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α-((TETRONOYL)OXY)- AND α-((TETRAMOYL)OXY)METHYL KETONE INHIBITORS OF THE INTERLEUKIN-1β CONVERTING ENZYME (ICE)

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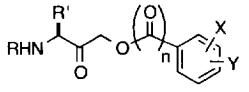
Abstract. Aryl-substituted tetronic acids, tetramic acids, and cyclic β-dicarbonyl moieties were evaluated as leaving groups in the peptidyl-$COCH_2$-X type inhibitor **iii**. Tripeptidyl aspartyl α-((tetronoyl)oxy)- and α-((tetramoyl)oxy)methyl ketone derivatives demonstrate potent time-dependent inhibition (k_{obs}/[I] 100,000-250,000 $M^{-1}s^{-1}$) of the cysteine protease ICE.

Peptidyl α-(aryloxy)- **i** and α-((aryl)acyloxy)methyl ketones **ii** represent a significant advance in the design of cysteine protease inhibitors.[1] These methyl ketone derivatives possess low chemical reactivity yet are potent, irreversible inactivators of cathepsin B and other cysteine proteases.[2] We[3] and others[4] have recently reported that aspartyl α-((aryl)acyloxy)methyl ketones (such as α-((2,6-dichlorobenzoyl)oxy)methyl ketones **1-3**) are irreversible inactivators of the interleukin-1β converting enzyme (ICE). Identification of potent and selective inhibitors may lead to a better understanding of the role that this cysteine protease plays in chronic and acute inflammatory diseases[5] and possibly apoptosis (programmed cell death).[6]

Figure 1



While α-((2,6-dichlorobenzoyl)oxy)methyl ketone **3** displayed potent time-dependent inactivation (k_{obs}/[I] > 400,000 $M^{-1}s^{-1}$) and >150-fold selectivity for ICE versus cathepsin B,[3a] we sought to identify new classes of quiescent affinity labels for ICE and other cysteine protease targets. Our interest in novel leaving groups was prompted by the report of Krantz and Smith who observed that the rate of cathepsin B inactivation by α-(aryloxy)- and α-((aryl)acyloxy)methyl ketones **i** and **ii** is strongly dependent on the pK_a of the phenol or benzoate leaving group.[1,2] However, the potency-pK_a correlation is not absolute.[7] This suggested to us that the inherent structure of the leaving group may be important for enzyme affinity. This communication describes our search for functionality other than benzoates and phenols to serve as leaving groups in the peptidyl-$COCH_2$-X type of inhibitor. While this report will focus primarily on the discovery and optimization of α-((tetronoyl)oxy)- and α-((tetramoyl)oxy)methyl ketone inhibitors, the SAR generated from this work led to the identification of additional inhibitor classes with improved selectivity, stability, and potency profiles.[8]

A variety of carboxylic acid derivatives (alkyl, heterocyclic, and fused aryl acids), aryl tetrazoles, aryl and heterocyclic thiols, phosphorous-based acids, and other structural classes were examined as potential leaving groups in the peptidyl-$COCH_2$-X type of inhibitor. Important selection criteria for this leaving group screening were estimated pK_a (pK_a < 6), availability, and that the functionality react cleanly in an S_n2 fashion with aspartyl bromomethyl ketones **4** (Scheme 1) in DMF using KF or K_2CO_3 as base. The benchmark inhibitors for these studies were the aspartyl α-((2,6-dichlorobenzoyl)oxy)methyl ketones **1-3**.[3a] In general, aspartyl

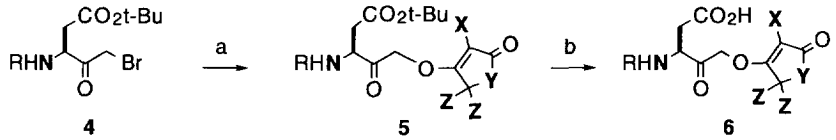
methyl ketones bearing these potential leaving groups (with the exception of certain phosphinic acids)[9] were either poor inhibitors of ICE or completely inactive.

