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1-PEPTIDYL-2-HALOACETYL HYDRAZINES AS ACTIVE SITE DIRECTED INHIBITORS OF PAPAIN AND CATHEPSIN B

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SUMMARY — *Fifteen 1-peptidyl-2-haloacetyl hydrazines, which can be considered halometanes of azapeptides containing Phe in P_2 and α-aza-Ala or α-aza-Gly in P_1, were synthesized and tested as models of cysteine-proteases inhibitors. By use of kinetic methods, they proved to irreversibly inactivate papain and cathepsin B* via *a reversible enzyme-inhibitor intermediate. Second-order rate constants of inactivation in the range 26-23000 $M^{-1}s^{-1}$ were observed for papain and 2000-39600 $M^{-1}s^{-1}$ for cathepsin B. K_I for the reversible EI adducts ranged from 230 to 0.16 μM for papain and from 11 to 0.37 μM for cathepsin B. Structure of possible reversible EI complex is proposed and used to discuss the effects of structural variation of the inhibitors on the kinetic parameters of inactivation. Title compounds proved to be selective for cysteine-proteases, since no inhibiting activity could be detected toward trypsin, chymotrypsin and porcine pancreatic elastase at 0.1 mM concentration, after 6 h incubation. Relatively low aspecific alkylating properties were also verified in tests using glutathione as the nucleophile.*

RIASSUNTO — *Quindici 1-peptidil-2-aloacetil idrazine che possono essere considerate alometilchetoni di azapeptidi contenenti Phe in P_2 e α-aza-Ala o α-aza-Gly in P_1 sono stati sintetizzati e provati come modelli di inibitori per le proteasi a cisteina. È stato dimostrato con metodi cinetici che essi inattivano la papaina e la catepsina B attraverso un intermedio reversibile enzima-inibitore. Le costanti del secondo ordine della velocità di inattivazione erano comprese tra 26 e 23000 $M^{-1}s^{-1}$ per la papaina e tra 2000 e 39600 $M^{-1}s^{-1}$ per la catepsina B. I valori di K_I per gli addotti reversibili EI variavano tra 230 e 0.16 μM per la papaina e tra 11 e 0.37 μM per la catepsina B. La struttura di un possibile complesso reversibile EI viene pro-*

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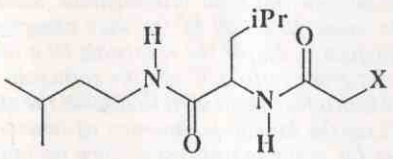
posta e impiegata per discutere gli effetti di varianti strutturali degli inibitori sui parametri cinetici di inattivazione. I composti esaminati hanno mostrato di essere inibitori selettivi per le proteasi a cisteina poiché non si è potuta rilevare alcuna inattivazione di proteasi a serina rappresentative come tripsina, chimotripsina e elastasi porcina pancreatica dopo 6 h di incubazione, con concentrazioni 0.1 mM di inibitore. Mediante tests che impiegano il glutatione come nucleofilo, è stato anche verificato che tali composti sono caratterizzati da relativamente modesta attività alchilante aspecifica.

Introduction

Cathepsin B, a lysosomal cysteine-protease present in many animal tissues [1], has been implicated in a number of pathological processes such as muscular distrophy, myocardial tissue damage [2], inflammation [3,4], tumor metastasis [5] and bone resorption [6]. A number of efficient and selective, synthetic, low molecular weight inhibitors have been developed for study and control of cathepsin B as well as other cysteine-proteases of the papain superfamily [7]. We have recently described a series of N-haloacetyl-aminoacid amides **1** as active site directed cysteine-proteases inhibitors [8]. They proved to irreversibly inactivate papain and cathepsin B via a reversible enzyme-inhibitor intermediate while no inhibiting activity could be detected toward trypsin, chymotrypsin and porcine pancreatic elastase at concentrations 100-1000-fold higher than those required to inhibit cysteine-proteases. In addition, their reactivity toward bionucleophiles like glutathione ranged from 1/2 to 1/200 that of peptidyl chloromethylketones.



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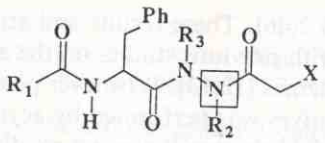
The mode of binding at the active site of papain, proposed to explain formation of reversible adducts (K_i 125-0.4 μM) and improvement of alkylation rates of Cysteine-25 thiolate by proximity effects, was based on the crystal structure of papain—E-64 complex [9,10]. As the consequence, in the reversible enzyme-inhibitor adduct, the amino acid amide moiety of the inhibitor should be oriented in a direction opposite to that of the enzyme substrate, presenting the trapping function at the amino rather than at the carboxy-terminal side of the molecule. This arrangement represents a serious disadvantage in view of the design of powerful and selective inhibitors of individual cysteine-proteases by variation of the peptidyl recognising moiety.

In this paper we describe the synthesis and the evaluation of 1-peptidyl-



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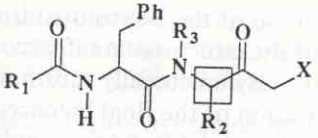

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N°	R_1CO	R_2	R_3	X	N°	R_1CO	R_2	R_3	X
2a	CH_3CO	H	H	Cl	4c	Z-Ala-Ala	H	H	I
2b	CH_3CO	H	H	Br	5a	Z	CH_3	H	Cl
2c	CH_3CO	H	H	I	5b	Z	CH_3	H	Br
3a	Z	H	H	Cl	5c	Z	CH_3	H	I
3b	Z	H	H	Br	6a	Z	H	CH_3	Cl
3c	Z	H	H	I	6b	Z	H	CH_3	Br
4a	Z-Ala-Ala	H	H	Cl	6c	Z	H	CH_3	I
4b	Z-Ala-Ala	H	H	Br					

2-haloacetyl hydrazines **2a-6c**. They can be regarded as isosters of the analogous peptidyl-halomethanes **7**. Since azapeptides are peptide analogs where the α-CH group of one or more aminoacids of the peptide chain is replaced by an N atom [11], haloacetyl derivatives **2-6** are formally related to **7** as halometanes of azapeptides where the amino acid residue in P_1 has been replaced by an α-azaaminoacid. In particular, **2a-4c** and **6a-6c** include an α-azaglycine and **5a-5c** an α-azaalanine unit. An advantage of this new model, with respect to haloacetyl aminoacid amides **1**, is the direct use of substrate specificities for the design of selective inhibitors, while low aspecific alkylating properties and inertness toward serine-proteases should be preserved.

Chemistry

The intermediate peptidyl or aminoacid hydrazides have been prepared by hydrazinolysis of the corresponding methylesters. Preparation of the isomeric inhibitors **5a-5c** and **6a-6c** required the separation of the two intermediate hydrazides Z-Phe-NH-NH-CO-CH_3 (**8**) and Z-Phe-N(CH_3)-NH_2 (**9**) which was readily achieved by silica gel chromatography. Acylation of methylhydrazine with Z-Phe methylester gave **8** as the prevailing product (58% isolated yield), while use of a more reactive mixed anhydride led to the formation largely of the other isomer **9** (43% isolated yield). The correct structure was attributed on the basis of ^1H NMR spectra: the methyl protons of the 1-substituted hydrazide **9** (δ 3.06) appear further downfield

than those of the 2-substuted isomer **8** (δ 2.46). These results and attribution of the structures are in accordance with previous studies on the acylation of unsymmetrically substituted hydrazines [12,13]. Whenever possible, preparation of the final haloacetyl derivatives was performed by acylation of the N-peptidyl-hydrazines under very mild Schotten-Baumann conditions. General application of this very convenient method was prevented by the extremely low solubility of some of the peptidyl hydrazines in common water immiscibile solvents. Mixed anhydrides of the haloacetic acids or haloacetyl N-hydroxy-succinimide esters [14] in aprotic solvents like DMF or THF were therefore used as an alternative. The tripeptide methylester Z-Ala-Ala-Phe-OCH_3 was obtained by coupling Z-Ala-Ala with phenylalanine methylester by the mixed anhydride method. All new compounds were homogeneous by TLC and gave satisfactory elemental microanalyses. The proposed structures are in accordance with their IR and ^{1}H NMR spectra.

