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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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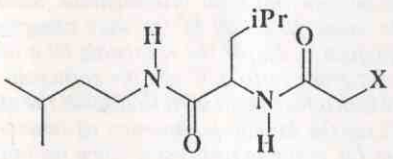
posta e impiegata per discutere gli effetti di varianti strutturali degli inibitori sui parametri cinetici di inattivazione. I composti esaminati hanno mostrato di essere inibitori selettivi per le proteasi a cisteina poiché non si è potuta rilevare alcuna inattivazione di proteasi a serina rappresentative 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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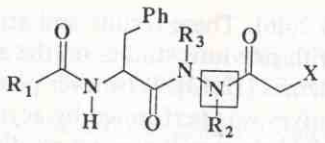
The mode of binding at the active site of papain, proposed to explain formation of reversible adducts (K_i 125-0.4 μM) and improvement of alkylation rates of Cysteine-25 thiolate by proximity effects, was based on the crystal structure of papain—E-64 complex [9,10]. As the consequence, in the reversible 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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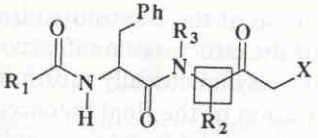

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N°	R_1CO	R_2	R_3	X	N°	R_1CO	R_2	R_3	X
2a	CH_3CO	H	H	Cl	4c	Z-Ala-Ala	H	H	I
2b	CH_3CO	H	H	Br	5a	Z	CH_3	H	Cl
2c	CH_3CO	H	H	I	5b	Z	CH_3	H	Br
3a	Z	H	H	Cl	5c	Z	CH_3	H	I
3b	Z	H	H	Br	6a	Z	H	CH_3	Cl
3c	Z	H	H	I	6b	Z	H	CH_3	Br
4a	Z-Ala-Ala	H	H	Cl	6c	Z	H	CH_3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