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Communications to the Editor

Pyridazinodiazepines as a High-Affinity, P_2-P_3 Peptidomimetic Class of Interleukin-1β-Converting Enzyme Inhibitor

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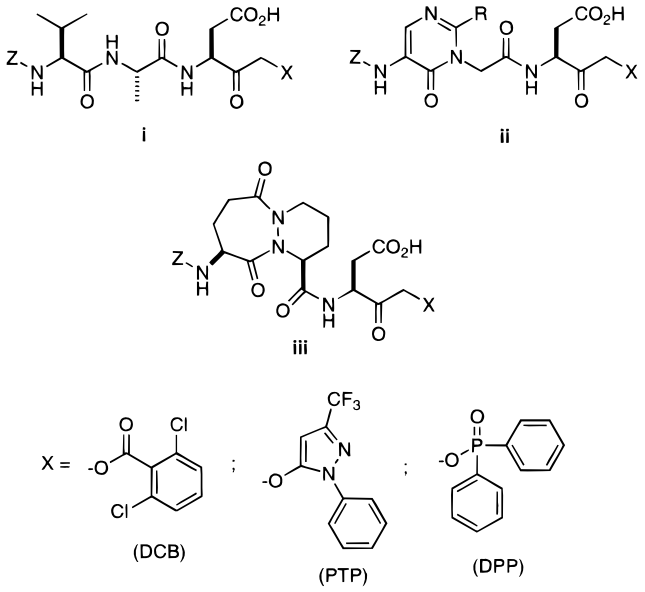
Interleukin-1β-converting enzyme (ICE) is the obligate enzyme for processing biologically inactive pro IL-1β to the biologically active cytokine, IL-1β.[1] Since this original discovery, the biological role of the enzyme has broadened to include the regulation of certain apoptotic processes, and a large family of homologs has been identified.[2] In a series of communications, we have chronicled our research efforts on the discovery of potent, selective, irreversible inhibitors of ICE.[3−8] These agents incorporate an aspartic acid-derived α-substituted methyl ketone as the essential enzyme recognition element.[7] The highest rates of inactivation, hence greatest potency, are observed in the tripeptide series **i**. Most recently, we disclosed the first examples of peptidomimetic inhibitors **ii** of the enzyme in which the Val-Ala unit (P_3-P_2 residues) was replaced by a pyrimidineacetic acid surrogate.[6] In this final communication, we describe the pyridazinodiazepines **iii** as a new peptidomimetic class of ICE inhibitor displaying exceptionally high affinity for the enzyme.



Previously, we documented the hydrogen-bonding pattern between ICE and its peptide-based inhibitors **i** by conducting an *N*-methyl scan of the tripeptide backbone.[4] These results demonstrated that the P_1 and P_3 amido hydrogens were required for high-affinity binding, leading to the replacement of P_3-P_2 residues in **i** with a pyrimidine acetyl mimetic[6,9] as in **ii**. Although the provision for correct hydrogen bonding exists in **ii**, the potency in this class did not strictly coincide with that of the tripeptide. For example in the tripeptide series, increased rates of inactivation are observed upon exchange of the N-terminal benzyloxycarbonyl (**1**: R = Z) to the 4-(methylthio)benzoyl group

