linear gradient elution from 0 to 20% solvent B (2 mL/min) for 3 min elutes, and suitably resolves, the other peptides in the following order (capacity factor indicated in parentheses): 4 (k_4 = 6.2), 3 (k_3 = 7.7), 6 (k_6 = 11.0), 5 (k_5 = 15), 8 (k_8 = 21), LH-RH ($k_{LH\text{-}RH}$ = 24), 10 (k_{10} = 27), 9 (k_9 = 44), 7 (k_7 = 60). The capacity factor k_n is a measure of the column retention volume for eluted compound n, as defined by the equation $k_n = (V_n - V_0)/V_0$, where V_n = retention volume from the time of injection to the peak maximum and V_0 = void volume or volume from the time of injection to the unretained solvent peak. In a separate experiment, isocratic elution with H_2O (0.4 mL/min) results in the emergence of suitably resolved fragments 1 (k_1 = 0.4) and 2 (k_2 = 2.0). The other peptides are strongly retained on the column and are removed by a 10-min gradient from 0 to 100% solvent B. To determine the LH-RH breakdown pattern, the eluent is collected in 1-min samples, except when 3 and 4 are eluted, at which time 0.5-min samples are collected. The radioactivity is measured by counting with Aquasol (New England Nuclear). Each radioactive peak obtained was assigned the identity of the peptide standared with which it cochromatographed.

Renal Homogenate Preparation and Incubations. Five female rats (Charles River) were decapitated and exsanguinated. The kidneys were removed, washed in buffer, and frozen at -80 °C. The defrosted kidneys were put through a tissue press at 4 °C. Five grams of pressed tissue was homogenized in 50 mL of a 10 mM phosphate buffer, pH 7.2. The homogenate was centrifuged at 105000g for 60 min. The supernatant was drawn off and stored at -80 °C. This supernatant was used for breakdown studies. [pGlu-3,4-^{3}H]LH-RH (10 μL, 0.2 mmol) was incubated at 37 °C with 40 μL of supernatant (2.6 mg of protein/mL) for 30 min. The reaction was terminated by the addition of 50 μL of CH_3CN, followed by centrifugation at 17300g for 10 min.

Incubation with Chymotrypsin. [pGlu-3,4-^{3}H]LH-RH (5 μL, 0.15 mol) was incubated for 1 h at 25 °C with α-chymotrypsin (40 ng) in a total volume of 50 μL of solution (pH 7.8) containing a final concentration of 0.05 M $CaCl_2$ and 0.04 M Tris-HCl. The reaction was terminated by the addition of CH_3CN (50 μL), followed by centrifugation at 17300g for 10 min. The supernatant was drawn off and frozen at -80 °C for later analysis.

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Synthesis of Thyrotropin-Releasing Hormone Analogues with Selective Central Nervous System Effects

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Thyrotropin-releasing hormone (TRH) analogues which show relative selectivity for action in the central nervous system have been recognized. Practical syntheses for three of these TRH analogues which show the greatest selectivity, <Aad-His-Tzl-NH_2 (5), <Glu-His-Pip-OMe (2), and <Aad-His-Pro-NH_2 (6), are described. The first two were prepared by solution methods of peptide synthesis. Compound 6 was prepared by the solid-phase method. Problems of histidine racemization, facile diketopiperazine formation, and instability of acylated thiazolidine carboxylic acid derivatives under acidic conditions have been minimized in order to attain optimal yields. Physical properties such as pK, NMR shifts, and circular dichroism have been examined as they might relate to biological activity and peptide conformation.

In a preliminary communication,[1] we have reported the synthesis and biological properties of a series of thyrotropin-releasing hormone (TRH) analogues. The primary objective of our studies of TRH analogues has been the dissociation of the hormone releasing properties[2] from the direct central nervous system (CNS) effects[3] which were thought to relate to antidepressant activity in humans.[4] The nature of the CNS effects of TRH as they relate to antidepressant activity has not been clear. Initial studies suggested that a dihydroxyphenylalanine (Dopa) potentiation activity might be important.[3] Another study has related CNS responses to enhanced turnover of noradrenalin,[5] while another relates them to cholinergic pathways.[6] These effects and their implications for therapy have been reviewed.[7]

In our initial structure–activity studies, CNS activity was measured by the ability to restore the anticonvulsant effect of methazolamide in reserpine-treated rats, a measure of noradrenergic stimulation.[8] The potency in this test has been compared to the potency of analogues for their ability to stimulate release of T_3 and T_4 using a standard type of in vivo assay.[2] As shown in Table I, structure modification

Table I. Biological Activities of TRH Analogues

analogue	activities[a] hormonal[b]	CNS[c]
<Glu-His-Pro-NH_2 (TRH, 1)	1	1
<Glu-His-Pip-OMe[d] (2)	0.2	1
<Aad-His-Pip-OMe[e] (3)	0.02	0.9
Ica-His-Pro-NH_2[f] (4)	0.1	0.35
<Aad-His-Tzl-NH_2[g] (MK 771, 5)	1	35
<Aad-His-Pro-NH_2 (6)	1	4
<Glu-His-Tzl-NH_2 (7)	0.2	2.3

[a] Potency relative to TRH. [b] Based on measurement of ^{125}I-labeled T_3 and T_4 after administration of varying doses of the analogue.[2] [c] Based on measurement of the ability of varying doses of the analogue to restore the anticonvulsant activity of methazolamide in reserpine-treated animals.[8,17] [d] Pip-OH = L-pipecolic acid. [e] Aad-OH = L-α-aminoadipic acid. [f] Ica-OH = L-2-oxoimidazolidine-4-carboxylic acid. [g] Tzl-OH = L-thiazolidine-4-carboxylic acid.

† Deceased November 12, 1980. The coauthors dedicate this paper to the memory of our friend and colleague.