Table 1. ICE Inhibition Data For α-((2,6-Dichlorobenzoyl)oxy)methyl Ketones **1-3**[3a]

Compound		$k_{obs}/[I]$ ($M^{-1}s^{-1}$)[a]
1	Cbz-Asp-CH_2-DCB[b]	7,100 ± 200
2	Cbz -Val-Asp-CH_2-DCB	41,000 ± 700
3	Cbz -Val-Ala-Asp-CH_2-DCB	407,000 ± 40,000

[a]Assay conditions as described in reference 3a (n = 3). [b]DCB = 2,6-dichlorobenzoyloxy.

Scheme 1. Synthesis of Aspartyl α-((Tetronoyl)oxy)- and ((Tetramoyl)oxy)methyl Ketones **7-20**.



4 **5** **6**

R = Cbz, Cbz-Val, Cbz-Val-Ala

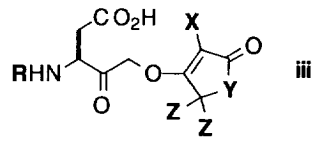
Key: (a) tetronic or tetramic acid (1.3 equiv), KF (2.5 equiv), DMF, 16 h, 70-95%;
(b) 25% TFA/CH_2Cl_2, 1 h, 80-95%

An additional structural class that met our selection criteria for leaving group screening was cyclic β-dicarbonyl compounds. Cyclic β-dicarbonyl-based leaving groups were first employed in benzisothiazolone-based inhibitors of the serine protease human leukocyte elastase (HLE).[10a] We set out to determine whether the use of these β-dicarbonyl leaving groups could be extended to the inhibition of cysteine proteases such as ICE.

A structurally-diverse group of cyclic β-dicarbonyls[10b] was incorporated into a selection of aspartic acid-derived peptide scaffolds using methodology described for the synthesis of aspartyl α-((2,6-dichlorobenzoyl)oxy)methyl ketones **1-3**.[3a] The synthesis of aspartyl α-((tetronoyl)oxy)- and α-((tetramoyl)oxy)methyl ketones **7-20** is described in Scheme 1. Aspartic acid bromomethyl ketone *tert*-butyl esters **4** (1 equiv) were subjected to direct displacement with the appropriate tetronic or tetramic acid (1.2 equiv) in the presence of potassium fluoride (2.5 equiv) followed by trifluoroacetic acid-mediated deprotection of the aspartyl sidechain carboxyl functionality.[11] The tetronic acids utilized for the preparation of compounds **7-9**, **16-18**, and **20** are commercially available. The tetronic and tetramic acid derivatives required for the preparation of compounds **10-13**,[12] **14**,[13] **15**,[14] and **19**[15] were prepared by literature methods.

Although a broad variety of β-dicarbonyl-based leaving groups resulted in picomolar inhibitors of elastase,[10] only narrow structural classes provided time-dependent inhibition of ICE. An early success was the discovery that the 2,6-dichlorobenzoate leaving group of **1** and **2** could be replaced with a 3-phenyltetronoate moiety. The second order rates of ICE inactivation for tetronate inhibitors **7** and **17** (Table 2) compared well with those of reference inhibitors **1** and **2**. Therefore, additional effort was made to explore the SAR for this class of leaving group. It quickly became apparent that the 3-phenyl substituent of **7** (**X** = C_6H_5) was important for efficient ICE inactivation. Replacement of the 3-phenyl substituent of **7** with a hydrogen (**8**, **X** = H) resulted in a 30-fold decrease in inactivation rate, while compound **9** (**X** = Cl) did not exhibit time-dependent inactivation in our enzyme assay.

Table 2. Inhibition and Stability Data for α-((Tetronoyl)oxy)- and ((Tetramoyl)oxy)methyl Ketones **7-20**.



