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Communications to the Editor

Aspartyl α-((1-Phenyl-3-(trifluoromethyl)-pyrazol-5-yl)oxy)methyl Ketones as Interleukin-1β Converting Enzyme Inhibitors. Significance of the P_1 and P_3 Amido Nitrogens for Enzyme–Peptide Inhibitor Binding

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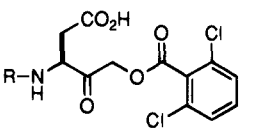
The production *in vivo* of the potent inflammatory cytokine interleukin-1β (IL-1β) requires proteolytic processing of a biologically inactive IL-1β precursor protein.[1] The cysteine protease, interleukin-1β converting enzyme (ICE), is now regarded as the processing enzyme.[2] The correlation of IL-1 levels with the severity of certain chronic inflammatory disease states has implicated ICE as a compelling target for therapeutic intervention.[3] We recently communicated that peptide α-((2,6-dichlorobenzoyl)oxy)methyl ketones **i** derived from aspartic acid are potent time-dependent inhibitors of ICE.[4] It was established that the enzyme has a strict preference for a P_1 aspartic acid residue in this class of inhibitor.[4,5] We now describe aspartyl α-((1-phenyl-3-(trifluoromethyl)pyrazol-5-yl)oxy)methyl ketones **ii** as a completely novel class of peptide-based ICE inhibitor. In addition, systematic incorporation of *N*-methyl amino acids into the peptide inhibitor backbone Val-Ala-Asp has enabled us to define the relative importance of the P_1–P_3 amido nitrogens required for high-affinity binding.

During the course of our study of the peptidic aspartyl ((arylacyl)oxy)methyl ketones,[4] we noted that the second-order inactivation rates of these inhibitors ranged



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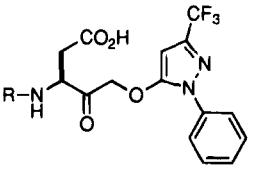

II

some 3 orders of magnitude.[6] The variation in potency was largely dependent on the type and pattern of aryl substitution, while independent of (arylacyl)oxy leaving group pK_a.[6,7] As a result, we elected to survey other functionality, structurally distinct from phenols and aryl carboxylic acids, for their ability to serve as leaving groups. One class of heterocycle examined was the 5-hydroxypyrazoles, in particular 5-hydroxy-1-phenyl-3-(trifluoromethyl)pyrazole.[8] This heterocycle was incorporated into a selection of aspartic acid-containing peptide scaffolds to afford a series of aspartyl ((1-phenyl-3-(trifluoromethyl)pyrazol-5-yl)oxy)methyl ketones **1**–**10**.[9a]

The peptide α-(pyrazoloxy)methyl ketones **1**–**10** were synthesized by one of two methods (Scheme 1).[9b] Borrowing directly from the methodology previously described for the synthesis of the aspartyl ((arylacyl)oxy)-methyl ketones,[4] aspartic acid bromomethyl ketone *tert*-butyl esters (1.0 equiv) were subjected to direct displacement with 5-hydroxy-1-phenyl-3-(trifluoromethyl)-pyrazole[8] (1.2 equiv) in the presence of potassium fluoride (2.5 equiv, DMF, 12 h, 25 °C) followed by treatment with trifluoroacetic acid (e.g., **11** → **12** → **1**). As an alternative method for inhibitor synthesis, peptide coupling of Z-protected amino acids with amine hydrochloride **13** and then TFA-mediated deprotection was readily achieved. This synthetic sequence is exemplified by the preparation of tripeptide **6**. Thus, amine **13** was obtained from *tert*-butyl ester **12** by catalytic hydrogenation in ethanol (10% Pd/C, 0.01 M solution of **12** in absolute ethanol containing 4 equiv of 6 M aqueous HCl, 1 atm H_2, 1 h, 25 °C). Subsequent TPTU-mediated coupling[10] of amine **13** to Z-(NMe)Val-Ala (1.1 equiv of dipeptide acid, 3.5 equiv of diisopropylethylamine, 1.1 equiv of TPTU, 1.3 equiv of HOBT, CH_2Cl_2, 0–25 °C, 4 h) furnished tripeptide **14**. Exposure of **14** to standard peptide TFA deprotection conditions (0.1 M solution of

