P_1 Aspartate-Based Peptide α-((2,6-Dichlorobenzoyl)oxy)methyl Ketones as Potent Time-Dependent Inhibitors of Interleukin-1β-Converting Enzyme

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Interleukin-1β-converting enzyme (ICE) is the processing protease responsible for the production of the potent inflammatory mediator interleukin-1β (IL-1β).[1] Inhibitors of this intracellular protease are viewed as potential therapeutic agents for the treatment of a variety of chronic inflammatory disease states.[2] The detection and characterization of ICE as the IL-1β processing enzyme was independently reported by research groups at Immunex[1a-d] and Merck.[1e,f] The human enzyme has subsequently been purified from THP-1 cells, the cDNA cloned, and the amino acid sequence determined.[1c,d,f] The enzyme is a heterodimer consisting of 20- and 10-kD noncovalently bound subunits and has been characterized as a thiol (cysteine) protease.[3] Interestingly, the primary structure of ICE bears no sequence homology with any known member of the cysteine (or serine) protease superfamily.[4]

ICE cleaves the biologically inactive human IL-1β precursor protein (pro-IL-1β) between residues Asp^{116} and Ala^{117} to release the biologically active mature cytokine.[1e,f,5] Peptide-based α-((2,6-dichlorobenzoyl)oxy)methyl ketones[6] **1-3** which contain a P_1 L-aspartic acid residue were synthesized and evaluated as time-dependent inhibitors of the enzyme. The selection of the P_2 Val and Ala in inhibitors **2** and **3** as hydrophobic residue replacements for the native P_2 His is based on previously reported peptide substrate data.[1f,5]

... - P4 - P3 - P2 - **P1** - **P1'** - P2' - P3' - P4' - ...

....Tyr·Val·His·**Asp^{116}**-**Ala^{117}**·Pro·Val·Arg-....

↑ ICE cleavage site on pro-IL-1β

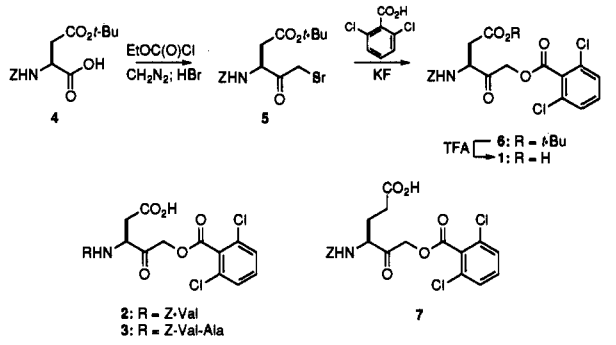
The synthesis of the requisite peptide α-((arylacyl)oxy)methyl ketones was carried out using methodology described by Krantz[6] and is exemplified by the synthesis of inhibitor **1** (Scheme 1). *N*-(Benzyloxycarbonyl)aspartic acid β-*tert*-butyl ester **4** (Z-Asp(OtBu)-OH; Bachem) was converted to Z-Asp(OtBu)-CH_2Br **5** upon (1) activation of the carboxylate as a mixed anhydride (1.4 equiv of *N*-methylmorpholine, 1.3 equiv of ethyl chloroformate, THF, -15 °C); (2) reaction of the mixed anhydride with diazomethane (2 equiv of CH_2N_2 in Et_2O, -15 °C → 25 °C); and (3) HBr-catalyzed decomposition of the *in situ* generated diazo ketone (excess glacial HOAc-45% HBr (1:1), 0 °C) in 75% yield. Noteworthy is the survival of the *tert*-butyl (side chain) ester during the acid-catalyzed decomposition of the diazo ketone. Treatment of **5** with 2,6-dichlorobenzoic acid (1.2 equiv of $2,6\text{-}Cl_2PhCO_2H$, 2.5 equiv of KF, DMF, 12 h, 25 °C) afforded the corresponding Z-Asp(OtBu)$CH_2OC(O)$-2,6-Cl_2Ph **6** in >95% yield. Deprotection of the *tert*-butyl ester in **6** to yield **1** occurred in quantitative yield following exposure of **6** to a solution of 25% trifluoroacetic acid (TFA) in CH_2Cl_2 (0.5 M solution of **6** in 25% v/v TFA/CH_2Cl_2). Similarly prepared were **2** and **3** from their respective carboxypeptides Z-Val-Asp(OtBu)-OH and Z-Val-Ala-Asp(OtBu)-OH. The homolog of **1**, Z-Glu-$CH_2OC(O)$-2,6-Cl_2Ph, **7**, was synthesized from Z-Glu-OH in analogous fashion.[7]

