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Using Peptidic Inhibitors to Systematically Probe the S1′ Site of Caspase-3 and Caspase-7

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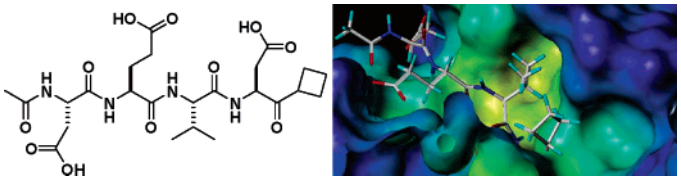
ABSTRACT



Fifteen ketone-containing peptides were designed, synthesized, and used to probe the effect of substitution at the P1′ position on caspase-3 and -7 inhibition. Even with the large bias of Ac-Asp-Glu-Val-Asp at the P4–P1 positions, certain peptides with cyclic functionality in the P1′ position show a dramatically reduced ability to inhibit these caspases. Additionally, trends toward isozyme selectivity were also uncovered for particular P1′ substituents. The data indicate that substitution in the P1′ position can drastically affect both caspase inhibition and selectivity.

The identification of compounds that selectively inhibit specific cellular enzymes is a major goal of modern medicinal chemistry and chemical biology. Such compounds not only have potential therapeutic value but can also be utilized as in vivo probes of protein function. However, many enzymes of interest exist as families of isozymes, and it is often extremely difficult to preferentially inhibit an individual isozyme in the presence of the others. An example of such an enzyme family is the caspases. The caspases are critical enzymes involved in signaling cascades that lead to both apoptosis and inflammation,[1] and it has proven to be difficult to specifically inhibit individual caspase isozymes.[2]

Caspases are cysteine proteases that recognize a tetrapeptide motif and catalyze the hydrolysis of peptide bonds C-terminal to aspartic acid residues in protein substrates. There are at least 11 different known human caspase isozymes, and these are typically categorized into three groups on the basis of the P4−P1[3] recognition sequence in the substrates that they cleave.[4] Group I caspases (caspases-1, -4, -5) catalyze the hydrolysis of substrates bearing a W/L-E-H-D recognition sequence. Group II caspases (caspases-3, -7) process D-E-V-D sequences, and Group III enzymes (caspases-8, -9, -10) proteolyze after L/V-E-X-D sequences.[5] In general, the Group I enzymes are thought to be critical to the inflammatory response,[6] whereas Group II and III caspases are responsible for the execution and initiation of apoptosis, respectively.

While caspase recognition of the P4−P1 residues has been thoroughly investigated in both substrate and inhibitor studies,[4,7] there is less information with regard to the effect of substitution at the position immediately after the scissile bond, the P1′ position.[8,9] In group II caspases, the S1′ site is considered to be a "hydrophobic bowl" that will accept either unbranched hydrophobic or aromatic amino acid sub-

(1) For reviews on caspases, see: (a) Denault, J. B.; Salvesen, G. S. *Chem. Rev.* **2002**, *102*, 4489−4500. (b) Nicholson, D. W. *Cell Death Diff.* **1999**, *6*, 1028−1042. (c) Fuentes-Prior, P.; Salvesen, G. S. *Biochem. J.* **2004**, *384*, 201−232.

(2) For reviews on caspase inhibition, see: (a) Wang, K. K. W. *Curr. Opin. Drug Discovery Dev.* **1999**, *2*, 519−527. (b) Talanian, R. V.; Brady, K. D.; Cryns, V. L. *J. Med. Chem.* **2000**, *43*, 3351−3371. (c) Concha, N. O.; Abdel-Meguid, S. S. *Curr. Med. Chem.* **2002**, *9*, 713−726.

(3) P denotes substrate position, and S denotes enzyme site or pocket.

(4) Thornberry, N. A.; Rano, T. A.; Peterson, E. P.; Rasper, D. M.; Timkey, T.; Garcia-Calvo, M.; Houtzager, V. M.; Nordstrom, P. A.; Roy, S.; Vaillancourt, J. P.; Chapman, K. T.; Nicholson, D. W. *J. Biol. Chem.* **1997**, 272, 17907−17911.

