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SYNTHESIS AND EVALUATION OF AZAPEPTIDE-DERIVED INHIBITORS OF SERINE AND CYSTEINE PROTEASES[1]

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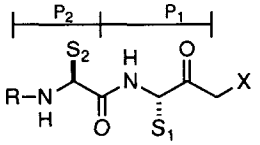
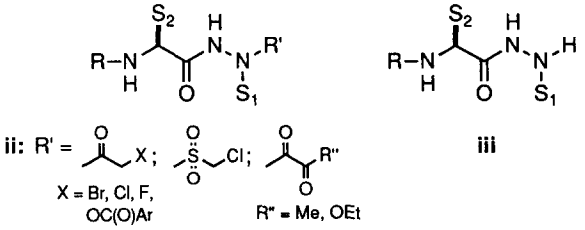
Abstract: Azapeptide analogues **ii** of the α-halomethyl ketones **i** were synthesized and evaluated as potential inhibitors of serine and cysteine proteases. Inhibitors **ii** discriminate between the two classes of protease, demonstrating selectivity for cysteine proteases. Azaglycines **1-3** and **5** displayed time-dependent inactivation ($k_{obs}/[I]$ = 200-1500 $M^{-1}s^{-1}$) of cathepsin B and calpain.

Peptide α–halomethyl ketones **i** are the classical active-site directed irreversible inhibitors of serine and cysteine proteases.[3] X-ray crystallographic analysis of the enzyme-inhibitor **i** complexes reveal that modification of both the catalytic serine and histidine residues occurs in the case of serine proteases while it is the catalytic cysteine residue which is solely modified in thiol proteases.[3] By attenuation of the inherent reactivity of **i**, from X = Cl to X = F[4] and more recently to X = OAr, OC(O)Ar,[5] class specific inhibitors of cysteine proteases have been discovered. However, there has been virtually no study of the effect of backbone modification in **i** on activity and specificity.[6,7] In this context, we have prepared and evaluated azapeptide analogues **ii**, where the P_1 α-carbon has been replaced by a nitrogen atom, as inhibitors of serine and cysteine proteases. The peptide side chains (S_n) of the azapeptide backbone in **ii** were varied to investigate inhibition against chymotrypsin, porcine pancreatic elastase, cathepsin B and calpain.[8] Our synthesis of **ii**, where the 1-peptidyl hydrazines **iii** were obligatory intermediates, permitted the incorporation of a variety of groups bearing functionality (R') capable of reversible or irreversible interaction with the protease's



i: X = halogen, OAr, OC(O)Ar



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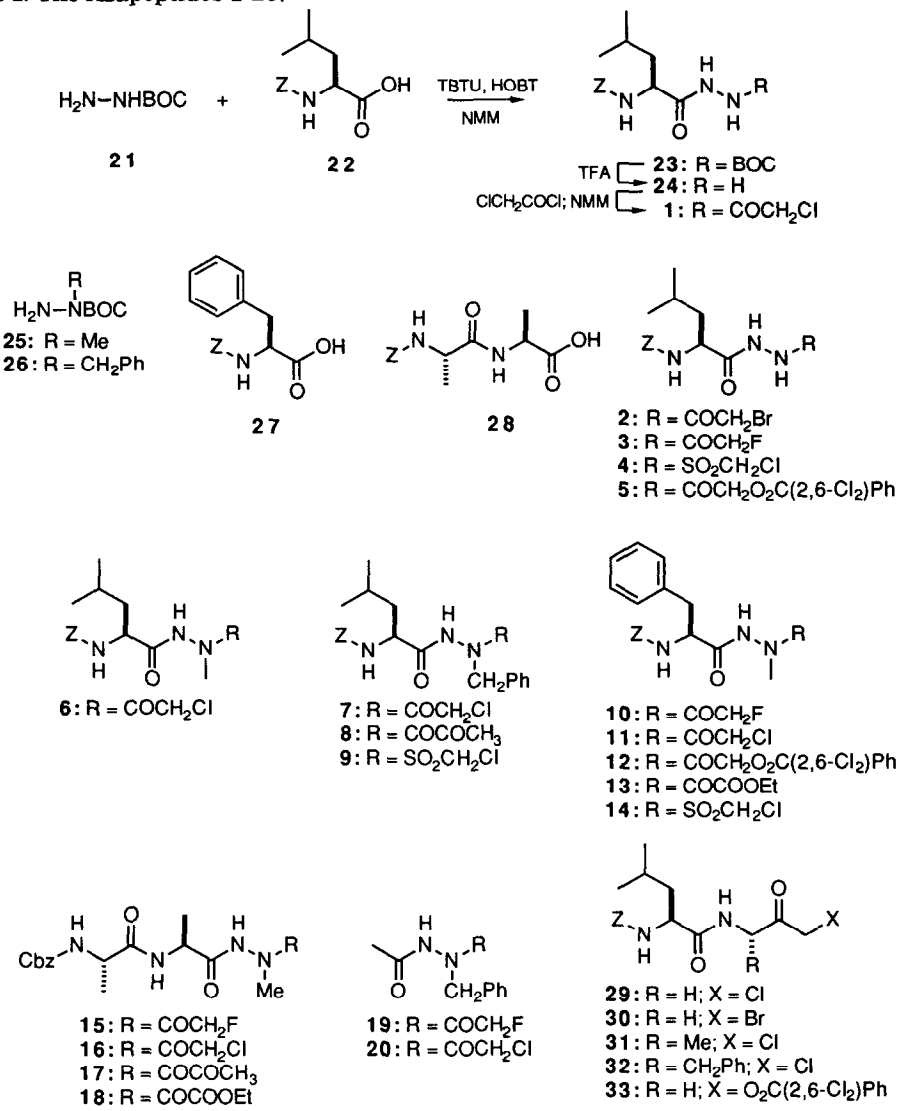
active-site serine/histidine or cysteine. These include for example, the chloromethyl sulfonyl moiety (**ii:** X = SO_2CH_2Cl), which is presented to the active site of these enzymes for the first time.

