Identification of Potent and Selective Small-Molecule Inhibitors of Caspase-3 through the Use of Extended Tethering and Structure-Based Drug Design

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The design, synthesis, and in vitro activities of a series of potent and selective small-molecule inhibitors of caspase-3 are described. From extended tethering, a salicylic acid fragment was identified as having binding affinity for the S_4 pocket of caspase-3. X-ray crystallography and molecular modeling of the initial tethering hit resulted in the synthesis of **4**, which reversibly inhibited caspase-3 with a $K_i = 40$ nM. Further optimization led to the identification of a series of potent and selective inhibitors with K_i values in the 20−50 nM range. One of the most potent compounds in this series, **66b**, inhibited caspase-3 with a $K_i = 20$ nM and selectivity of 8−500-fold for caspase-3 vs a panel of seven caspases (1, 2, and 4−8). A high-resolution X-ray cocrystal structure of **4** and **66b** supports the predicted binding modes of our compounds with caspase-3.

Introduction

Cysteine proteases comprise a distinct mechanistic class of proteolytic enzymes that have been widely implicated as potential therapeutic targets. To date, three structural classes of cysteine proteases are known, (i) the ICE (interleukin-1β converting enzyme) class, (ii) the papain class (e.g., cathepsins), and (iii) the picornovirus 3C-protease class. A number of reviews have been published.[1−3]

Of specific interest to us are the caspases (cysteinyl aspartate specific proteases), which play key roles in both cytokine maturation and programmed cell death (apoptosis).[4,5] Common to all caspases is the presence of a catalytic diad comprised of a nucleophilic cysteine and histidine imidazole ring. All caspases cleave their respective peptide substrates after an aspartic acid residue; it is this near absolute requirement for aspartic acid at the P_1 position[6] of their substrates that makes the caspases some of the most specific proteases known. This specificity can be largely attributed to favorable hydrogen-bonding interactions between the charged aspartate residue of the substrate and the three amino acid residues Arg64, Gln161, and Arg207 in the caspase active site (caspase-3 numbering adopted from previous studies).[7] Studies using a positional-scanning combinatorial library have demonstrated that the S_4 subsite is the single most important determinant of specificity among the caspases.[8,9] To date, at least 12 human caspase members have been identified. On the basis of their sequence homology, substrate specificity, and structural similarities, the caspases can be divided into two major subfamilies. Those related to ICE (caspase-1, -4, -5, and -13) are involved in inflammation (cytokine maturation). Caspase-1 was the first caspase member to be identified and is responsible for the cleavage of the Asp116−Ala117 bond in the inactive 31 kDa cytokine pro-IL-1β in monocytes to the active 17 kDa cytokine IL-1β, a key inflammatory mediator. The second subfamily is comprised of caspases involved in mediating programmed cell death or apoptosis. The upstream members in the apoptotic signaling cascade are referred to as initiator caspases (caspase-2, -8, -9, and -10) while downstream members are referred to as effector caspases (caspase-3, -6, and -7). Initiator caspases are activated in response to proapoptotic signals and are responsible for activating the effector caspases, which in turn are responsible for the proteolytic cleavage of substrates involved in the final stages of cell disassembly.[5,10] It is worth noting that the exact roles and functions of individual caspase members in the apoptotic signaling pathway have not been fully deciphered at this time.

Not surprisingly, as a result of their roles in inflammation and apoptosis, the caspases have generated substantial interest from both the pharmaceutical and the biotechnology sector as potential targets for drug discovery. Inflammatory diseases such as rheumatoid arthritis and diseases involving dysregulated apoptosis such as myocardial infarction, stroke, traumatic brain injury, sepsis, Alzheimer's disease, and Parkinson's disease represent a large unmet medical need and could benefit from potent and selective caspase inhibitors.[11−13] Studies involving knockout mice as well as numerous proof-of-concept animal models have demonstrated that regulation of caspase activity may be a viable therapeutic approach.[2,14,15]

In addition to being potential therapeutic agents, potent and selective caspase inhibitors would be invaluable tools in the study and delineation of the roles that individual caspases play in the complex apoptosis signaling pathways. The majority of cell-based assays and animal models studying the neuroprotective effects of caspase inhibitors make use of peptide-derived inhibitors, both reversible and irreversible. Although quite potent, most reversible peptide inhibitors are not highly