(5) Although their substrate specificities are different from the ones listed, on the basis of the length of their propeptide domains, caspase-6 is often placed in Group II, and caspase-2 is generally placed in group III. See: Shi, Y. *Mol. Cell* **2002**, *9*, 459−470.

(6) Caspase-1 has also been implicated in the apoptotic pathway. See: Choi, C.; Jeong, E.; Benveniste, E. N. *J. Neurooncol.* **2004**, *67*, 167−176.

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strates.[10,11] Recently reported inhibitors incorporate this type of functionality at the P1′ position, and a structural examination of a series of these inhibitors defines a general trend for caspase-3 of increasing inhibition with increasing surface area.[10] However, this trend was seen using inhibitors with very similar P1′ groups and varied P4−P1 substituents. Thus, to provide a clearer understanding of the P1′ position and its importance to caspase inhibitor design, we report the systematic substitution of the P1′ position and the effects of the resulting peptides upon inhibition of caspase-3 and caspase-7.

To systematically probe the effect of P1′ substitution on inhibition, we designed a series of inhibitors of the general structure shown in Figure 1 that hold the optimal Group II

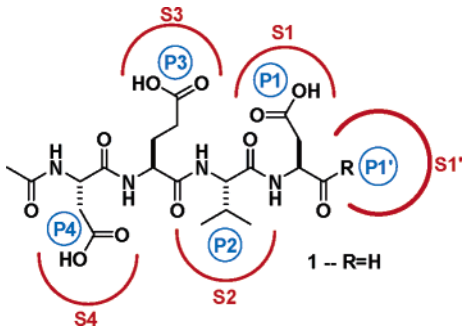

Figure 1. General ketone-containing Ac-DEVD peptide and its interaction with the various substrate-binding sites (S4−S1′) of caspase-3 and -7. By holding the P4−P1 amino acids constant, the effect of substitutions in the P1′ position can explicitly be probed and compared to aldehyde **1**.

caspase recognition sequence (DEVD) constant while changing only the P1′ position. In this design, a ketone replaces the amide carbonyl of the scissile bond; the active site cysteine of caspases is known to attack such ketones to reversibly form thio-hemiacetals.[11] A range of R groups can then project from the ketone into the S1′ pocket. Convenient access to the desired compounds was envisioned through the coupling of various β-amino-γ-keto acids to an appropriately protected DEV tripeptide, followed by deprotection.

The crystal structures of caspase-3[12] and caspase-7[13] bound to aldehyde **1** were used to guide selection of appropriate P1′ substituents. The length of functionality in the P1′ position was limited to groups that would best interact with the S1′ pocket, shown in Figure 2. There are slight differences in the size and shape of the S1′ site between caspase-3 and caspase-7; caspase-3 appears to have a more shallow yet broad S1′ pocket, while caspase-7 has a deeper and narrower S1′ site. It was envisioned that these differences might allow for some isozyme selectivity in the caspase inhibition.