iii

Compd	R	X	Y	Z	k_{obs}/[I] ($M^{-1}s^{-1}$)[a]	Stability ($t_{1/2}$, h)[b]
7	Cbz	C_6H_5	O	H	5,700 ± 1000	0.86 ± 0.07[c]
8	Cbz	H	O	H	188 ± 16	---[d]
9	Cbz	Cl	O	H	< 25	---
10	Cbz	4-Cl C_6H_4	O	H	4,100 ± 1200	---
11	Cbz	4-OMeC_6H_4	O	H	4,000 ± 1200	---
12	Cbz	4-Me C_6H_4	O	H	4,500 ± 500	---
13	Cbz	3,4-Cl_2 C_6H_3	O	H	640 ± 230	---
14	Cbz	C_6H_5	O	CH_3	260 ± 20	---
15	Cbz	C_6H_5	CH_2	H	3,070 ± 80	3.9 ± 0.4
16	Cbz	CH_2-C_6H_5	O	H	2,850 ± 70	9.8 ± 0.8
17	Cbz-Val	C_6H_5	O	H	27,900 ± 2,300	---
18	Cbz-Val	CH_2-C_6H_5	O	H	21,200 ± 2000	---
19	Cbz-Val	C_6H_5	NH	H	8,500 ± 300	95 ± 10
20	Cbz-Val-Ala	CH_2-C_6H_5	O	H	252,000 ± 11,000	20 ± 3

[a]Assay conditions as described in reference 3a (n = 3). [b]Solutions for stability studies were prepared by addition of a 0.1 mL DMSO solution of inhibitor (2 mg/mL) to 0.9 mL of assay buffer (RPMI 1640 media, 1% FBS, pH 7.4). Aliquots were removed and analyzed by HPLC. Half-lives were determined by linear regression of the natural logarithm of peak area versus time. [c]95% confidence interval. [d]Not determined.

The fact that the 3-phenyl substituent of **7** (**X** = C_6H_5) appeared important for inhibition of ICE suggested that a Topliss tree approach may be instructive for optimization of this series. The Topliss decision tree is a commonly used approach to determine the optimum substitution on a benzene ring in an active lead compound.[16] The initial group of four analogs used in this decision tree (**10-13**) were prepared and tested for inhibitory activity. Unfortunately, the inhibitory potencies of the initial group did not show sufficient spread to permit a meaningful analysis using this approach.

Instability of tetronoate inhibitors **7**, and **10-13** in assay buffers is one possible reason for the inconclusive results of the Topliss approach. Rates of inactivation for tetronoate **7** compared well to that of the corresponding 2,6-dichlorobenzoate derivative **1** in our primary enzyme assay. However, tetronate **7** did not inhibit the release of mature IL-1β in our whole-cell monocyte assay[17] in contrast to inhibitors **1** and **2** (IC_{50} 10 μM and 1 μM respectively). Stability studies (Table 2) revealed that the tetronoate-based inhibitor **7** had an unexpectedly short half-life ($t_{1/2}$ of inhibitor **7** = 0.86 h) in assay buffers such as that employed in the whole-cell assay (RPMI 1640, 1% FBS, pH 7.4, 4-6 h incubation period).

We suspected the tetronoate leaving group and not the peptide scaffold was in some manner responsible for the poor buffer stability of **7** since 2,6-dichlorobenzoate derivatives **1-3** did not share this liability.[18] We reasoned that buffer instability of **7** may arise from susceptibility of the vinylogous carbonate moiety embedded in the tetronoate to hydrolysis or reaction with nucleophiles in assay media.[19] Several strategies were investigated to increase stability of the leaving group: introduction of gem-dimethyl substitution (**14**); removal of the phenyl ring from conjugation (**16**); and replacement of the vinylogous carbonate by the less reactive vinylogous ester (**15**) and carbamate (**19**) functionality. While ICE inactivation rates for these compounds (**14-16**, and **19**) were generally 2-3x lower than the corresponding 3-phenyl tetronoate inhibitors

7 and **17**, all of these modifications did show improved buffer stability.[20] 3-Benzyl tetronoate analog **16** and 3-phenyl tetramoate analog **19** displayed the best buffer stability ($t_{1/2}$ = 9.8 h and 95 h, respectively). Thus these two leaving groups were chosen for additional study.[21]