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Scheme 1. Synthesis of the α-((1-Phenyl-3-(trifluoromethyl)pyrazol-5-yl)oxy)methyl Ketones **1**–**10** (Absolute Stereochemistry Is as Shown)

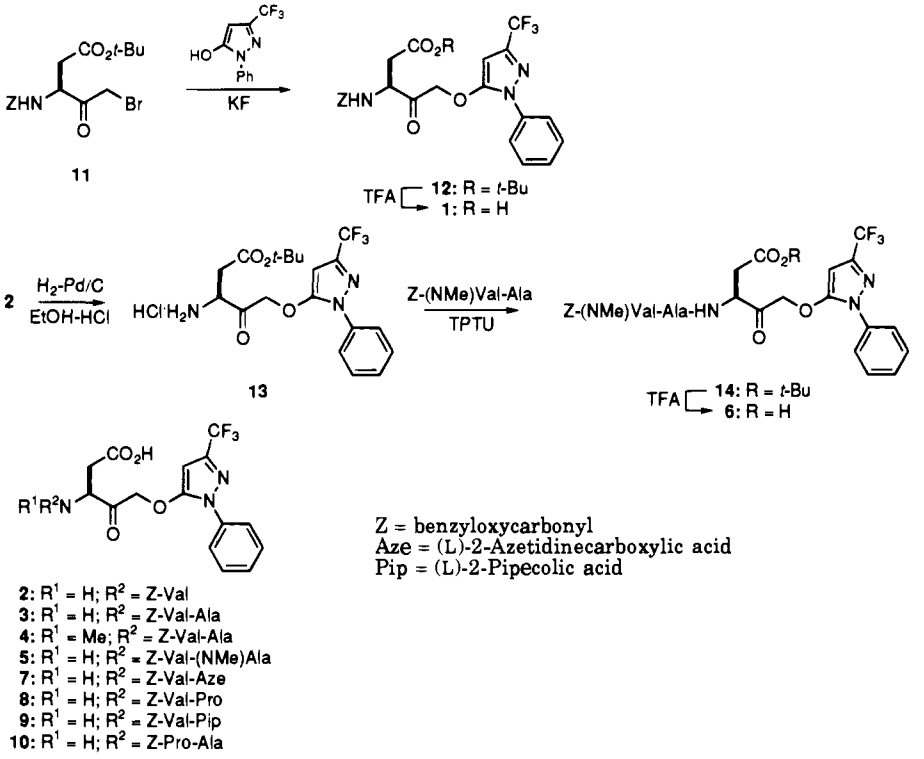

Table 1. Evaluation of Inhibitors **1**–**10** against ICE

inhibitor	ICE[a] k_{obs}/[I] (M^{-1} s^{-1})
Z-Asp-CH_2PTP[b] (**1**)	11 000 ± 200
Z-Val-Asp-CH_2PTP (**2**)	20 000 ± 700
Z-Val-Ala-Asp-CH_2PTP (**3**)	280 000 ± 40 000
Z-Val-Ala-N(Me)Asp-CH_2PTP (**4**)	<300
Z-Val-N(Me)Ala-Asp-CH_2PTP (**5**)	233 000 ± 700
Z-N(Me)Val-Ala-Asp-CH_2PTP (**6**)	8 600 ± 600
Z-Val-Aze-Asp-CH_2PTP (**7**)	46 200 ± 600
Z-Val-Pro-Asp-CH_2PTP (**8**)	116 100 ± 200
Z-Val-Pip-Asp-CH_2PTP (**9**)	271 000 ± 35 000
Z-Pro-Val-Asp-CH_2PTP (**10**)	<300

[a] For a description of the assay, see ref 4. [b] PTP = (1-phenyl-3-(trifluoromethyl)pyrazol-5-yl)oxy.

14 in 25% v/v TFA/CH_2Cl_2) gave inhibitor **6** (Scheme 1) in *ca.* 60% overall yield from intermediate **12**.