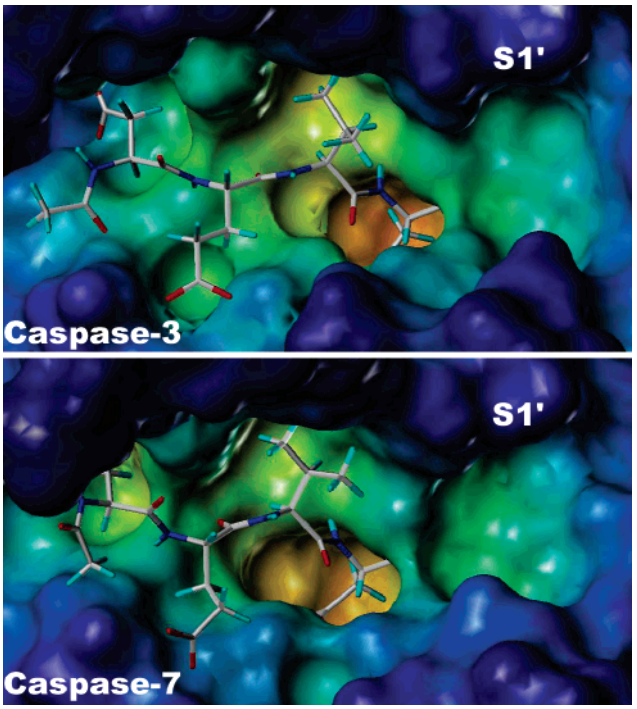

Figure 2. X-ray crystal structures of peptide **1** bound to caspase-3 and -7.[12,13] The S1′ sites of these enzymes have differences in both size and shape. Blue represents surface-exposed regions.

To synthesize the designed inhibitors, a common protected peptide trimer was coupled to various β-amino-γ-keto acids. The protected peptide DEV trimer **5** could easily be obtained in good yield and high purity using the FAAST solution-phase peptide synthesis technique (Scheme 1).[14] The requisite β-amino-γ-keto acid modules **6a**−**o** were synthesized either through a method previously developed in our laboratory based on Grignard addition to protected L-homoserine[15] or through a method based on the cuprate displacement of a

(7) (a) Talanian, R. V.; Quinlan, C.; Trautz, S.; Hackett, M. C.; Mankovich, J. A.; Banach, D.; Ghayur, T.; Brady, K. D.; Wong, W. W. *J. Biol. Chem.* **1997**, *272*, 9677−9682. (b) Chereau, D.; Kodandapani, L.; Tomaselli, K. J.; Spada, A. P.; Wu, J. C. *Biochemistry* **2003**, *42*, 4151−4160.

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(10) Becker, J. W.; Rotonda, J.; Soisson, S. M.; Aspiotis, R.; Bayly, C.; Francoeur, S.; Gallant, M.; Garcia-Calvo, M.; Giroux, A.; Grimm, E.; Han, Y.; McKay, D.; Nicholson, D. W.; Peterson, E.; Renaud, J.; Roy, S.; Thornberry, N.; Zamboni, R. *J. Med. Chem.* **2004**, *47*, 2466−2474.

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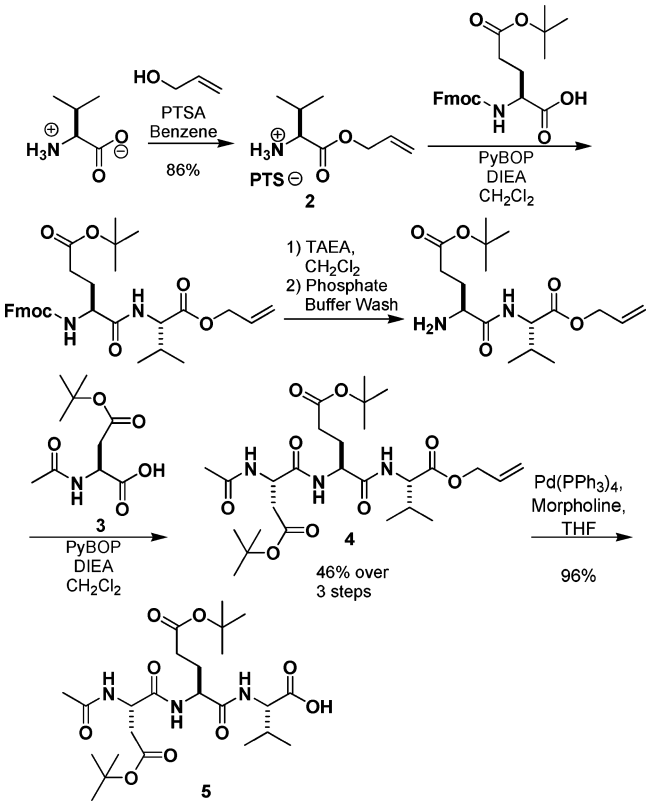
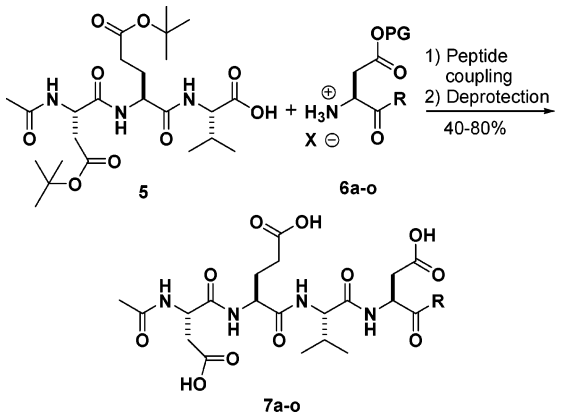
Scheme 1. Synthesis of Ac-DEV Trimer