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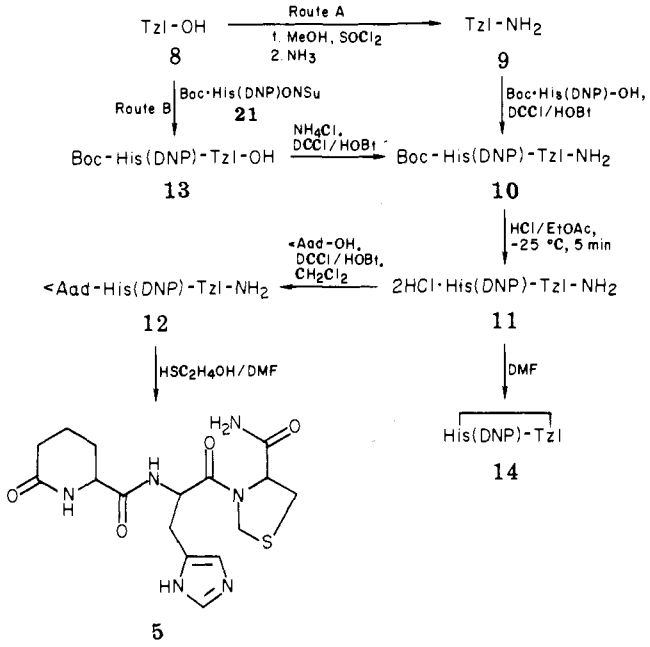
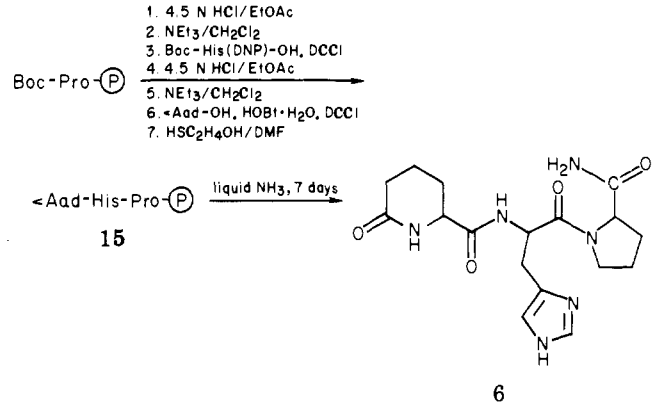
Scheme I



of TRH resulted in a relative CNS selectivity obtained by either selective reduction of hormonal effects (compounds **2**–**4**) or by selective enhancement of the CNS response (compounds **5**-**7**). Ring expansion of the pyroglutamic acid to six members enhances the CNS activity but not the hormonal potency (**6**). The same effect is seen when a sulfur replaces one methylene group in the carboxy-terminal proline as in compound **7**. These two means of selective enhancement of CNS potency can be combined in a single molecule to give an analogue which is 35 times more potent than TRH in this measure of CNS activity while remaining equipotent to TRH in hormonal activity (**5**). Enlargement of the carboxy-terminal ring to six members, coupled with replacement of the amide by a methyl ester, results in a selective reduction of the hormonal potency (compound **2**). Combining these two modifications with enlargement of the pyroglutamyl ring to six members results in a further selective reduction of the hormonal potency (compound **3**). Replacement of a methylene group of the pyroglutamyl residue by NH reduces both activities, but the hormonal response is reduced to a greater degree (compound **4**). Further compounds which show the general structure–activity relationships are given in ref 1. Those giving the best selectivities have been summarized above and in Table I.

We wish to report in particular the details of the synthesis and the physical properties of three of the TRH analogues which showed the greatest biological potency and selectivity: <Aad-His-Tzl-NH_2 (**5**), <Aad-His-Pro-NH_2 (**6**), and <Glu-His-Pip-OMe (**2**). Of this group, compound **5**, being of unusually high potency for CNS activity, is of special interest and has been the subject of detailed biological evaluations.[7-10] Practical syntheses for these

Scheme II



analogues were devised, overcoming the problems of racemization of histidine during couplings with poor nucleophiles, facile diketopiperazine formation of dipeptides containing carboxy-terminal *N*-alkyl amino acids, and instability of acylated thiazolidine carboxylic peptides during acidolytic removal of the *tert*-butyloxycarbonyl blocking group. Some physical properties of these analogues have been studied in the hope of correlating a physical property with the biological activities.

The most active analogue, <Aad-His-Tzl-NH_2 (**5**), was synthesized by stepwise solution chemistry starting with either thiazolidine-4-carboxylic acid (Tzl-OH, **8**) or thiazolidine-4-carboxamide (**9**) (Scheme I). In the preferred route, A, Tzl-NH_2 (**9**) was condensed with Boc-His(DNP)-OH by the action of dicyclohexylcarbodiimide (DCCI) in the presence of hydroxybenzotriazole[11] (HOBt) to give dipeptide amide **10** from which unreacted starting material could be removed by extractive procedures. Acidolytic removal of the Boc protecting group from the dipeptide with HCl at 0 °C or trifluoroacetic acid at 25 °C resulted in various amounts of more polar byproducts. This decomposition of the acylated Tzl-NH_2 during acid deblockings could be minimized by lowering the temperature for the HCl/EtOAc treatment to −25 °C and using the minimum time for complete deblocking. The coupling of dipeptide amide **11** with piperidonecarboxylic acid (<Aad-OH) using DCCI/HOBt proceeded in high yield when carried out in CH_2Cl_2. In DMF, however, the major reaction product was the diketopiperazine *cyclo*[His(DNP)-Tzl] (**14**). Facile diketopiperazine formation from carboxy-terminal proline dipeptide esters is well documented[12] and was also encountered in the syntheses of **2** and **6**, but such rapid cyclization of a dipeptide amide is less common.[13] Removal of the DNP blocking group was accomplished with mercaptoethanol in DMF, to give the desired analogue **5** after purification by silica gel chromatography. The dipeptide amide, Boc-His(DNP)-Tzl-NH_2 (**10**), was also prepared by route B starting with thiazolidine-4-carboxylic acid (**8**). Condensation of Tzl-OH with Boc-His(DNP)-ONSu gave dipeptide acid **13**, which