Chemical Experimental Section

Melting points (Büchi oil bath apparatus) are uncorrected. IR spectra were obtained with a Perkin-Elmer 521 spectrophotemeter. ^{1}NMR spectra were recorded on a Varian EM 390 spectrometer using TMS as internal standard. $[\alpha]_D$ were determined with a Schmidt-Haensch 1604 polarimeter. Elemental microanalyses (C,H,N) of all new compounds were within $\pm 0.4\%$ of the calculated values.

N-Carbobenzyloxy-L-alanyl-L-alanyl-L-phenylalanine methylester

A solution of N-Carbobenzyloxy-L-alanyl-L-alanine (1.47 g, 5 mmol) and N-methylmorpholine (0.55 ml, 5 mmol) in 25 ml of anhydrous THF was cooled to -15°C and i-butylchloroformate (683 mg, 5 mmol) was added dropwise under stirring. After 30 min, L-phenylalanine methylester (0.90 g, 5 mmol, prepared from the hydrochloride, 1.08 g, by addition of N-methylmorpholine, 0.55 ml, in 25 ml THF) was added slowly, under stirring, at the same temperature. The resulting reaction mixture was stored for 15 h at 4°C, allowed to warm to room temperature and filtered. Solution was diluted with 75 ml AcOEt, washed with 1 N HCl, saturated $NaHCO_3$ and brine. After drying over Na_2SO_4, the solvent was removed under reduced pressure. Crystallization of the crude material from MeOH gave 2.15 g (95%) of the pure product: mp 192-193°C; $[\alpha]_D^{22} = -5°$(1;DMF).

IR (KBr): main peaks at 3301, 1743, 1687, 1642, 1537, 1453, 1260 cm^{-1}.

^{1}H-NMR (DMSO) δ1.10 and 1.20(two s,6H,CH_3), 2.90-3.09(m,2H,PheCH_2), 3.59(s,3H,OCH_3), 3.94-4.64(m,3H,αCH), 5.04(s,2H,Z-CH_2), 7.26 and 7.37(two s,10H,C_6H_5), 7.90(d,1H,NH,J=7.5Hz), 8.28(d,1H,NH,J=7.5Hz).

N-Carbobenzyloxy-L-alanyl-L-alanyl-L-phenylalanine hydrazide

To a solution of N-carbobenzyloxy-L-alanyl-L-alanyl-L-phenylalanine methylester (1.1 g, 2.4 mmol) in THF/EtOH 7:3 (150 ml), hydrazine hydrate (2.4 ml, 48 mmol) was added and the mixture was kept at room temperature for 6 days. The crystalline hydrazide was collected by filtration, washed with EtOH and dried under vacuum. Recrystallization from DMF-Et_2O gave 1.0 g (91%) of the pure product: mp 243-245°C; $[\alpha]_D^{22} = -5°$(1;DMF).

IR (KBr): main peaks at 3289, 1688, 1641, 1601, 1535, 1260, 1227 cm^{-1}.

^{1}H-NMR (DMSO) δ1.12 and 1.18(dd,6H,CH_3,J=6Hz), 2.47-3.07(m,2H,PheCH_2), 3.91-4.65(m,3H,αCH), 4.22(s,2H,NH_2), 5.03(s,2H,ZCH_2), 7.25 and 7.38(two s,10H,C_6H_5), 7.98(d,2H,NH,J=7.5), 9.20(s,1H,NH).

1-(N-Carbobenzyloxy-L-phenylalanyl)-2- methyl hydrazine (8)

A solution of N-carbobenzyloxy-L-phenylalanine methylester (2.2 g, 7 mmol) and methylhydrazine (1.84 ml, 35 mmol) was kept at room temperature for 24 h. After removal of the solvent at reduced pressure, the residue was purified by silica gel chromatography ($CHCl_3$). Crystallization from $CHCl_3$/Et_2O gave the pure product as white crystals: 1.82 g (58%); mp 138-140°C; $[\alpha]_D^{22} = -2°$(2;MeOH).

IR (KBr): main peaks at 3297, 3214, 1688, 1531, 1304, 1270 cm^{-1}.

^{1}H-NMR ($CDCl_3$) δ2.46(s,3H,CH_3), 2.92-3.09(m,2H,PheCH_2), 4.20-4.53(m, 1H,αCH), 5.03(s,2H,ZCH_2), 5.82(d,1H,NH,J=9Hz), 7.08-7.35(m,10H,C_6H_5), 8.05(bs,1H,NH).

1-(N-Carbobenzyloxy-L-phenylalanyl)-1-methyl hydrazine (9)

A solution of N-Carbobenzyloxy-L-phenylalanine (2.1 g, 7 mmol) and N-methylmorpholine (0.77 ml, 7 mmol) in anhydrous $CHCl_3$ (35 ml) was cooled to −15°C and i-butylchloroformate (956 mg, 7 mmol) was added dropwise, under stirring. After 30 min, a solution of methylhydrazine (0.37 ml, 7 mmol) in $CHCl_3$ (10 ml) was added slowly while the temperature of −15°C was maintained. The reaction mixture was stored for 15 h at 4°C in a refrigerator, allowed to warm to room temperature and filtered. The solution was washed with saturated $NaHCO_3$ and brine, dried over Na_2SO_4 and the solvent removed under reduced pressure to give the crude product. The desired isomer was separated by silica gel chromatography ($CHCl_3$). Crystallization from benzene-hexane gave the pure product as white crystals: 1.0 g (43%); mp 122-124°C; $[\alpha]_D^{22}$= 37°(1;$CHCl_3$).

IR (KBr): main peaks at 3343, 1693, 1658, 1535, 1261 cm^{-1}.

^{1}H-NMR ($CDCl_3$) δ2.80-3.10(m,2H,PheCH_2), 3.06(s,3H,CH_3), 3.33(s,2H,NH_2), 5.10(s,2H,ZCH_2), 5.50-5.75(m,1H,αCH), 7.11-7.51(m,11H,$C_6H_5$and NH).

Preparation of 1-Petidyl-2-haloacetyl-hydrazines. General procedure A.

To an ice cooled suspension (solution) of the required hydrazide (1 mmol) in $CHCl_3$ (5 ml), in the presence of 1 M aqueous $NaHCO_3$ (2.4 ml), a solution of the appropriate haloacetyl chloride (1.2 mmol) in $CHCl_3$ (2.4 ml) was rapidly added under vigorous stirring. Reaction mixture was allowed to warm to room temperature while stirring was continued for 30 min. The product separated as a white solid which was filtered, washed with water and $CHCl_3$ and dried.

When derivatives of methylhydrazine were employed, both the reagent and the product were soluble in $CHCl_3$. Isolation of the crude product was then achieved by dilution of the reaction mixture with $CHCl_3$ (20 ml), washing with saturated $NaHCO_3$, 1 N HCl and brine. Drying over anhydrous Na_2SO_4 and removal of the solvent under reduced pressure gave the crude product.