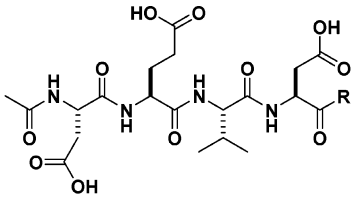
thioester in protected L-aspartic acid (see Supporting Information).[16] The coupling between these β-amino-γ-keto acid building blocks and the protected DEV peptide **5** furnished fully protected products, which were deprotected to provide the target molecules **7a**−**o** in >95% purity (Scheme 2).

Scheme 2. Coupling of Trimer to β-Amino-γ-keto Acids



Enzyme inhibition assays were then performed using human caspases-3 and -7, which were recombinantly expressed in *E. coli* and purified as described.[17] Real-time assessment of the inhibition was carried out for all compounds using the Ac-DEVD-*p*NA substrate. This substrate provides the *p*-nitroaniline chromophore with a λ_{max} at 405 nm after caspase-catalyzed hydrolysis. Assays were conducted in triplicate in 384-well plates. Apparent K_I values[18] were obtained for all 15 compounds versus caspase-3 and caspase-7, as well as for Ac-DEVD-aldehyde inhibitor **1** for a comparison control (Table 1).

Table 1. Inhibition of Caspases-3 and -7 with Ketonic Peptides



	R	K_I caspase-3	K_I caspase-7
1	H	5.8 nM	19.7 nM
7a		5.3 nM	41.7 nM
7b		3.3 nM	19.7 nM
7c		1.1 nM	18.2 nM
7d		1.3 nM	12.2 nM
7e		94.2 nM	652.8 nM
7f		1.5 nM	14.5 nM
7g		3.5 nM	16.4 nM
7h		8.3 nM	97.7 nM
7i		0.7 nM	3.0 nM
7j		0.2 nM	4.0 nM
7k		172.0 nM	420.3 nM
7l		2.1 nM	3.5 nM
7m		272.3 nM	572.5 nM
7n		2255.1 nM	15842.0 nM
7o		904.6 nM	5993.0 nM

Inspection of the data in Table 1 reveals several interesting features about the inhibition properties of these peptidic ketones. In the straight-chain aliphatic series **7a**−**d**, there is a general trend of increasing inhibition with increasing chain length, agreeing with previously reported data.[10] Excluding the phenyl derivative **7o**, the general trend for the unsubstituted aromatic P1′ inhibitors **7f**−**h** is increasing potency with

decreasing linker length. Both substituted derivatives **7i** and **7j** have increased potency over the parent phenethyl derivative **7g**, and the addition of a second methyl substituent slightly enhances inhibition of caspase-3.

Unexpectedly, a large detrimental effect on inhibition was observed for substituents possessing α or β branching in the P1′ position. For example, there is about a 500-fold difference between the benzyl **7f** and phenyl **7o**. With the exception of cyclobutyl **7l**, compounds **7k**−**o** with cyclic substituents in the P1′ position are between 20- and 800-fold less potent than the parent aldehyde **1**. The dramatic lack of inhibition of compounds such as **7n** was surprising, as the presence of the Ac-DEVD peptide places a large bias on the system toward caspase inhibition, and this effect has not been noted in previous studies of the P1′ site.[19]

Interestingly, a modulation in inhibition selectivity between caspase-3 and caspase-7 can occur by simply varying the functionality occupying the P1′ position. The parent aldehyde **1**, and thus the incorporated Ac-DEVD recognition sequence, has a ∼4-fold selectivity for caspase-3. Through modulations in the P1′ position, this selectivity could be elevated to ∼20-fold for caspase-3, as in the case of **7j**. A reversal of this trend was found for the inhibitors possessing cyclic P1′ substituents (**7k**−**m**), although these compounds still inhibit caspase-3 slightly better than caspase-7.