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Table II. Racemization of Histidine in the <Glu-His-N_3 + Pip-OMe Reaction

additives[e] (equiv): pH:[c]	none 8-9	none 7.0[b]	HONSu (2) 7.0	HOBt (2)[d] 7.0	HONSu (1) + HOBt (1) 7.0
96 D-His[a]	45	20-25	6	27	9

[a] Analyzed by HPLC as <Glu-D-His-Pip-OMe. [b] Azide rearrangement was more favored and a major side reaction at low pH than at high pH. [c] As measured by application to moist pH paper, range 6-8. [d] <Glu-His diketopiperazine (18) was a major byproduct. [e] HOBt = N-hydroxybenzotriazole hydrate; HONSu = N-hydroxysuccinimide.

Table III. Characterization Data of TRH Analogues

no.	amino acid anal.[a]	$[\alpha]^{25}_D$, deg (c 0.5, 50% HOAc)	pK_a	R_f[b] (system)[c]	CD, nm ($10^{-3}[\Theta]$, deg cm²/dmL) pH 9	CD, nm ($10^{-3}[\Theta]$, deg cm²/dmL) 98% dioxane
5	<$Aad^{d}_{1.04}$-$His_{0.99}$-$Tzl^{e}_{0.98}$-$(NH_2)_{1.04}$	-109.5	6.10	0.16 (A), 0.26 (B), 0.37 (C)	235 (-4.24), 214 (7.00)	224 (-16.4)
6	<$Aad_{0.98}$-$His_{1.00}$-$Pro_{1.01}$-$(NH_2)_{1.01}$	-56.4	6.18	0.15 (A), 0.24 (B), 0.23 (C)	238 (-0.70), 212 (11.0)	230 (-6.8)
2	<$Glu_{1.01}$-$His_{1.01}$-$Pip_{0.98}$-OMe	-86.5	6.18	0.36 (A), 0.53 (B), 0.52 (C)	234 (-3.21), 215 (8.60)	238 (-1.84)

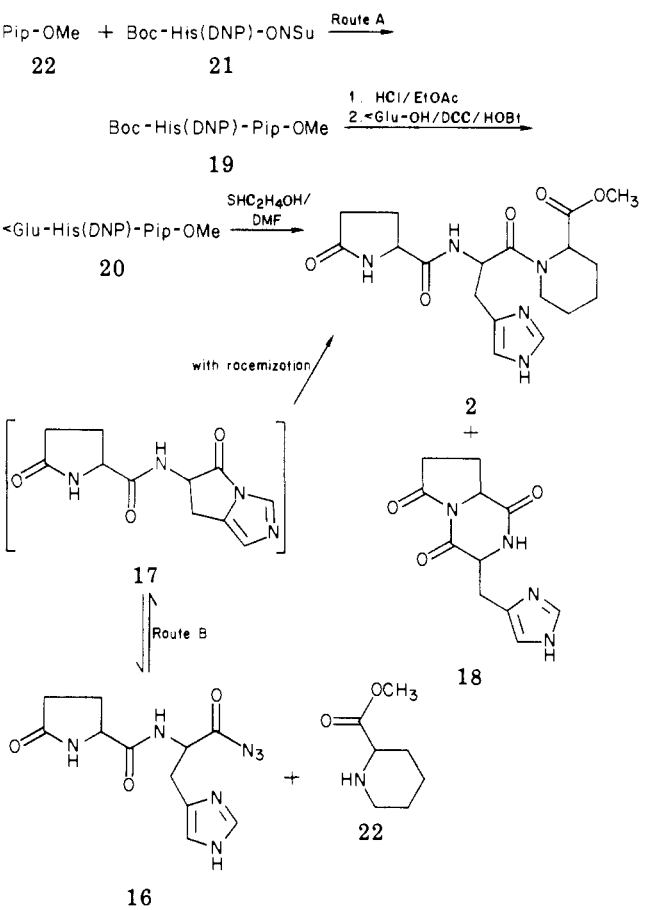
[a] After 20-h hydrolysis at 110 °C with 6 N HCl. [b] Silica gel, Analtech. [c] A = 80:20:2 CMW; B = 80:20:2 CMA; C = 10:5:1:3 EPAW. [d] Analyzed as α-aminoadipic acid. [e] Value consists of $Tzl_{0.73}$ + $Cys_{0.25}$.

was converted to the amide using ammonium chloride and DCCI activation in the presence of HOBt. Contamination of the product by Boc-His(DNP)-NH_2 necessitated purification by silica gel chromatography at this point and, therefore, made this route less favorable than route A.

<Aad-His-Pro-NH_2 (6) was synthesized (Scheme II) by the solid-phase method[14] using fewer washings, shorter washing times, and shorter coupling times, along with smaller excesses of blocked amino acids compared to usual protocols. Formation of diketopiperazine from the dipeptide resin was minimized by adding the <Aad-OH in a mixture of DMF and CH_2Cl_2 containing HOBt to the dipeptide resin and following immediately with the addition of the required amount of DCCI. By this procedure, the exposure of dipeptide ester to carboxylic acid catalyzed diketopiperazine formation conditions[12] was kept to a minimum. After the DNP group was removed, the peptide was released from the resin as the amide by treatment with liquid ammonia. After purification by chromatography, the tripeptide 6 was isolated in 93% yield based on starting amino acid content on the resin. Following the more normal time course of additions (2–5 min equilibration of blocked amino acid with deblocked peptide resin before addition of DCCI) resulted in a final product yield of only 63%.

