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Evaluation of the Inhibitory Activity on Serine and Aspartic Proteases of 4-Amino-4H-1,2,4-triazole and 5-Aminothiazole Derivatives Structurally Related to β-Lactam Antibiotics

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Abstract—Twenty new derivatives of 4-amino-4H-1,2,4-triazole and 5-aminothiazole have been examined for their inhibitory potential towards serine and aspartic proteases. Upon prolonged incubation with enzyme, the phenylacetylaminothiazolium salts exhibit progressive, time-dependent inhibition of chymotrypsin according to a first-order process. The formation of a tetrahedral transition state-like complex by attack of the active-site serine at the C2-position of the pseudobase form of the thiazolium may be responsible for the observed effect. Triazolium salts appeared to be simple competitive inhibitors of this enzyme, effective in the mM range concentration. Poor inhibitions of trypsin and pepsin were also obtained in the triazolium series. In spite of their structural analogy with β-lactams, the selected derivatives failed to inhibit human leucocyte elastase.

Proteases are implicated in a wide variety of biological and pathological processes and constitute targets for suitable therapeutic inhibitors. Leucocyte elastase, a serine protease released by the azurophilic granules of polymorphonuclear neutrophils (PMN) has attracted considerable interest. Under certain pathological circumstances, a human leucocyte elastase (HLE)-antiprotease imbalance can occur and results in degenerative diseases including emphysema (Janoff 1985), atherosclerosis (Keeley et al 1989) and rheumatoid arthritis (Bender et al 1986). Recently, substituted cephalosporin esters and amides (Doherty et al 1986) and monocyclic β-lactams (Firestone et al 1990; Maillard et al 1990; Wakselman et al 1991) have been developed as mechanism-based inhibitors of HLE. We present here new potential inhibitors of elastase and other proteases structurally related to β-lactams. 5-Acylaminothiazolium salts, 4-acylamino-4H-1,2,4-triazolium salts, 4-acylamino-1,2,4-triazoline-3-thiones and 4-acylamino-1,2,4-triazoline-3-ones, bearing a free carboxylate function in accordance with β-lactam antibiotics, have been previously selected for their potential inhibitory activity on bacterial serine DD-peptidases (Pirotte 1990) and some of these 5-phenylacetylaminothiazolium salts were found to be antistaphylococcal agents (Pirotte et al 1991).

Several of the compounds selected for the present study contain a *tert*-butoxycarbonylmethyl moiety as do the known β-lactam *tert*-butyl esters described as powerful HLE inhibitors (Doherty et al 1986). Interaction of 4-acylamino-4H-1,2,4-triazolium salts **1–10** and 5-phenylacetylaminothiazolium salts **11–13** (Fig. 1) with a serine protease may lead to the formation of a transition-state analogue by nucleophilic addition of the active serine at the 5- or 2-position of the azolium ring, respectively. Triazolinethiones **14–17** and triazolinones **18–20** are conceived as inhibitors possibly leading to thiocarbamoyl-enzyme and carbamoyl-enzyme, respectively.

To check the selectivity of action on HLE and because some of them bear structural features allowing a potential recognition by other proteases, their interaction was examined towards serine proteases of elastic (HLE, porcine pancreatic elastase (PPE)), tryptic (trypsin, thrombin) and chymotryptic (chymotrypsin) specificity, and towards an aspartic protease (pepsin).

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Materials and Methods

Materials

Human leucocyte elastase (HLE), porcine pancreatic elastase (PPE), bovine α-chymotrypsin, porcine pepsin and bovine trypsin and human thrombin were obtained from Elastin Products Co, Serva, Cooper Biochemicals, Sigma and Boehringer-Mannheim, respectively. The commercial sources of the chromogenic substrates were Bachem, methoxysuccinyl-alanyl-alanyl-prolyl-valyl *p*-nitroanilide (MeO-Suc-Ala_2-Pro-Val-NA) for HLE; Sigma, succinyl-alanyl-alanyl-analyl *p*-nitroanilide (Suc-Ala_3-NA) for PPE; Serva, succinyl-alanyl-alanyl-prolyl-phenylalanine *p*-nitroanilide (Suc-Ala_2-Pro-Phe-NA) for chymotrypsin; Boehringer-Mannheim, benzoyl-arginyl *p*-nitroanilide (Bz-Arg-NA) for trypsin; Kabi Vitrum, D-phenylalanine-L-pipecolyl-L-arginine *p*-nitroanilide (S-2238) for thrombin; Protogen AG, pyroglutamyl-histidyl-*para*-nitrophenyl-alanyl-phenylalanyl-alanyl-alanyl-leucinamide (Pyr-His-Phe(NO_2)-Phe-Ala_2-Leu-NH_2) for pepsin. Active-site titrations were carried out with *N*-benzyloxycarbonyl-alanyl-alanyl-prolyl-azaalanyl-*p*-nitrophenyl ester (Z-Ala-Ala-Pro-Aala-Ala-ONp) for both elastases (Powers & Gupton 1977), *p*-nitrophenylacetate (PNPA) for chymotrypsin (Bender et al 1966) and *p*-nitrophenyl *p'*-guanidinobenzoate (NPGB) for trypsin and thrombin (Chase & Shaw 1970). PNPA and NPGB were from Sigma; Z-Ala-Ala-Pro-Aala-Ala-ONp was a kind gift

