peptide substrates at secondary binding sites, such as S_1, S_2, S_3 and S_1', S_2', S_3', etc., are essential. (For S_1, S_2, etc. nomenclature, see ref 9.) It has already been demonstrated that tripeptidic chloromethyl ketones can provide highly selective affinity labels for trypsin-like enzymes.[4-6] They derive their discriminatory power principally due to secondary binding interactions at S_1, S_2, and S_3 subsites of the target enzyme, which are complimentary to the enzyme's natural substrate. These results prompted us to examine the possibility that esters derived from *p*-guanidinobenzoic acid (which satisfies the primary specificity requirement of trypsin-like enzymes) might be made more selective if the alcohol portion had a peptide structure. This portion of the inhibitor would occupy S_1', S_2', and S_3' secondary binding sites of the "departing group" region of the active center of the target enzyme. This region is not explored by peptidic chloromethyl ke-

†Research carried out at Brookhaven National Laboratory under the auspices of the U.S. Department of Energy.

(1) E. Reich, D. Rifkin, and E. Shaw, Eds., "Proteases and Biological Control", Cold Spring Harbor Press, Cold Spring Harbor, NY, 1975.
(2) E. Shaw, *Bayer Symp.*, **5**, 531–540 (1974).
(3) G. Glover, C. C. Wang, and E. Shaw, *J. Med. Chem.*, **16**, 262 (1973).
(4) C. Kettner and E. Shaw, *Biochemistry*, **17**, 4778 (1978).
(5) C. Kettner and E. Shaw, *Thrombosis Res.*, **4**, 696 (1979).
(6) C. Kettner, S. Springhorn, and E. Shaw, *Hoppe*-Seyler's *Z. Physiol. Chem.*, **359**, 1183 (1978).
(7) C. A. Bauer, *Biochemistry*, **17**, 375 (1978).
(8) H. A. Scheraga, in "Chemistry and Biology of Thrombin", Proceedings of a Conference on the Chemistry and Biology of Thrombin, Mayo Clinic and Foundation, Rochestor, MN, Mar 31–Apr 1, 1977, R. L. Lundblod, J. W. Fenton II, and K. G. Mann, Eds., Ann Arbor Science Publishers, Ann Arbor, MI, 1977, pp 145–159.
(9) D. Atlas, *J. Mol. Biol.*, **93**, 39 (1975).

0022-2623/81/1824-0698$01.25/0

Table I. Depsipeptides of *p*-Guanidinobenzoic Acid

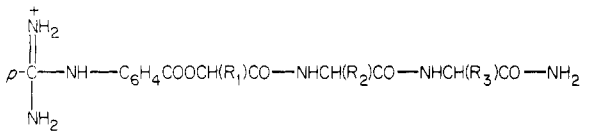


compd	R	R_f in BuOH–HOAc–H_2O	anal.	formula
PG-1	C_2H_5	0.69		$C_{12}H_{17}N_3O_4$
PG-3	CH_2CO-NH_2	0.44	C, H, N	$C_{12}H_{16}N_4O_5 \cdot H_2O$
PG-4	CH_2CO-Val-NH_2	0.55	C, H, N	$C_{17}H_{25}N_5O_6 \cdot H_2O$
PG-5	CH_2CO-Phe-NH_2	0.62	C, H; N[b]	$C_{21}H_{25}N_5O_6 \cdot H_2O$
PG-6	CH_2CO-Gly-Gly-NH_2	0.24	C, H; N[c]	$C_{16}H_{22}N_6O_7 \cdot 1.5H_2O$
PG-7[d]	CH_2CO-Ala-Phe-NH_2	0.53	C, H; N[e]	$C_{24}H_{30}N_6O_7 \cdot 1.5H_2O$
PG-8[f]	CH_2CO-Phe-Pro-NH_2	0.50	C, H, N[g]	$C_{26}H_{32}N_6O_7 \cdot 1.5H_2O$
PGA-1	$CH(CH_3)CO$-Phe-NH_2	0.59	C, H, N[h]	$C_{22}H_{27}N_5O_6 \cdot H_2O$

[a] All esters upon hydrolysis in 6 N HCl yielded at least 90% *p*-guanidinobenzoic acid measured spectrophotometrically at λ_{259} (log ϵ 11 520 in 1 mM HCl). [b] N: calcd, 15.17; found, 14.74. [c] N: calcd, 19.22; found, 18.78. [d] Amino acid analysis: Ala, 1.0; Phe, 1.04. [e] C: calcd, 53.21; found, 53.87. N: calcd, 15.51; found, 14.77. [f] Amino acid analysis: Phe, 1.0; Pro, 1.02. [g] N: calcd, 14.81; found, 15.24. [h] N: calcd, 14.73; found, 15.16.