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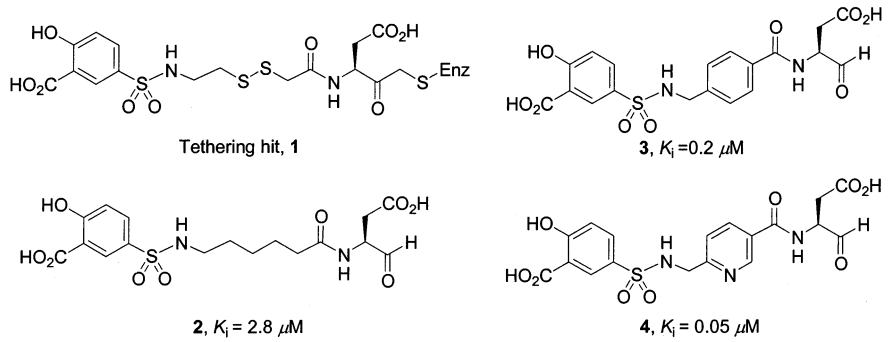
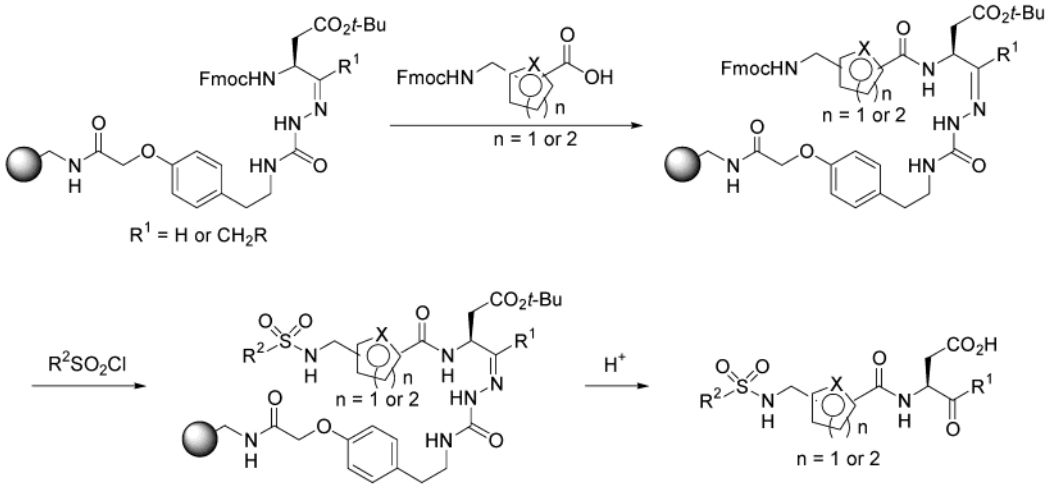


Figure 1. Initial leads developed from an extended tethering screen.

Scheme 1. General Scheme for Analogue Synthesis



specific for any one caspase. For example, the tetrapeptide inhibitor DEVD-CHO, which contains the caspase-3 consensus cleavage sequence, inhibits caspase-3 with a K_i value of 0.23 nM but also inhibits caspase-1 with a K_i value of 18 nM.[16] Irreversible inhibitors have been shown to be effective in a variety of proof-of-concept pharmacological models. However, because of the fact that these are irreversible inhibitors, they potentially lack selectivity and the observed results do not necessarily imply inhibition of caspases only.[17]

Recently, we reported the use of "extended tethering", a fragment-based ligand discovery technology, to identify a novel pharmacophore that was successfully combined with a known fragment to create a unique nonpeptidic inhibitor of caspase-3.[18] In this strategy, a known binding fragment of the target, in this case an aspartyl group, is covalently attached to a binding pocket in the protein active site. The bound fragment bears a free thiol group, which is allowed to react reversibly with a library of disulfide-containing compounds. In doing so, the "extender" probes the disulfide library for additional binding elements for proximal binding pockets of the active site. In the case of caspase-3, conversion of initial tethering hit **1** to a reversible small-molecule compound resulted in inhibitor **2** with low micromolar affinity (K_i = 2.8 μM) (Figure 1). Rigidification of the acyclic linker with a phenyl linker resulted in compound **3**, which displayed 14-fold increase in binding affinity (K_i = 0.20 μM).