Table 1. Results of ICE and Cathepsin B Assays with Inhibitors 1-3 and 7

inhibitor	ICE[a] k_{obs}/[I] (M^{-1} s^{-1})	cathepsin B[b] k_{obs}/[I] (M^{-1} s^{-1})	selectivity ICE:cathepsin B
Z-Asp-CH_2DCB[c] (1)	7,100 ± 200	<10	>700:1
Z-Val-Asp-CH_2DCB (2)	41,000 ± 700	380 ± 80	108:1
Z-Val-Ala-Asp-CH_2DCB (3)	406,700 ± 38,400	2250 ± 40	180:1
Z-Glu-CH_2DCB (7)	*d*	*e*	

[a] ICE was partially purified from THP-1 cells using the DEAE-Sephacel and Sephadex G-75 steps described by Black (ref 1b). The ICE assay contained 10 mM HEPES (pH 7.5), 25% glycerol, 1 mM dithiotreitol (DTT) and 10 μM Suc-Tyr-Val-Ala-Asp-AMC (Bachem) in a volume of 30 μL in a polystyrene 96-well microtiter plate. Progress curves were obtained at 37 °C over 30 min. Kinetic data were obtained on a Fluoroskan II fluorescence plate reader under control of an Apple Macintosh computer running the DeltaSoft data aquisition program (BioMetallics, Inc.). Nonlinear progress curves were analyzed as described by Tian and Tsou (ref 13). [b] Bovine spleen cathepsin B was purchased from Sigma and assayed using Z-Phe-Arg-AMC (Bachem) under the conditions described by Barrett (ref 14). Adapted for use in continuous assays at 37 °C. [c] DCB = (2,6-dichlorobenzoyl)oxy. [d] No inhibition at 20 μM inhibitor. [e] Not determined.

Scheme 1. Synthesis of α-((2,6-Dichlorobenzoyl)oxy)methyl Ketone **1** and the Structures of Ketones **2**, **3**, and **7** (Absolute Stereochemistry Is as Shown)



Z = benzyloxycarbonyl

Evaluation of inhibitors **1-3** against ICE revealed these compounds to be potent time-dependent inactivators[8] of the enzyme (Table 1). Inhibitor **1**, Z-Asp-$CH_2OC(O)$-2,6-Cl_2Ph, which contains only a single amino acid residue, was sufficient for relatively rapid inactivation of enzyme. The k_{obs}/[I] of 7100 M^{-1} s^{-1} for **1** is the greatest value ever reported for a single amino acid-based affinity label against a cysteine protease.[9] Typically, a dipeptide unit is the smallest recognition sequence needed to achieve inactivation rates >1000 M^{-1} s^{-1} for this enzyme class.[10] Inclusion of the P_2 Val and P_3-P_2 Val-Ala residues into **1** enhances inhibitory potency in this series. The tripeptide analog Z-Val-Ala-Asp-$CH_2OC(O)$-2,6-Cl_2Ph, **3**, possesses

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a second-order rate constant equal to 406 700 M^{-1} s^{-1}. This rate is substantially faster than the *ca.* 16 500 M^{-1} s^{-1} rate observed for the inactivation of ICE with the tetrapeptide diazo ketone, Ac-Tyr-Val-Ala-Asp-CHN_2.[1f,11] Furthermore, enzyme specificity for an aspartic acid residue at P_1 in this class of inhibitor was clearly demonstrated as α-((2,6-dichlorobenzoyl)oxy)methyl ketone **7**, the side chain homolog of **1**, was devoid of enzyme affinity. This specificity for aspartic acid and the high inactivation rate observed for **1** suggests that the discriminating primary binding site for ICE is the S_1 subsite, atypical for a cysteine protease.[10]

Inhibitors **1–3** were also examined for their selectivity against cathepsin B (Table 1). ((Arylacyl)oxy)methyl ketone **1** displayed the highest selectivity for ICE versus cathepsin B (>700:1), while the more potent methyl ketones **2** and **3** demonstrated *ca.* 100–180-fold preference for ICE.[12]

