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# A SUBSTRATE COMBINATORIAL ARRAY FOR CASPASES

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**Abstract:** An efficient strategy for the synthesis of a tetrapeptidyl substrate combinatorial array directed toward the caspases is described. Testing of this array with caspases 1 and 4 gave substrate hydrolytic profiles characteristic of each caspase, and permitted the identification of efficiently processed substrates. A comparison of this approach to that using a positional scanning library is presented. 

Caspases represent a large family of cysteinyl aspartate-specific proteinases that are intimately involved in the highly orchestrated process of programmed cell death (apoptosis).[1] All members of this protease family are characterized by their unique requirement for an aspartic acid residue at the $P_1$ position of the substrate cleavage site. Interleukin-1β converting enzyme (ICE, caspase 1) was the first characterized member of this family. The roles of ICE and subsequently identified caspases in inflammation and apoptosis have been and continue to be the subject of intense investigation.[2]

Discrimination between caspase family members in such studies would be facilitated with protease-selective substrates and inhibitors. As such, a facile method for the identification of selective substrates for individual caspases is desirable. Merck has published the elegant use of a positional scanning (PS) library to determine the consensus sequences of the optimal tetrapeptide substrates for individual caspases.[3,4] PS allows one to efficiently scan diversity space and is made possible by assaying a small number of large mixtures of substrates. However, the evaluation of substrate mixtures precludes an accurate comparison of individual substrates, because neighboring effects (e.g., conformational biases) associated with a particular peptide sequence may lead to non-additive effects. An alternative, but complementary approach toward substrate profiling is the use of a combinatorial array (CA) of individual substrates. In contrast to a PS library, a CA is less efficient at covering diversity space (because individual peptides rather than mixtures are synthesized), but allows for the accurate determination of the consequences of single amino acid changes within a particular sequence. In addition, detailed characterization of individual peptide substrates, including the determination of kinetic parameters, can be carried out without the requirement for resynthesis.

This communication describes our strategy for the preparation of a 100-member array of individual tetrapeptide substrates directed toward the caspases. The utility of this approach is demonstrated by comparing

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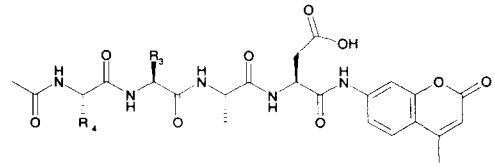
the relative rates of hydrolysis of individual array members by caspases 1 and 4, two closely related family members.[5] Our results show that both approaches (CA and PS) lead to the identification of similar substrate sequences that are efficiently processed by caspase 1 and caspase 4. However, the dependence of hydrolytic activity on the substrate sequence when measured directly by the CA approach can differ significantly from that inferred from the PS approach.

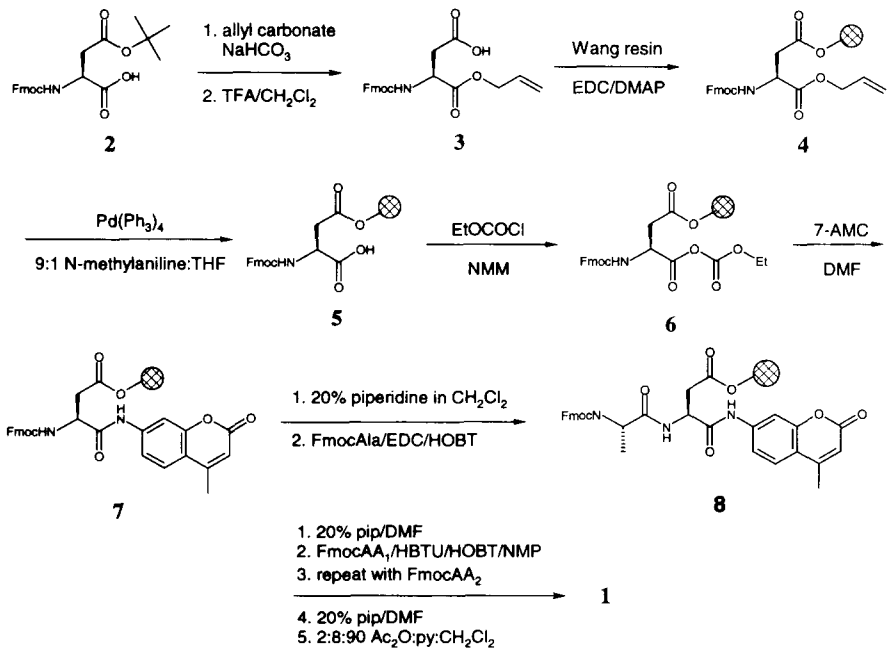
The CA consists of a series of potential fluorogenic tetrapeptide substrates with the general structure **1**. Since a $P_1$ aspartic acid is recognized (in fact, required) by all caspases, this residue was left invariant throughout the array.[2] It had been demonstrated that caspase 1 can accommodate a variety of residues in position $P_2$ with minor variations in activity.[6] Therefore, alanine was incorporated into this position throughout the library, initially to limit its size. Since a large determinant of recognition had been attributed to positions $P_3$ and $P_4$,[7] amino acid diversity was introduced into these positions.



