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SYNTHESIS AND EVALUATION OF DIACYLHYDRAZINES AS INHIBITORS OF THE INTERLEUKIN-1ß CONVERTING ENZYME (ICE)

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Abstract. Diacylhydrazines ([azaAsp[1]] derivatives) were prepared and shown to inactivate ICE in a time-dependent manner. Inactivation rates for most of these diacylhydrazines were 10-fold slower than their α-substituted methylketone congeners. Rates for **6** and **7** (ca. 18,000 $M^{-1}s^{-1}$) compared favorably to those reported for Ac-Tyr-Val-Ala-Asp-CH-N_2 and azapeptide inhibitors for other cysteine proteases.

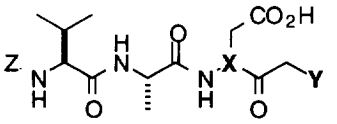
The cytokine interleukin-1β (IL-1β) is believed to be an important mediator of the biochemical cascade leading to inflammation.[2] Interleukin-1β Converting Enzyme, or ICE, cleaves the biologically inactive human IL-1β precursor protein (p-IL-1β) between residues Asp^{116} and Ala^{117} to release the biologically active cytokine.[3-7] Drugs that inhibit ICE might therefore attenuate IL-1β production and thus represent a new therapeutic approach for the treatment of a variety of inflammatory disease states.[8] Recent lines of evidence also suggest that ICE, or a related homolog, may play a role in the regulation of programmed cell death (apoptosis).[9] Potent and selective inhibitors of ICE may lead to a better understanding of the role that ICE plays in both inflammation and programmed cell death.

The discovery of potent, selective inhibitors for ICE is an active area of research. Highly effective inhibitors of ICE such as aspartyl aldehydes,[3,10,11] ketones[12] and α-(acyloxy)methyl ketones[13] have already been described. The crystal structure of ICE has recently been solved[14,15] and should aid future inhibitor design efforts. We have recently communicated that peptidyl α-((2,6-dichlorobenzoyl)oxy)-,[16] α-((tetronoyl)oxy)-,[17] α-((1-phenyl-3-(trifluoromethyl)pyrazol-5-yl)oxy)-,[18] α-((diphenylphosphinyl)oxy)methyl ketones[19] derived from aspartic acid (such as **2-5**, Table 1) are potent time-dependent inactivators of ICE. While these compounds displayed rapid time-dependent inactivation ($k_{obs}/[I]$ > 100,000 $M^{-1}s^{-1}$) and >150-fold selectivity for ICE versus the cysteine protease cathepsin B, it was not clear what effect replacement of the P_1 aspartic acid residue of **1-5** with a P_1 azaaspartic acid residue, [azaAsp[1]], would have on the characteristics of these inhibitors.

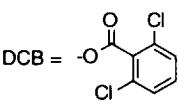
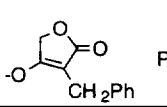
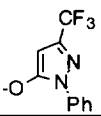
This α-CH ⇒ N substitution was of interest to us for the following reasons. First, ICE has a strict requirement for an L-aspartic acid residue in the P_1 position of substrates.[20] To our surprise, however, the [D-Asp[1]] analogs of **2-4** were equipotent in our *in vitro* ICE assay.[20] Interpretation of these data was complicated by the fact that the P_1 Asp residue of inhibitors **2-4** epimerized (complete epimerization in 5-16 h, HPLC) when inhibitors were incubated in assay buffer (10 mM Hepes, pH = 7.5, 25% glycerol, 1 mM dithiothreitol).[21] Epimerization would no longer be a complication if the α-CH (sp^3 center) of these inhibitors was replaced with a nitrogen atom (sp^2 center, **6-11**). Exactly what effect this substitution would have on inhibitory potency was not known.

Second, azaamino acid residues have been successfully employed to increase drug potency and metabolic/proteolytic stability.[22] The results of an N-methylation scan experiment with inhibitor **4** revealed the