Compound number	R_1	R_2	R_3	X
A				
1[a]	H	H	H	Br
2[a]	H	H	$C(CH_3)_3$	Br
3[a]	CH_3	H	$C(CH_3)_3$	Br
4[b]	CH_3CO	H	-	-
B				
5[c]	CH_3CO	-	$C(CH_3)_3$	-
6[a]	CH_3CO	CH_3	$C(CH_3)_3$	Br
7[b]	$C_6H_5CH_2CO$	H	-	-
8[c]	$C_6H_5CH_2CO$	-	$C(CH_3)_3$	-
C				
9[a]	$C_6H_5CH_2CO$	CH_3	H	Br
10[a]	$C_6H_5CH_2CO$	CH_3	$C(CH_3)_3$	Br
11	$C_6H_5CH_2CO$	H	H	Br
12	$C_6H_5CH_2CO$	H	CH_3	Br
13	$C_6H_5CH_2CO$	H	$C(CH_3)_3$	Br
14	H	H	$C(CH_3)_3$	-
15	CH_3CO	H	$C(CH_3)_3$	-
16	CH_3CO	CH_3	$C(CH_3)_3$	-
17	$C_6H_5CH_2CO$	H	$C(CH_3)_3$	-
18	H	H	$C(CH_3)_3$	-
19	CH_3CO	H	$C(CH_3)_3$	-
20	$C_6H_5CH_2CO$	H	$C(CH_3)_3$	-

[a] Isolated as the bromide B form; [b] isolated as the zwitterionic A form; [c] isolated as the zwitterionic C form.

FIG. 1. Structures of the compounds investigated.

from Dr J. Bieth, Université Louis Pasteur, F-67400 Illkirch, France. The concentrations (in molarities) of enzyme solutions have been expressed as active site concentrations determined as described above except for pepsin (spectrophotometric determination using $A^{1\%}_{280}$ of 1·46 (Pohl et al 1984)). All kinetic studies were performed using a Lambda 5 Perkin Elmer spectrophotometer equipped with a thermostated cell holder. The synthesis of the investigated compounds was previously reported (Pirotte & Delarge 1990; Pirotte et al 1992) except for **5**, **8** and **10** (see below). As a probable result of free rotation at the amide C-N bond, the tertiary amide compounds **6**, **9**, **10** and **16** showed no apparent E/Z mixtures of stable rotamers.

Chemical synthesis

*4-Acetylimino-1-*tert*-butoxycarbonylmethyl-4H-1,2,4-triazolium inner salt (5).* A solution of 4-amino-*tert*-butoxycarbonylmethyl-4H-1,2,4-triazolium bromide (**1**) (Pirotte et al 1992) (2·0 g) in acetic anhydride (12 mL) was heated at 60°C for 1 h. The solution was stirred with water (100 mL) for 15 min and then concentrated under reduced pressure. The residue was dissolved in water (50 mL) and neutralized with $NaHCO_3$. The resulting solution was extracted thrice with chloroform (100 mL) and the organic solution was dried over Na_2SO_4, filtered and concentrated. The solid residue was crystallized in toluene. Compound **5**. (0·88 g, 51%), mp 174–175°C; ^{1}H NMR (DMSO-d_6,HMDS) δ 1.35 (9H, s, $C(CH_3)_3$), 1.70 (3 H, s, CH_3CO), 5.10 (2 H, s, N-CH_2-), 8.95(1H, s, H_3),10.55 (1 H, s, H_5); anal. calcd for $C_{10}H_{16}N_4O_3$: C 49.99, H 6.71, N 23.32; found: C 49.69, H 6.74, N 23.53.