The preparation of Z-leucine-azaglycine chloroacetyl hydrazide **1** (Scheme I) is representative of the synthesis of azapeptides **1-20**. The BOC-protected hydrazine **21**[7] was coupled to commercially available Z-leucine **22** using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate[9] (TBTU; 1.2 equiv TBTU, 1.0 equiv 1-hydroxybenzotriazole (HOBT), 3.5 equiv N-methyl morpholine (NMM) in DMF) furnishing hydrazide **23**. Subsequent treatment with trifluoroacetic acid (25% v/v TFA-CH_2Cl_2, 2 h, 25 °C) and removal of solvent afforded the TFA salt of hydrazine **24**. In situ neutralization of TFA salt **24** (2.5 equiv NMM, CH_2Cl_2, 0 °C, 2 min)[10] followed by condensation with $ClCH_2COCl$ (1.2 equiv $ClCH_2COCl$, 1.5 equiv NMM, CH_2Cl_2, 25 °C, 30 min) gave hydrazide **1** (mp 192-193 °C (CH_2Cl_2-hexane)). The overall yield for the three-steps was ca. 30%. Condensation of **24** with $BrCH_2COCl$, $(FCH_2CO)_2O$, and $ClCH_2SO_2Cl$ gave target inhibitors **2-4**, respectively. Analogous preparation of **6-11, 13-18** from BOC-hydrazines **25** or **26**[9] was carried out by coupling with Z-leucine **22**, Z-phenylalanine **27**, or Z-alanine-alanine **28**, deprotection, and then reaction with the electrophiles described for the preparation of **1-4**, EtO_2CCOCl and $CH_3COCOCl$. In the case of **19** and **20**, BOC-hydrazine **26** was first acetylated (1.2 equiv $(CH_3CO)_2O$, 1.5 equiv NMM) and was then treated with either $ClCH_2COCl$ or $(FCH_2CO)_2O$ in the presence of NMM. For the preparation of inhibitors **5** and **12**, a solution of the corresponding bromide in DMF was displaced with 1.5 equiv 2,6-dichlorobenzoic acid using KF as a base.[5] Peptide halomethyl ketones **29-32** and aryl acyloxymethyl ketone **33** were prepared using standard literature procedures.[3,5]

Hydrazides **15-18** and **9**, **19** and **20** contain Ala-Ala-Ala and Phe complementary recognition elements for elastase and chymotrypsin (serine proteases).[3b] Neither the chloroacetyl-, fluoroacetyl- nor the chloromethylsulfonyl hydrazides were inhibitors of these enzymes at concentrations up to 10 μM (Table I). Although, azapeptides **17** and **18** may interact in a reversible fashion with the active-site serine hydroxyl via hemiketal formation or ester exchange, no inhibition of enzyme was observed for **17** and **18** at the highest concentration (10 μM) assayed (Table I).

