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# Identification of Potent and Novel Small-Molecule Inhibitors of Caspase-3

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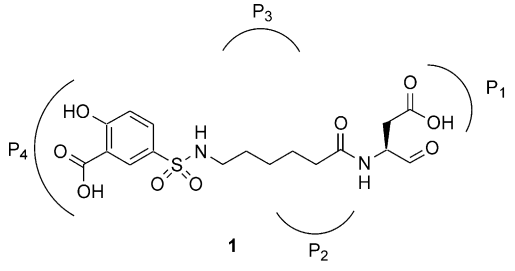
**Abstract**—The design and synthesis of a series of novel, reversible, small molecule inhibitors of caspase-3 are described.


Caspases (cysteinyl aspartate-specific proteases) have been implicated in various disease states involving dysregulated apoptosis such as myocardial infarction, stroke, traumatic brain injury, and sepsis. Caspase-3 lies at a key junction in the apoptotic cascade, mediating apoptosis from both the intrinsic and extrinsic activation pathways. Therefore, caspase-3 inhibition may prove to be a valuable therapeutic approach to treating these diseases.[1] As part of our ongoing efforts to discover small molecule caspase-3 inhibitors through use of Tethering[2] with extenders, we identified compound **1** as a low micromolar ($K_i = 4.0$ μM) inhibitor.[3] Molecular modeling of **1** bound to the caspase-3 active site suggested that the molecule interacts with the protein via the catalytic cysteine (Cys163) as well as with the $S_1$ and $S_4$ binding subsites of caspase-3, and reproduces the binding mode observed in crystal structures of earlier analogues.[3] Compounds derived from the DEVD tetrapeptide sequence have been shown to be potent inhibitors of Caspase-3, and the $P_2$ isopropyl side chain of the valine residue is important for inhibitory activity.[4] Because the proposed model of **1** bypasses the $S_2$ recognition site, we reasoned that the inhibitory potency of **1** could be increased by incorporation of a $P_2$ binding element. The present report describes one of our approaches in developing SAR around this series of compounds by the systematic examination of $P_2$ and $P_4$ substituents, scaffold rigidification and aldehyde warhead replacement.

## Chemistry

For synthetic ease, we initially prepared the compounds as diastereomeric mixtures at $P_2$. Syntheses of alkyl analogues **2**–**7** is described in Scheme 1. The $P_1$ amino acid and warhead portions of the molecule were installed using




**1** **Ac-DEVD-CHO**

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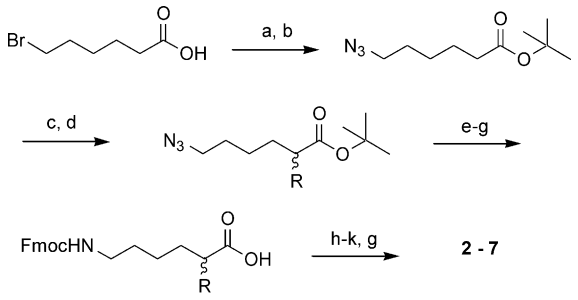
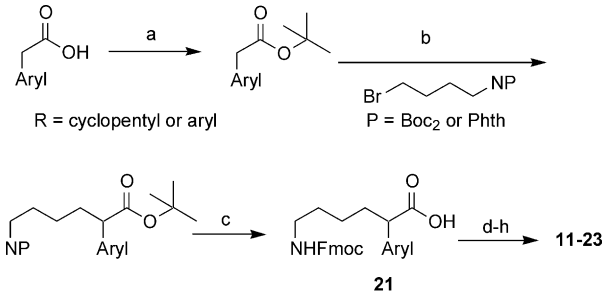
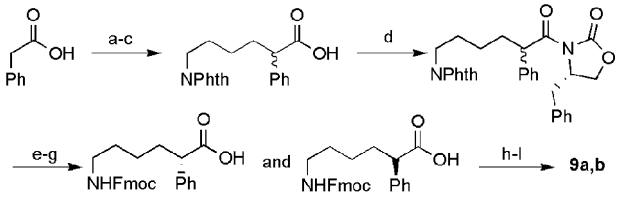