References

(1) (a) Black, R. A.; Kronheim, S. R.; Cantrell, M.; Deeley, M. C.; March, C. J.; Prickett, K. S.; Wignall, J.; Conlon, P. J.; Cosman, D.; Hopp, T. P.; Mochizuki, D. Y. Generation of Biologically Active Interleukin-1β by Proteolytic Cleavage of the Inactive Precursor. *J. Biol. Chem.* **1988**, *263*, 9437. (b) Black, R. A.; Kronheim, S. R.; Sleath, P. R. Activation of Interleukin-1β by a Co-induced Protease. *FEBS Lett.* **1989**, *247*, 386. (c) Cerretti, D. P.; Kozlosky, C. J.; Mosley, B.; Nelson, N.; Ness, K. V.; Greenstreet, T. A.; March, C. J.; Kronheim, S. R.; Druck, T.; Cannizzaro, L. A. Molecular Cloning of the Interleukin-1β Converting Enzyme. *Science* **1992**, *256*, 97. (d) Kronheim, S. R.; Mumma, A.; Greenstreet, T.; Glackin, P. J.; Van Ness, K.; March, C. J.; Black, R. A. Purfication of Interleukin-1β Converting Enzyme, the Protease that Cleaves the Interleukin-1β Precursor. *Arch. Biochem. Biophys.* **1992**, *296*, 698. (e) Kostura, M. J.; Tocci, M. J.; Limjuco, G.; Chin, J.; Cameron, P.; Hillman, A. G.; Chartrain, N. A.; Schmidt, J. A. Identification of a Monocyte Specific Pre-interleukin 1β Convertase Activity. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5227. (f) Thornberry, N. A.; Bull, H. G.; Calaycay, J. R.; Chapman, K. T.; Howard, A. D.; Kostura, M. J.; Miller, D. K.; Molineaux, S. M.; Weidner, J. R.; Aunins, J.; Elliston, K. O.; Ayala, J. M.; Casano, F. J.; Chin, J.; Ding, G. J.-F.; Egger, L. A.; Gaffney, E. P.; Limjuco, G.; Palyha, O. C.; Raju, S. M.; Rolando, A. M.; Salley, J. P.; Yamin, T.-T.; Lee, T. D.; Shively, J. E.; MacCross, M.; Mumford, R. A.; Schmidt, J. A.; Tocci, M. J. A Novel Heterodimeric Cysteine Protease is Required for Interleukin-1β processing in Monocytes. *Nature* **1992**, *356*, 768.

(2) (a) Dinarello, C. A.; Wolff, S. M. The Role of Interleukin-1 in Disease. *N. Engl. J. Med.* **1993**, *328*, 106. (b) Dinarello, C. A. Interleukin-1 and Interleukin Antagonism. *Blood* **1991**, *77*, 1627.

(3) Cysteine 285 of the p20 kD subunit is believed to be the active-site cysteine as determined by affinity labelling using [^{14}C]iodoacetate. See ref 1f.

(4) (a) Barrett, A. J. In *Proteinase Inhibitors*; Barrett, A. J., Salvesen, G., Eds.; Elsevier Science Publishers B. V.: Amsterdam, 1986; Chapter 1 and references therein. (b) The primary structure of ICE bears no sequence homology with any known protein as determined by searching the amino acid and nucleotide ICE sequences against PIR-Protein and GenBank.

(5) (a) Sleath, P. R.; Hendrickson, R. C.; Kronheim, S. R.; March, C. J.; Black, R. A. Substrate Specificity of the Protease That Processes Human Interleukin-1β. *J. Biol. Chem.* **1990**, *265*, 14526. (b) Howard, A. D.; Kostura, M. J.; Thornberry, N.; Ding, G. J. F.; Limjuco, G.; Weidner, J.; Salley, J. P.; Hogquist, K. A.; Chaplin, D. D.; Mumford, R. A.; Schmidt, J. A.; Tocci, M. J. IL-1-Converting Enzyme Requires Aspartic Acid Residues For Processing of the IL-1β Precursor at Two Distinct Sites and Does Not Cleave 31-kDa IL-1α. *J. Immunol.* **1991**, *147*, 2964.

(6) Peptide α-((arylacyl)oxy)- and α-(aryloxy)methyl ketones are class specific inhibitors of cysteine proteases. (a) Krantz, A.; Copp, L. J.; Coles, P. J.; Smith, R. A.; Heard, S. B. Peptidyl (Acyloxy)methyl Ketones and the Quiescent Affinity Label Concept: The Departing Group as a Variable Structural Element in the Design of Inactivators of Cysteine Proteases. *Biochemistry* **1991**, *30*, 4678 and references therein. (b) Robinson, V. J.; Pauls, H. W.; Coles, P. J.; Smith, R. A.; Krantz, A. Carbon-13 NMR Characterization of the Papain Adduct Formed by Peptidyl Acyloxy-, Aryloxy-, and Chloromethyl Ketone Irreversible Inhibitors. *Bioorg. Chem.* **1992**, *20*, 42.