The synthesis of <Glu-His-Pip-OMe (2) (Scheme III) by coupling of <Glu-His-azide with methyl pipecolate (route B) resulted in 25% of D-His tripeptide formation as described previously.[15] Further studies have now shown that racemization in this reaction can be suppressed to a minimum of 6% D-His containing tripeptide by the addition of hydroxysuccinimide (HONSu) to the azide coupling (Table II). The racemization of histidine has been shown to result via the more easily racemizable cyclic acylimidazole 17 in the slow coupling with methyl pipecolate.[15] The suppression of racemization by adding HONSu is consistent with trapping "out" the racemizing intermediate as the hydroxy succinimide ester which, although still a good acylating agent, is much less prone to racemization than 17. An alternate and more practical method was devised which resulted in product with >98% optical purity. The tripeptide was synthesized by the stepwise solution method as shown in route A (Scheme III). Histidine was introduced as the DNP derivative to give 19. Extreme ease of diketopiperazine formation was again observed after removal of the Boc protecting group at the

Scheme III



dipeptide ester stage. The cyclization was minimized by the addition of <Glu-OH to the dipeptide followed by immediate activation with DCCI. Even though the carboxy-terminal methyl ester showed extreme lability toward intramolecular aminolysis to form diketopiperazine, the final tripeptide methyl ester 2 showed high stability toward intermolecular ammonolysis which allowed purification by chromatography on silica gel using a solvent system containing ammonium hydroxide without loss of product due to amide formation. The final product was found to contain <2% of the D-His diastereomer as shown by high-pressure liquid chromatography (HPLC) and Manning–Moore[16] analysis.

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The methods described above all give practical yields and have been used to prepare quantities of the tripeptides in excess of 10 g each.

Physical and Conformational Properties. Various physical properties of these TRH analogues have been examined in the hope of finding a correlation between the physical or conformational properties of the analogues and their biological activities.

Titration of the imidazole of histidine showed pK_a values very similar to TRH (Table III), pointing out that neither hormonal nor CNS potencies can be correlated with the pK_a of histidine.[18]

CD measurements (Table III) in H_2O and dioxane of the two C-terminal amide analogues **5** and **6** gave spectra similar to TRH.[19] In dioxane, similar to TRH, intramolecular hydrogen bonding between an amide proton of the C-terminal amino acid and the histidine carbonyl can be postulated based on the strong negative ellipticity at 224 and 230 nm in **5** and **6**, respectively. The spectrum of analogue **2**, a C-terminal ester, is similar to TRH (**1**) and analogues **5** and **6** in H_2O. On the other hand, it does not show the increased negative ellipticity in dioxane seen for the C-terminal amides **5** and **6**. The hydrogen-bonded conformation of amides proposed in ref 19 can be postulated to be more essential for hormonal action on the pituitary than for CNS activity, explaining the selective lowering of hormonal action of the esters **2** and **3** (Table I).

Attempts to correlate the selective CNS activities of the analogues with solution conformational features as measured by proton NMR in Me_2SO-d_6 and D_2O were unsuccessful. The three analogues showed many of the typical features of TRH,[20] such as the nonequivalence of the protons next to the nitrogen in the C-terminal residues, the nonequivalence of the histidine β-hydrogens, and the low-field "anti" trans carboxamide protons in **5** and **6**. The chemical-shift differences between the geminal hydrogens of the CH_2 group next to the nitrogen in the carboxy-terminal amino acid were more pronounced in Me_2SO-d_6 than in D_2O and were equal in magnitude to those observed in TRH for analogue **6** and larger than in TRH for analogues **5** and **2**. <Aad-His-Tzl-NH_2 (**5**) showed chemical-shift differences of 0.7 and 0.5 ppm in Me_2SO-d_6 and D_2O, respectively. A surprisingly large difference of 1.72 ppm in Me_2SO-d_6 was observed for the ϵ protons of the minor (cis) isomer in the pipecolate analogue **2**. The major (trans) isomer showed a chemical-shift difference of 0.94 ppm. The low vicinal coupling constants (0 and 4 Hz) of the α protons in both the cis and the trans isomers of the pipecolic acid residue in **2** are evidence for the α proton being in an equatorial position and the bulky methyl carboxylate group therefore having to assume an axial position. This preferred axial conformation of an α substituent in an acylated piperidine has been observed previously.[21]

The cis/trans peptide bond ratio of the C-terminal amino acid in the analogues was found to be similar to that observed in TRH. A lower coalescence temperature of 47 °C was observed for interconversion of the rotamers of the most active analogue **5**, corresponding to a free energy of activation of 16.8 kcal.[22] In TRH the barrier to rotation about this amide bond is greater ($\Delta G^{\ddagger}$ = 20.1 kcal), showing a coalescent temperature around 100 °C.

In spite of these interesting observations in the NMR spectra which indicate conformational preferences in solution, there is no basis for any correlation with the biological activities. There is also no reason to believe that the facilitated C–N bond rotation seen in **5** has any biological implication.

Increased lipophilic character of these analogues compared to TRH is indicated by consistently greater R_f values on silica gel chromatography in a variety of solvent systems. The greater observed CNS activities could relate to facilitated transport into the CNS. Again, however, no direct evidence for this exists.