Preparation of 1-Peptidyl-2-haloacetyl-hydrazines. General procedure B

A solution of the required haloacetic acid (1 mmol) and N-methylmorpholine (1 mmol) in anhydrous $CHCl_3$ (5 ml) was cooled to −15°C and i-butylchloroformate (1 mmol) was added dropwise, under stirring. After 30 min, a solution of the required hydrazine derivative (1 mmol) in $CHCl_3$ (5 ml) was added

slowly while the temperature of −15°C was maintained. The reaction mixture was then stored for 15 h at 4°C in a refrigerator, allowed to warm to room temperature and filtered. The solution was washed with 1 N HCl, saturated $NaHCO_3$ and brine. After drying over Na_2SO_4, the solvent was removed under reduced pressure to give the crude product.

Preparation of 1-Peptidyl-2-haloacetyl-hydrazines. General procedure C

A solution of the required hydrazine derivative (1 mmol) and haloacetyl N-hydroxysuccinimide ester (2 mmol) was allowed to react at room temperature for 30 min. The product was precipitated by progressive addition of water (10 ml) under stirring, filtered, washed with water and dried under vacuum.

*1-(N-Acetyl-L-phenylalanyl)-2-chloroacetyl hydrazine (**2a**)*

Reaction of N-acetyl-L-phenylalanyl hydrazide (222 mg, 1 mmol) with chloroacetyl chloride (136 mg, 1.2 mmol) according to procedure A and crystallization of the crude material from EtOH, gave the pure product as white crystals: 201 mg (67%); mp 208-210°C; $[\alpha]_D^{22} = 8°$(1;DMF).

IR (KBr): main peaks at 3258, 3205, 1600, 1549, 1486, 1373 cm^{-1}.

^{1}H-NMR (DMSO) δ1.75(s,3H,CH_3CO), 2.68-3.06(m,2H,PheCH_2), 4.13(s,2H, CH_2Cl), 4.40-4.80(m,1H,αCH), 7.30(s,5H,C_6H_5), 8.20(d,1H,NH,J=9.0Hz), 10.38(bs,2H,NH).

*1-(N-Acetyl-L-phenylalanyl)-2-bromoacetyl hydrazine (**2b**)*

N-Acetyl-L-phenylalanyl hydrazide (111 mg, 0.5 mmol) and bromoacetic acid (70 mg, 0.5 mmol) were reacted according to procedure B. Crystallization of the crude material from EtOH gave the pure product as white crystals: 134 mg (78%); mp 206-208°C (dec); $[\alpha]_D^{22} = -2°$(2;DMF).

IR (KBr): main peaks at 3261, 3207, 1602, 1549, 1493, 1373 cm^{-1}.

^{1}H-NMR (DMSO) δ1.78(s,3H,CH_3CO), 2.70-3.20(m,2H,PheCH_2), 3.96(s,2H, CH_2Br), 4.44-4.83(m,1H,αCH), 7.32(s,5H,C_6H_5), 8.20(d,1H,NH,J=9.0Hz), 10.32 and 10.41(two s,2H,NH).

*1-(N-Acetyl-L-phenylanyl)-2-iodoacetyl hydrazine (**2c**)*

N-Acetyl-L-phenylalanyl hydrazide (111 mg, 0.5 mmol) and iodoacetic acid (95 mg, 0.5 mmol) were reacted according to procedure B. Crystallization of the crude material from DMF-EtOH gave the pure product as white crystals: 144 mg (73%); mp 209-210°C; $[\alpha]_D^{22} = -3°$(2;DMF).

IR (KBr): main peaks at 3259, 3207, 1595, 1548, 1493, 1373 cm^{-1}.

^{1}H-NMR (DMSO) δ1.76(s,3H,CH_3CO), 2.68-3.06(m,2H,PheCH_2), 3.74(s,2H,CH_2I), 4.44-4.78(m,1H,αCH), 7.30(s,5H,C_6H_5), 8.08-8.34(m,1H,NH), 8.31 and 8.35(two s,2H,NH).

*1-(N-Carbobenzyloxy-L-phenylalanyl)-2-chloroacetyl hydrazine (**3a**)*

Reaction of N-carbobenzyloxy-L-phenylalanyl hydrazide (156 mg, 0.5 mmol) with chloroacetyl chloride (68 mg, 0.6 mmol) according to procedure A and crystallization of the crude material from MeOH gave the pure product as white crystals: 181 mg (93%); mp 202-204°C; $[\alpha]_D^{22} = -26°$(1;MeOH).

IR (KBr): main peaks at 3267, 3206, 1694, 1605, 1538, 1487, 1267 cm^{-1}.

^{1}H-NMR (DMSO) δ2.70-3.05(m,2H,PheCH_2), 4.15(s,2H,CH_2Cl), 4.23-

4.58(m,1H,αCH), 4.96(s,2H,ZCH_2), 7.15-7.48(m,10H,C_6H_5), 7.56-7.74(m,1H,NH), 10.42(bs,2H,NH).

*1-(N-Carbobenzyloxy-L-phenylalanyl)-2-bromoacetyl hydrazine (**3b**)*

Reaction of N-carbobenzyloxy-L-phenylalanyl hydrazide (156 mg, 0.5 mmol) with bromoacetyl chloride (95 mg, 0.6 mmol) according to procedure A and crystallization of the crude material from MeOH gave the pure product as white crystals: 186 mg (86%); mp 191-193°C; $[\alpha]_D^{22} = -10°$(1;DMF).

IR (KBr): main peaks at 3267, 3198, 1696, 1606, 1542, 1485, 1267 cm^{-1}.

^{1}H-NMR (DMSO) δ2.72-3.05(m,2H,PheCH_2), 3.96(s,2H,CH_2Br), 4.10-4.54(m,1H,αCH), 4.95(s,2H,ZCH_2), 7.12-7.45(m,10H,C_6H_5), 7.50-7.58(m,1H,NH), 10.40(bs,2H,NH).

*1-(N-Carbobenzyloxy-L-phenylalanyl)-2-iodoacetyl hydrazine (**3c**)*

N-Carbobenzyloxy-L-phenylalanyl hydrazide (156 mg, 0.5 mmol) and iodoacetic acid (95 mg, 0.5 mmol) were reacted according to procedure B. Crystallization of the crude material from DMF-EtOAc gave the pure product as white crystals: 198 mg (82%); mp 211-212°C; $[\alpha]_D^{22} = -8°$(2;DMF).

IR (KBr): main peaks at 3265, 3211, 1694, 1600, 1542, 1484, 1268 cm^{-1}.

^{1}H-NMR (DMSO) δ2.74-3.07 (m,2H,PheCH_2), 3.75(s,2H,CH_2Br), 4.20-4.53(m,1H,αCH), 5.00(s,2H,ZCH_2), 7.15-7.47(m,10H,C_6H_5), 7.48-7.70(m,1H,NH), 10.40(bs,2H,NH).

*1-(N-Carbobenzyloxy-L-alanyl-L-alanyl-L-phenylalanyl)-2-chloro acetyl hydrazine (**4a**)*

N-Carbobenzyloxy-L-alanyl-L-phenylalanyl hydrazide (300 mg, 0.66 mmol) and O-chloroacetyl-N-hydroxysuccinimide (255 mg, 1.32 mmol) were reacted according to procedure C. Crystallization of the crude material from DMF-EtOH gave the pure product as white crystals: 320 mg (91%); mp 234-235°C; $[\alpha]_D^{22} = -13°$(1;DMF).

IR (KBr): main peaks at 3053, 1687, 1642, 1612, 1531, 1492, 1452, 1253, 1051 cm^{-1}.