Potent time-dependent inactivation of ICE was seen for the Z-Asp, Z-Val-Asp, and Z-Val-Ala-Asp peptide inhibitors **1**–**3** containing the (1-phenyl-3-(trifluoromethyl)pyrazol-5-yl)oxy (or -PTP) leaving group (Table 1).[11] The k_{obs}/[I] of 11 100 M^{-1} s^{-1} for **1** compares well to the k_{obs}/[I] of 7100 M^{-1} s^{-1} for the corresponding *N*-(benzyloxycarbonyl)-L-aspartic acid α-((2,6-dichlorobenzoyl)oxy)methyl ketone reported previously by us.[4] Sequential increase in the number of the amino acids present in the peptidic inhibitors affords an expected increase in the rate of enzyme inactivation. Thus, Z-Val-Asp-CH_2PTP (**2**) and Z-Val-Ala-Asp-CH_2PTP (**3**) possess second-order rate constants equal to 20 000 M^{-1} s^{-1} and 280 000 M^{-1} s^{-1}, respectively.

In further studies, an *N*-methyl scan of the Z-Val-Ala-Asp backbone in inhibitor **3** was conducted to assist in defining the relative importance of the P_1, P_2, and P_3 amido nitrogens in ICE–inhibitor binding. The significance of the amido nitrogens as well as a detailed understanding of the overall enzyme–peptide inhibitor binding geometry for both the papain superfamily[12a]

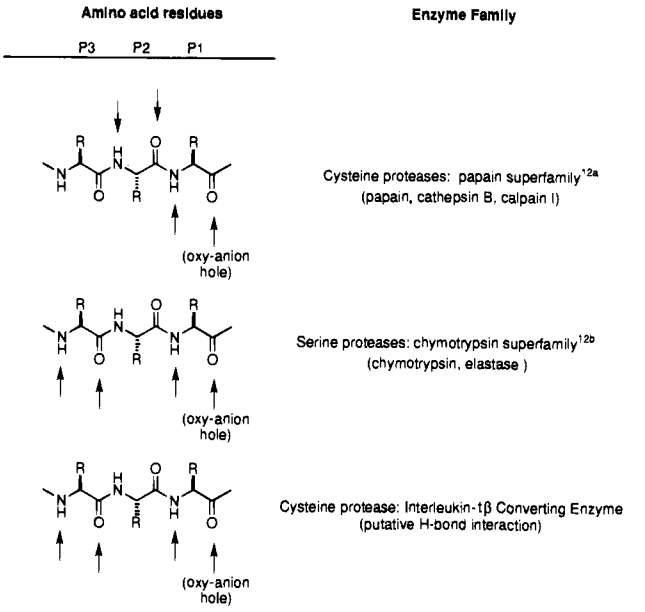

Figure 1. Comparative representation of the critical sites along the peptide inhibitor backbone which are engaged in hydrogen-bonding as observed for peptide inhibitor binding to cysteine proteases (papain superfamily), serine proteases (chymotrypsin superfamily), and ICE (arrows indicate sites of hydrogen bonding).

(cysteine proteases, including papain, cathepsin B, and calpain I) and the chymotrypsin superfamily[12b] (serine proteases, including chymotrypsin and elastase) is reviewed in Figure 1. Evidence gleaned from substrate/inhibitor binding studies in conjunction with X-ray crystal structures of cysteine protease– and serine protease–inhibitor complexes reveals that these enzyme families bind their peptide inhibitors through two distinct hydrogen-bonding motifs. For the papain superfamily, it is the inhibitor P_1 and P_2 amido nitrogens

which are necessary for productive enzyme binding. The inhibitor P_1 and P_3 amido nitrogens are the important NH functionality engaged by many serine proteases yielding an antiparallel β-sheet structure.[13] ICE is a cysteine protease which bears no primary sequence homology with any known cysteine or serine protease. The purpose of the *N*-methyl scan of **3** was to determine which hydrogen-bonding motif is utilized by ICE.