Thus, we have examined molecular properties of these interesting TRH analogues involving each amino acid residue individually as well as the whole taken collectively in the form of overall physical properties. These properties have been examined in both water and organic solvents which might more closely represent the receptor environment. We have not been able to find a single property which we can correlate with the biological properties, even though many of these properties have been previously proposed to have some role in at least one of the biological properties of TRH and analogues. Our failure to find correlations of the physical properties studied to the biological selectivity and potency is probably a consequence of the complex interactions in vivo involving absorption, metabolism, transport, distribution, and receptor interactions. For whatever reasons, these analogues do show interesting pharmacologic properties different from those of TRH. The properties as a CNS stimulant both of a cholinergic and adrenergic type of analogue (**5**) compared to TRH have been reported in detail,[8-10] as well as the possible therapeutic applications of compounds showing these properties.[7]

Experimental Section

Analtech silica gel plates were used for TLC; the following solvent systems were used: CMW, $CHCl_3$–MeOH–H_2O; CMA, $CHCl_3$–MeOH–concentrated NH_4OH; EPAW, EtOAc–pyridine–HOAc–H_2O. Spots were visualized by chlorination of amide bonds using *tert*-butyl hypochlorite in hexane (1%). Excess reagent was destroyed by heating at 100 °C for 4 min. Dark blue spots develop with 1% starch/KI solution. HPLC analyses of <Glu-His-Pip-OMe were carried out using a Whatman Pell ODS precolumn and a Waters μBondapak C_{18} column as the stationary phase and 0.01 M NH_4OAc, pH 4.3/CH_3CN (93:7) as the mobile phase. Elution times were 7.71 and 9.46 min for <Glu-D-His-Pip-OMe and <Glu-L-His-Pip-OMe, respectively.

Tzl-NH_2 (9). To a suspension of Tzl-OH (**8**; 10 g, 0.075 mol) in 100 mL of MeOH was added at 0 °C 20 mL of thionyl chloride. The suspension was stirred for 24 h at 25 °C. Evaporation of the solvent and trituration of the residual oil with benzene yielded 13.41 g (97% yield) of Tzl-OMe·HCl, mp 164–166.5 °C.

A solution of Tzl-OMe·HCl (5 g, 0.027 mol) in 30 mL of liquid ammonia in a sealed vessel was kept at 25 °C for 24 h. The ammonia was allowed to evaporate, and the crude amide product was partially dissolved in *i*-PrOH–MeOH, filtered, evaporated, and purified by chromatography using a 700-g silica gel 60 (E. Merck) column which was eluted with CMW (90:10:1) at a rate of 70 mL/45 s per fraction. Fractions containing product with

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an R_f of 0.32 (90:10:1 CMW) were combined and evaporated to a residual oil, which crystallized upon cooling. Trituration with benzene yielded 3.03 g (98% yield) of pure product, mp 96–98.5 °C (lit.[23] 97–99 °C).

Boc-His(DNP)-Tzl-NH_2 (10). Route A. To a mixture of **9** (132 mg, 1 mmol), Boc-His(DNP)-OH[24] (421 mg, 1 mmol) and HOBT (162 mg, 1.2 mmol) in 5 mL of CH_2Cl_2 was added DCCI (206 mg, 1 mmol), and the suspension was stirred at 25 °C for 4 h. The precipitated dicyclohexylurea was removed by filtration, and the filtrate was extracted four times with saturated bicarbonate solution, three times with 5% citric acid solution, and once with saturated bicarbonate solution. The CH_2Cl_2 solution was dried with $MgSO_4$ and filtered, and the filtrate was evaporated to give 511 mg (96% yield) of dipeptide **10**: R_f 0.47 (90:10:1 CMW), 0.6 (90:10:1 CMA); IR ($CHCl_3$) 2.98, 3.09, 3.27 (NH), 5.95, 6.02 (C=O), 6.22, 6.51, 7.47 (DNP) μm; NMR ($CDCl_3$) δ 1.45 [s, 9, $C(CH_3)_3$], 3.05–3.5 (m, 4, His β-CH_2, Tzl β-CH_2), 4.2 (d, 1, J = 9 Hz, 1 SCH_2N), 4.7–4.9 (m, 2, His α-CH, 1 SCH_2N), 5.1 (m, 1, Tzl α-CH), 5.7 (d, 1, J = 8 Hz, NH), 6.06 (s, 1, NH), 7.05 and 7.67 (2 s, 1, 1, imidazole CH), 7.82, 8.67, 8.9 (d, dd, d, 1, 1, 1, J = 9, 9 and 2, and 2 Hz, DNP aromatic CH).

Boc-His(DNP)-Tzl-NH_2 (10). Route B. Preparation of Boc-His(DNP)-ONSu (21): A solution of Boc-His(DNP)-OH (4.21 g, 10 mmol) and *N*-hydroxysuccinimide (1.15 g, 10 mmol) in 24 mL of THF was cooled in an ice bath, DCCI (2.27 g, 11 mmol) was added, and the suspension was stirred at 5 °C for 2.5 h. Dicyclohexylurea started to precipitate within 3 min. The mixture was filtered and the filtrate was evaporated to a viscous oil. Trituration of the oil with two portions of ether gave **21** as a gummy solid, which showed slight contamination with DCCI (IR band at 4.8 μm). The crude product was dissolved in 20 mL of CH_2Cl_2, filtered, and precipitated with cyclohexane. The cloudy supernatant was decanted and the residual oil was dried in vacuo to constant weight (5.06 g). The IR spectrum of the amorphous product in $CHCl_3$ was consistent with the structure of **21**, showing characteristic IR bands in $CHCl_3$ at 5.55, 5.65, 5.80, 5.90 μm. Several preparations of **21** contained various amounts of Boc-His(DNP)-OH. This contaminant in the active ester made isolation of Boc-His(DNP)-Tzl-NH_2 (**10**) considerably more difficult.