Time-dependent inactivation of cathepsin B and calpain (cysteine proteases) was observed for hydrazides **1-3** and **5** which contain the azaglycine residue at P_1 and the hydrophobic leucine residue at P_2 (Table I).[11] Direct comparison of the second-order rate constants ($k_{obs}/[I]$) for the inactivation of cathepsin B by **1-3** and **5** verses their P_1 α-carbon congeners **29**, **30** and **33** are some 3000, 220 and 62 fold lower. The substantial reduction of ($k_{obs}/[I]$) for the series is believed to be linked to differences in the binding affinity (K_i) of the two classes of inhibitors as opposed to differences in the chemical step (k_{inact}).[12] The two fold increase in ($k_{obs}/[I]$) for **2** against calpain versus cathepsin B may be a reflection of the preferred specificity for Leu at P_2 for calpain.[13] Exchange of the chloroacetyl functional group

Scheme I. The Azapeptides **1-20**.



for the chloromethylsulfonyl group, results in an inactive compound against both enzymes, despite the electrophilic character of this species. Introducing a methyl (**6**) or benzyl (**7**) group at S_1 in the aza series also results in a complete loss of inhibitory activity, in contrast to the well tolerated Gly to Ala or Phe substitution in the chloromethyl ketone series (**29** → **31** and **32**). Particularly striking is the isosteric replacement of Ala with Aala in Z-Leu-Ala-$COCH_2Cl$ **31** (**31** → **6**) for inhibition against cathepsin B.

Table I. Summary of Kinetic Data for Azapeptides **1-20** and Reference Inhibitors **29-33.**

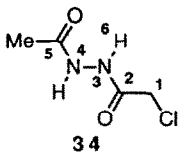
Inhibitor	Chymotrypsin[a] k_{obs}/I ($M^{-1}s^{-1}$)	Elastase[a] k_{obs}/I ($M^{-1}s^{-1}$)	Cathepsin B[a] k_{obs}/I ($M^{-1}s^{-1}$)[c]	Calpain[b] k_{obs}/I ($M^{-1}s^{-1}$)[c]
Z-Ala-Ala-Aala-$COCH_2F$ (**15**)	-[d]	ni[e]	-	-
Z-Ala-Ala-Aala-$COCH_2Cl$ (**16**)	-	ni	-	-
Z-Ala-Ala-Aala-COCOMe (**17**)	-	ni	-	-
Z-Ala-Ala-Aala-$COCO_2Et$ (**18**)	-	ni	-	-
Ac-Aphe-$COCH_2F$ (**19**)	ni	--	-	-
Ac-Aphe-$COCH_2Cl$ (**20**)	ni	-	-	-
Z-Leu-Agly-$COCH_2Cl$ (**1**)	ni	-	184	536
Z-Leu-Agly-$COCH_2Br$ (**2**)	ni	-	875	1500
Z-Leu-Agly-$COCH_2F$ (**3**)	-	-	ni	ni
Z-Leu-Agly-$COCH_2DCB$ (**5**)[f]	-	-	288	742
Z-Leu-Agly-SO_2CH_2Cl (**4**)	-	-	ni	ni
Z-Leu-Aala-$COCH_2Cl$ (**6**)	-	-	ni	ni
Z-Leu-Aphe-$COCH_2Cl$ (**7**)	ni	-	ni	ni
Z-Leu-Aphe-COCOMe (**8**)	ni	-	ni	ni
Z-Leu-Aphe-$COCO_2Et$ (**9**)	ni	-	ni	ni
Z-Phe-Aala-$COCH_2F$ (**10**)	-	-	ni	ni
Z-Phe-Aala-$COCH_2Cl$ (**11**)	-	-	ni	ni
Z-Phe-Aala-$COCH_2DCB$ (**12**)	-	-	ni	ni
Z-Phe-Aala-COCOMe (**13**)	-	-	ni	ni
Z-Phe-Aala-$COCO_2Et$ (**14**)	-	-	ni	ni
Z-Leu-Gly-$COCH_2Cl$ (**29**)	-	-	517000	31500
Z-Leu-Gly-$COCH_2Br$ (**30**)	-	-	194000	3800
Z-Leu-Gly-$COCH_2DCB$ (**33**)	-	-	17900	-
Z-Leu-Ala-$COCH_2Cl$ (**31**)	-	-	88650	6200
Z-Leu-Phe-$COCH_2Cl$ (**32**)	-	-	2670	5300
Z-Phe-Ala-$COCH_2Cl$[g]	-		45300	-