The inhibition data for the N-methylated inhibitors **4**–**6** against ICE are presented in Table 1. For inhibitors **4** and **6**, where the P_1 and the P_3 amido nitrogens have been independently methylated, there is a dramatic loss (*ca.* 30 to >1000×) in potency. However, comparison of Z-Val-(NMe)Ala-Asp-CH_2PTP (**5**) to **3**, in which the P_2 NH hydrogen-bonding ability has been removed through N-methylation, reveals that **5** is essentially equal potent with the reference inhibitor **3** ($k_{obs}/[I]$ for **5** = 233 000 M^{-1} s^{-1} versus 280 000 M^{-1} s^{-1} for **3**). These data clearly demonstrate the importance of the P_1 and P_3 amido nitrogens versus the P_2 amido nitrogen for hydrogen bonding to the active site.[14,15]

The inhibition constants obtained for **4**–**6** have led us to postulate that a β-sheet type hydrogen-bonding recognition pattern is employed by ICE to bind this class of inhibitor (Figure 1). This hydrogen-bonding network is unprecedented for the papain superfamily and is reminiscent of the hydrogen-bonding interactions seen in crystal structures of serine protease–inhibitor complexes (Figure 1). Additional support for the β-sheet model comes from incorporating cyclic amino acids at positions P_2 and P_3 in inhibitors **7**–**10**. Again the analogous trend in the inhibitor profile is seen where the P_2 Aze (L-2-azetidinecarboxylic acid), Pro and Pip (L-2-piperidinecarboxylic acid) residues are tolerated (k_{obs}/I = 46 200, 116 000, and 271 000 M^{-1} s^{-1}, respectively) by the enzyme, while the P_3 Pro residue is disfavored (k_{obs}/I = <300 M^{-1} s^{-1}).[16] These data are consistent with the fact that the Z-Pro at position P_3 is unable to offer a free NH group, a necessary requirement for high-affinity binding. Verification of this putative β-sheet model and complete assessment of the hydrogen-bonding network must await an X-ray crystal structure determination of an ICE–inhibitor complex.

In summary, aspartic acid α-((1-phenyl-3-(trifluoromethyl)pyrazol-5-yl)oxy)methyl ketones have been discovered as a novel class of ICE inhibitor. The second-order rate constants for enzyme inactivation of the series **1**–**3** compare favorably to the analogous aspartic acid α-((2,6-dichlorobenzoyl)oxy)methyl ketones reported previously.[4] The results of the *N*-methyl scan of the Z-Val-Ala-Asp-CH_2PTP (**3**) peptide backbone have revealed that the P_1 and P_3 amido nitrogens are critically involved in the hydrogen bonding of the inhibitor to the active site of the enzyme. Demonstration of ICE inhibition by *crm*A protein (a viral serpin),[17] the aspartic acid S_1 subsite specificity determinant,[2,4,5,18] and now the putative hydrogen-bonding inhibitor recognition pattern (Figure 1) underscore the "serine protease-like" character of this unique enzyme. The elucidation of the β-sheet hydrogen-bonding motif exhibited between ICE and inhibitor **3** has important ramifications for peptidomimetic design, and our research activities in this area will be reported in due course.

Note Added in Proof: After submission of our manuscript, the X-ray crystal structures of ICE-(Ac-YVAD-H) (Wilson, K. P.; et al. *Nature* **1994**, *370*, 270–252) and ICE-(Ac-YVAD-CH_2Cl) (Walker, N. P. C.; et al. *Cell* **1994**, *78*, 343–352) were reported. The X-ray diffraction studies substantiate our putative ICE–inhibitor β-sheet model.

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procedure described by: McDermott, J. R.; Benoiton, N. L. N-Methylamino Acids in Peptide Synthesis. II. A New Synthesis of N-Benzyloxycarbonyl,N-Methylamino Acids. *Can. J. Chem.* **1973**, *51*, 1915–1919.

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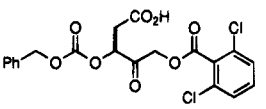
(11) The inactivation event is predicted to be irreversible by analogy with the inactivation of cysteine proteases by peptidyl ((arylacyl)-oxy)methyl ketones, see ref 7.

(12) (a) For a leading reference, see: *Mechanisms of Protease Action*; Polgar, L., Ed.; CRC Press, Inc.: Boca Raton, FL, 1989; Chapter 3; (b) Chapter 4.