A useful design feature of this affinity label approach is the ability to exploit a tighter binding affinity element to help compensate for leaving groups of lower reactivity.[22] As anticipated, sequential addition of amino acid residues to the peptidic scaffold increased the rate of enzyme inactivation for a series of analogs employing both leaving groups. The approximate 10-fold rate enhancement per added peptide residue for tetronoate analogs **16**, **18**, and **20** is similar to that observed for α-((2,6-dichlorobenzoyl)oxy)methyl ketones **1**-**3** and α-((1-phenyl-3-(trifluoromethyl)pyrazol-5-yl)oxy)methyl ketones.[8] In the tetramoate series, addition of an appropriate third amino acid residue to the binding affinity element of tetramoate **19** generally produced a 15- to 20-fold rate enhancement. While this 15- to 20-fold potency increase is nearly double that observed with either α-((2,6-dichlorobenzoyl)oxy)methyl ketones (**2** vs. **3**) and α-((1-phenyl-3-(trifluoromethyl)pyrazol-5-yl)oxy)methyl ketone series,[8] it is less than that observed (170-fold) for the corresponding aspartyl aldehyde series of reversible inhibitors (K_i 1,900 nM vs. 11 nM for Cbz-Val-Asp-H and Cbz-Val-Ala-Asp-H, respectively).[23] Potent, time-dependent inhibition and improved buffer stability were observed for tripeptidyl 3-phenyl tetronoate analogs such as **20** ($k_{obs}/[I]$ = 252,000 $M^{-1}s^{-1}$, $t_{1/2}$ 20 h)[24] and certain tripeptidyl 3-phenyl tetramoate analogs ($k_{ob}/[I]$ > 100,000 $M^{-1}s^{-1}$, $t_{1/2}$ > 150 h). Like related series of aspartyl α-substituted methyl ketones such as **1**-**3**,[25] peptide-based inhibitors bearing 3-phenyl tetronoate or 3-phenyl tetramoate leaving groups would be expected to demonstrate high ICE selectivity owing to the strict requirement of ICE for a P_1 aspartic acid residue. Additional details for inhibitors bearing 3-phenyltetronoate and related leaving groups will be communicated at the appropriate time.

In summary, this is the first report demonstrating the utility of cyclic β-dicarbonyl-based leaving groups for the irreversible inhibition of a cysteine protease. Exploratory studies also suggest that β-dicarbonyl-based leaving groups may find broad applicability for inhibition of other cysteine proteases as well.[26] Further, the commercial availability of diverse cyclic β-dicarbonyls and the ease with which these groups are incorporated into potential inhibitors make this approach attractive to emerging combinatorial or high-throughput chemistry paradigms. Structure activity relationships gleaned from these β-dicarbonyl-based leaving groups subsequently led directly to the discovery of additional novel classes of phenyl-substituted heterocyclic leaving groups (i.e., 5-hydroxy-1-phenylpyrazoles).[8] As a result of these and related efforts, a proprietary set of structurally-diverse leaving groups was identified. These leaving groups served as a key design component in our multi-pronged strategy to identify potent, selective, and orally-bioavailable inhibitors of ICE. Parallel efforts to replace the Val-Ala (P_3-P_2) residues of these inhibitors with conformationally restricted dipeptide surrogates which retained the critical hydrogen-bonding functionality (P_1- and P_3-NH)[8] were also successful. Irreversible and reversible surrogate-based inhibitors became the focus of our discovery effort and are the topic of recent[27] and future reports.

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7. Subtle or specific effects of both the departing group and the peptide scaffold can influence inhibitor potency. This is evident by the data present in Figure 2 in ref 2a. At leaving group pK_a of 3.5, cathepsin B inactivation rates range some 200-fold. Potency is also dependent on the type and pattern of aryl substitution in the aspartyl α-((aryl)acyloxy)methyl ketone class of ICE inhibitor, unpublished observation. In constrast, Thornberry et al. (Tables 1 and 2, ref 4a) have shown that the second order rate of inactivation is independent of the leaving group pK_a for certain tri- and tetrapeptide aspartyl α-((aryl)acyloxy)methyl ketones analogs.
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11. All new compounds have physical and spectroscopic data consistent with their structure.

    For **5** (R = $PhCH_2OCO$, X = Ph, Y = O, Z = H): ^1H NMR (300 MHz, $CDCl_3$) δ 7.82 (d, *J* = 7.57 Hz, 2H), 7.41-7.36 (m, 8H), 7.60 (d, *J* = 8.0 Hz, 2H), 5.12-5.08 (m, 4H), 4.71-4.66 (m, 2H), 4.40 (ddd, *J* = 8.0, 5.1, 4.4 Hz, 1H), 3.08-3.00 (dd, *J* = 17.7, 4.4 Hz, 1H), 2.73-2.67 (dd, *J* = 17.7, 5.1 Hz, 1H), 1.43 (s, 9H).