*1-*tert*-Butoxycarbonylmethyl-4-phenylacetylimino-4H-1,2,4-triazolium inner salt (8).* A solution of 4-phenylacetylamino-4H-1,2,4-triazole (Pirotte et al 1992) (5·0 g) and *tert*-butyl bromoacetate (9·65 g) in nitromethane (75 mL) was heated at 80°C for 3 h. After elimination of the solvent under reduced pressure, the residue was partitioned between aqueous 2% (w/v) $NaHCO_3$ (50 mL) and chloroform (150 mL). The organic layer was washed with water (50 mL), dried over Na_2SO_4, filtered and concentrated. The solid residue was crystallized in toluene. Compound **8**. (5·8 g, 74%), mp 184–186°C; ^{1}H NMR (DMSO-d_6, HMDS) δ 1·35 (9 H, s, $C(CH_3)_3$), 3·25 (2 H, s, CH_2-Ph), 5·10 (2 H, s, N-CH_2-), 7·20 (5H, m, C_6H_5), 9·03 (1 H, s, H_3), 10·60 (1 H, s, H_5); anal. calcd for $C_{16}H_{20}N_4O_3$: C 60.74, H 6.37, N 17.71; found: C 60.46, H 6.64, N 17.53.

*1-*tert*-Butoxycarbonylmethyl-4-(*N*-methyl-*N*-phenylacetylamino-)4H-1,2,4-triazolium bromide (10).* A solution of 4-(*N*-methyl-*N*-phenylacetylamino)-4H-1,2,4-triazole (Pirotte et al 1992) (2·0 g) and *tert*-butyl bromoacetate (3·6 g) in nitromethane (20 mL) was heated at 70°C for 6 h. Diethyl ether (300 mL) was added and the oil which separated was isolated after decantation. It was partitioned between water (20 mL) and chloroform (150 mL). The aqueous layer was extracted once again with chloroform (100 mL) and the combined organic layers were dried over Na_2SO_4, filtered

and concentrated to give an oil. Trituration of the oily residue with dry diethyl ether (50 mL) gave a white solid which was filtered under anhydrous conditions, washed with dry diethyl ether and dried under vacuum. Compound **10**. (2·38 g, 63%), mp 86–90°C; ^{1}H NMR (DMSO-d_6, HMDS) δ 1.40 (9H,s,C(CH_3)$_3$), 3·60 (3H,s,N-CH_3), 3.85 (2H,s,CH_2-Ph), 5.45 (2H,s, N-CH_2-) 7·25 (5H,s,C_6H_5), 9·62 (1H,s,H_3), 10.70 (1H,s,H_5); anal. calcd for $C_{17}H_{23}N_4O_3Br$. 1.5 H_2O:C 46.58, H 5.98, N 12.78; found:C 46.44, H 5.73, N 12.91.

Enzyme activity assays

The amidolytic activities of the various proteases were determined at 25°C towards the appropriate chromogenic substrate. The total assay volume was 1 mL in the following buffers: 0·1 M Tris (pH 8·0), 0·01% (v/v) Brij 35, 0·02% NaN_3 for HLE, 0·1 M Tris (pH 8·0) for PPE, 0·1 M HEPES (pH 7·5), 0·5 M NaCl for chymotrypsin, 0·05 M Tris (pH 8·0), 0·01 M $CaCl_2$ for trypsin, 0·05 M Tris (pH 7·5), 0·04 M NaCl, 0·1% (v/v) PEG 6000 for thrombin and 0·1 M sodium acetate (pH 5·0) for pepsin. The concentration of the chromogenic substrate was usually 70–100 μM for HLE and PPE, 30–60 μM for chymotrypsin, 400 μM for trypsin, 16·7 μM for thrombin, 0·5 mM for pepsin, and the enzyme concentration was: 160 nM (PPE), 13–54 nM (HLE), 20–40 nM (chymotrypsin), 70 nM (trypsin), 0·5 nM (thrombin) and 20 nM (pepsin).

Enzyme inhibition procedure

Method A. The enzymes were assayed spectrophotometrically at 25°C by continuous monitoring (405 nm) of the release of *p*-nitroaniline from the chromogenic substrate using the incubation mixture that contained test compounds in the appropriate buffer (see above). The experimental conditions are summarized in Table 1. The effect of a 10-min preincubation before the addition of the chromogenic substrate was also investigated. When non specified, the percentage of DMSO (v/v) varied from 2 to 10% for reagents **8**, **10**, **14–16**, **18–20** in the two methods.