To a suspension of Tzl-OH (**8**; 16.1 g, 0.121 mol) and Boc-His(DNP)-ONSu (**21**; 62.5 g, 0.121 mol) in 400 mL of DMF was added portionwise a total of 24 mL (0.165 mol) of NEt_3 over a 7-h period, keeping the pH of the reaction at 7.2–7.6 (moist pH paper, range 6–8). The reaction mixture was stirred at 25 °C for 24 h, after which period all starting material had dissolved. The solution was evaporated in vacuo to a thick oil, dissolved in 1 L of CH_2Cl_2, and extracted with two 500-mL portions of 5% aqueous citric acid and two 500-mL portions of water. The CH_2Cl_2 layer was dried over anhydrous $MgSO_4$ and filtered, and the filtrate was evaporated to a gum (63 g). The crude Boc-His(DNP)-Tzl-OH [**13**; R_f 0.3 (80:20:2 CMA)] contained a large amount of Boc-His(DNP)-OH (R_f 0.25).

Crude dipeptide acid **13** (63 g) was dissolved in concentrated NH_4OH and lyophilized to give 56.9 g of the crude ammonium salt of **13**. The IR spectrum in $CHCl_3$ showed carbonyl bands at 5.89 and 6.12 μm and an increase in intensity of the absorption band at 6.22 μm (COO^-).

To a mixture of the crude ammonium salt of **13** (29.59 g, 0.0525 mol), HOBt–H_2O (12.3 g, 0.08 mol), and NH_4Cl (14.3 g, 0.267 mol) in 250 mL of CH_2Cl_2 was added with stirring at 25 °C a solution of DCCI (15 g, 0.073 mol) in CH_2Cl_2 (45 mL). Adjustments of pH to 7.2 (as measured by moist pH paper, range 6–8) were made periodically by the addition of a total of 13.8 mL (0.095 mol) of NEt_3. After 1.5 h, the suspension was filtered, the volume of the filtrate was adjusted to 1.5 L by the addition of CH_2Cl_2, and the solution was extracted 3 times with 350-mL protions of H_2O. The CH_2Cl_2 layer was dried over $MgSO_4$ and filtered, and the filtrate was evaporated to a thick oil. Precipitation of the crude product three times from CH_2Cl_2 with petroleum ether yielded 28.4 g of crude dipeptide amide **10** [R_f 0.47 (90:10:1 CMW)] contaminated with Boc-His(DNP)-NH_2 [R_f 0.39 (90:10:1 CMW)] and dicyclohexylurea. The dipeptide amide **10** was isolated by chromatography using 2.2 kg of silica gel 60 (E. Merck) and the solvent mixture $CHCl_3$–*i*-PrOH–H_2O (90:10:0.5) as eluent. Fractions containing single-spot product, as shown by TLC, were combined and evaporated to give 12.95 g (46.3%) of **10**, identical (IR, NMR, TLC) with product obtained by route A. Side fractions gave an additional 3.12 g (11.1%) of **10** containing trace amounts of impurities as indicated by TLC analysis. In a separate experiment using purified dipeptide acid **13**, conversion to amide proceeded in 87.5% isolated yield.

His(DNP)-Tzl-NH_2·2HCl (11). A solution of **10** (12.7 g, 0.024 mol) in 318 mL of EtOAc in a 1-L round-bottom flask equipped with drying and gas inlet tubes was cooled to −25 °C. Hydrogen chloride gas was bubbled through the solution at −25 °C for 5 min with vigorous agitation (manual swirling) of the solution. A large amount of the HCl was removed by a 5-min-long vigorous N_2 purge at 0 °C, during which period most of the product precipitated. Petroleum ether (600 mL) was added to the suspension, the mixture was filtered, and the solid was washed several times with ether. The product (11.9 g), R_f 0.35 (80:20:2 CMA), was dried in vacuo for 2 h and used immediately in the preparation of **12**.

<Aad-His(DNP)-Tzl-NH_2 (12). To a mixture of **11** (11.9 g, 0.0234 mol), <Aad-OH[25] (3.72 g, 0.026 mol), and HOBT·H_2O (5.96 g, 0.039 mol) in 450 mL of CH_2Cl_2 was added DCCI (6.95 g, 0.034 mol). After the mixture stirred at 25 °C for 8 min, triethylamine (7.5 mL, 0.052 mol) was added, and after an additional 5 min the solution was adjusted to pH 7.2 (as measured with moist pH paper, range 6–8) with an additional 6.5 mL (0.045 mol) of NEt_3. After a total reaction time of 1 h, the granular dicyclohexylurea was removed by filtration, the filtrate was evaporated in vacuo to a volume of 75 mL, and the product was precipitated with 250 mL of petroleum ether. Precipitation of product with petroleum ether after dissolution in CH_2Cl_2 was done twice to remove most of unreacted DCCI. Crude **12** (28.5 g) was obtained in the form of a gum. The crude tripeptide was purified by chromatography using a silica gel 60 (E. Merck) column (silica gel/peptide, 75:1) packed in CMW (75:25:2.5). The column was eluted with this solvent mixture at a rate of 70 mL/45 s per fraction. Fractions containing product with R_f 0.6 (80:20:2 CMW) were combined and side-fractions were rechromatographed. A combination of two reactions starting with 12.7 g (0.0237 mol) and 11.4 g (0.0213 mol) of **10** yielded 17.13 g (68%) of **12**.

When a reaction was carried out in DMF, instead of CH_2Cl_2, the major product was the diketopiperazine of His(DNP)Pro (**14**), R_f 0.8 (80:20:2, CMW), negative ninhydrin reaction (no amino group), and having an NMR spectrum consistent with diketopiperzine.

<Aad-His-Tzl-NH_2 (5). A solution of **12** (17 g, 0.0304 mol) in 540 mL of DMF and 60 mL of mercaptoethanol was kept at 25 °C for 30 min. The solvents were evaporated in vacuo to give 29 g of oily residue, which was chromatographed on a 2.2-kg silica gel 60 (E. Merck) column by eluting with CMA (70:30:3). Fractions containing product with R_f 0.26 (80:20:2 CMA) were combined, evaporated to a small volume, and lyophilized to give 10.72 g (90%) of product, 99% pure (TLC quantitation),[26] and an additional 1.00 g (8.4%) of product from side-fractions. The IR spectrum in $CHCl_3$ showed an absence of the characteristic bands for the DNP group (6.22, 6.51 and 7.48 μm). Further characterization data are given in Tables III and IV.