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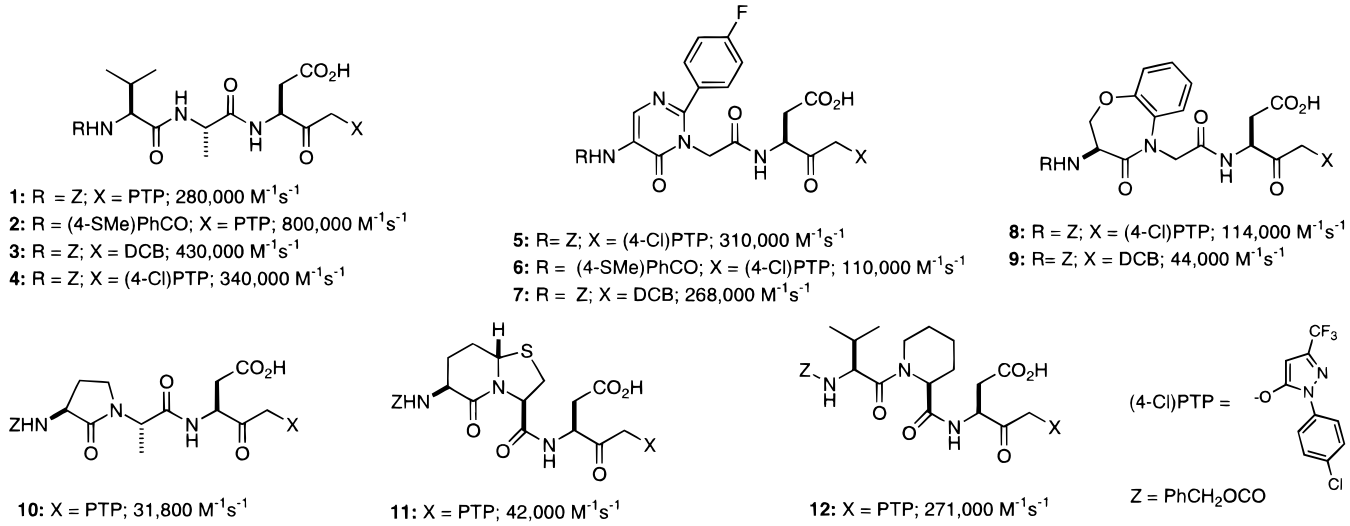
Chart 1. Second-Order Rate Constants of Inactivation (k_{obs}/[I]) of ICE for Peptidomimetic Inhibitors **5**−**11** and Reference Peptides **1**−**4** and **12**

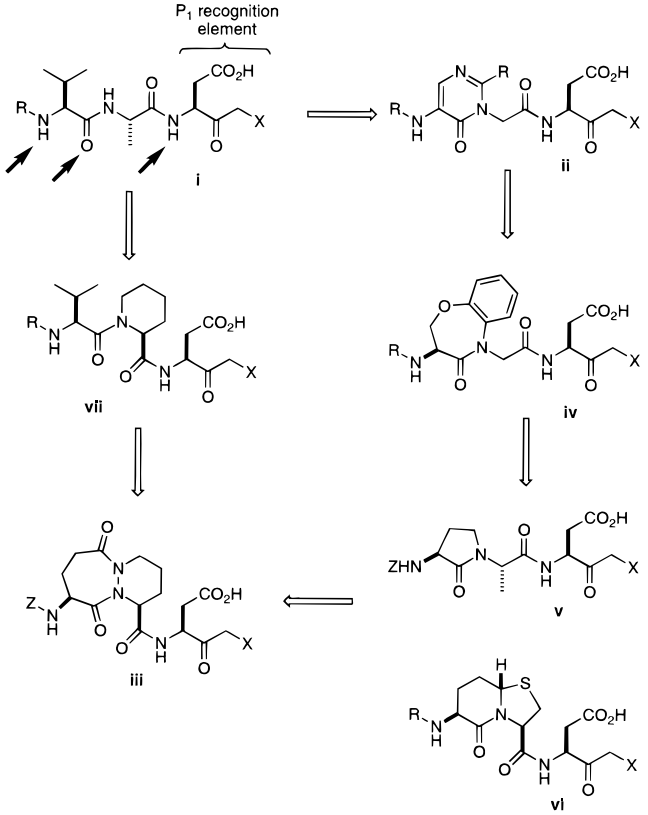


(**2**: R = 4-SMePhC(O)), but a similar trend in potency is not observed in the analogous pyrimidine acetyl pair **5** and **6** (Chart 1). One explanation for the divergence in SAR was thought to be due to the conformational constraint imposed by the sp^2 center in the pyrimidine acetic acid series, which may lead to suboptimal presentation of the P_3 amide and side chain to the enzyme.[10a]