**1**

The preparation of the CA was initiated with aspartic acid derivative **2** (Scheme 1). C-Terminal allylation, and sidechain deprotection (TFA/$CH_2Cl_2$) yielded acid **3**. The acid (2 equiv) was then efficiently coupled to Wang resin (2 equiv EDC, 1 equiv DMAP, $CH_2Cl_2$) to afford the resin-bound ester **4**. Removal of the allyl ester was effected with $Pd(PPh_3)_4$ in 10% N-methylaniline/THF for 12 h,[8] followed by extensive washing of the resin (pyridine, NMP and $CH_2Cl_2$) to remove the catalyst. Activation of acid **5** (NMM/EtOCOCl/$CH_2Cl_2$/ -20 $^{O}$C) furnished the activated ester **6** as a resin bound intermediate which was stable to repeated washings with $CH_2Cl_2$ (IR 1835 $cm^{-1}$). Reaction of **6** with 7-AMC in DMF for 24 h provided the coumaride **7**.[9] This product is clean by magic angle spinning $^1$H NMR, and resin cleavage (50% TFA/$CH_2Cl_2$) yielded a single product. The conversion of allyl ester **4** to coumaride **7** occurs in essentially quantitative yield. Fmoc deprotection and coupling with Fmoc-alanine afforded the resin bound dipeptide **8**.

The parallel syntheses of tetrapeptide substrates **1** were performed on an Advanced Chemtech 396.[10] A diverse set of 10 out of the 20 naturally occurring amino acids was chosen for the preparation of a 10 by 10 CA. Standard solid-phase Fmoc amino acid chemistry was employed for two rounds of amino acid couplings to **8** (Fmoc deprotection followed by amino acid coupling with HBTU/HOBT/NMM). An N-acetyl capping group was introduced (10% solution of 4/1 pyridine/acetic anhydride in $CH_2Cl_2$), and cleavage of the individual substrates from the resin was effected with a 5% solution of 1/3/1 1,2-ethanedithiol/anisole/dimethyl sulfide in 50% TFA/$CH_2Cl_2$. Each substrate was isolated by precipitating the peptide from its cleavage solution with cold ether. The presence of each array product was confirmed by mass spectrometry, and a dozen randomly selected array components were characterized by $^1$H NMR.




**Scheme 1**

Individual members of the array were evaluated as potential substrates for caspases 1 and 4 using a standard fluorometric assay.[11] Figure 1 depicts the relative rates of hydrolysis by these proteases for the 100 member substrate array assayed at a fixed concentration of 100 μM. Ac-WEAD-AMC is the most efficiently processed substrate for both proteases. It is also evident from these plots that caspase 4 has a much more stringent substrate specificity than caspase 1 and that these two closely related family members can be easily distinguished by these profiles.

A large, hydrophobic residue such as Phe or Trp is preferred at position $P_4$ by caspase 1, and correlates well with the sequences found at the cleavage sites of the natural caspase 1 substrate proIL-1β (YVHD and FEAD).[12] Caspase 4 also has a strong preference for Trp, but is much more discriminating relative to caspase 1 as evidenced by the slow hydrolysis of of substrates with Phe in position $P_4$. Both caspases display a strong preference for glutamic acid in position $P_3$ and select *against* the basic residues lysine and arginine. Subsequent to these studies, the crystal structure of caspase 3 complexed with Ac-DEVD-CHO was published[4] and revealed the reason for this preference: an electrostatic interaction between the $P_3$ glutamic acid and Arg341, a residue that is conserved among all family members. Additionally, the presence of Arg341 may account for the poor hydrolytic rates associated with substrates possessing basic residues in position $P_3$ because of the unfavorable

electrostatic interaction that would likely be created. The results also show that valine in this position yields substrates which are preferentially processed, consistent with the YVHD cleavage site of proIL-1β.