^{1}H-NMR (DMSO) δ1.14(d,6H,CH_3,J=7Hz), 2.66-3.07(m,2H,PheCH_2), 3.94-4.40(m,2H,AlaαCH), 4.13(s,2H,CH_2Cl), 4.43-4.76(m,1H,PheαCH), 5.02(s,2H,ZCH_2), 7.08-7.55(m,11H,$C_6H_5$and NH), 7.77-8.13(m,2H,NH), 10.32(bs,2H,NH).

*1(N-Carbobenzyloxy-L-alanyl-L-alanyl-L-phenylalanyl)-2-bromoacetyl hydrazine (**4b**)*

N-Carbobenzyloxy-L-alanyl-L-alanyl-L-phenylalanyl hydrazide (282 mg, 0.62 mmol) and O-bromoacetyl-N-hydroxysuccinimide (295 mg, 1.24 mmol) were reacted according to procedure C. Crystallization of the crude material from DMF-EtOH gave the pure product as white crystals: 310 mg (87%); mp 211-213°C; $[\alpha]_D^{22} = -12°$(1;DMF).

IR (KBr): main peaks at 3048, 1687, 1641, 1609, 1530, 1486, 1452, 1256, 1050 cm^{-1}.

^{1}H-NMR (DMSO) δ1.15(d,6H,CH_3,J=7Hz), 2.66-3.06(m,2H,PheCH_2), 3.76-4.44(m,2H,AlaαCH), 3.96(s,2H,CH_2Br), 4.47-4.81(m,1H,PheαCH), 5.01(bs,2H, ZCH_2), 7.02-7.50(m,11H,$C_6H_5$and NH), 7.96(bs,2H,NH),10.36(bs,2H,NH).

*1-(N-Carbobenzyloxy-L-alanyl-L-alanyl-L-phenylalanyl)-2-iodoacetyl hydrazine (**4c**)*

N-Carbobenzyloxy-L-alanyl-L-alanyl-L-phenylalanyl hydrazide (280 mg, 0.62 mmol) and O-iodoacetyl-N-hydroxysuccinimide (353 mg, 1.24 mmol) were reacted according to procedure C. Crystallization of the crude material from DMF-EtOH gave the pure product as white crystals: 349 mg (85%); mp 202-204°C; $[\alpha]_D^{22} = -11°$(1;DMF).

IR (KBr): main peaks at 3034, 1687, 1642, 1608, 1531, 1486, 1452, 1253, 1049 cm^{-1}.

^{1}H-NMR (DMSO) δ 0.98-1.30(m,6H,CH_3), 2.65-3.10(m,2H,Phe CH_2), 3.75(s,2H,CH_2I), 3.94-4.42(m,2H,AlaαCH),4.46-4.82(m,1H,PheαCH), 5.04(bs,2H, ZCH_2), 7.05-7.55(m,11H,C_6H_5 and NH), 7.94(bs,2H,NH), 10.35(s,2H,NH).

*1-(N-Carbobenzyloxy-L-phenylalanyl)-2-methyl-2-chloroacetyl hydrazine (**5a**)*

Reaction of 1-(N-carbobenzyloxy-L-phenylalanyl)-2-methyl hydrazine (163 mg, 0.5 mmol) with chloroacetyl chloride (68 mg, 0.6 mmol) according to procedure A and crystallization of the crude material from dichloroethane-hexane gave the pure product as white crystals: 184 mg (91%); mp 123-125°C; $[\alpha]_D^{22} = -4°$(1;$CHCl_3$).

IR ($CHCl_3$) main peaks at 3428, 1710, 1497, 1384, 1293, 1241 cm^{-1}.

^{1}H-NMR ($CDCl_3$) δ 2.95(s,3H,CH_3N), 3.10(d,2H,PheCH_2,$J = 7.5$Hz), 3.74(s,2H,CH_2Cl), 4.33-4.63(m,1H,αCH), 5.08(s,2H,ZCH_2), 5.65(d,1H,NH, $J = 9.0$Hz), 7.15-7.46(m,10H,C_6H_5), 8.83(s,1H,NH).

*1-(N-Carbobenzyloxy-L-phenylalanyl)-2-methyl-2-bromoacetyl hydrazine (**5b**).*

1-(N-carbobenzyloxy-L-phenylalanyl)-2-methyl hydrazine (163 mg, 0.5 mmol) and bromoacetic acid (70 mg, 0.5 mmol) were reacted according to procedure B. Crystallization of the crude material from dichloroethane-hexane gave the pure product as white crystals: 175 mg (78%); mp 125-128°C; $[\alpha]_D^{22} = -5°$(1;$CHCl_3$). IR ($CHCl_3$): main peaks at 3429, 1711, 1498, 1388, 1291, 1248 cm^{-1}.

^{1}H-NMR ($CDCl_3$) δ 2.98(s,3H,CH_3N), 3.10(d,2H,PheCH_2,$J = 7.5$Hz), 3.58(s,2H,CH_2Br), 4.33-4.63(m,1H,αCH), 5.10(s,2H,ZCH_2), 5.70(d,1H,NH,$J = 9.0$Hz), 7.15-7.46(m,10H,C_6H_5) 9.12(d,1H,NH,$J = 6$Hz).

*1-(N-Carbobenzyloxy-L-phenylalanyl)-2-methyl-2-iodoacetyl hydrazine (**5c**)*

1-(N-Carbobenzyloxy-L-phenylalanyl)-2-methyl hydrazine (163 mg, 0.5 mmol) and iodoacetic acid (95 mg, 0.5 mmol) were reacted according to procedure B. Purification of the crude material by silica gel chromatography ($CHCl_3$-EtOAc 9:1) and cystallization from CH_2Cl_2-hexane gave the pure product as white crystals: 133 mg (52%); mp 133-135°C; $[\alpha]_D^{22} = -10°$(1;$CHCl_3$).

IR ($CHCl_3$) main peaks at 3428, 1711, 1497, 1382, 1292, 1245 cm^{-1}.

^{1}H-NMR ($CDCl_3$) δ2.95(s,3H,CH_3N), 3.11(d,2H,PheCH_2 $J = 7.5$Hz), 3.45(two s,2H,CH_2I), 4.33-4.68(m,1H,αCH), 5.10(s,2H,ZCH_2), 5.60(d,1H,NH,$J = 7.5$Hz), 7.18-7.46(m,10H,C_6H_5), 8.65(d,1H,NH,$J = 6.0$Hz).

*1-(N-Carbobenzyloxy-L-phenylalanyl)-1-methyl-2-chloroacetyl hydrazine (**6a**)*

Reaction of 1-(N-carbobenzyloxy-L-phenylalanyl)-1-methyl hydrazine (196 mg, 0.6 mmol) with chloroacetyl chloride (81 mg, 0.72 mmol) according to procedure A and purification of the crude material by florisil chromatography ($CHCl_3$-hexane

9:1) gave the pure product as a colourless thick oil: 233 mg (96%); $[\alpha]_D^{22}=22°$ (1;$CHCl_3$).

IR ($CHCl_3$) main peaks at 3427, 1711, 1678, 1501, 1393, 1261 cm^{-1}.

^{1}H-NMR ($CDCl_3$) δ2.90-3.10(m,2H,PheCH$_2$), 3.16(s,3H,CH_3N), 4.02(s,2H, CH_2Cl), 4.70-5.20(m,3H,αCH and ZCH$_2$), 5.65(d,1H,NH,J=7.5Hz), 7.12-7.48(m,10H,C_6H_5), 8.70(bs,1H,NH).