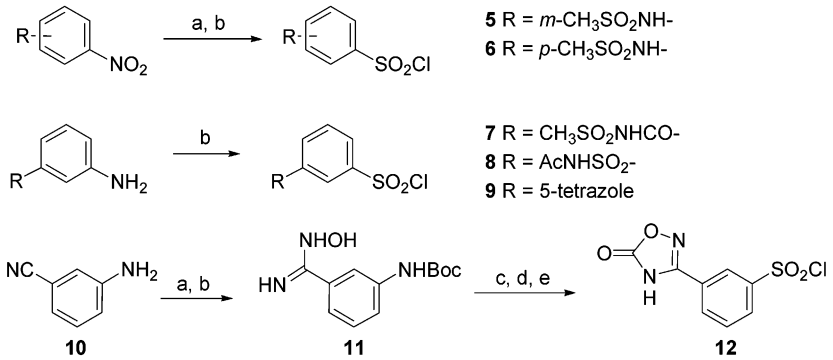
In this paper, we describe the optimization of compound **3** through modification of the salicylic acid group, the active site electrophile, and the linker region. These efforts have produced some of the most specific nonpeptidic caspase-3 inhibitors reported to date, which display nanomolar potencies. In addition, X-ray crystallographic studies have yielded crystal structures, which support the predicted binding modes of our compounds with caspase-3.

Chemistry

Synthetic Strategy. Unless otherwise noted, aspartyl aldehyde and ketone-based inhibitors were prepared utilizing previously reported methods (Scheme 1). *N*-Fluorenylmethoxycarbonyl(Fmoc)-protected aspartyl aldehyde or thiomethyl ketone, prepared from the *N*-Fmoc-Asp(O*t*-Bu)-OH, is attached to support using a semicarbazide linker to provide support-bound semicarbazone.[19,20] Linking through the invariant carbonyl is ideal because it protects the carbonyl from nucleophilic attack as well as racemization at the α-stereocenter.[21,22] Removal of the Fmoc group under basic conditions, followed by acylation with Fmoc-protected heterocycles, introduces a rigid linker, which links the aspartyl group to the S_4 binding fragment (e.g., salicylic acid). Removal of the newly incorporated Fmoc group followed by acylation with sulfonyl chlorides introduces the S_4 binding element. Finally, acidic cleavage of the semicarbazone linker releases the fully functionalized aldehyde/ketone inhibitors from the support.

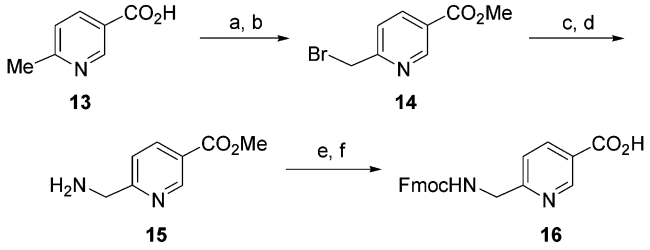
Synthesis of Substituted Benzenesulfonyl Chlorides. The synthesis of noncommercially available substituted benzenesulfonyl chlorides used in the preparation of compounds in Tables 1 and 5 is shown in Scheme 2. Starting with an appropriately substituted

Scheme 2. Synthesis of Sulfonyl Chlorides[a]



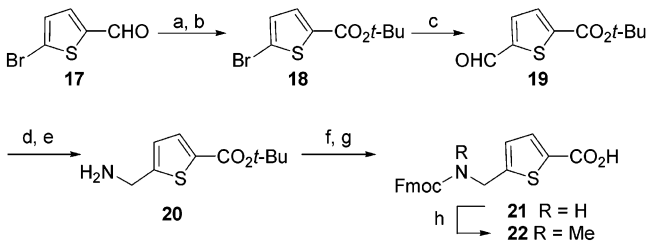
[a] Reagents and conditions (reactions 1 and 2): (a) 10% Pd/C, H_2, EtOH, HCl. (b) $NaNO_2$, SO_2, $CuCl_2$, HCl, HOAc. Reagents and conditions (reaction 3): (a) Boc_2O, Et_3N, THF, 60 °C. (b) Aqueous NH_2OH, EtOH, 80 °C. (c) (i) 2-Ethylhexyl chlororformate, pyridine, THF, 0 °C; (ii) xylene, reflux. (d) 4.0 N HCl, room temperature. (e) $NaNO_2$, SO_2, $CuCl_2$, HCl, HOAc.

Scheme 3. Synthesis of 2,5-Disubstituted Nicotinic Acid[a]



[a] Reagents and conditions: (a) $SOCl_2$, MeOH. (b) NBS, $(PhCO_2)_2$, HOAc. (c) NaN_3, DMF, 70 °C. (d) 10% Pd/C, H_2, EtOH. (e) Aqueous LiOH, dioxane. (f) Fmoc-OSu, $NaHCO_3$.