In an attempt to find a mimetic with enhanced potency relative to **ii**, we synthesized the benzoxapine-acetamides[11,12] **iv** (Chart 2). One key difference between the compounds **iv** and those of series **ii** is that the terminal NH is attached to an sp^3 center. This is more analogous to the presentation of the P_3 valine residue in the tripeptide inhibitors. However, the second-order rate constants of inactivation for the benzoxapine series are poorer than either series **i** or **ii** (Chart 1). Upon direct comparison, the inactivation rates for the benzoxapine-based inhibitors **8** and **9** range from *ca.* one-third to one-tenth that of the pyrimidineacetamide (**5** and **7**) and tripeptide analogs (**3** and **4**).[13] A possible rationale for the attenuated performance of the benzoxapine acetic acid and the pyrimidine acetic acid mimetics relative to the tripeptides **i** (Chart 2) may lie with the absence of a *formal* P_2 side chain. The fused aryl ring in the benzoxapine and the 2-aryl ring in the pyrimidine series are hypothesized to project into the surrounding solvent with minimal interaction with the enzyme's S_2 binding pocket.[10b,14] It was thought that it may be desirable to have such appendages displayed to the enzyme directly from the α-carbon of an amide backbone, thereby more closely resembling the topography of the P_2 residue. For this reason, we opted to consider constrained dipeptides as second-generation mimetics having putative P_2 and P_3 side chain functionality.

As a starting point for mimetic selection, inhibitors containing a Freidinger lactam[15] **v** and a bicyclic turned dipeptide (BTD)[16] **vi** were synthesized[12] (Chart 2) and evaluated against ICE. Both of these mimetics satisfied our design criteria with regard to hydrogen-bonding functionality and P_2 and P_3 α-side chain functionality. Once again, however, the k_{obs}/[I] values were disappointing. Inhibitors **10** and **11** display inactivation rates of approximately 50 000 M^{-1} s^{-1}, compared with the corresponding tripeptide **4** of 340 000 M^{-1} s^{-1} (Chart 1). Because the NH and side chain requirements were believed to be adequately represented by structures **10** and **11**, it became apparent that the bioactive conformation of the amide backbone in the tripeptide series **i** is not achieved in **10** and **11**, and a further survey of P_2−P_3 surrogates was necessary.

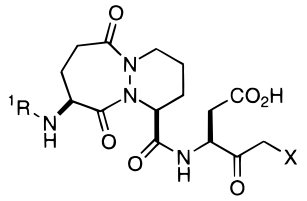
Chart 2. Evolution in Peptidomimetic Design Leading to the Pyridazinodiazepine-based ICE Inhibitors[a]



[a] Bold arrows indicate H-bond functionality required for high affinity binding.

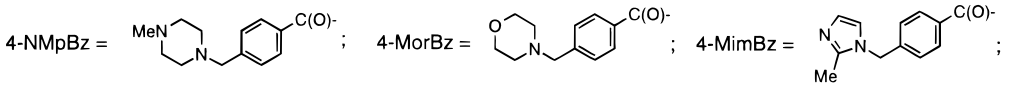
Concomitant with this peptidomimetic effort, we continued our studies in the tripeptide series. Upon introducing cyclic amino acids into the P_2 position, it was discovered that the pipecolic acid-containing tripeptide **12** (k_{obs}/[I] = 270 000 M^{-1} s^{-1}) is equally potent to Z-Val-Ala-Asp-CH_2PTP (k_{obs}/[I] = 280 000 M^{-1} s^{-1};

Table 1. Second-Order Rate Constants for Inactivation of ICE by Inhibitors **13**−**21** and Reference Peptides **1**, **3**, **4**, and **22**−**24**