Table 1. Structural and ICE Inhibition Data For Inhibitors **1**-**11**

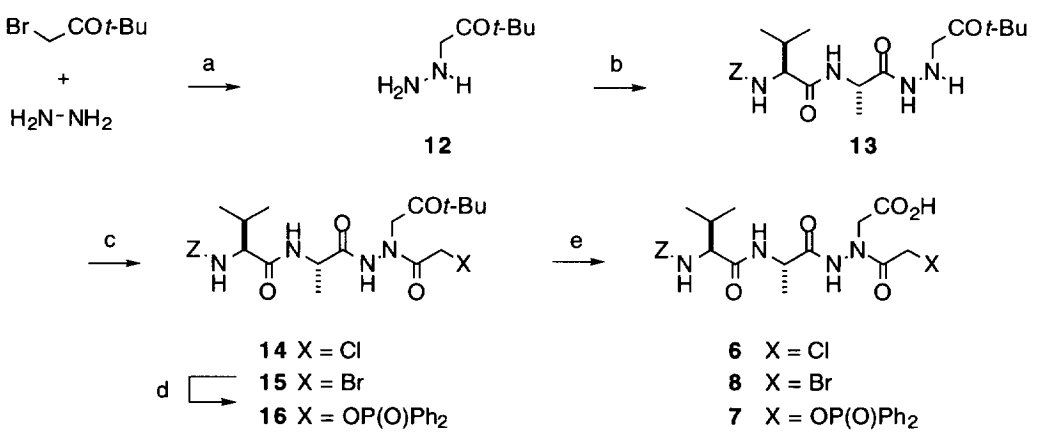


Compound	X	Y	k_{obs} / [I] ($M^{-1}s^{-1}$)[a]
1	CH	Cl	ND[b]
2	CH	DCB	407,000±38,000
3	CH	BTA	290,000±35,000
4	CH	PTP	280,000±38,000
5	CH	DPP	118,000±16,000
6	N	Cl	27,300±7400
7	N	DPP	19,400±1000
8	N	Br	1,640±400
9	N	DCB	700±50
10	N	PTP	470±30
11	N	BTA	120±10

DCB =  BTA =  PTP =  DPP = -OP(O)Ph_2

[a] Assay conditions as described in reference 16 (n = 3). [b] Not determined (low buffer stability, $t_{1/2}$ ~ 15 min)

Scheme 1. Preparation of [azaAsp[1]] Inhibitors **6**, **7**, and **8**.

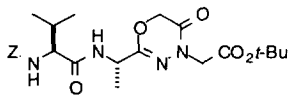


Key: (a) Niedrich, H., ref. 23; (b) Z-Val-Ala-OH, EDC, HOBT, DMF, 6 h, 90%; (c) chloro- or bromo-acetylchloride, NMM, CH_2Cl_2, 0 °C, 2 h, 95%; (d) $HOP(O)Ph_2$, KF, DMF, 65 °C, 15 h, 64%; (e) 25% TFA / CH_2Cl_2, 2 h, 90-98%.

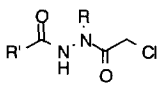
importance of the P_3 and P_1 amido NH functionality for productive hydrogen bonding of **4** to the ICE active site.[18] The acidity of the hydrazide NH group of an azaamino acid residue is higher than in a typical peptide bond and thus should favor stronger H-bonding between enzyme and inhibitor.[22] Thus, incorporation of an [azaAsp^1] residue might increase potency as well as impart proteolytic stability to the peptide-based scaffold of **1-5**.

Finally, diazomethane is required in a key step for preparation of inhibitors **1-5**.[16] Diazomethane is toxic, potentially explosive and not amenable to scaleup. In contrast, we anticipated that [azaAsp^1] inhibitors **6-11** were synthetically accessible without diazomethane. In this communication, we report the preparation and evaluation of diacylhydrazines **6-11** ([azaAsp^1] derivatives) as inhibitors of the interleukin-1ß converting enzyme (ICE).

The preparation of diacylhydrazines **6-11** is described in Scheme 1. Hydrazine **12** was prepared from 70% hydrazine hydrate and *t* -butylbromoacetate using the conditions of Niedrich.[23] The acylhydrazine **13** was obtained by coupling hydrazine **12** with Z-Val-Ala-OH using a water-soluble carbodiimide protocol. Acylation of the primary amino functionality of **12** was predicted based on literature precedent.[24] Treatment of acylhydrazine **13** with either chloro- or bromoacetyl chloride provided the corresponding chloro- and bromoacetyl derivatives **14** and **15** in excellent yield. As anticipated, KF-assisted conversion of the bromoacetyl hydrazide **15** to the corresponding phosphinic acid ester derivative **16** required higher temperature (65 °C) and longer reaction duration (15 h) than that necessary for the preparation of phosphinic acid ester **5** from the corresponding bromomethylketone (25 °C, 5-10 h).[25] Preparation of the analogous DCB, PTP and BTA derivatives (**16**, X = DCB, PTP, BTA) required similar conditions with yields ranging from 40-85%. At this temperature, the rate of intramolecular cyclization of bromoacetyl hydrazide **15** (or **16**) can compete with that of KF-assisted intermolecular displacement. As a result, peptidyl-1,3,4-oxadiazine **17** was often obtained as the major byproduct (5-40%). Finally, treatment of *t*-butyl esters **14**, **15**, and **16** with a solution of trifluoroacetic acid and dichloromethane (1:3) provided the desired [azaAsp^1] derivatives **6-8** as stable white amorphous solids in near quantitative yield.[26]