tones. The esters would thus be depsipeptides that might derive selectivity from differences among trypsin-like enzymes in this part of the active center that would influence reagent affinity and/or rate of transfer of the guanidinobenzoic group to the active center serine. In some cases, the resultant acyl-enzyme is quite stable. For example, the half-time for deacylation of *p*-guanidinobenzoylplasmin is 36 h.[10]

In the work we report here, depsipeptides of type I have



I

been synthesized and their effectiveness to act as affinity labels for trypsin-like proteases has been studied. The sequence of amino acids which extend from the scissile bond toward the C-terminal end of the peptide substrate for various trypsin-like enzymes generally contains an amino acid in the P_1' position with a secondary α carbon. Thus, for R_1 of I to occupy the S_1' site on the target enzyme and to exert interactions comparable to the enzyme's natural substrate, synthesis of depsipeptides with a secondary alcoholic carbon would be desirable. Due to synthetic difficulties, we have examined simpler depsipeptides with R_1 = H or CH_3.

Chemistry. Depsipeptides evaluated in this study (Table I) were prepared by condensing zwitterionic *p*-guanidinobenzoic acid (PGBA) with the bromoacetyl derivative of an amino acid amide or bromoacetyl derivative of a dipeptide amide.

Results and Discussion

The new peptidyl esters of *p*-guanidinobenzoic acid caused a progressive inactivation of the trypsin-like enzymes as acyl-enzyme accumulated. At the inhibitor concentrations studied, the loss in activity followed first-order kinetics characteristic of affinity-labeling reagents acting on a catalytically essential group. It was feasible, therefore, to apply the usual kinetic treatment in which the first-order rate constant of inactivaiton, k_{app}, is divided by the inhibitor concentration to provide a reactivity constant with the dimensions of a second-order rate constant (min^{-1} M^{-1}). Table II summarizes the reactivity of

Table II. Comparison of Reactivities of Depsipeptides in the Inactivation of Trypsin-like Proteases Reactivity[a]

ester	trypsin	HPK	plasmin	uro-kinase	boar acrosin
PG-1	259	26.0		11.00	200 000
PG-3	17 000	2800	644	300	117 000
PG-4	11 200	2000	441	5.00	114 000
PG-5	46 000	8600	753	21.0	207 000
PGA-1	14 200	3000	743	32.00	ND
PG-6	10 200	2000	ND	ND	ND
PG-7	13 400	1400	ND	ND	ND
PG-8	32 000	2600	ND	ND	ND

[a] Reactivity is defined as k_{app} divided by inhibitor concentration. ND = not determined.

Table III. Comparison of the Individual Rate Constants of Esters in the Inactivation of Trypsin and Human Plasma Kallikrein

	trypsin		HPK	
ester	k_2, min^{-1}	$K_m \times 10^5$, M	k_2, min^{-1}	$K_m \times 10^5$, M
PG-1	1.89	729		
PG-3	0.523	3.07	1.25	44.5
PG-4	0.74	6.58	0.87	42.5
PG-5	3.24	7.06	0.74	8.6
PGA-1	3.7	26.0	0.1	3.29
PG-6	0.832	8.13	0.344	16.71
PG-7	0.748	5.58	0.84	59.0
PG-8	1.27	4.0	0.83	31.05

individual depsipeptides on various enzymes. With the exception of acrosin, all the depsipeptides show higher reactivity than the ethyl ester. The compound PG-5 had the highest reactivity of all the enzymes examined (with the exception of urokinase). In the case of plasma kallikrein, for example, 10^{-5} M PG-5 gave a half-time of inactivation of 8.8 min. Rat urinary and human urinary kallikreins were not susceptible to these depsipeptides.