a) All compounds were dissolved in DMSO. Assays for bovine pancreatic chymotrypsin, porcine pancreatic elastase and bovine spleen cathepsin B were performed in a final volume of 200 µL in polystyrene 96-well plates using a Fluoroskan II fluorescence plate reader The chymotrypsin assay contained 0 1 HEPES (pH 7.5), 2 M NaCl, 0.4 mM EDTA, 0.005% Triton X-100, 50 µM Suc-ala-ala-pro-phe-AMC, and 1 nM chymotrypsin. Elastase assays contained 0 2 M Tris-HCl (pH 8.0), 0 1 mM EDTA, 0.005% Triton X-100, 250 µM MeOsuc-ala-ala-pro-val-AMC, and 2 nM elastase. The cathepsin B assay contained 0.1 M MES (pH 6 0), 0.1 mM EDTA, 0.1% Brij 35 with 100 µM Z-Phe-Arg-AMC as substrate and approx 0 2 nM cathepsin B. Assays were initiated by the addition of enzyme, and progress curves were monitored for 15 to 40 min at 25 °C. Progress curveswere fit by the method of Tian and Tsou (Tian, W -X., Tsou, C -L. *Biochemistry* **1982**, *21*, 1028 b) The human erythrocyte calpain assay was carried out 8 °C. Details of the assay will be presented elsewhere (Harris, A. et al. *Biochemistry*, manuscript submitted). See ref 13. c) Replicate determinations gave standard deviations <25% d) Not determined. e) No inhibition observed at 10 µM inhibitor. f) DCB = $O_2C(2,6\text{-}Cl_2)Ph$ g) Data taken from Rasnick, D. *Anal Biochem.* **1985**, *149*, 461.

Comparison of the low energy conformations of model compounds, diacyl hydrazines **34** and **35** and the chloromethyl ketone **36**, indicates subtle differences in bond lengths, bond angles and torsion angles between to the two structural classes (Figure I). These physiochemical differences may contribute toward the attenuated inhibitory activity of **1-3** and **5** and the complete lack of affinity of **4, 6-14** against cathepsin B and calpain, with respect to **29-33**. Substituting the P1 α-carbon with a nitrogen atom in these inhibitors results in the conversion

of a sp^3 center to a sp^2 center and a shortening of the bond between C2 and N3 (C3) by 0.15 Å with a concomitant increase in bond angle of some 10° (Figure I).[14] It is evident from the iso-energy contour plots (Figure II) that the newly created N-N linkage in **34** and **35** imparts conformational constraint relative to **36**. Simultaneous rotation of ψ and ϕ torsion angles in **34-36** provides few common low energy populations. This suggests that the 1-peptidyl α-haloacetyl hydrazines may have difficulty in achieving the productive, bioactive orientation readily attained by the chloromethyl ketones and may be forced to occupy different space in the active site.[15]

Figure I. Some of Physiochemical Data for Model Inhibitors **34**, **35** and **36**.[14, 16]



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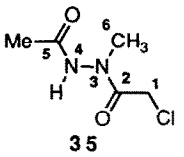

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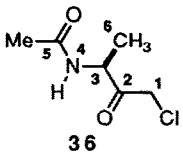
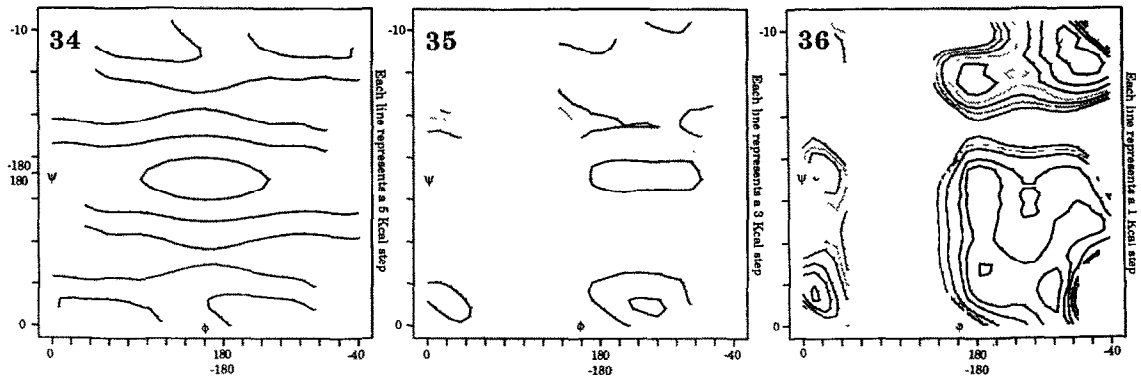