17



18

The potency of inhibition and the inherent chemical reactivity of diacylhydrazines **6-11** were determined. Table 1 summarizes the results of our inhibition studies. Time-dependent inactivation of ICE was observed with all compounds (**1-11**).[27] However, the activity of the [azaAsp^1] inhibitors **6-11** was disappointing. In the case of [azaAsp^1] inhibitors **9-11**, the second-order rates of inactivation, k_{obs}/[I], were much slower (100-1000x) than those of their α-substituted methylketone parents (**2**, **3** and **4**). However, despite the lack of P_1 chirality, [azaAsp^1] inhibitors **6** and **7** show inactivation rates greater than 18,000 $M^{-1}s^{-1}$. These rates are comparable to those of a recently reported tetrapeptide diazomethylketone (Ac-Tyr-Val-Ala-Asp-CH-N_2, 16,500 $M^{-1}s^{-1}$)[3] and azapeptide inhibitors reported for other cysteine proteases.[28,29]

Successful application of the quiescent nucleofuge strategy[30] requires selective irreversible reaction of the inhibitor with the active site thiolate of the target cysteine protease and not with other bionucleophiles present *in vivo*. Giordano has reported that compared to chloromethyl ketone derivatives, chloroacetyl hydrazides such as **18** have attenuated reactivity toward bionucleophiles such as glutathione.[28] By analogy, [azaAsp^1] inhibitors **6-11** ought to demonstrate greater stability than inhibitors **1-5**. Stability studies with chloromethyl ketone **1**, α-

((diphenylphosphinyl)oxy)methyl ketone **5**, and [azaAsp^1] inhibitors **6** and **7** support this idea. Table 2 lists the experimentally determined half-lives for compounds **1**, **5-7** in a typical ICE assay buffer containing 1 mM dithiothreitol at 25 °C and with assay buffer containing 1 mM glutathione at 70 °C. While the observed half-life of chloromethyl ketone **1** in assay buffer with 1 mM dithiothreitol at 25°C was quite short (0.25 h), 89% of **6** and 98% of **7** remained intact after 5 days under identical conditions. Because little degradation had occurred, the half-lives of **6** and **7** could not be accurately determined. However, we conclude that the inherent reactivity of [azaAsp^1] inhibitors **6** and **7** is attenuated at least 1000-fold compared to that of chloromethyl ketone **1**. Just as stability of chloroacetyl hydrazide **6** was greater than that of chloromethyl ketone **1**, the stability of α-((diphenylphosphinyl)oxy)acetyl hydrazide **7** (half-life = 130 h) was more than 100-fold greater than that of the corresponding α-((diphenylphosphinyl)oxy)methyl ketone **5** in a stressed stability study (assay buffer, 1 mM glutathione, 70 °C). In fact, the observed half-life of inhibitor **7** under these conditions was the same with or without 1 mM glutathione.[31] This observation again highlights the stability of inhibitor **7** to thiol nucleophiles. Clearly, non-specific alkylation of bionucleophiles by [azaAsp^1] inhibitors **6-11** should be minimal under physiological conditions. The lower ICE inactivation rates for [azaAsp^1] inhibitors **6-11** may be due in part to the enhanced chemical stability of these inhibitors compared to the α-substituted methyl ketones **1-5**.

Table 2. Half-lives of Inhibitors **1**, **5**, **6**, and **7** in ICE assay buffer (1 mM dithiothreitol) at 25 °C and with assay buffer (1 mM glutathione) at 70 °C.

	Assay buffer, 1 mM dithiothreitol, 25 °C[a,c,d]	Assay buffer, 1 mM glutathione, 70 °C[b,c,d]
Inhibitor	$t_{1/2}$ (h)	$t_{1/2}$ (h)
1	0.25	---[e]
5	---	0.96
6	>>120 h (89% intact after 120 h)	3.5
7	>>120 h (98% intact after 120 h)	130

[a] Solutions for stability studies were prepared by addition of a 0.1 mL DMSO solution of inhibitor (1 mg/mL DMSO) to 0.9 mL of buffer (10 mM Hepes, pH = 7.5, 25% glycerol, 1 mM dithiothreitol, purged with N_2 and sealed). [b] Solutions for stability studies were prepared by addition of a 0.1 mL DMSO solution of inhibitor (1 mg/mL DMSO) to 0.9 mL of buffer (10 mM Hepes, pH = 7.5, 25% glycerol, 1 mM glutathione, purged with N_2 and sealed). [c] Aliquots were removed and analyzed by HPLC over a 5 day period. Half-lives were determined by linear regression of the natural logarithm of peak area versus time. [d] 95% confidence interval ± 10%. [e] Not determined.