Inhibitor No.	R^1	X	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)[a]
13	Z[b]	DCB[c]	572,000
14	Z	PTP[c]	413,000
15	Z	(4-Cl)PTP[c]	437,000
16	4-NMpBz[b]	DCB	210,000
17	4-MorBz[b]	DCB	425,000
18	4-MimBz[b]	DCB	340,000
19	4-DMaBz[b]	(4-Cl)PTP	162,000
20	4-TacBz[b]	(4-Cl)PTP	1,220,000
21	4-CetBz[b]	(4-Cl)PTP	800,000
1	Z-Val-Ala-Asp-CH_2PTP		280,000[4]
3	Z-Val-Ala-Asp-CH_2DCB		432,000[3]
4	Z-Val-Ala-Asp-CH_2(4-Cl)PTP		340,000
22	4-NMpBz-Val-Ala-Asp-CH_2PTP		200,000
23	4-MorBz-Val-Ala-Asp-CH_2PTP		186,000
24	4-TacBz-Val-Ala-Asp-CH_2PTP		960,000

(a) Assay as described in ref. 25; triplicate determinations; standard error <10%. (b) Z = benzyloxycarbonyl;



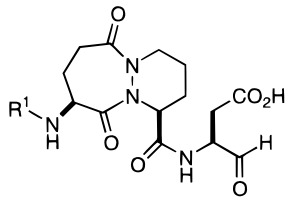
(c) DCB = (2,6-dichlorobenzoyl)oxy; PTP = (1-phenyl-3-(trifluoromethyl)pyrazol-5-yl)oxy; (4-Cl)PTP = (1-(4-chlorophenyl)-3-(trifluoromethyl)pyrazol-5-yl)oxy.

Chart 1).[4] This observation then led us to consider the pyridazinodiazepine **iii** as a potential P_2−P_3 peptidomimetic for ICE (Chart 2).[17] Mimetics **iii** possess the salient aspartic acid P_1 residue and an optimal six-membered cyclic residue at P_2 to which is fused the P_3 side chain. Inhibitor class **iii** also displays the requisite P_1 and P_3 amide nitrogens necessary for productive hydrogen bonding in the active site.[4,10c]

Rapid time-dependent inactivation of ICE was observed for the class of pyridazinodiazepines **13**−**21**, with rates comparable to or exceeding those determined for the tripeptides (Table 1).[12] The second-order rate constant of inactivation for the DCB analog **13** is 572 000 M^{-1} s^{-1} versus the DCB tripeptide **3** of 430 000 M^{-1} s^{-1}. Similar rates of inactivation are observed for analogs in the PTP (**14**, 413 000 M^{-1} s^{-1} versus **1**, 280 000 M^{-1} s^{-1}) and (4-Cl)PTP analog series (**15**, 440 000 M^{-1} s^{-1} versus **4**, 340 000 M^{-1} s^{-1}). The SAR of the P_3 N-terminal capping groups in series **iii** parallels the tripeptides, providing evidence that series **i** and **iii** share a common binding orientation in the active site. N-Terminal groups containing basic functionality[18] result in attenuated inactivation rates for both the pyridazinodiazepines (**16**−**18** versus **13**; **19** versus **15**) and the tripeptides (**22**−**23** versus **1**). In contrast, enhanced inactivation rates are observed for N-terminal groups possessing acidic functionality in both series (**20**, **21**, versus **15** and **24** versus **1**). In fact, the pyridazinodiazepine **20** with the 4-[(carboxymethyl)thio]benzoyl capping group displays an exceptionally rapid inactivation rate of 1 220 000 M^{-1} s^{-1}.[21]