    For **7**: ^1H NMR (300 MHz, Me_2SO-d_6) δ 7.98 (d, *J* = 7.6 Hz, 2H), 7.87 (d, *J* = 7.15 Hz, 2H), 7.43-7.27 (m, 8H), 5.34 (s, 2H), 5.11 (s, 2H), 4.90 (m, 2H), 4.87 (ddd, *J* = 7.6, 7.1, 5.8 Hz, 1H), 2.80 (dd, *J* = 16.9, 5.8 Hz, 1H), 2.64 (dd, *J* = 17.0, 7.1 Hz, 1H). FABMS (nba) *m/z* 440.4 (M^++H). Anal. calcd for $C_{23}H_{21}NO_8$. 0.25 H_2O: C, 62.23; H, 4.88; N, 3.16. Found: C, 62.20; H, 4.89; N, 3.07.

    For **20**: ^1H NMR (300 MHz, Me_2SO-d_6) δ 8.85 (d, 1H), 8.20 (d, 1H), 7.25 (m, 10H), 5.25 (dd, 1H), 5.15 (dd, 1H), 5.0 (m, 2H), 4.70 (d, 1H), 4.60 (d, 1H), 4.40 (m, 1H), 4.15 (m, 1H), 3.80 (m, 1H), 3.30 (d, 1H), 3.15 (d, 1H), 2.80 (dd, 1H), 2.55 (dd, 1H), 1.20 (d, 3H), 0.90 (d, 3H), 0.82 (d, 3H). FABMS (nba) *m/z* 624.7 (M^++H). Anal. calcd for $C_{32}H_{37}N_3O_{10}$. 1.0 H_2O: C, 59.90; H, 6.13; N, 6.55. Found: C, 59.52; H, 5.81; N, 6.70.

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18. One might anticipate that the 2,6-dichlorobenzoate derivative ought to exhibit less buffer stability than the 3-phenyltetronoate analog based on leaving group pK_a alone (2,6-dichlorobenzoic acid, pK_a 1.72 (ref 2a); 3-phenyltetronic acid, pK_a 3.4 (Charton, *J. Org. Chem.* **1965**, 974)).
19. O-, S-, and N-based nucleophiles are known to react with alkyl tetronates and other vinylogous carbonates under a variety of nonphysiological conditions (see Shandala, M. Y.; Ayoub, M. T.; Mohammad, M. J. *J. Heterocycl. Chem.* **1984**, *21*, 1753; Croxall, W. J.; Freimiller, L. R.; Shropshire, Y. *J. Am. Chem. Soc.* **1950**, *72*, 4275; De Benneville, P. L.; Macartney, J. H. *J. Am. Chem. Soc.* **1950**, *72*, 3725).
20. The buffer stability of analog **14** was not determined since the geminal disubstitution was poorly tolerated by the enzyme (see Table 2).
21. As potency and buffer stability improved, the tetronoate-based inhibitors began to inhibit the release of mature IL-1β in the whole-cell monocyte assay (**18**, IC_{50} 10 μM).
22. See ref 2a. In fact, Thornberry has demonstrated that binding and not the S_N2 displacement of leaving group is the rate-determining step in ICE inactivation for a series of *tetra*peptide α-((aryl)acyloxy)- and (aryloxy)methyl ketones (see ref 4a).
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24. Inhibitor **20** has not been tested in a cell-based assay.
25. Series of aspartyl methyl ketones bearing 2,6-dichlorobenzoate,[3a] 5-hydroxyl-1-phenyl-3-(trifluoromethyl)pyrazole,[8] and diaryl phosphinic acids[9] as leaving groups have all demonstrated >100-fold selectivity for ICE versus the cysteine protease cathepsin B. However to our knowledge, none of these compounds have been tested for selectivity versus the emerging ICE/ced-3 family of cysteine proteases which includes ICE rel-II, ICE rel-III, Nedd-2/ICH-1 and CPP-32.[6b]
26. For example, 3-phenyltetronic acid (PTA) is also a viable leaving group for cathepsin B with Cbz-Phe-Ala-CH_2-PTA showing ca 90% inhibition of cathepsin B at 50 nM.[9]
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