In addition, structural modifications brought about by the α-CH ⇒ N substitution presumably also play a role in the attenuated potency of the [azaAsp^1] inhibitors **6-11**. Computationally-determined low energy conformations of a model N-alkylated diacylhydrazine and a chloromethylketone indicate differences in bond lengths, bond angles, and torsion angles between the two structural classes.[29] This suggests that the [azaAsp^1] inhibitors **6-11** may have difficulty achieving the bioactive orientation readily attained by inhibitors **1-5**.

In conclusion, diacylhydrazines **6-11** were prepared as inhibitors of the interleukin-1ß converting enzyme. These compounds do not require diazomethane for preparation, show attenuated reactivity with bionucleophiles, and cannot epimerize at the P_1 position. While inactivation rates for most of these diacylhydrazines were much

slower (100-1000x) than their α-substituted methylketone parent, [azaAsp[1]] inhibitors **6** and **7** inactivated ICE at rates greater than 18,000 $M^{-1}s^{-1}$. These rates compare favorably to that of a reported tetrapeptide diazomethylketone, Ac-Tyr-Val-Ala-Asp-CH-N_2, and with the best azapeptide inhibitors reported for other cysteine proteases. The substantial reduction of inactivation rates for the [azaAsp[1]] series is presumably due to differences in the binding affinity (Ki) and/or differences in the chemical alkylation step (k_{inact}).

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21. While the mechanistic details of the epimerization are not known with certainty, our experimental data (not shown) supports intramolecular abstraction of the α-proton by the ß-carboxylate oxygen. Intramolecular side-chain mediated epimerization of a P_1 Lys residue of an acyloxymethylketone inhibitor has also been observed by Krantz et al. (Wagner, B. M.; Smith, R. A.; Coles, P. J.; Copp, L. J.; Ernest, M. J.; Krantz, A. *J. Med. Chem.* **1994**, *37*, 1833-1840).

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25. α-Chloroamides and esters are at least an order of magnitude less reactive toward nucleophilic displacement than α-chloroketones. See references 28, 29 and references therein.

26. All new compounds have physical and spectroscopic data consistent with their structure. For **7**: ^{1}H NMR (300 MHz, DMSO-d_6)δ 8.18 (d, J 5.3 Hz, 1H), 7.75 (m, 4H), 7.5 (m, 7H), 7.32 (m, 5H), 7.15 (bs, 1H), 5.00 (d, J = 12.7 Hz, 1H), 4.94 (d, J = 12.7 Hz, 1H), 4.6 (m, 4H), 4.07 (m, 1H), 3.80 (t, J = 8.1 Hz, 1H), 1.85 (m, 1H), 1.07 (d, J = 5.8 Hz, 3H), 0.76 (d, J = 6.8 Hz, 3H), 0.73 (d, J = 6.8 Hz, 3H). FAB MS (nba) *m/z* 653 (M^++H). Anal. Calcd for $C_{32}H_{37}N_4O_9P{\cdot}0.25H_2O$: C, 58.49; H, 5.75; N, 8.53. Found: C, 58.23; H, 5.87; N, 8.41. For intermediate **15**: ^{1}H NMR (300 MHz, DMSO-d_6)δ 8.26 (d, J = 4.7 Hz, 1H), 7.3 (m, 7H), 4.97 (m, 2H), 4.2 (m, 4H), 3.94 (d, J = 12.5 Hz, 1H), 3.83 (t, J = 7.9 Hz, 1H), 1.93 (m, 1H), 1.38 (s, 9H), 1.20 (d, J = 7.0 Hz, 3H), 0.82 (d, J = 6.8 Hz, 3H), 0.78 (d, J = 6.7 Hz, 3H).

27. The inactivation event is presumed to be irreversible by analogy with the known mechanism of irreversible inactivation by peptide α-halo- and α-(acyloxy)methyl ketones.

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31. This suggests that the major mechanism of degradation of **7** under these conditions does not involve nucleophilic displacement of the leaving group by the thiol functionality of glutathione or one of its decomposition products. While no attempt was made to determine the mechanism(s) of degradation for [azaAsp^1] inhibitor **7**, it is tempting to propose a mechanism involving intramolecular cyclization of **7** leading to an 1,3,4-oxadiazine structure (analogous to **17**).

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