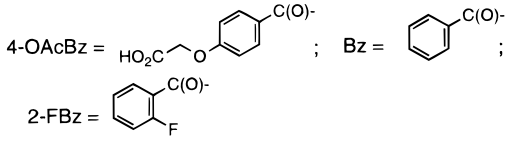
A selection of aspartic acid aldehydes **25**−**28** containing the pyridazinodiazepine mimetic were also synthesized[12a,19] and evaluated as reversible inhibitors of ICE. As revealed in Table 2, aldehydes **25**−**28** are potent reversible inhibitors with inhibition constants ranging from 1 to 25 nM. Inhibitors **26** and **28** (K_i = 1.0 nM, each) are 15-fold more potent than the corresponding

Table 2. Reversible Inhibition Constants for Inhibitors **25**−**28** and Reference Peptides **29**−**30** against ICE



Inhibitor No.	R^1	K_i (nM)[a]
25	Z[b]	25
26	4-OAcBz[b]	1
27	Bz[b]	5
28	2-FBz[b]	1
29	Z-Val-Ala-Asp-H	15[20]
30	Ac-Tyr-Val-Ala-Asp-H[b]	6[20]

(a) Assay as described in ref. 25; triplicate determinations; standard error <10%. (b) Z = benzyloxycarbonyl; Ac = acetyl;



tripeptide aldehyde and some 6-fold more potent than the tetrapeptide **30**. Within the mimetic class, potency enhancement is observed for acidic N-terminal functionality (**25** K_i = 25 nM versus **26** K_i = 1 nM), analogous to the irreversible agents. A 5-fold increase in affinity is seen for the 2-fluorobenzamide **28** (K_i = 1 nM) versus the unsubstituted benzamide **27** (K_i = 5 nM).[21,22]

In further studies, aldehyde **27** was selected as a candidate for oral administration in the dog. Compound **27** is stable in ex vivo liver and intestinal slice assays and possesses a plasma clearance rate in the dog of *ca.* 7 (mL/min)/kg. In a standard iv/po dog model, inhibitor **27** is orally bioavailable to the extent of 12% and 16% (determination in two dogs).[23] Pyridazinodiazepine **27** is a selective inhibitor of IL-1β versus IL-1α, TNF-α, and IL-6 in monocytes (IC_{50} = 1.0 μM).[24a] It also inhibits IL-1β production by >95% in a mouse model of biochemical efficacy at a single 100 mg/kg dose given by ip administration.[24b]

In summary, the pyridazinodiazepine is a suitable P_2−P_3 mimetic for the construction of high-affinity ICE inhibitors. The utility of the mimetic is demonstrated in both irreversible **13**−**21** (Table 1) and reversible inhibitors **25**−**28** (Table 2). The defining experiments leading to the discovery of the pyridazinodiazepine mimetic were 3-fold. First, an *N*-methyl scan of the amide backbone in the tripeptide series **i** established the importance of the P_1 and P_3 amido hydrogens for enzyme binding. On the basis of this observation, the hypothesis that the enzyme engages its peptide substrate via a β-sheet was developed.[4] Second, a cyclic amino acid scan at position P_2 in the tripeptide series established that pipecolic acid was an efficient surrogate for valine.[4] Finally, a survey of constrained dipeptide mimetics whose selection criteria satisfied the β-sheet hypothesis was conducted. Specifically, the selected mimetics displayed the essential P_3 NH, P_3 C(O), and P_1 NH hydrogen-bonding functionality,[4] ultimately relying on pipecolic acid as a template for pyridazinodiazepine synthesis.

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(10) (a) The ψ_3 angle (P_3-P_2 N/N dihedral angle) is 180° by virtue of the pyrimidine ring. This requires the P_3-P_2 residues in the series **i** to be bound as an extended antiparallel β-sheet in the active site. (b) The ψ_3 angle for the calculated (MM2) benzoxapine acetamides is 170°, a somewhat more relaxed extended β sheet. (c) The ψ_3 angle is 167°: see ref 17a.