Due to the higher reactivity of these esters in the inactivation of trypsin and human plasma kallikrein (HPK), a detailed kinetic analysis was performed to determine k_2 and K_m (Table III). Increased binding of these depsipeptides to trypsin makes them generally more reactive than the ethyl ester. Esters PG-5 and PG-8 are more effective against trypsin than PG-3, primarily due to a more rapid acylation (k_2). The parameters k_2 and K_m for PG-5 with trypsin are fivefold greater and twofold smaller than for PG-3, respectively. On the other hand, in the case of human plasma kallikrein, PG-5 is superior to other

(10) T. Chase and E. Shaw, *Biochemistry*, 8, 2212 (1969).

reagents not because of a more rapid acylation constant, k_2, but mainly due to a lower K_m (Table III). A greater reactivity of PG-4 toward HPK was anticipated due to the presence of valine in the P_2' position [in kininogen, P_2' is occupied by a valine residue]. However, the superiority of PG-5 to PG-4 suggests the considerably hydrophobic character of the S_2' site. Lower reactivity of PGA-1 with both trypsin and HPK indicates that an ester of a primary alcoholic carbon is preferred. The importance of hydrophobic interactions at the S_2' site is further suggested by the lower reactivity of PG-6 and PG-7, in which the side chain of phenylalanine either was not present (PG-6) or was moved to P_3' (PG-7). Insertion of a proline residue into the P_3' position, as in PG-8, did not enhance the reactivity of PG-5 toward trypsin or HPK. This may be due to a lesser importance of the S_3' binding site or to the unsuitability of the proline side chain for interactions there. Due to the limited number of structures examined, interpretations must remain tentative.

The reactivity of PG-5 in the inactivation of plasma kallikrein is in marked contrast to its inertness toward the urinary kallikreins. This is unexpected in view of the similar specificities of these enzymes as proteases and may be useful in distinguishing them in physiological situations in which it is not clear whether a plasma or tissue kallikrein is acting. This is currently the situation with respect to the identity of the protease responsible for the activation of prorenin, for example.[11] Peptides of arginyl chloromethyl ketones that contain a suitable sequence, such as Pro-Phe-Arg-CH_2Cl, are effective against both plasma and tissue (urinary) kallikreins.

Earlier studies on the reversible inactivation of target enzymes by aromatic esters showed the contribution in variation of the positively charged group or nature of the aralkyl skeleton toward the development of the selective affinity labels for plasmin, thrombin, or urokinase.[2,12,13] *p*-Nitrophenol was generally the departing group. In the present work, we have demonstrated the utility of depsipeptide derivatives of an aryl acid, *p*-guanidinobenzoic acid, which forms a stable acyl-enzyme derivative. The peptidyl portion promoted binding and acylation. This introduces a new structural parameter to exploit in seeking selectivity or improved reactivity. For example, modification of *p*-nitrophenyl *p*-amidinobenzenesulfonate, a known selective inhibitor of thrombin,[12] to a depsipeptide of the same acid might further enhance its properties.

Experimental Section

Enzyme Inactivation Studies. The sources of the enzymes used in this study were the same as described earlier.[4,6,12] A typical inactivation experiment consisted of incubating, at 25 °C, 5 to 50 μL of enzyme solution (10^{-8} to 10^{-9} M final), 20 to 80 μL of inhibitor dissolved in water (10^{-3} to 10^{-6} M final), and 50 mM Pipes (pH 7.0, 200 mM NaCl) buffer to give a final volume of 1 mL. Timed portions (100 μL) were removed for assay with Z-Lys-thiobenzyl ester, as described earlier.[14] Mares-Guia and Shaw[15] have already demonstrated that *p*-guanidinobenzoyltrypsin is extremely stable and that the kinetics of inactivation by ethyl *p*-guanidinobenzoate obeys the Kitz and Wilson relationship (eq 1). The pseudo-first-order rate constants (k_{app}) were obtained

$$E + I \underset{K_m}{\rightleftharpoons} EI \xrightarrow[k_2]{} E\text{-}I$$

$$1/k_{app} = 1/k_2 + 1/I(K_m/k_2) \quad (1)$$

from a semilogarthmic plot of percent control activity vs. time. The k_2 and K_m were then obtained by plotting $1/k_{app}$ against $1/I$ and performing a linear regression.

Synthesis of Reagents. Due to the hygroscopic nature of the final products, no attempt was made to determine the melting point. The elemental analysis was performed only on the final product (ester). The amino acid amides were purchased from Sigma Chemical Co.; the dipeptidic amides were either purchased from commercial sources or prepared by standard methods. Zwitterionic *p*-guanidinobenzoic acid (PGBA) and its ethyl ester hydrochloride were prepared by the method of Chase and Shaw[16] and Mares-Guia and Shaw,[15] respectively. Bromoacetyl *N*-hydroxysuccinimide ester was prepared by the method of Wilchek and Givol.[17] α-Bromopropionyl *N*-hydroxysuccinimide ester was also prepared by the identical method, mp 76–79 °C.