In an effort to understand the interesting effects of the cyclic derivatives **7k**−**n** both in terms of their detrimental effect on inhibition and their reduction of isozyme selectivity, molecular modeling studies were conducted using the crystal structures of **1** bound to caspase-3 and caspase-7.[20] Within this framework, the ketonic side chains of **7k**−**n** were constructed, and the complex was then minimized (see Supporting Information for details). The modeling suggests that these cyclic compounds are unable to reach the S1′ pocket and instead project above the active site over the aspartic acid moiety (see Figure 3 for modeling with caspase-3). This conformation likely minimizes the steric interactions between the ring system and the cysteine/histidine active site pair. The cyclopropyl **7k** ring system in this orientation has the two β-carbons in the ring in close proximity to the active site residues, thus hindering inhibition. While this is relieved as the ring becomes larger, new steric interactions with Tyr338, and the valine side chain in the P2 position begin to hinder inhibitor interaction with the enzyme. The cyclobutyl ring in **7l** thus appears to be optimal, minimizing steric interactions and enabling significant caspase inhibition.

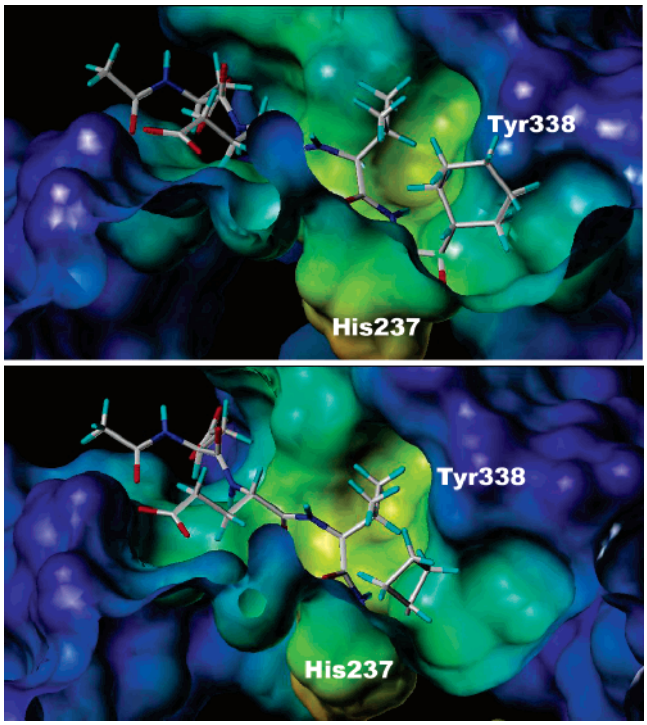


Figure 3. Comparison of molecular models of inhibitors **7n** (top) and **7l** (bottom) minimized with caspase-3. The view is a partly cut-away surface to facilitate visualization of the P1′ groups. In compound **7n**, the cyclohexyl ring is forced into close proximity with the nearby P2 side chain and amino acids His237 and Tyr338. In contrast, the cyclobutyl ring of **7l** fits between His237 and Tyr338 with little steric interaction.

In conclusion, the P1′ position of the Ac-DEVD peptide has been systematically probed for its effect on caspase-3 and caspase-7 inhibition. It was found that cyclic groups in this position in general hinder inhibition, most likely due to unfavorable steric interactions with amino acids in and above the active site, and not in the S1′ pocket. Also, certain substituents provide slight selectivity between the two caspase isozymes. The data from these studies indicate that substitution in the P1′ position could be used in synergy with other elements to obtain highly potent and isozyme-selective caspase inhibitors.

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Supporting Information Available: Experimental procedures, characterization data, and inhibition curves. This material is available free of charge via the Internet at http://pubs.acs.org.

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