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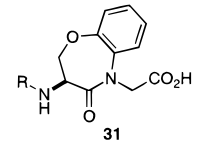
(12) (a) All new compounds gave physical and spectroscopic data consistent with their structure. (b) The general synthesis of the ICE inhibitors described herein follows the modular-based approach as previously described in ref 6. It involves coupling a peptidomimetic carboxylic acid (**31**−**34**) to an aspartylamine **35**−**37** followed by treatment with trifluoroacetic acid to remove the *tert*-butyl protecting group to give **8**−**11**, and **13**−**15**. For the synthesis of benzoxapine acetic acid **31**, see ref 11. For the synthesis of mimetic **32**, see ref 15. For the synthesis of BTD **33**, see ref 16. For the synthesis of mimetic **34**, see ref 17. For the synthesis of amines **35**−**37**, see ref 6.



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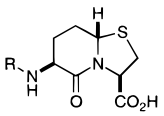

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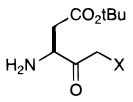

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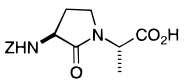
36: X = PTP
37: X = (4-Cl)PTP



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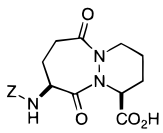

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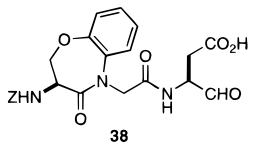
(13) Analogous to the decreased affinity (as measured by inactivation rate) in the benzoxapine series **iv** and the pyrimidine mimetic series **ii**, relative to the tripeptide series, the aldehydes **38** and **39** are also poor binders (reversible K_i values of >1 μM) versus tripeptide aldehyde **29** (Table 1).



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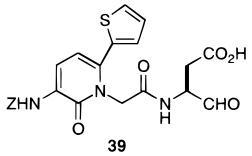

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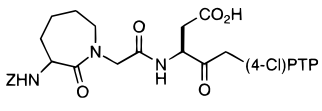
(14) (a) As evidenced by the X-ray crystal structures of ICE−inhibitor complexes, the P_2 side chain is solvent exposed, although it plays a key role in binding (see: Wilson, K. P.; Black, J. F.; Thomson, J. A.; Kim, E. E.; Griffith, J. P.; Navia, M. A.; Murcko, M. A.; Chambers, S. P.; Aldape, R. A.; Raybuck, S. A.; Livingston, D. J. Structure and Mechanism of Interleukin-1β Converting Enzyme. *Nature* **1994**, *370*, 270−275. Walker, N. P. C.; Talanian, R. V.; Brady, K. D.; Dang, L. C.; Bump, N. J.; Ferenz, C. R.; Franklin, S.; Ghayur, T.; Hackett, M. C.; Hammill, L. D.; Herzog, L.; Hugunin, M.; Houy, W.; Mankovich, J. A.; McGuiness, L.; Orlewicz, E.; Paskind, M.; Pratt, C. A.; Reis, P.; Summani, A.; Terranova, M.; Welch, J. P.; Xiong, L.; Moller, A.; Tracey, D. E.; Kamen, R.; Wong, W. W. Crystal Structure of The Cysteine Protease Interleukin-1β Converting Enzyme: A $(p20/p10)_2$ Homodimer. *Cell* **1994**, *78*, 343−352). For example, the $k_{obs}/[I]$ for Z-Val-Gly-Asp-DCB is 37 000 M^{-1} s^{-1} (see ref 7) versus that of the P_2 Ala analog **3**, 430 000 M^{-1} s^{-1}. (b) Attenuation in inactivation rates are seen with the exchange of the 4-fluorophenyl ring for a methyl group at position 2 of the pyrimidine acetic acid mimetic (see ref 6). Likewise, the caprolactam-based inhibitor **40**, an analog of benzoxapine **8** where the aryl ring is absent, shows a decreased inactivation rate (54 000 $M^{-1}s^{-1}$ versus 114 000 M^{-1} s^{-1} for **8**).