Table 1. Experimental conditions for the evaluation of compounds **1–18** according to method A.

Enzyme	Enzyme (nM)	Substrate (μM)	Compound C (mM)	Compound C Number
HLE	13	70	1–4	All except **2**, **14–18**
	54	15	4	**14–16**, **18**
	54	15	50	**2**
	54	100	0·15	**17**
PPE	35.10^3	30	0·7–3	All except **17**
	27.10^3	100	0·15	**17**
Chymotrypsin	38	29	5	All except **8**, **10**, **17**
	13·8	25–60	1–6	**8**
	13·8	25–60	1–25	**10**
	13·8	25–60	0·15	**17**
Trypsin	66	250	1	All except **2**, **17**
	66	250	56	**2**
	66	250	0·15	**17**
Thrombin	0·5	16·7	1·5–4	**2**, **14–16**, **18**
	0·5	16·7	0·15	**17**
Pepsin	20	500	5	All except **17**
	20	500	0·15	**17**

Method B. The enzyme was preincubated with the inhibitor at 25°C and aliquots (10 μL) were checked at intervals for remaining activity. This method was used for compounds which presented an inhibition effect (real or suspected) after 10 min of preincubation (see above, method A). HLE (1·3 μM) and PPE (16 μM) were treated at 25°C and pH 8·0 (0·1 M Tris, and 0·02% NaN_3, 0·01% Brij 35 HLE), 5% (HLE) and 10% (PPE) v/v DMSO by the reagents **14**, **18** (1·3–5 mM) and **17** (150 μM). The reaction was stopped by dilution in 0·4 or 1 mL of the appropriate standard assay mixture. For the other enzymes, the concentrations were: chymotrypsin 0·4 μM, **8**, **10–13** 1–10 mM with 2% (v/v) DMSO for **8** and **10**, trypsin 2·8 μM, **14–16** 2·8 mM, **17** 150 μM in 5% (v/v) DMSO. Enzyme activity was compared with that of a blank containing the same percentage of DMSO but without reagent, to correct for spontaneous inactivation of the enzyme. For chymotrypsin, the inhibition first-order rate constant, k_{obs}, was obtained from the slope (in the initial phase) of the semilogarithmic plot of remaining enzyme activity vs time.

Determination of the hydrolysis rate constants

The disappearance of reagents **11–13** was followed at 324 nm in 0·1 M HEPES, 0·5 M NaCl at pH 7·5 and 25°C. The resulting curve-fit to an exponential function allowed the determination of the corresponding first-order rate constants.

Results and Discussion

Interaction of the investigated compounds with HLE and PPE

HLE and PPE were exposed to compounds belonging to the 4 series using either the preincubation method in which the enzymes were incubated for a maximum period of 60 min with large amounts of reagents (5 mM for **18** for example), or the method in which the reagent competes with the chromogenic substrate.

As expected, compounds bearing a free carboxylate function are not inhibitors of HLE. HLE is known to cleave peptide linkages between small hydrophobic amino acids. Doherty et al (1986) noted that neutral cephalosporins devoid of a free carboxylate at position 4 are considerably more potent inhibitors of HLE than compounds bearing that function, even remote from the cephem nucleus. Similarly, clavulanate does not inhibit HLE while benzyl clavulanate is an elastase inhibitor (Finke et al 1990). No inhibition was observed with neutral derivatives of triazolinethiones (**14–17**) and triazolinones (**18–20**) bearing an ester instead of a free carboxylate function. Positively charged compounds and zwitterionic compounds (**1–13**) failed to inhibit HLE and PPE.