(13) (a) Peptide inhibitors are bound in an antiparallel β-sheet with a single main chain strand at the active site for both chymotrypsin and elastase.[11a] For the serine proteases subtilisin and proteinase K, the same P_1 and P_3 NH groups are used for binding, although the inhibitor is complexed between two peptide strands in the active site: Betzel, C.; Singh, T. P.; Visanji, M.; Peters, K.; Fittkau, S.; Saenger, W.; Wilson, K. S. Structure of the Complex of Proteinase K with a Substrate Analogue Hexapeptide Inhibitor at 2.2-Å Resolution. *J. Biol. Chem.* **1993**, *268*, 15854–15858 and references therein. (b) There are examples of serine protease-inhibitor complexes where the P_1 NH appears not to be hydrogen-bonded, see: Delbaere, L. T.; Brayer, G. D. The 1.8 Å Structure of the Complex Between Chymostatin and *Streptomyces griseus* Protease A. A Model for Serine Protease Catalytic Tetrahedral Intermediates. *J. Mol. Biol.* **1985**, *183*, 89–103. Takahashi, L. H.; Radhakrishnan, R.; Rosenfield, R. E., Jr.; Meyer, E. F., Jr.; Trainor, D. A. Crystal Structure of the Covalent Complex Formed by a Peptidyl α,α-Difluoro-β-Keto Amide with Porcine Pancreatic Elastase at 1.78-Å Resolution. *J. Am. Chem. Soc.* **1989**, *111*, 3368–3374. Edwards, P. D.; Meyer, E. F., Jr.; Vijayalakshmi, J.; Tuthill, P. A.; Andisik, D. A.; Gomes, B.; Strimpler, A. Design, Synthesis and Kinetic Evaluation of a Unique Class of Elastase Inhibitors, the Peptidyl α-Ketobenzoxazoles, and the X-ray Crystal Structure of the Covalent Complex Between Porcine Pancreatic Elastase and Ac-Ala-Pro-Val-2-Benzoxazole. *J. Am. Chem. Soc.* **1992**, *114*, 1854–1863.

(14) (a) The peptide structure–activity relationship observed for ICE is similar in some respects to that observed for porcine pancreatic and human leukocyte elastase. For example, in the elastase tripeptide inhibitor Ac-Ala-Ala-Ala-CH_2Cl, replacing the P_2 Ala with Pro gives an inhibitor of comparable activity, while substituting the P_3 Ala with Pro gives a chloromethyl ketone which does not bind to the enzyme: Tuhy, P. M.; Powers, J. C. Inhibition of Human Leukocyte Elastase by Peptide Chloromethyl Ketones. *FEBS Lett.* **1975**, *50*, 359. Powers, J. C.; Tuhy, P. M. Active-Site Specific Inhibitors of Elastase. *Biochemistry* **1973**, *12*, 4767–4774. One principal difference between ICE and elastase is that an inhibitor series incorporating a P_2 Pro residue has enhanced (>4×) potency against elastase (see references above). However, for a given inhibitor series active against ICE, the opposite trend is true (e.g., 2× loss for **7** versus **3**). It is concluded from our work that Pip at the P_2 position is the preferred cyclic P_2 residue for ICE. (b) The P_2 N-alkylated amino acids are also well tolerated by human leucocyte elastase. This is consistent with the fact that the P_2 NH is not involved in hydrogen bonding to the enzyme: Skiles, J. W.; Fuchs, V.; Chow, G.; Skoog, M. Inhibition of Human Leucocyte Elastase by N-Substituted Tripeptide Trifluoromethyl Ketones. *Res. Commun. Chem. Pathol. Pharmacol.* **1990**, *68*, 365–374.

(15) (a) The lack of affinity for the N(Me)Asp analog **4** is not believed to be simply a result of steric occlusion as carbonate **15** (also unable to hydrogen bond) does not inhibit the enzyme (k_{obs}/[I] <100 M^{-1} s^{-1}), unpublished observation. (b) The k_{obs}/[I] for Z-N(Me)Asp-CH_2PTP is 12 ± 0.7 M^{-1} s^{-1} while Z-Ala-Asp-CH_2-PTP is 12 000 ± 200 M^{-1} s^{-1}.



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(18) Serine proteases use the S_1 subsite to discriminate against substrates and inhibitors (e.g., trypsin, S_1 subsite specificity for Arg). The S_2 subsite is the specificity determinant for cysteine proteases.[11]