<Aad-His-Pro-[P][27] (15). Boc-Pro-resin (50 g, 54 mmol of Boc-Pro esterified[14] to 2% cross-linked polystyrene–divinylbenzene) was treated according to the following scheme for

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(24) Boc-His(DNP)-OH (Bachem) contains varying amounts of EtOH which forms Boc-His(DNP)-OEt as a minor byproduct during the coupling. Evaporation (2×) of the reagent with toluene removes all solvated EtOH.

(25) Greenstein, J. P.; Winitz, M. *Chem. Amino Acids* **1961**, *3*, 2411. L-Aad-OH was obtained by resolution of DL-Aad-OH using a modification of the procedure described in ref 25, p 1874. Ac-DL-Aad-OH (21.5 g) was hydrolyzed at 40 °C with 1.0 g of hog kidney acylase (NBC) in the presence of 0.01 N $CoCl_2$ at pH 6.85 for 20 h to give 6.9 g of L-Aad-OH.

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(27) Because of problems with the composition system, the usual symbol for polymer support resin, a circled P, will be represented in the text as [P].

Table IV. ^{1}H NMR Data[a] of TRH Analogues

	<Aad-His-Tzl-NH$_2$ (5)		<Aad-His-Pro-NH$_2$ (6)		<Glu-His-Pip-OMe (2)	
	Me_2SO	D_2O	Me_2SO	D_2O	Me_2SO	D_2O
% cis bond:	16	23	14	16	30	25
coalescence temp, °C:	47	80	105	>95	117	>95
C-terminal amino acid						
CH_2N, trans	4.30 (d, 8)	4.51 (d, 9)	3.22 (m)	3.54 (dd, 10, 7)	3.04 (t, 13)	3.12 (t, 13)
	4.96 (d, 8)	4.95 (d, 9)	3.56 (m)	3.85 (dd, 10, 7)	3.98 (d, 13)	4.00 (d, 13)
CH_2N, cis					2.54 (t, 13)	2.74 (t, 12)
					4.26 (d, 13)	4.28 (d, 12)
α-CH, trans	4.70 (m)	4.82 (m)	4.20 (m)	4.41 (dd,[b] 9, 5)	5.20 (d, 4)	5.23 (d, 3)
α-CH, cis					4.92 (s)	4.89 (s)
NH_2	7.24		7.02			
	8.42		8.00			
His α-CH, trans	4.78 (m)	5.00 (t, 9)	4.62 (q, 7)	4.94 (t, 7)	5 (m)	5.22 (t, 7)
His β-CH_2, trans	2.87 (dd, 14, 6)	3.14 (dd, 15, 8)	2.80[c]	3.02 (dd, 15, 8)	2.70 (dd, 15, 8)	2.98 (dd, 15, 8)
	3.06 (dd, 14, 8)	3.09 (dd, 15, 8)	2.96 (15, 8)	3.14 (15, 7)	2.90 (dd, 15, 5)	3.12 (dd, 15, 6)

[a] NMR data were obtained at 300 MHz using a Varian SC 300 spectrometer. Chemical shifts are given in parts per million downfield from Me_4Si in Me_2SO and DSP in D_2O; splitting patterns and coupling constants are given in parentheses. [b] Apparent splitting. [c] No structure.

solid-phase peptide synthesis: (1) CH_2Cl_2 wash, 3 × 2 min; (2) 4.5 N HCl–EtOAc deprotection, 2 and 15 min; (3) EtOAc, MeOH, CH_2Cl_2 washes, 1 × 2 min each; (4) 1:9 (v/v) NEt_3–CH_2Cl_2 neutralization, 1 and 3 min; (5) CH_2Cl_2 washes, 6 × 2 min; (6) blocked amino acid in a minimum volume of CH_2Cl_2 or DMF–CH_2Cl_2, followed by DCCI in CH_2Cl_2 (1:1 v/v), 15 min; (7) DMF, MeOH, CH_2Cl_2 washes, 1 × 2 min each.

All washes were 400 mL. For the preparation of dipeptide-resin, 2.5 equiv of Boc-His(DNP)-OH were needed for complete coupling; for the preparation of tripeptide-[P], <Aad-OH (10.75 g, 75 mmol) was dissolved in 80 mL of DMF–CH_2Cl_2 (1:3 v/v) containing HOBt·H_2O (8.75 g, 57 mmol), the solution was added to the peptide-[P], and the DCCI–CH_2Cl_2 solution (50 mL, 121 mmol) was added immediately. The dinitrophenyl blocking group was removed by the treatment of the tripeptide-[P] with NEt_3–DMF (1:9 v/v), 1 × 2 min; three DMF washes, and mercaptoethanol–DMF (1:9 v/v), 1 × 30 min. After final washings of the peptide-[P] with DMF (2×), CH_2Cl_2 (2×), HOAc (3×), MeOH (4×), and CH_2Cl_2 (3×), 58.9 g of <Aad-His-Pro-[P] was obtained.

<Aad-His-Pro-NH$_2$ (6). A suspension of <Aad-His-Pro-[P] (58.9 g) in 210 mL of liquid ammonia was kept at 25 °C for 7 days. The ammonia was allowed to evaporate. The product–resin mixture was suspended in MeOH and filtered, and the filtrate was evaporated to give 25.5 g of tripeptide amide (6), which was purified by chromatography using 2.2 kg of silica gel 60 (E. Merck). Product was eluted with CMW (70:30:3), 70-mL fractions were taken every 45 s, and product fractions were combined after TLC evaluation. Fractions containing a single-spot product yielded 10.6 g of product 6. Side-fractions were rechromatographed to give an additional 8.18 g (total yield 92.8%) of product of >99% purity as judged by TLC.[26] Further characterization data are given in Tables III and IV.