*1-(N-Carbobenzyloxy-L-phenylalanyl)-1-methyl-2-bromoacetyl hydrazine (**6b**)*

Reaction of 1-(N-carbobenzyloxy-L-phenylalanyl)-1-methyl hydrazine (245 mg, 0.75 mmol) with bromoacetyl chloride (142 mg, 0.90 mmol) according to procedure A and purification of the crude material by florisil chromatography ($CHCl_3$-hexane 9:1) gave the pure product as a colourless thick oil: 329 mg (98%); $[\alpha]_D^{22}=20°$(1;$CHCl_3$).

IR ($CHCl_3$) main peaks at 3426, 1710, 1678, 1501, 1393, 1247, cm^{-1}.

^{1}H-NMR ($CDCl_3$) δ2.87-3.09(m,2H,PheCH$_2$), 3.16(s,3H,CH_3N), 3.73(s,2H,CH_2Br), 4.63-5.18(m,3H,αCH, and Z CH_2), 5.88(d,1H,NH,J=7.5Hz), 7.07-7.51(m,10H,C_6H_5), 9.10(bs,1H,NH).

*1-(N-Carbobenzyloxy-L-phenylalanyl)-1-methyl-2-iodoacetyl hydrazine (**6c**)*

1-(N-Carbobenzyloxy-L-phenylalanyl)-1-methyl hydrazine (262 mg, 0.8 mmol) and iodoacetyl chloride [15] (196 mg, 0.96 mmol) were allowed to react according to procedure A. Washing with 3% aqueous $Na_2S_2O_5$ was included in this case. Drying over Na_2SO_4 and evaporation of the solvent under reduced pressure gave the pure product as a pale yellow thick oil: 384 mg (97%); $[\alpha]_D^{22}=29°$(1;$CHCl_3$).

IR ($CHCl_3$) main peaks at 3429, 1709, 1678, 1503, 1394, 1249, cm^{-1}.

^{1}H-NMR ($CDCl_3$) δ2.90-3.20(m,2H,PheCH$_2$), 3.14(s,3H,CH_3N), 3.65(s,2H,CH_2I), 4.80-5.15(m,3H,αCH and ZCH$_2$), 5.97(d,1H,NH,J=7.5Hz), 7.18-7.38 (m,10H,C_6H_5), 9.31(bs,1H,NH).

Biochemistry

Compounds **2a-6c** were tested as inhibitors of papain and cathepsin B catalyzed hydrolysis of N-carbobenzyloxyglycine-*p*-nitrophenylester (Z-Gly-ONp). The activity of both enzymes was irreversibly and completely inhibited by all the compounds.

Inactivation of proteases with substrate analog inhibitors proceeds according to equation (1):

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \xrightarrow{k_2} E\text{-}I \qquad (1)$$

where EI,E-I, k_{+1} and k_{-1} represent the reversible complex, the irreversibly inactivated enzyme and the rate constants for non-covalent reaction step respectively; k_2 is the rate constant of the formation of covalently modified enzyme. Recently, Tian and Tsou [16] introduced a convenient method of evaluation of inactivation rates in the presence of a substrate according to equation (2) where substrate and inhibitor are competing

$$\begin{array}{ccccc} I & \rightleftharpoons & EI & \xrightarrow{k_2} & E\text{-}I \\ + & & & & \\ E & & & & \\ + & & & & \\ S & \rightleftharpoons & ES & \xrightarrow{k_{cat}} & E+P \end{array} \quad (2)$$

for the enzyme binding site. According to this method, the decrease in enzyme concentration during the reaction obeyed pseudo-first-order kinetics. Reactions were followed by measuring the decrease in the formation of *p*-nitrophenol at $[S] >> K_m$, at various inhibitor concentrations. The temporal dependence of the absorbance change was exponential and well fitting to a first-order rate law [16]. Non-linear regression analysis of the absorbance versus time data to the equation (3), where A and A_∞ are

$$A = A_\infty\{1\text{-}e^{-k_{obs}[II]}\} \quad (3)$$

the absorbances at t and t_∞ respectively, yielded the pseudo-first-order rate constant k_{obs} for the formation of the inhibited enzyme. Data were collected for no less than three half-times to demonstrate the complete inactivation of the enzyme by the inhibitor tested. Non-linear regression analysis of the progress curve data to the equation (3) is considered the method of choice for calculation of kinetic parameters because it yields accurate values for the inhibition constants under a variety of circumstances [17]. In case of complexing competitive inhibitors, it has been shown [16,18,19] that the following equation is valid:

$$k_{obs} = \frac{k_2 K_a}{1 + \frac{[S]}{K_m} + K_a\,[I]} \quad (4)$$

where K_a is the inhibitor association constant $1/K_i$. Therefore a plot of $1/[I]k_{obs}$ versus $1/[I]$ will give k_2 as the reciprocal of the intercept on the ordinate and K_i from the slope $1/K_a k_2(1 + [S]/K_m)$. Progress curves at constant substrate and different inhibitor concentrations yielded different k_{obs} values (Fig. 1). Kinetic parameters reported in Table I were determined by fitting k_{obs} values in a plot of $1/[I]k_{obs}$ versus $1/[I]$ according to equation (5) which is a rearrangement of equation (4).

$$\frac{1}{k_{obs}\,[I]} = \frac{1 + [S]/K_m}{K_a\,k_2}\,\frac{1}{[I]} + \frac{1}{k_2} \quad (5)$$

All progress curves examined reached a steady A_∞ absorbance within the time of the experiment indicating that inhibition was always complete. When concentrated solutions (20-50-fold that used in progress curves) of papain or cathepsin B were incubated (10-20 min) with the inhibitors in the concentration range of Table I, enzymatic activity could not be restored by

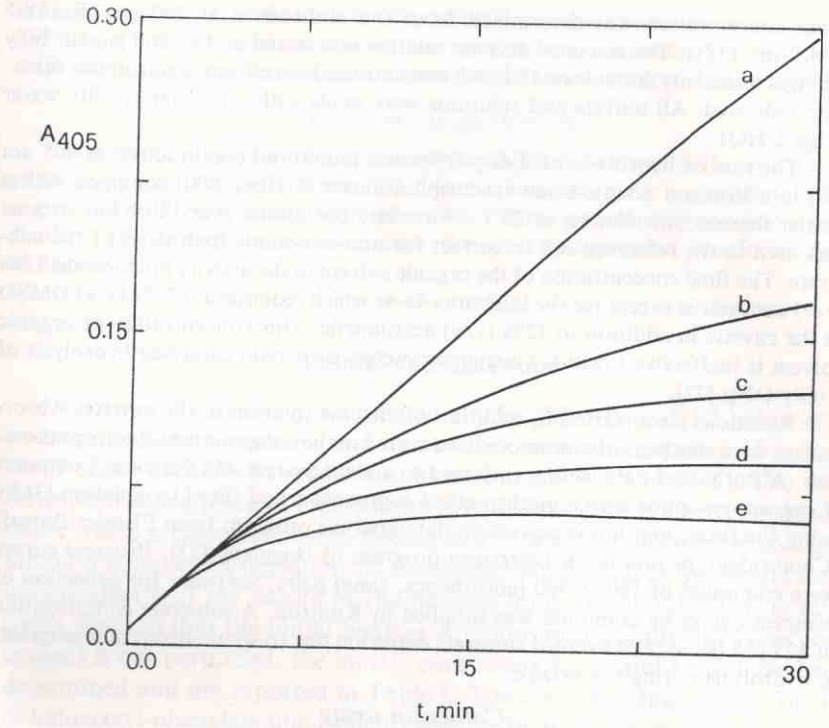

Fig. 1

Papain inactivation by compound **4c**[a].
[a]*Reaction conditions: [E]* $= 7 \times 10^{-9}$ *M, [ZGlyONp]* $= 125$ *μM, 100 mM phosphate buffer, pH 6.8,4 mM EDTA,* CH_3CN *12% (v/v), 25 °C. Inhibitor concentrations were 0.0, 1.3, 3.0, 4.4 and 6.7 μM for curves a,b,c,d,e. Reactions were started by addition of the enzyme.*

20-50-fold dilution. These data suggest that 1-peptidyl-2-haloacetyl hydrazines **2a-6c** are not hydrolytically attacked at an appreciable extent, under the range of concentrations used, since complete inhibition was independent on [E], and that they behave as irreversible inhibitors since the final enzyme-inhibitor complex was not reversible upon dilution.