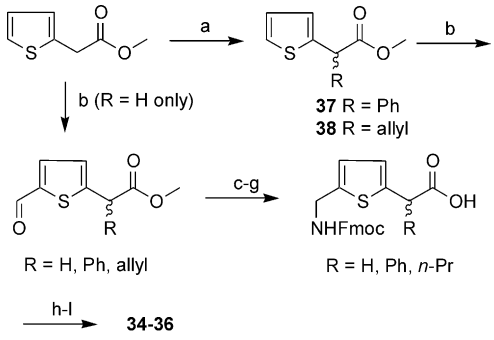

**Scheme 1.** (a) DMF di-*t*-butyl acetal/benzene/reflux;[7] (b) $NaN_3$/DMSO; (c) LDA/THF/−78 °C; (d) alkyl halide; (e) $H_2$/Pd/C; (f) Fmoc-OSu/$NaHCO_3$/dioxane/$H_2O$; (g) 1:3 TFA/$CH_2Cl_2$; (h) $H_2N$–Asp(*O*-*t*-Bu)–CHNNHCONH– resin/EDC/DMF; (i) 1:4 piperidine/DMF; (j) 5-chlorosulfonyl salicylic acid/DIEA/$CH_2Cl_2$; (k) 5:1:1:0.25 THF/AcOH/MeCHO/TFA.




**Scheme 2.** (a) DMF di-*t*-butyl acetal/benzene/reflux or $H_2SO_4$/$Mg_2SO_4$/$CH_2CH_2$/*t*-BuOH[8]; (b) NaH/DMF or LDA/THF; (c) for P = Phth; (i) $N_2H_4$/EtOH/reflux; (ii) Fmoc-OSu/$NaHCO_3$/dioxane/$H_2O$; (iii) 1:3 TFA/$CH_2Cl_2$; for P = $Boc_2$, (i) 1:3 TFA/$CH_2Cl_2$; (ii) Fmoc-OSu/$NaHCO_3$/dioxane/$H_2O$; (d) $H_2N$-Asp(O-*t*-Bu)–CHNNH-CONH–resin/EDC/DMF; (e) 1:4 piperidine/DMF; (f) 5-chlorosulfonylsalicylic acid/DIEA/$CH_2Cl_2$; (g) 5:1:1:0.25 THF/AcOH/MeCHO/TFA; (h) 1:3 TFA/$CH_2Cl_2$.




**Scheme 3.** (a) $H_2SO_4$/$Mg_2SO_4$/$CH_2CH_2$/*t*-BuOH; (b) NaH/DMF; (c) 1:4 TFA/$CH_2Cl_2$; (d) (i) Oxalyl chloride/cat. DMF/EtOAc; (ii) *n*-BuLi/(*S*)-(−)-4-benzyl-2-oxazolidinone; (iii) flash chromatography (25/75 EtOAc/hexane); (e) LiOH/$H_2O_2$/THF/$H_2O$; (f) $N_2H_4$/EtOH/reflux; (g) Fmoc-OSu/$NaHCO_3$/dioxane/$H_2O$; (h) $H_2N$-Asp(O-*t*-Bu)-CHNNHCONH- resin/EDC/DMF; (i) 1:4 piperidine/DMF; (j) 5-chlorosulfonylsalicylic acid/DIEA/$CH_2Cl_2$; (k) 5:1:1:0.25 THF/AcOH/MeCHO/TFA; (l) 1:3 TFA/$CH_2Cl_2$.




**Scheme 4.** (a) For R = Ph, PhBr/NaHDMS/$Pd(OAc)_2$/ligand/THF; for R = Pr; (i) NaH/allyl bromide/DMF; (b) $Cl_2CH(OMe)/SnCl_4$/$CH_2Cl_2$;[13] (c) $HONH_2$/EtOH; (d) Zn/AcOH; (e) NaOH/MeOH; (f) Fmoc-OSu/$NaHCO_3$/dioxane/$H_2O$; (g) for R = Pr only $H_2$/Pd/C/EtOAc; (h) $H_2N$-Asp(*O*-*t*-Bu)–CHNNHCONH–resin/EDC/DMF; (i) 1:4 piperidine/DMF; (j) 5-chlorosulfonylsalicylic acid/DIEA/$CH_2Cl_2$; (k) 5:1:1:0.25 THF/AcOH/MeCHO/TFA; (l) 1:3 TFA/$CH_2Cl_2$.

solid phase synthesis. A resin-bound semicarbazide[5] was treated with Fmoc-Asp(O*t*-Bu)-CHO[6] to give the support-bound semicarbazone. Fmoc removal (piperidine/DMF) was then followed by amide formation with the desired acid. A second Fmoc deprotection followed by treatment with 5-chlorosulfonylsalicylic acid provided the resin-bound sulfonamide. Cleavage from the resin gave the aldehyde and removal of the Asp *t*-butyl ester gave the final products **2**–**7** which were purified via reverse-phase preparatory HPLC.