Effect on trypsin-like and aspartic proteases

Almost all the investigated compounds failed to inhibit significantly trypsin and thrombin. For example, using a large molar excess of **2** (1 mM) over enzyme (0·5 nM), the observed decrease of the hydrolysis rate of S-2238 catalysed by thrombin was not significant. A larger effect ($\sim$ 50%) was observed for trypsin at the following concentrations of inhibitors: E 66 nM, **2** 56 mM, pH 8·0 and 25°C. Owing to its positive charge, **2** is potentially able to bind to thrombin and

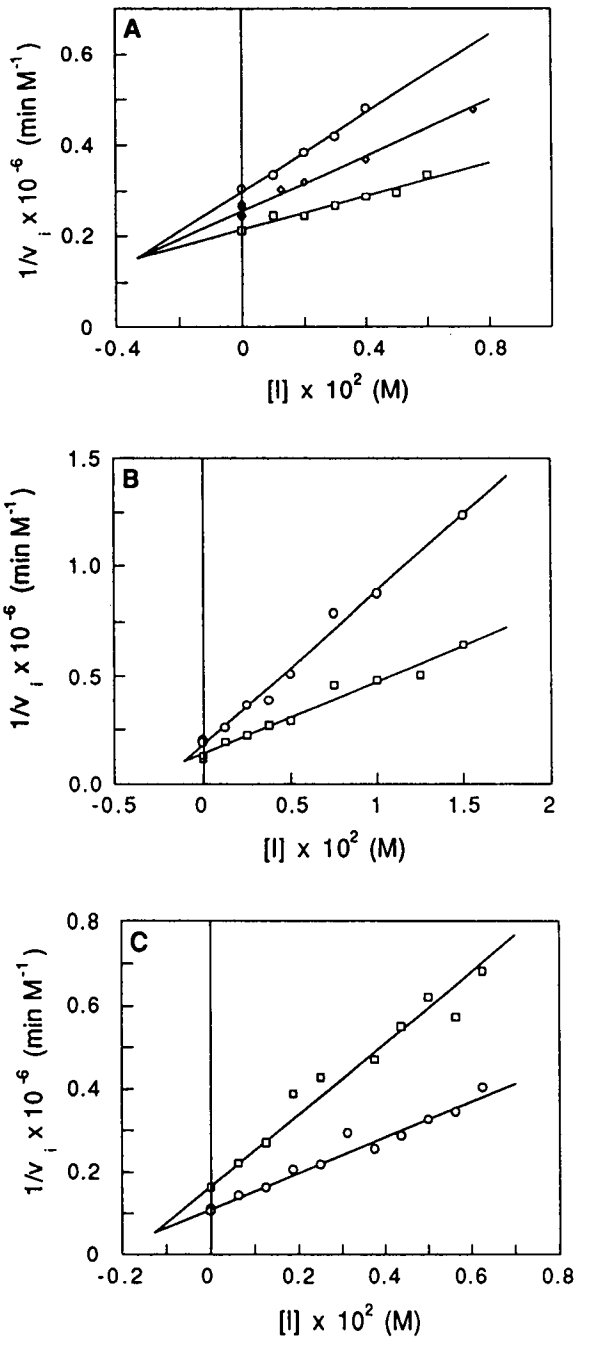


FIG. 2. Dixon plot of the inhibition of chymotrypsin by **8** (A), **10** (B) and **13** (C) with Suc-Ala_2-Pro-Phe-NA as substrate. The initial rates were determined in 0·1 M HEPES, 0·5 M NaCl, pH 7·5 at 25°C, 8 (**8** and **10**) and 10% (**13**) (v/v) DMSO. The enzyme concentration was 13·8 nM. The substrate concentration (μM) was: 25 (○), 35 (◇), 45 (□) in A; 35 (○), 60 (□) in B; 25 (○); 45 (□) in C.