Biochemical Experimental Section

Papain assay

Papain EC 3.4.22.2 was obtained from Sigma, product no P-4762, and solutions were made fresh daily by incubating the enzyme (1.5-2 mg) for 45 min at room temperature in 25 ml of 50 mM phosphate buffer pH 6.8 containing 2 mM EDTA and 0.5 mM L-cysteine according to Thompson [20] with minor modifications. En-

zyme concentration was determined from the absorbance at 280 nm ($E = 58.5$ $mM^{-1}cm^{-1}$) [21]. The activated enzyme solution was stored at 4°C and papain fully retained its activity for at least 10 h. All reagents used were from Sigma unless otherwise indicated. All buffers and solutions were made with ultra high quality water (Elga UHQ).

The rate of hydrolysis of Z-Gly-ONp was monitored continuously at 405 nm [22] in a Kontron double-beam spectrophotometer (Uvikon 860) equipped with a Peltier thermocontroller set at 25°C. A cuvette containing everything but enzyme was used in the reference cell to correct for non-enzymatic hydrolysis of the substrate. The final concentration of the organic solvent in the activity mixture was 12% (v/v) acetonitrile except for the inhibitors **4a-4c** which required a 0.75% (v/v) DMSO in the cuvette in addition to 12% (v/v) acetonitrile. This concentration of organic solvent is ineffective towards kinetic parameters of papain catalysed hydrolysis of Z-Gly-ONp [22].

Reactions were started by addition of enzyme solution in the cuvette. Absorbances were continuously monitored and stored in the computerized spectrophotometer. Absorbances data were transferred to a Data System 450 Personal Computer (Kontron) equipped with a mathematical coprocessor and fitted to equation (3) by using Enzfitter, non-linear regression data analysis program from Elsevier-Biosoft (Cambridge), or non-linear regression program of Duggleby [23]. Progress curves were composed of 180 to 360 (absorbance, time) pairs. Software for collection of progress curves on computer was supplied by Kontron. A substrate concentration of 125 μM ($K_m \times 12.5$) avoided substrate depletion due to spontaneous and enzymatic hydrolysis during the assays.

Cathepsin assay

Cathepsin B EC 3.4.22.1 from bovine spleen, product no C-6286 was purchased from Sigma and solutions were made fresh daily by incubating the enzyme under the same conditions as for papain. Enzyme concentration was determined from absorbance at 280 nm ($E^{1\%} = 20$ cm^{-1}) [24]. Enzyme activity was tested as described for papain [25].

Chymotrypsin and trypsin assay

Chymotrypsin EC 3.4.21.1 from bovine pancreas, product no 27270 was obtained from Fluka. Enzyme was dissolved in 50 mM phosphate buffer pH 6.8 and concentration determined from the absorbance at 280 nm ($E = 50$ $nM^{-1}cm^{-1}$) [26]. Chymotrypsin was assayed by monitoring the hydrolysis of N-benzoyl-L-tyrosine ethyl ester at 256 nm [27]. Trypsin EC 3.4.21.4 from bovine pancreas, code TRTPCK, was from Worthington Enzyme (Cooper Biomedical). The enzyme was dissolved in 0.001 N HCl and concentration determined from the absorbance at 280 nm [28]. Trypsin was assayed by monitoring the hydrolysis of N-benzoyl-L-arginine ethyl ester at 253 nm [29]. Preincubation at 25°C for 6 h with 0.1 mM iodoacetyl derivative **3c** with both the enzymes did not inhibit their activity.

Porcine pancreatic elastase assay

Porcine pancratic elastase EC 3.4.21.11, product no E-0127, was obtained from Sigma. Enzyme was dissolved in 50 mM phosphate buffer, pH 7.0 and concentration determined from $E^{1\%} = 22$ cm^{-1} at 280 nm [30]. Activity was monitored by fol-

lowing the hydrolysis of Boc-L-Ala-ONp at 347 nm [31] in 50 mM phosphate buffer, pH 7.0 at 25°C. No inactivation was observed following 6 h incubation with iodoacetyl derivatives **3c** under the same conditions as for chymotrypsin and trypsin.

Alkylation of glutathione

The rate of alkylation of glutathione was measured following the thiol disappearance by using Ellman's reagent [32]. Observations were made in phosphate buffer at pH 7.4 and 37°C. Concentration of organic solvent and all other conditions were similar to that employed in the experiments for enzyme inactivation. Results are listed in Table II.

Results and discussion

Likewise N-haloacetyl-aminoacid amides [8], 1-peptidyl-2-haloacetyl-hydrazines **2a-6c** completely and irreversibly inactivate papain and cathepsin B under pseudo-first-order conditions, at pH 6.8 and 25°C. The concentration dependence of inactivation revealed saturation kinetics in accordance with a preassociation step leading to a reversible, non-covalent enzyme-inhibitor complex EI (equation 1). Assuming that the rate of the second step, independently of the nature of the halogen atom of the inhibitor, is relatively slow, so that the initial binding equilibrium (typically diffusion controlled) is not perturbed, the kinetic parameters K_i, k_2 and k_2/K_i have been determined and are reported in Table I. The corresponding values for the N-haloacetyl-phenylalanine-isopentylamides **1a-1c** [8] are included for comparison purposes.

The proposed structure of the EI reversible complex between papain and 1-Z-Phe-2-methyl-2-haloacetyl hydrazines **5** is shown in Fig 2 ($R = CH_3$). All the hydrophobic bonding interactions and hydrogen bonds reported are inferred from the X ray crystallographic analysis of the papain—Z-Phe-Ala-CH_2Cl complex [33]. In fact haloacetyl hydrazine derivatives **5** differ from the halometylketone studied by Drenth only for the N-2 atom in place of the alanine α-CH group. In this isoelectronic exchange, atomic distances and bond angles are not seriously modified and all the interactions with the enzyme active site of the unchanged part of the molecule are expected to be substantially retained. The main structural changes due to the mobility of the electron pair of the new nitrogen atom are represented by loss of the α-CH chiral centre, a more extended area of planarity and a lower conformational mobility. At last, the enhanced acidity of the hydrazide NH group, relatively to peptide NH, should favour stronger H-bonding with the CO of Asp-158 and increase the stability of the EI adduct.

This arrangement of the 1-peptidyl-2-haloacetyl-hydrazines at the active site of papain seems to explain their behaviour of active site directed inhibitors and is in accordance with irreversible inactivation by alkylation

TABLE I

Inactivation of papain and cathepsin B by 1-peptidyl-2-haloacetyl-hydrazines[a]. [a]Conditions: 100 mM phosphate buffer, pH 6.8; $CH_3CN = 12\%$ (v/v); $[E] = 7 \times 10^{-9}$ M; [S] = 125 μM. Replicate determinations indicate standard deviations for the kinetic parameters less than 20%.