Derivatives **8**–**20** were synthesized as described in Scheme 2. Alkylation of the requisite ester with either bis-Boc or phthalimide protected amino butyl bromide furnished protected amino acids **21**, which were fully elaborated in a similar manner as described above.

Since direct chromatographic separation of the diastereomers of **9** was not possible, the enantiomers of an advanced intermediate, derivatized as acyloxazolidinone diastereomers, were separated via column chromatography (Scheme 3). Cleaving the chiral auxiliary and subsequent elaboration gave the final products **9a** and **9b**. Unfortunately analysis via $^1H$ NMR indicated that each diastereomer was contaminated with 20% of the other diastereomer, indicating that partial epimerization had occured in one of the later synthetic steps.

Syntheses of sulfonyl chlorides needed for compounds **22**–**29** were accomplished either from direct chlorosulfonylation with $ClSO_3H$ or from the treatment of the requisite aryl diazonium salt with $SO_2$ and a copper catalyst according to literature procedure.[9] The thiomethyl[10] and oxazolyl ketones[11] of Asp were coupled with the requisite acid to give compounds **31**–**33**.

The syntheses of thiophene analogues **34**–**36** are described in Scheme 4. Alkylation of methyl 2-thiopheneacetate with allyl bromide or palladium catalyzed arylation[12] provided substituted acetates **37** and **38**, which were fully elaborated as shown.

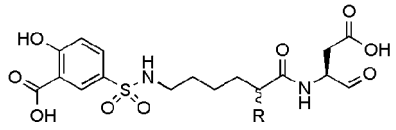
## Results and Discussion

Our initial series of compounds were designed to explore the effect of a $P_2$ binding element by installing substituents alpha to the amide carbonyl of **1** (Table 1). In general, alkyl substitutions with increasing steric bulk resulted in greater caspase-3 inhibitory activity relative to **1**. An aromatic substituent gave the largest increase in potency. For example, phenyl analogue **9** displayed a 40-fold gain in activity. Analogues with simple changes to the aromatic ring had activity similar to **9**, with compounds **14** and **19** being the most potent of the ser-

ies. As previously mentioned, all analogues were synthesized as diastereomeric mixtures at $P_2$ for synthetic convenience. However, once general SAR trends were established we went back and examined the importance of stereochemistry at the $P_2$ position. Molecular modeling of **9** (Fig. 1) indicated that both diastereomers were capable of orienting the phenyl ring into the $S_2$ subsite of caspase-3. To this end, the two diastereomers **9a** and **9b** were prepared and isolated as 80:20 mixtures and evaluated for caspase-3 inhibitory activity (Table 2). Diastereomer **9a** appeared to be 5-fold more active than diastereomer **9b**, showing the importance of stereochemistry at the $P_2$ positon. However, we did not attempt to determine the absolute configuration of **9a** or **9b**.

We previously had determined that the hydroxyl and the carboxylic acid group of the salicylic acid moiety are involved in key binding interactions with amino acid residues of the caspase-3 $S_4$ binding subsite.[3] We selected compound **9** as a starting point for additional analogue synthesis to investigate SAR around the $P_4$ group. A number of analogues containing several known carboxylic acid bioisosteres[15] were synthesized with binding results summarized in Table 3. Among the analogues evaluated, only oxadiazolone **27** exhibited inhibitory activity comparable to that of salicylic acid analogue **9**. The decreased potency of the corresponding *N*-methyl oxadiazolone **28** appears to indicate the importance of an acidic proton in retaining good inhibitory activity.[16] We also briefly examined the replacement of the sulfonamide in **9** with an amide. The 4-fold loss in potency of amide **29** indicates the importance of the ability of the sulfonamide linkage to adopt a *cis*