Methyl Pipecolate Hydrochloride (22). To 270 mL of methanol at 0 °C was added dropwise with stirring 54 mL of thionyl chloride. L-Pipecolic acid *l*-tartrate[28] (27 g) was added, and the suspension was stirred for 24 h at 25 °C. The solvents were evaporated in vacuo, and benzene (toluene recommended as alternate) was evaporated off the oily residue 3 times to remove water. The crude product was redissolved in methanol and ether was added to turbidity. A small amount of amorphous solid was filtered off and the filtrate was kept at 5 °C for 24 h. A first crop of 8.70 g [mp 177–180 °C; $[\alpha]^{25}_D$ –9.80 (*c* 1, 6 N HCl), –6.2° (*c* 2, H_2O) (lit.[29] mp 168–170 °C; $[\alpha]_D$ –6.9 (c 10, H_2O))] was obtained and additional crops of a total of 6.2 g with identical rotations were obtained from the mother liquor by further addition of ether.

Boc-His(DNP)-Pip-OMe (19). A mixture of methyl L-pipecolate hydrochloride (22; 19.1 g, 0.106 mol), Boc-His(DNP)-ONSu (21; 63.85 g, 0.123 mol), and 10 mL of NEt_3 in 150 mL of CH_2Cl_2 was stirred for 96 h at 25 °C. The pH of the reaction was adjusted periodically to 7.6 (moist pH paper, range 6–8) with a total of 15 mL of NEt_3. The suspension was filtered, and the volume of the filtrate was adjusted to 800 mL by the addition of CH_2Cl_2 and extracted with 300 mL of H_2O, 15 400-mL portions of saturated bicarbonate solution, 2 400-mL portions of 1% citric acid solution, and 400 mL of H_2O. The CH_2Cl_2 layer was dried over $MgSO_4$ and after evaporation of solvent gave 50.3 g (87% yield) of product 19: R_f 0.7 (90:10:1 CMA); IR ($CHCl_3$) 2.98 (NH), 5.78, 5.87, 6.10 (C=O), 6.22, 6.52, 7.47 (DNP) μm; NMR ($CDCl_3$) δ 1.35–1.9 [m, 14, $C(CH_3)_3$, Pip γ- and δ-CH_2, 1 Pip β-CH_2], 2.16 (d, 1, *J* = 12 Hz, 1 Pip β-CH_2), 2.6–3.4 (m, 3, His β-CH_2, 1 Pip ε-CH_2), 3.71 (s, 3, OCH_3), 3.97 (d, 1, *J* = 14 Hz, 1 Pip ε-CH_2), 5.06 (m, 1, His α-CH), 5.3–5.65 (m, 2, Pip α-CH, NH), 7.05 and 7.67 (2 s, 1, 1, imidazole CH), 7.8, 8.6, 8.88 (d, dd, d, 1, 1, 1, *J* = 9, 9 and 2, and 2 Hz, DNP aromatic CH).

<Glu-His(DNP)-Pip-OMe (20). Boc-His(DNP)-Pip-OMe (19; 19.0 g, 34.8 mmol) in 150 mL of EtOAc was cooled to 0 °C. A vigorous stream of HCl was allowed to bubble through the solution for 9 min, followed by a stream of N_2 for 10 min. Deblocked dipeptide was precipitated with 200 mL of ethyl ether, filtered, and dried for 2 h in vacuo to give 17.5 g (98% yield) of His(DNP)-Pip-OMe·2HCl. This product could only be visualized on TLC as the diketopiperazine [R_f 0.4 (90:10:1 CMA), 0.4 (90:10:1 CMW), no terminal amino group (ninhydrin)].

To a suspension of HOBT·H_2O (8.1 g, 53 mmol) and <Glu-OH (9.1 g, 70 mmol) in 315 mL of acetonitrile at 0 °C was added dipeptide dihydrochloride (17.5 g, 33.7 mmol), followed immediately by the addition of 29.1 mL of DCCI solution (0.5 g/mL of CH_2Cl_2) and NEt_3 (20.5 mL). The pH of the mixture was adjusted to 7.2–7.6 with an additional 2.5 mL of NEt_3, and the reaction mixture was allowed to warm to room temperature. After 1 hour the suspension was filtered, and the volume of the filtrate was adjusted to 2 L by the addition of CH_2Cl_2 and extracted with four 400-mL portions of saturated bicarbonate solution. The organic layer was evaporated to a volume of about 100 mL, 400 mL of ether was added, and the product was extracted into a total of 600 mL of 2.5% aqueous citric acid. The acidic layers were combined and backwashed once with 100 mL of ether and then neutralized with $NaHCO_3$. The oily product was extracted into a total of 1.5 L of EtOAc, and the organic layers were dried over $MgSO_4$ and evaporated to 17.2 g (89% yield) of <Glu-His(DNP)-Pip-OMe (20): R_f 0.43 (90:10:1 CMA).

<Glu-His-Pip-OMe (2). Route A. The DNP blocking group was removed by dissolving the blocked tripeptide 20 (17.2 g) in 170 mL of mercaptoethanol–DMF (1:9 v/v) and keeping the solution at 25 °C for 30 min. The solution was evaporated in vacuo, and the residual oil was purified by chromatography on a 2-kg column of silica gel 60 by elution with CMA (85:15:1.5). Fractions containing product [R_f 0.2 (90:10:1 CMA)] were combined, evaporated in vacuo, and lyophilized to give 7.6 g (63% yield) of tripeptide 2 (single spot by TLC) and 2.33 g (19.3%)

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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.

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

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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-

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