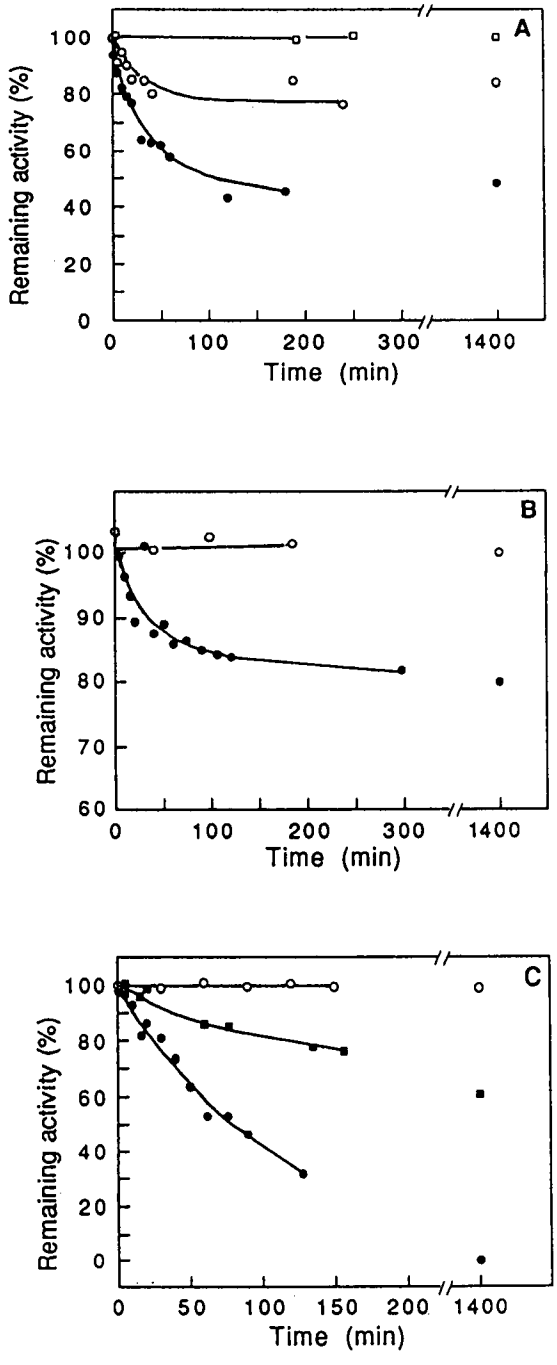


FIG. 3. Evolution of the activity of bovine chymotrypsin (0·4 μM) in the absence (□ A; ○ B and C) and in the presence of **11** (○ 2 mM; ● 10 mM) (A), **12** (● 1 mM) (B) and **13** (■ 0·5 mM; ● 5 mM) (C) at 25°C and pH 7·5 (0·1 M HEPES, 0·5 M NaCl). Activity remaining was determined against Suc-Ala_2-Pro-Phe-NA at pH 7·5 and 25°C as described in Materials and Methods.

trypsin active sites but large amounts of inhibitor are necessary to detect an appreciable inhibitory effect on trypsin. This suggests a poor affinity of this enzyme for the reagent.

Pepsin was inhibited in the presence of **8**, **10** and **19**. The enzyme activity was decreased by 20% (**8** and **10**) and by 15% (**19**) in the presence of 5 mM inhibitor at pH 5 and 25°C. Pepsin is able to cleave Phe-X peptide bonds. The presence of the phenylacetyl (**8** and **10**) or *tert*-butyl side chains may be responsible for the observed poor binding to the active site.

Inhibition of chymotrypsin by thiazolium (11–13) and triazolium (8,10) salts

The effect of the investigated compounds on chymotrypsin activity was examined. An inhibition process was observed with compounds **8** and **10–13** (Figs 2, 3). The chymotrypsin-

catalysed hydrolysis of Suc-Ala_2-Pro-Phe-NA was slower in the presence of **8**, **10** and **13**. As can be seen in Fig. 2, the pattern of the Dixon plots indicates that in all cases a competitive inhibition is observed (inhibition constants, K_i of ~3·3 (**8**), 1·0 (**10**) and 1·5 (**13**) mM). **7** and **9**, the corresponding acids of **8** and **10**, poorly inhibited chymotrypsin; 22 and 17% inhibition was observed at 5 mM, respectively. In this 1,2,4-triazolium series, only *N*-phenylacetylated substituted reagents display an inhibitory efficiency (in particular, no effect is observed with the acetylated compounds). No noticeable inhibition of chymotrypsin was observed using the other reagents.

In the thiazolium series, the immediate competitive effect is followed by a slow inactivation process as evidenced using the preincubation method (Fig. 3). The activity losses followed pseudo first-order kinetics characterized by the rate constants k_{obs}. These rate constants divided by the lowest inhibitor concentration gave a measure of the efficiency of inactivation $k_{obs}/[I] = \sim 2.2$ (**11**), 3.4 (**12**) and 5 (**13**) M min^{-1} at pH 7·5 and 25°C, and display a tendency to level off at higher concentrations. The order of inactivation efficiency is *t*-Bu > CH_3 > H (R_3 substituent).