**Table 1.** In vitro caspase-3 inhibition assay results for compounds **1**–**20**. The effectiveness of compounds against the activity of human recombinant caspase-3 was measured by collecting kinetic data over a 20-min fluorescence-based assay using Ac-DEVD-AFC as substrate. All $K_{i(apparent)}$ values were calculated using the following equation: $K_i = IC_{50}/(1 + [substrate]/K_m)$[3]

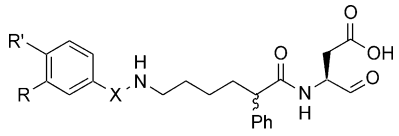


| Compd | R = | $K_{i(app)}$, μM |
|---|---|---|
| **1** | H | 4.0 |
| **2** | Me | 6.4 |
| **3** | Et | 1.8 |
| **4** | *n*-Pr | 0.8 |
| **5** | *i*-Pr | 0.7 |
| **6** | *i*-Bu | 0.6 |
| **7** | $PhCH_2$ | 4.4 |
| **8** | Cyclopentyl | 1.3 |
| **9** | Ph | 0.1 |
| **10** | 2-FPh | 0.1 |
| **11** | 3-FPh | 0.2 |
| **12** | 4-FPh | 0.2 |
| **13** | 2-ClPh | 0.2 |
| **14** | 3-ClPh | 0.07 |
| **15** | 4-ClPh | 0.2 |
| **16** | 2-MePh | 0.2 |
| **17** | 3-MePh | 0.1 |
| **18** | 4-MePh | 0.6 |
| **19** | 2-Thienyl | 0.07 |
| **20** | 3-Thienyl | 0.1 |

**Table 2.** In vitro caspase-3 inhibition assay results for the enriched diastereomeric mixtures of **9**

| Compd | Diastereomer ratio (a/b) | $K_{i(app)}$, μM |
|---|---|---|
| **9** | 50:50 | 0.1 |
| **9a** | 80:20 | 0.04 |
| **9b** | 20:80 | 0.2 |

**Table 3.** In vitro caspase-3 inhibition assay results for compounds **22**–**29**



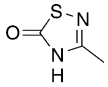
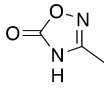
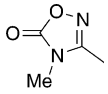
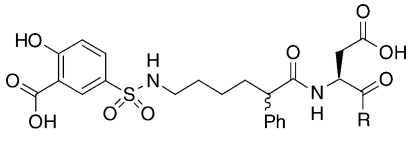
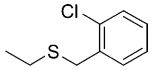
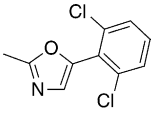
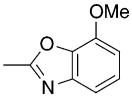
| Compd | R = | R′ = | X = | $K_{i(app)}$, μM |
|---|---|---|---|---|
| **9** | COOH | OH | $SO_2$ | 0.1 |
| **22** | H | H | $SO_2$ | 5.8 |
| **23** | H | OH | $SO_2$ | 2.0 |
| **24** | COOH | H | $SO_2$ | 6.8 |
| **25** |  | H | $SO_2$ | 1.4 |
| **26** |  | H | $SO_2$ | 0.5 |
| **27** |  | H | $SO_2$ | 0.1 |
| **28** |  | H | $SO_2$ | 1.8 |
| **29** | COOH | OH | CO | 0.4 |

**Table 4.** In vitro caspase-3 inhibition assay results for compounds **30**–**33**



| Compd | R = | $K_{i(app)}$, μM |
|---|---|---|
| **9** | H | 0.1 |
| **30** | $-CH_2SCH_3$ | 0.6 |
| **31** |  | 0.3 |
| **32** |  | 3.3 |
| **33** |  | 1.0 |