(7) All new compounds gave physical and spectroscopic data consistent with their structure. For **6**: ^{1}H NMR ($CDCl_3$) δ 7.36 (m, 9H, Ar), 5.90 (d, 1H, J = 8.0 Hz, NH), 5.20 (m, 4H, $ArCH_2O$ and $COCH_2O$), 4.67 (m, 1H, CH), 3.00 and 2.75 (doublet of doublets, 1H each, J = 4.8 and 17.0 Hz, CH_2COO), 1.42 (s, 9H, *t*-Bu); mass spectrum m/z (relative intensity) 510 (M + H, 10). For **1**: ^{1}H NMR (d_6-DMSO) δ 7.90 (d, 1H, J = 8.0 Hz, NH), 7.60 (m, 3H, 2,6-Cl_2-Ar), 7.30 (m, 5H, Ar), 5.27 and 5.19 (doublets, 1H each, J = 17.0 Hz, $COCH_2O$), 5.05 (s, 2H, $ArCH_2O$), 4.51 (m, 1H, CH), 2.80 (dd, 1H, J = 6.0 and 17.0 Hz, C*H*H'COO), 2.60 (dd, 1H, J = 7.5 and 17.0 Hz, CH*H'*COO); mass spectrum m/z (relative intensity) 454 (M + H, 15).

(8) The inactivation event is predicted to be irreversible. See ref 6b.

(9) For leading references, see: (a) Rich, D. H. In *Proteinase Inhibitors*; Barrett, A. J., Salvesen, G., Eds.; Elsevier Science Publishers B. V.: Amsterdam, 1986; Chapter 4. (b) Powers, J. C.; Harper, J. W. *Ibid.* Chapter 3.

(10) (a) *Mechanisms of Protease Action*; Polgar, L., Ed.; CRC Press, Inc.: Boca Raton, FL, 1989; Chapter 4. (b) The potency of **1** and **2** also contrasts with the reported minimal tetrapeptide sequence, Ac-Tyr-Val-Ala-Asp-NHMe, needed for substrate affinity.[1f] Within the peptide aldehyde series Z-Asp-H, Z-Ala-Asp-H, and Z-Val-Ala-Asp-H, only the tripeptide aldehyde displays sub-micromolar activity: Graybill, T.; Dolle, R. E.; Helaszek, C. T.; Miller, R. E.; Ator, M. A. The Preparation and Evaluation of Peptidic Aspartyl Hemiacetals as Reversible Inhibitors of Interleukin-1β Converting Enzyme (ICE). *Int. J. Peptide Protein Res.* in press. (c) Z-Phe-Ala-$CH_2OC(O)$-2,6-Cl_2Ph, the potent inactivator of cathepsin B,[6a] is inactive against ICE.

(11) Significant differences in the rates of inactivation of cathepsin B by (acyloxy)methyl ketones versus diazo ketones have been observed previously: Pliura, D. H.; Bonaventura, B. J.; Smith, R. A.; Coles, P. J.; Krantz, A. Comparative Behavior of Calpain and Cathepsin B Toward Peptidyl Acyloxymethyl Ketones, Sulfonium Methyl Ketones and Other Potential Inhibitors of Cysteine Proteases. *Biochem. J.* **1992**, *288*, 759.

(12) (a) Inhibitor **3** is a selective inhibitor of IL-1β versus IL-1α, TNF-α, and IL-6 in monocytes, IC_{50} = 0.5 μM: Uhl, J.; Brophy, L. S.; Dolle, R. E.; Helaszek, C.; Miller, R.; Ator, M. Presented at The American Society for Cell Biology; New Orleans, LA: 1993. (b) The ethyl ester of Z-Val-Ala-(D,L)Asp-$CH_2OC(O)$-2,6-Cl_2Ph is reported to selectively inhibit the release of IL-1β in THP-1 cells with an IC_{50} = 0.1 μM (Heng, R.; Payne, T. G.; Revesz, L.; Weidmann, B. PCT Application, WO 93/09135, 1993). Presumably, the ethyl ester is hydrolyzed *in vivo* to give the free acid which inhibits ICE. No enzyme inhibition data is given.

(13) Tian, W.-X.; Tsou, C.-L. Determination of the Rate Constant of Enzyme Modification by Measuring the Substrate Reaction in the Presence of the Modifier. *Biochemistry* **1982**, *21*, 1028.

(14) Barrett, A. J.; Kembhavi, A. A.; Brown, M. A.; Kirschke, H.; Knight, C. G.; Tamai, M.; Hanada, K. L-*trans*-Epoxysuccinyl-leucylamido-(4-guanidino)butane (E-64) and Its Analogues as Inhibitors of Cysteine Proteinases Including Cathepsins, B, H, and L. *Biochem. J.* **1982**, *201*, 189.