To analyse the observed time-dependent inhibition, the stability of compounds **10**–**13** was examined in buffer solution by recording the UV spectra of the reaction mixtures at regular intervals in the presence and absence of chymotrypsin. The spectrum of the starting molecules (Fig. 4) agreed with the existence in aqueous solution of an equilibrium between the starting thiazolium structure (**10**–**13**) and a stable pseudobase **21** (Scheme 1). It was previously demonstrated that for 5-phenylacetylaminothiazolium salts in aqueous solution the nucleophilic addition of the hydroxide ion at the 2-position of the ring led to the reversible formation of a stable pseudobase with apparent pK values of

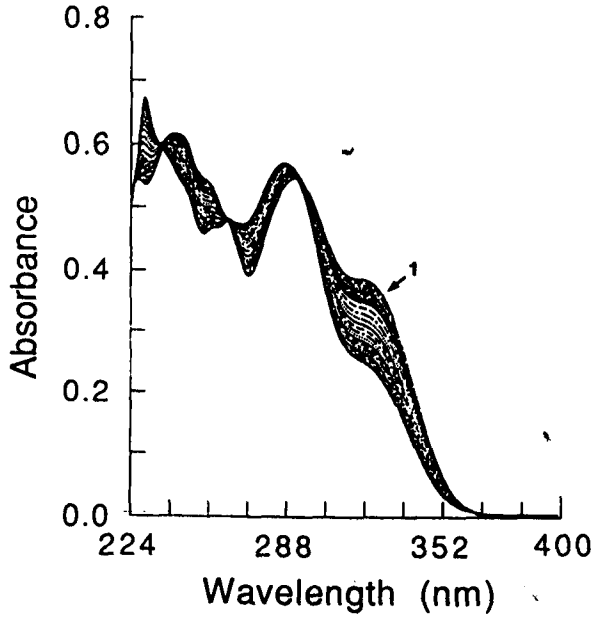
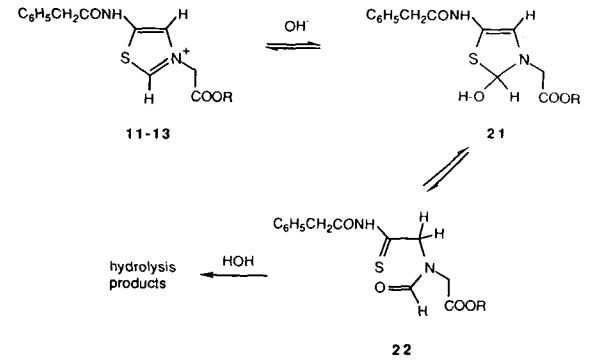


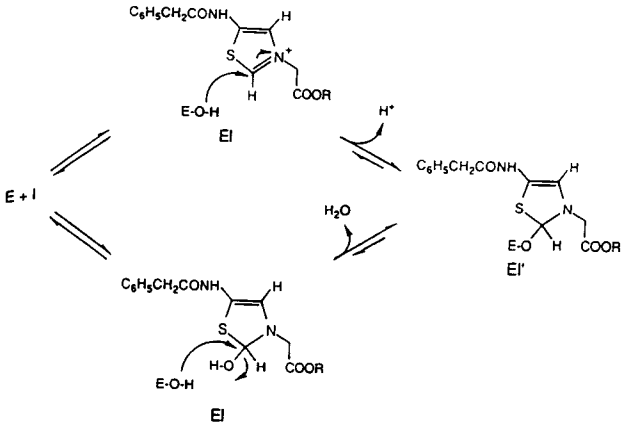
FIG. 4. Evolution of UV spectra **12** (10^{-4} M) in the presence (or in the absence) of 0·4 μM chymotrypsin at pH 7·5 (0·1 M HEPES, 0·5 M NaCl) and 25°C. The first spectrum (1) was recorded after 15 s, the next eight at intervals of 7 min and the others at intervals of 20 s.



SCHEME 1

7·85, 7·41 and 7·44 for compounds **11**, **12** and **13**, respectively (Pirotte & Delarge 1990). At pH 7·5, the pseudobase form predominates in the case of **12** (55·2%) and **13** (53·4%) but not in that of **11** (30·9%). An extremely slow spectral evolution was observed for **11** and **13** ($t_{\frac{1}{2}} = \sim 14$ h for **13** and > 14 h for **11** at pH 7·5 and 25°C) while a complex evolution was evidenced for **12**, indicating successive reactions with a first-order process characterized in the initial phase by a half-life of ~75 min. The slow decrease in absorbance at 320 nm (Fig. 4) for the disappearance of the pseudobase form (Pirotte & Delarge 1990), suggests, as observed for related compounds (Begtrup 1979), a slow ring opening leading to transient thioamide **22** and subsequent hydrolysis products (Scheme 1). No modification in the spectral evolution for compounds **10**–**13** was observed in the presence of chymotrypsin (0·4 μM).