Enzyme and inhibitor		Range	K_I	k_2	k_2/K_I
N°	Structure	(μM)	(μM)	(min^{-1})	($M^{-1}s^{-1}$)
Papain					
1a	Cl-CH_2-CO-Phe-NH-*i*-C_5H_{11}[b]	125-1000	50	0.20	67
1b	Br-CH_2-CO-Phe-NH-*i*-C_5H_{11}[b]	2.5-50	0.9	0.40	7400
1c	I-CH_2-CO-Phe-NH-*i*-C_5H_{11}[b]	2.0-20	0.4	0.40	16700
2a	Ac-Phe-NH-NH-CO-CH_2Cl	10-50	4.40	0.42	1590
2b	Ac-Phe-NH-NH-CO-CH_2Br	0.8-6	0.16	0.13	13540
2c	Ac-Phe-NH-NH-CO-CH_2I	0.7-7.5	0.24	0.13	9030
3a	Z-Phe-NH-NH-CO-CH_2Cl	11-48	5	0.30	1000
3b	Z-Phe-NH-NH-CO-CH_2Br	1-10	0.50	0.40	13330
3c	Z-Phe-NH-NH-CO-CH_2I	0.5-4	0.18	0.25	23150
4a	Z-Ala-Ala-Phe-NH-NH-CO-CH_2Cl	7.0-50	2.10	0.62	4920
4b	Z-Ala-Ala-Phe-NH-NH-CO-CH_2Br	1.3-10	1	0.9	15000
4c	Z-Ala-Ala-Phe-NH-NH-CO-CH_2I	1.3-10	0.91	0.73	13770
5a	Z-Phe-NH-N(CH_3)-CO-CH_2Cl	400-1200	230	0.36	26
5b	Z-Phe-NH-N(CH_3)-CO-CH_2Br	30-200	10	0.35	583
5c	Z-Phe-NH-N(CH_3)-CO-CH_2I	15-75	5.9	0.33	932
6a	Z-Phe-N(CH_3)-NH-CO-CH_2Cl	400-1200	70	0.10	24
6b	Z-Phe-N(CH_3)-NH-CO-CH_2Br	60-400	12.0	0.33	458
6c	Z-Phe-N(CH_3)-NH-CO-CH_2I	50-200	14.8	0.30	338
Cathepsin B					
2c	Ac-Phe-NH-NH-CO-CH_2I	0.7-6	1.70	1.2	11760
3a	Z-Phe-NH-NH-CO-CH_2Cl	1.5-16	2.40	0.96	6670
3b	Z-Phe-NH-NH-CO-CH_2Br	0.2-2	0.46	0.84	30430
3c	Z-Phe-NH-NH-CO-CH_2I	0.2-2	0.37	0.88	39640
4c	Z-Ala-Ala-Phe-NH-NH-CO-CH_2I	1.3-10	0.83	0.86	17270
5c	Z-Phe-NH-N(CH_3)-CO-CH_2I	6-50	11.0	1.3	1970
1c	I-CH_2-CO-Phe-NH-*i*-C_5H_{11}	5.9-44.4	1.8	0.3	2800

of the Cys-25 thiolate (*) [8,34,35]. On the basis of this simple hypothesis, replacement of chlorine by bromine or iodine in the inhibitory ligand was expected to cause: i) a considerable increase of the first order rate constant k_2 for the thiolate alkylation as a consequence of enhanced leaving group ability and ii) no substantial change of the affinity binding constant K_i since recognising at the active site appears to be essentially confined to the azapeptidyl moiety of the inhibitor. On the contrary, a decrease rather than an increase of k_2 and a 23 and 40-fold increase of binding affinity were observed by halogen replacement in the series **5a, 5b, 5c**. Similar trends of k_2 and K_i values were shown also by the other series of 1-peptidyl-2-haloacetyl hydrazines **2, 3, 4** and **6** and, as far as K_i is concerned, by haloacetylaminoacid amides like **1a-1c** [8].

The increase of affinity in the series Cl < Br < I can be explained in terms of hydrophobicity considerations according to Cullis and coworkers. They have recently demonstrated [36] that binding affinity of each of a family of 3-halo-3-deoxy-chloramphenicol derivatives to chloramphenicol acetyltransferase does increase in the series F < Cl < Br < I and that there is a linear free energy relationship between the dissociation constants for binding and an empirical hydrophobicity scale derived from reverse-phase HPLC retention times.

For papain inactivation by 1-peptidyl-2-haloacetyl hydrazines, it should be assumed that an appropriate hydrophobic region of the enzyme active site could accommodate a substituent as bulky as a iodo group in the EI reversible complex of Fig. 2. Replacement of chlorine by bromine or iodine will simply increase the hydrophobic character of the inhibitory ligand, causing a reduction of the energetic cost of removing it from water solvent to the hydrophobic active site. It should be remarked that the effect on K_i here observed is higher than that reported for 3-halo-3-deoxychloramphenicol derivatives (K_i = 19, 7 and 3.4 μM for the 3-chloro, 3-bromo and 3-iodo derivatives respectively).

To explain the trend of k_2 in the series Cl > Br > I we assume that active site and ligand conformations in the EI reversible adduct are productive for binding, but unfavourable, in terms of distance and orientation of the reacting centres, for the C-S bond formation. It is intended that these unfavourable effects should increase in the series Cl < Br < I as a consequence of the enhanced steric requirements of the halogen accommodated within the proposed hydrophobic cavity. We point out that, while applying hydrophobic considerations to explain the effects on K_i may be quite satisfactory, it is less to asses the effect on k_2, probably involving precise positions and orientations of the carbon-halogen bond of the inhibitory ligand.

(*) Addition of Cys-25 thiolate to the haloacetyl carbonyl carbon with formation of a tetrahedral adduct, followed by thiolate transposition to the terminal methylene group accompanied by halide removal can not be excluded.

Since the increase of affinity here observed exceeds that reported for the 3-halo-3-deoxychloramphenicol derivatives, it is possible that, in addition to the aspecific hydrophobic effect, the halogen atom may directly intervene in specific interactions [37] with appropriate groups of the enzyme active site. In particular, "halogen-bonds" of the type C—X---Y, where C—X reprensents a carbon-halogen bond and Y an electron donor group, may represent an attractive proposal. While the precise nature of the bond is discussed, its specific character, bond angles and interatomic distances have been determined. Stronger interactions were observed with amine nitrogen rather than ether oxygen or aromatic groups as electron donors and with iodo rather than with bromo or chloro alkanes. Thus, the reversible adduct EI reprensented in Fig 2 could include a specific C—X---Y interaction (**) to explain further improvement of the binding affinity and the increasing importance of the unfavourable orientation of the alkylating group by replacement of chlorine by bromine or iodine.

The strength of the proposed papain—1-Z-Phe-2-methyl-2-iodoacetyl hydrazine complex ($K_i = 6\mu M$) is similar to that of typical enzyme-substrate complexes ($K_m = 9\mu M$ for papain—Z-Gly p-nitrophenylester) [38]. Both K_i and k_2/K_i for papain inactivation, however, are some 15-fold worse for 1-Z-Phe-2-methyl-2-iodoacetyl hydrazine **5c** than for the previously reported N-iodoacetyl-L-phenylalanine isopentylamide **1c**. Some structural variations were therefore considered and their effects on the kinetic parameters examined.

Replacement of the methyl group by hydrogen at the hydrazine N-2 leads to the haloacetyl derivatives **3** containing an α-azaglycine rather than an α-azaalanine unit in the P_1 position. They showed an average improvement of about 30-fold of both K_i and k_2/K_i.