Synthesis of Bromoacetyl Derivatives of Amino Acid Amides and Dipeptidic Amides. To 1 mmol of an amino acid amide hydrochloride or a dipeptidic amide hydrochloride in a 50-mL recovery flask was added the minimum amount of water needed to obtain solution. Sodium bicarbonate, 1.2 equiv, was then added, followed by 25 mL of dimethoxyethane. To this was added with stirring 1.1 equiv of bromoacetyl succinate ester dissolved in 10 mL of dimethoxyethane, and the stirring was continued for an additional 1–4 h. Later the reaction mixture was acidified to pH 2.0 and DME was evaporated off on a rotary evaporator. A white precipitate was obtained, which was thoroughly washed with water and dried under vacuum. It was found advisable to use the bromoacetyl derivative without further purification. Bromoacetylvaline amide crystallized from hot acetonitrile, mp 185–190 °C, as did bromoacetylphenylalanine amide, mp 165–167 °C, and bromoacetylalanylphenylalanine amide, mp 194–197 °C. Bromoacetylphenylalanylproline amide is a hygroscopic solid, mp 55–60 °C, and gives a single spot on silica gel TLC in ethyl acetate, R_f 0.15. Bromoacetylglycylglycine amide was synthesized by reacting bromoacetyl bromide (1.9 mmol) and glycylglycine amide (1.0 mmol) at 0 °C in the presence of 2 equiv of base: mp of white solid, 215–220 °C dec; mp of α-bromopropionylphenylalanine amide, 203–206 °C.

Synthesis of Depsipeptide Ester (PG-4 thru PG-8 and PGA-1). In a 25-mL recovery flask, 1 mmol of zwitterionic PGBA, 1 mmol of bromoacetyl derivative, and 6 mL of dry DMF were combined. The reaction was stirred at 60–80 °C in an oil bath under a reflux for 24 h. The solvent was evaporated off under vacuum at 40 °C to yield an oily residue. Trituration with ethyl acetate followed by acetonitrile yielded a yellowish solid, which was dissolved in water and filtered to remove unreacted bromoacetyl derivative, and the filtrate was acidified with 1 equiv of 1 N HCl. Lyophilization yielded a pale yellow solid. For a better separation of the product from unreacted acid on an AG1-X2 (Ac^-) column, it was found useful to have a common anionic form of PGBA and ester. The pale yellow solid was dissolved in water, appied to an AG1-X2 (Cl^-) column (1.2 × 20 cm), and eluted with water. Fractions containing the product were detected on a fluorescent silica gel plate (E. Merck silica gel 60 F_{254}), pooled (elution volume 40 mL), and lyophilized to yield a white solid. This solid was dissolved in water and applied on an AG1-X2 (Ac^-) column (1.2 × 20 cm) and eluted with water. Fractions containing product as detected by silica gel TLC in BuOH–AcOH–H_2O (4:1:1) were combined (elution volume 20 mL) and lyophilized. Later fractions contain unreacted PGBA. The product, an acetate salt, is a white fluffy solid which is highly soluble in water and shows the presence of water of hydration upon analysis. Synthesis of the glycolamide ester (PG-3) was achieved by reacting zwitterionic PGBA with chloroacetamide. In general, 50 mg of chromatographically purified ester was obtained from 250–400 mg of guanidinobenzoic acid.

Acknowledgment. The technical assistance of Ms. Francis Brown is gratefully acknowledged.

(11) F. H. M. Derkx, B. N. Bouma, M. P. A. Schalekamp, and M. A. D. H. Schalekamp, *Nature (London)* **289**, 315–316 (1979).
(12) S. C. Wong, G. D. J. Green, and E. Shaw, *J. Med. Chem.*, **21**, 456 (1978).
(13) S. C. Wong and E. Shaw, *Arch. Biochem. Biophys.*, **176**, 113 (1976).
(14) G. D. J. Green and E. Shaw, *Anal. Biochem.*, **93**, 223 (1979).
(15) M. Mares-Guia and E. Shaw, *J. Biol. Chem.*, **242**, 5782 (1967).
(16) T. Chase and E. Shaw, *Methods Enzymol.*, **19**, 22–23 (1970).
(17) M. Wilchek and D. Givol, *Methods Enzymol.*, **46**, 153 (1977).