36

	34	35	36
Bond length:			
Atoms 2-3	1.33 Å	1.36 Å	1.51 Å
Bond angle:			
Atoms 2-3-6	120.5°	119.7°	109.4°
Dihedral angles:			
Atoms Cl-1-2-3 (ω)	178.3°	-121.1°	105.3°
Atoms 1-2-3-4 (ψ)	179.5°	0.6°	126.9°
Atoms 2-3-4-5 (ϕ)	-175.1°	-110.2°	-65.2°

Figure II. Iso-energy ψ,ϕ Contour Plots for Model Inhibitors **34**, **35** and **36**.[14]



With respect to the discrimination of the azapeptide-based inhibitors for cysteine over serine proteases, there may be two important factors to consider. First, conversion of a ketone carbonyl to an amide carbonyl results in a carbonyl which is much less prone to hemiketal formation upon attack by an active site hydroxyl or thiol, a possible pre-requisite to high affinity, particularly for serine proteases.[3b] Second, based on stereoelectronic considerations, α-chloro ketones are an order of magnitude more reactive toward nucleophilic displacement

verses α-chloro esters, -nitriles, and -sulfones.[17] Thus, the combination of the decreased reactivity of the α-haloacetyl hydrazides and the poor nucleophilicity of the hydroxyl group relative to thiol may give rise to class selectivity. This latter attribute serves as the basis for exclusive cysteine protease selectivity for the peptide aryloxy- and arylacyloxymethyl ketones.[5]

REFERENCE AND NOTES

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6. Isosteric replacement of the α-CH for N in peptides has been successfully applied in other cases: (a) Gupton, B. F.; Carroll, D. L.; Tuhy, P. M.; Kam, C.-M.; Powers, J. C. *J. Biol. Chem.* **1984**, *259*, 4279. (b) Greenlee, W. J.; Thorsett, E. D.; Springer, J. P.; Patchett, A. A.; Ulm, E. H.; Vassil, T. C. *Biochem. Biophys. Res. Commun.* **1984**, *122*, 791. (c) Leqog, A.; Marraud, M.; Aubry, A. *Tetrahedron Lett.* **1991**, *32*, 2765.
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10. In other instances it was neccessary to isolate the free base through CH_2Cl_2 extraction of the TFA salt from 7% aqueous K_2CO_3.
11. The inactivation event is predicted to be irreversible by analogy with the mechanism of irreversible inactivation incurred by peptide α-halomethyl ketones.
12. Although no k_{inact} or K_i data were obtained for **1-3** and **5**, support for this assumption rests with the fact that k_{inact} is the slow step in the inactivation process and a narrow range of values for k_{inact} (~0.002-0.064 s^{-1}) have always been found, independent of inhibitor type. The values determined for K_i however, vary by as much as 10^4. See ref 5 above and citations therein.
13. Due to auto-catalysis, it was neccessary to perform the human erythrocyte calpain assay at 8 °C. The second-order rate constants have been estimated to be 2x-3x higher if the determinations could be conducted at 25 °C. (Harris, A.; et al. *Biochemistry*, manuscript submitted).
14. Low energy conformations for compounds **34**, **35** and **36** and iso-energy contour plots were obtained using the SYBYL Molecular Modeling System (version 5.3.0), TRIPOS Assoc., St. Louis, MO. The authors thank Dr. Adi Treasurywala, Department of Biophysics & Computational Chemistry, SWPRD, for obtaining these plots. Bond angles, bond lengths and dihedral angles ω, ψ and ϕ for **34** and **35** are in excellent agreement with data secured from X-ray crystallographic analysis of closely related structures. See the Cambridge Crystallographic Data Base, codes DIACHZ and SISYAU. The authors thank Dr. Joseph Salvino, Department of Medicinal Chemistry, SWPRD, for retrieving this data.
15. This is true for cysteine protease inhibitors E-64, Tos-Lys-CH_2Cl and Z-Phe-Ala-CH_2Cl, each of which have a unique orientation in the active site of papain. See the Brookhaven Protein Crystallographic Data Base, codes 4PAD (Tos-Lys-CH_2Cl) and 6PAD (Z-Phe-Ala-CH_2Cl). For E-64 see: Yamamoto, D.; Ishida, T.; Inoue, M. *Biochem. Biophys. Res. Commun.* **1990**, *171*, 711.
16. The ω torsion angle of 105.3° in structure **36** is consistant with the gauche-conformation predominately observed for chloromethyl ketones. Olivato, P. R. Guerrero, S. A. *J. Chem. Soc., Perkin Trans. II* **1983**, 1053.
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