These data suggest two main possible mechanisms for the observed inhibition with **10**–**13**: formation of a stable acyl-enzyme implicating either the phenylacetyl or the -$COOR_3$ moiety; or formation of a stabilized enzyme-inhibitor complex. An inactivation due to the acylation of active site serine 195 by the reagents is unlikely. For the chymotrypsin-catalysed hydrolysis of amides, the deacylation step is fast compared with the acylation step (Zerner & Bender 1964); the opposite is observed for esters. Thus, the accumulation of a phenylacetyl-enzyme is not expected. Moreover, an inhibition would have been observed also with compounds bearing the same phenylacetyl side chain (**7**, **9**, **17** and **20**). The half-life of the phenylacetyl-enzyme hydrolysis was reported to be 30 s at pH 7·56 and 25°C (Dupaix et al 1970). An even poorer stability of the resulting acyl-enzyme is expected for a nucleophilic attack of the ester moiety -$COOR_3$ by active serine due to a larger electron-withdrawing effect of the 4-amino-4H-1,2,4-triazole and 5-aminothiazole rings compared with the phenyl substituent. The order of magnitude of this effect can be indirectly appreciated from the comparison of the NMR chemical shifts generally reported for the methylene protons of C_6H_5-CH_2-COOR or -CONRR′ (< 4·0 ppm) and (heterocyclic) N-CH_2-COOR (> 5·0 ppm) (see NMR data of compound **10**). The acid compound **11** devoid of the corresponding ester, inactivates chymotrypsin: such a result is not consistent with the formation of a stable acyl-enzyme. However, in the case of **12**, the formation of transient acyl-enzymes implicating slowly generated degradation products derived from the ring opening of the thiazolium or pseudobase (Scheme 1) may be envisaged but



SCHEME 2

only after a significant amount of the initial form of the reagent has been transformed (a perturbation of the initial first-order process is clearly observed after 2 h). The good stability of **11** and **13** in buffer solution at pH 7·5 seems to exclude analogous side reactions for these compounds.

The experimental results with compounds **11**–**13** are consistent with the formation of a stabilized covalent adduct between enzyme and inhibitor. The mechanism shown in Scheme 2 implicating a nucleophilic attack by an active-site amino acid residue (proably serine 195) at the C2-position of either the thiazolium or pseudobase form may be evoked. The formation of a tetrahedral transition state analogue is expected from such an interaction. In the first step, the inhibitor is weakly bound. This first Michaelis complex EI is then converted to a more tightly bound complex E_I according to a first-order process. By moderate dilution (Fig. 3), the enzyme remains inhibited when previously treated with **11**–**13** (but not with **8** and **10**) suggesting that the enzyme-inhibitor complex is not dissociated to free enzyme and inhibitor. The IC50 value (730 μM) obtained after 18 h incubation of **13** with the enzyme is lowered by a factor of ~ 4 compared with that obtained without a previous incubation. The complex EI may correspond to a preassociation enzyme-inhibitor complex while EI' corresponds to a covalent adduct. The mechanism of Scheme 2 assumes initially competitive inhibition. This inhibition process was evidenced by determining steady-state velocities for the inhibition of chymotrypsin by **13** at two substrate concentrations and over the range of inhibitor concentration (Fig. 2). The conversion of a Michaelis complex to a stabilized covalent adduct has been reported for the formation of transition state analogues with serine proteases using peptide aldehydes (Kuramochi et al 1979) or boronic acids (Kettner & Shenvi 1984). We tentatively conclude that the active species on chymotrypsin could be the pseudobase since the interaction of the positively charged thiazolium moiety with the hydrophobic primary binding site of chymotrypsin is less probable.

In conclusion, among the different compounds that have been evaluated against serine proteases, the most promising agents were found to be the cationic heterocycles, thiazolium or triazolium salts, bearing in most cases the phenylacetylamino side chain. A previous report on the antistaphylococcal activity of 5-phenylacetylaminothiazolium salts (Pirotte et al 1991) explored their possible interaction with the bacterial serine DD-peptidases according to a new inhibitory mechanism. The moderate activity of the same thiazolium salts against chymotrypsin suggests a comparable mode of inhibition of the two serine enzymes. Moreover, the most active compound of this series in the two biological models was found to be the *tert*-butyl ester. A careful examination of compounds structurally related to the examined series must be done in the hope of selecting more powerful inhibitors of serine and aspartic proteases.

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