40

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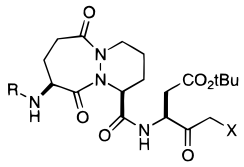
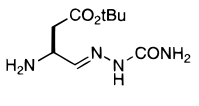
(18) For N-capping modifications in the pyridazinodiazepines **16**−**21**, intermediates **41** and **42** are hydrogenated to remove the Z-protecting group yielding amines **43** and **44**. The amines in turn are acylated with a desired carboxylic acid and treated with TFA to give the N-terminal modified inhibitor. The conditions used for these transformations were identical to those described in ref 6.



41: X = DCB; R = Z
42: X = (4-Cl)PTP; R = Z
43: X = DCB; R = H
44: X = (4-Cl)PTP; R = H

(19) The aldehydes **25**−**28** were prepared by coupling **34** with the semicarbazone **45**, yielding **46**, followed by acid hydrolysis to furnish **25**. Alternatively, hydrogenation to remove the Z-protecting group (**46** to **47**), acylation, and then acid hydrolysis provides aldehydes **26**−**28**. For the synthesis of the semicarbazone **45** and the respective reaction conditions, see ref. 20.



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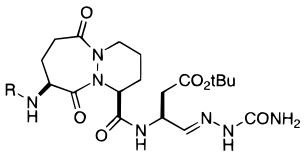
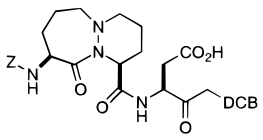


46: R = Z
47: R = H

(20) Graybill, T. L.; Dolle, R. E.; Helaszek, C. T.; Miller, R. E.; Ator, M. A. Preparation and Evaluation of Peptide Aspartyl Hemiacetals as Reversible Inhibitors of Interleukin-1β Converting Enzyme (ICE). *Int. J. Pept. Protein Res.* **1994**, *44*, 173−182.

(21) The corresponding 7-deoxy analog **48** is also active ($k_{obs}/[I]$ = 438 000 M^{-1} s^{-1}).



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(22) The fluorine atom in **28** is likely to be intramolecularly H-bonded to the P_3 NH in solution. Our purpose for introducing the fluorine substitution was to present a pseudo-conformationally-locked benzamide ring to the enzyme in an attempt to facilitate cell penetration and potentially overall binding.

(23) Unpublished observations. The tripeptide aldehyde **29** was not orally bioavailable.

(24) (a) Uhl, J.; Krasney, P.; Brophy, L.; Arnold, R.; Dolle, R. E.; Helaszek, C.; Miller, R.; Gilman, S.; Ator, M. Secretion of Human Monocyte Mature IL-1β: Optimization of Culture Conditions and Inhibition by ICE Inhibitiors. *Inflammation Res.* **1995**, *44*, S211−16. (b) Miller, B. E.; Krasney, P. A.; Gauvin, D. M.; Holbrook, K. B.; Koonz, D. J.; Abruzzese R. V.; Miller, R. E.; Pagani, K. A.; Dolle, R. E.; Ator, M. A.; Gilman, S. C. Inhibition of Mature IL-1β Production in Murine Macrophages and a Murine Model of Inflammation by WIN 67694, an Inhibitor of Il-1β Converting Enzyme. *J. Immunol.* **1995**, *154*, 1331−1338.

(25) Assay: ICE was partially purified from THP-1 cells using the DEAE-Sephacel and Sephadex G-75 steps described by Black (Black, R. A.; Kronheim, S. R.; Sleath, P. R. Activation of Interleukin-1β by a Co-induced Protease. *FEBS Lett.* **1989**, *247*,

386). The ICE assay contained 10 mM HEPES (pH 7.5), 25% glycerol, 1 mM dithiothreitol (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 acquisition program (Biometallics, Inc.). Nonlinear progress curves were analyzed to obtain inactivation rates as described by Tian and Tsou (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*, 1028−1032). K_i values for reversible inhibitors were determined as described in reference 20.

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