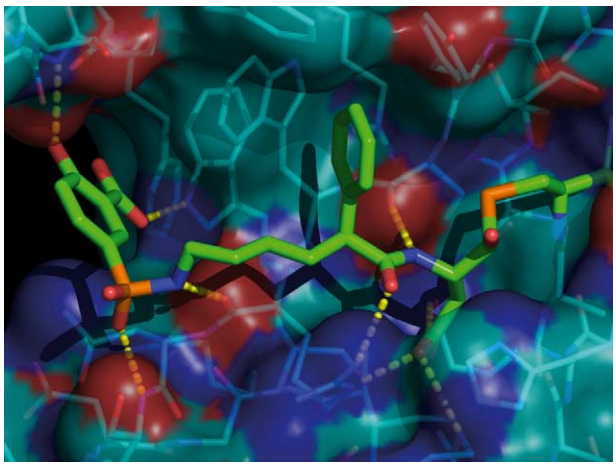


**Figure 1.** Theoretical binding conformation of the (*S*,*S*) diastereomer of **9** in the caspase-3 active site..[14]

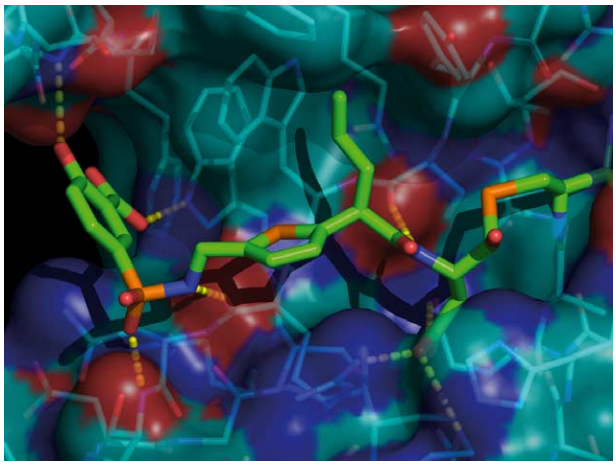


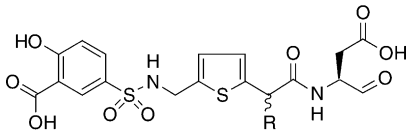
**Figure 2.** Theoretical binding conformation of the (*S*,*S*) diastereomer of **35** in the caspase-3 active site.

conformation in orienting the salicylic acid group in the caspase-3 $S_4$ binding subsite.

Due to potential therapeutic liabilities of an aldehyde warhead, we decided to investigate warheads based upon less reactive ketones. Both thiomethyl and oxazolyl ketones have been shown to be suitable warheads in peptidomimetic based caspase inhibitors.[9,10] We prepared several analogues of compound **9** with the results shown in Table 4. Incorporation of either thiomethyl or oxazolyl ketones onto **9** yielded inhibitors with reduced potency relative to the parent aldehyde. Among the ketone analogues in this series, the thiomethyl ketones **30** and **31** exhibited the best potency, indicating the reactivity of the carbonyl is more important than potential binding contacts in the $S_1'$ subsite.

We then turned our attention to replacing the flexible scaffold of **9** with a more rigid framework. We reasoned that replacement of three of the methylene groups of the main chain by an aromatic ring could improve potency by eliminating several rotational degrees of freedom. We chose the 2,5-disubstituted thiophene system to test this hypothesis since molecular modeling suggested this system should best approximate the geometry for presenting the $P_2$ and $P_4$ binding elements as seen in **9** (Fig. 2). The inhibitory activities for thiophene analogues **34**–**36** are shown in Table 5. Simply rigidifying the flexible chain resulted in a compound (**34**) equipotent to **1**. Adding a $P_2$ binding element gave a 10-fold increase in potency, yet the compounds were not better inhibitors than **9**. These results suggest that although the strategy of rigidifying the flexible linker of **9** is valid, a thiophene ring does not appear to allow the ideal binding geometry between the salicylic acid, a large $P_2$ substituent and the aspartyl side chain acid that is seen in **9**.

**Table 5.** In vitro caspase-3 inhibition assay results for compounds **34**–**36**



| Compd | R = | $K_{i(app)}$, μM |
|---|---|---|
| **34** | H | 4.0 |
| **35** | *n*-Pr | 0.3 |
| **36** | Ph | 0.3 |

In summary, we have discovered a novel series of reversible,[17] potent in vitro caspase-3 inhibitors through the use of our extended tethering technology. Beginning with an early lead, compound **1**, we developed SAR by systematically examining both $P_2$ and $P_4$ binding elements, aldehyde warhead replacements and scaffold rigidification. From our work described in this paper, we feel there is potential for further optimization of this series and subsequent work will be reported in due course.

## Acknowledgements

We would like to thank Stuart Lam, Thomas Webb and Alex Hsi for their assistance with preparatory HPLC purifications.

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17. Representative analogues were tested for reversible inhibition by incubation of compound with Caspase-3 followed by analysis by MS.