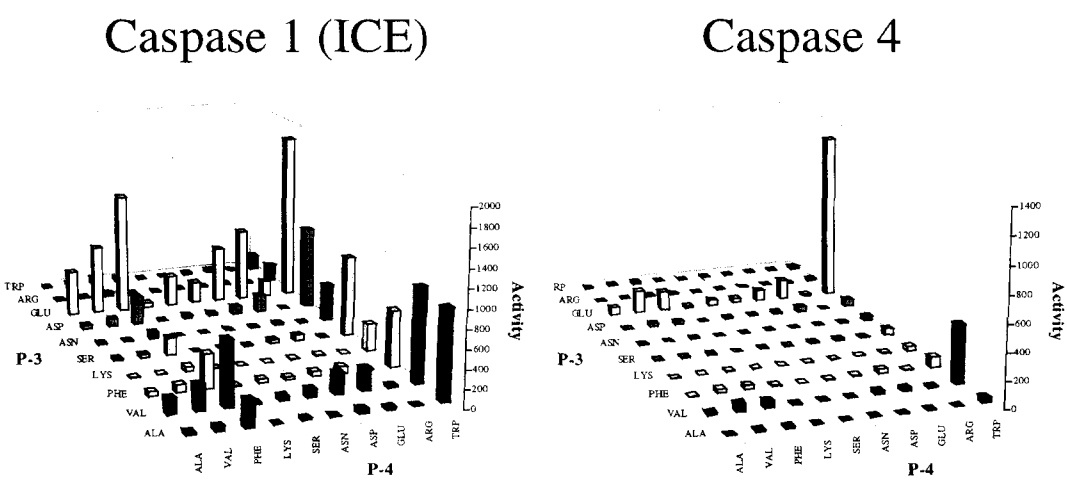



**Figure 1**

The advantage of a CA approach is exemplified by comparing the caspase 1 hydrolytic profiles derived from the two approaches. For the sequence Ac-$P_4$EAD-AMC, substrates corresponding to $P_4$ = Ala, Val, Phe, Asp, or Glu are hydrolyzed at a lower, but still significant, rates relative to Ac-WEAD-AMC. In the PS study, $P_4$ = Phe appears to be the only one of these five substrates hydrolyzed at an appreciable rate.[3a] This discrepancy is due to the fact that large mixtures of substrates are screened in the PS approach, and the results are subject to non-additive effects. For example, the relative effects of different amino acids at position $P_4$ on the rate of substrate hydrolysis will vary with the $P_3P_2$ sequence of the peptide. Thus, in contrast to the CA approach, the profile derived from a PS library may not adequately quantify the relative importance of individual amino acids within a particular sequence. The more detailed information offered by the CA approach is critical not only for identifying optimal substrates but for designing inhibitors that are both potent and selective for the individual caspases.

Table 1 lists the measured kinetic parameters for several of the better substrates determined for caspase 1. Ac-WVAD-AMC possesses a similar profile to Ac-YVAD-AMC,[13] whereas Ac-WEAD-AMC exhibits both a lower $K_m$ and higher $V_{max}$. Therefore, the enhanced hydrolytic susceptibility of Ac-WEAD-AMC relative to the YVAD sequence is primarily the result of the $P_3$ Val to Glu substitution.

Ac-YVAD-AMC has been the traditional substrate for determining caspase 1 activity and was the most efficiently processed caspase 1 substrate known prior to the evaluation of substrate libraries. Screening of this array resulted in the identification of Ac-WEAD-AMC, which caspase 1 processes four times more efficiently than Ac-YVAD-AMC as determined from the $V_{max}/K_m$ values (Table 1). The PS approach yielded an even better substrate, Ac-WEHD-AMC, which is processed another four-fold more efficiently than Ac-WEAD-AMC.[3a] The results from both approaches suggest similar substrate preferences by caspase 1; however, they also highlight the advantage of the PS approach in terms of its ability to identify the best substrate, which was enabled by a more thorough coverage of diversity space. The inability of the CA approach to identify Ac-WEHD-AMC as an optimal caspase 1 substrate was based upon the decision to limit the size of the array by making the $P_2$ position invariant. This was done to illustrate the utility of the CA strategy, not to identify the best out of all possible substrates. Simultaneous variation of $P_2$-$P_4$ would still yield a library of reasonable size and allow optimization on that basis, provided proper diversity is maintained among all three positions.

**Table 1. Kinetic Constants of Substrates for Caspase 1**

| Substrate | Vmax* (FU/μM) | Km (μM) | Vmax/Km* (FU/min/μM) |
|---|---|---|---|
| Ac-YVAD-AMC | 1060 | 20 | 53 |
| Ac-WVAD-AMC | 1300 | 27 | 48 |
| Ac-WEAD-AMC | 1840 | 9 | 204 |

*FU = fluorescent units

In conclusion, an efficient method for the solid phase preparation of tetrapeptidyl combinatorial arrays of substrates tailored to the caspases has been detailed in this report. The method yielded characteristic hydrolytic profiles for both caspases 1 and 4 and identified efficiently processed substrates for each. Although the positional scanning approach is superior for the identification of the optimal substrates because of its exhaustive coverage of diversity space, the complementary strategy reported here (CA) provides a more detailed picture of the consequences of single residue changes within a substrate peptide sequence. Information of this nature is critical for the mapping of binding determinants in the enzyme active site not only for identifying the optimal substrate(s) but also for the design of potent and selective inhibitors. The screening of this and additional arrays prepared by the methods described herein against other members of the caspase family will be the subject of a subsequent report.

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13. A previous publication (ref 3a) states that Ac-WVAD-AMC is four-fold better than Ac-YVAD-AMC ($k_{cat}/K_m$). That was not the case in our study. The source of this discrepancy is unclear. Nevertheless, Ac-WVAD-AMC was identified as an efficiently processed substrate in both studies.