In the attempt to define the role of the N-2 methyl group, 1-methyl-2-haloacetyl hydrazines **6**, positional isomers of **5**, were synthesized and tested as papain inhibitors. As shown in Table I, K_i increases from 0.2 μM for the unsubstituted hydrazine **3c** to 5.9 μM for the 2-methyl derivative **5c** and 15 μM for the 1-methyl derivative **6c**. These values can be referred to the proposed structure of the EI complex of Fig 2, taking into account the effects of substitution on the interactions with the enzyme active site and on the conformational preferences of the free ligands. Tridimensional structure of 1,2-diacylhydrazines by X-ray crystallography has been scarcely studied. It is known, however, that the coplanar disposition of the two R-CO-N planes of 1,2-diformylhydrazine [39,40] is turned to orthogonal for N-substituted derivatives bearing at least an alkyl group [41]. Thus an orthogonal orientation of the two hydrazine R-CO-N planes for the N-methyl

(**) His-159 could be suggested as a possible candidate for electron donor groups Y at the papain active site. We are seeking to better address this matter by molecular graphics.

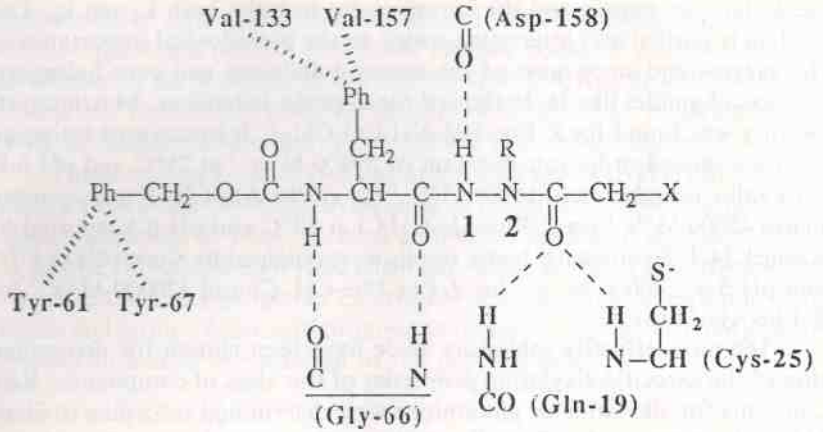

Fig. 2

Schematic representation of the proposed binding of 1-peptidyl-2-haloacetyl hydrazines to the active site of papain. Groups belonging to the enzyme are given in bold.

derivatives **5a-6c** and a planar one for the unsubstituted derivatives **2a-4c** can probably be assumed.

Independently on the precise conformational preferences involved, the 75-fold increase of K_i by replacement of the N-1 H of **3c** with the methyl group, as in **6c**, should be attributed to loss of the H-bond with the Asp-158 carbonyl and rising of steric strain. Hydrogen replacement by methyl at the other nitrogen N-2 causes a 30-fold increase of K_i in **5c** relative to **3c**. This effect could probably be attributed to loss of an H-bond (Fig 2 R = H) with an appropriate electron donor group of the active site, rather than to steric strain, since it is known that papain can readily accommodate both Ala or Gly in the P_1 position.

The effect on K_i and k_2/K_i of shortening or lengthening the recognising peptidyl moiety of 1-peptidyl-2-haloacetyl hydrazines was also examined. More significant changes were observed for k_2 than for K_i in the shortened inhibitors **2a-2c** relatively to **3a-3c**. As a result, peptide shortening leads to an improvement of the second order rate constant for chloroacetyl derivatives (**2a** more effective than **3a**) and to a decrease for iodoacetyl derivatives (**2c** less effective than **3c**). Extension of peptidyl chain as far as P_3 (inhibitors **4a-4c**) caused an increase of both K_i and k_2 for papain and, notably, a 3-fold improvement of the second order rate constants of inactivation for the chloroacetyl derivative **4a** relative to **2a**. No significant changes were observed instead for the corresponding bromo and iodoacetyl derivatives.

A representative selection of the discussed papain inhibitors was tested also toward cathepsin B. All of them showed to be more effective for cathep-

sin B than for papain and the improvement includes both K_i and k_2. This finding is particularly interesting owing to the pathological importance of the enzyme and since most of the known inihibitors and even haloacetyl aminoacid amides like **1a-1c** showed the opposite behaviour. Maximum efficiency was found for Z-Phe-NH-NH-CO-CH_2-I. It inactivated cathepsin B with a second-order rate constant of 39600 $M^{-1}s^{-1}$ at 25°C and pH 6.8. This value is higher than 16200 $M^{-1}s^{-1}$ for Z-Phe-Ala-CH_2-F and approximates 45000 $M^{-1}s^{-1}$ for Z-Phe-Ala-CH_2Cl at 28°C and pH 6.5 reported by Rasnick [42]. Significantly better results were obtained by Shaw [43] at 37°C and pH 5.4: 540000 $M^{-1}s^{-1}$ for Z-Phe-Phe-CH_2-Cl and 320000 $M^{-1}s^{-1}$ for Z-Phe-Ala-CH_2-F.

The most effective inhibitors **3a-3c** have been chosen for determination of the aspecific alkylation properties of this class of compounds. Rate constants for alkylation of glutathione were determined according to Shaw [44] and are reported in Tab. II together with some reference data. As expected, their reactivity is similar to that of N-haloacetyl-aminoacid amides **1a-1c**. Thus both chloroacetyl derivatives **3a** and **1a** are some 3-fold as reactive toward glutathione as fluoromethylketones and iodoacetyl derivatives **3c** and **1c** are only 1/2 as reactive as chloromethylketones.

TABLE II

Comparative reactivity of 1-peptidyl-2-haloacetyl hydrazines toward gluthatione[a]. [a]Tests were carried out in phosphate buffer at pH 7.4 and 37°C. [b]Acetonitrile; [c]1% DMSO was also present; [d]See Ref. 44

Conc. of	Inibitor		Conc. of org.	k
GSH (mM)	Structure	Conc (mM)	solvent[b] (v/v)	($M^{-1}s^{-1}$)
0.04	**3a**	0.4	13	$3.1x10^{-2}$
0.04	**3b**	0.2	13	1.4
0.008	**3c**	0.05	13[c]	3.0
0.04	**1a**	0.4	13	$2.9x10^{-2}$
0.04	**1b**	0.2	13	1.70
0.04	**1c**	0.2	13	2.30
0.05	Z-Phe-CH_2Cl[d]	0.1	0	6.16
3.3	Ala-Phe-Lys-CH_2F[d]	6.7	5	$1.1x10^{-2}$

The inhibiting activity of 1-peptidyl-2-haloacetyl-hydrazines toward

representative serine-proteases was also verified. No inhibition of trypsin chymotrypsin and porcine pancreatic elastase could be detected after 6 h incubation in the presence of Z-Phe-NH-NH-CO-CH_2-I **3c** at 0.1 mM concentration. This finding confirms the previously observed, remarkable selectivity of haloacetamide derivatives toward cysteine-proteases.

The described 1-peptidyl-2-haloacetyl hydrazines rival effectiveness of known fluoro and chloromethylketones as inhibitors of cysteine-proteases, namely cathepsin B. They represent an interesting class of inhibitors in view of simple, direct and high yield chemical synthesis. Their effectiveness and aspecific alkylating properties can readily be modulated by variation of the haloacetyl group. Work is in progress to improve enzyme selectivity among cysteine-proteases by variation of the peptidyl unit.

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