Scheme 4. Synthesis of 2,5-Disubstituted Thiophene Linker[a]



[a] Reagents and conditions: (a) $NaClO_2$. (b) *t*-BuOH, EDC, DMAP. (c) *n*-BuLi, −78 °C and then 1-formylpiperidine. (d) Aqueous NH_2OH. (e) Zn, HOAc. (f) Fmoc-OSu, $NaHCO_3$. (g) TFA. (h) CH_2O, TFA and then triethylsilane.

aniline or nitrobenzene precursor, sulfonyl chlorides **5**−**9** were obtained by either (i) a nitro group reduction via catalytic hydrogenation followed by chlorosulfonylation (compounds **5** and **6**) or (ii) a single chlorosulfonylation step (compounds **7**−**9**). The corresponding 5-oxo-1,2,4-oxadiazole **12**[23] was prepared in five steps in good yield from commercially available 3-amino benzonitrile **10**.

Synthesis of Fmoc-Protected Heterocyclic Linkers. Fmoc-protected heterocyclic linkers used in the synthesis of target compounds shown in Table 3 were prepared according to Schemes 3−5. Esterification of commercially available 6-methyl nicotinate **13** followed by bromination via halogenation with *N*-bromosuccinimide (NBS) gave **14**. Bromide displacement with sodium azide followed by catalytic hydrogenation gave primary amine **15**. Final saponification with aqueous lithium hydroxide followed by Fmoc protection afforded **16**. The 2,5-disubstituted pyrazine used in the synthesis of **63a**,**b** was prepared in the same manner as shown for the substitution of **16** except for the substitution of 5-methyl-2-pyrazinecarboxylate for 6-methyl nicotinate.

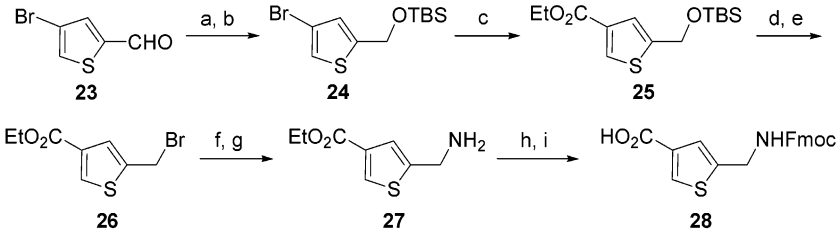
The 2,5-disubstituted thiophene linker **21** was prepared in seven steps starting from commercially available 2-bromothiophene-5-carboxaldehyde **17** according to Scheme 4. Aldehyde oxidation with $NaClO_2/NaH_2PO_4$ followed by esterification with *tert*-butyl alcohol gave the corresponding *t*-butyl ester **18**. Lithium−halogen metal exchange with *n*-butyllithium followed by trapping with 1-formylpiperidine provided aldehyde **19**. Conversion of **19** to the oxime with aqueous hydroxylamine followed by reduction with zinc dust gave primary amine **20**. Subsequent Fmoc protection and ester hydrolysis afforded linker **21**. Linker **21** was converted to the *N*-methylated linker **22** in one pot using formaldehyde followed by reduction with triethylsilane. The 3,5-disubstituted thiophene used in the synthesis of **68a**,**b** was prepared in the same manner as shown for **21** except for the substitution of 3-bromothiophene-5-carboxaldehyde for 2-bromothiophene-5-carboxaldehyde.

The corresponding 2,4-disubstituted thiophene linker **28** was prepared from aldehyde **23** as outlined in Scheme 5. Sodium borohydride reduction and *tert*-butyldimethylsilyl (TBS) protection of the primary alcohol gave the silyl ether **24**. Treatment of **24** with *n*-butyllithium followed by trapping with ethyl chloroformate provided ethyl ester **25**. Conversion of **25** to bromide **26** was accomplished by first TBS deprotection with fluoride ion followed by bromination with CBr_4 and triphenylphosphine. Treatment of **26** with sodium azide and subsequent catalytic hydrogenation furnished amine **27**. Saponification followed by Fmoc protection gave linker **28**.

The corresponding Fmoc-protected pyrimidine, thiazole, and isoxazole used in the synthesis of **62a**,**b**, **64a**,**b**, and **65a**,**b**, respectively, were prepared according to literature methods.[24−29] The disubstituted furan linker used in the preparation of **70a**,**b** was synthesized using the same method as linker **28** except for the substitution of commercially available 5-chloromethyl-furan-2-carboxylic acid ethyl ester for intermediate **26**.

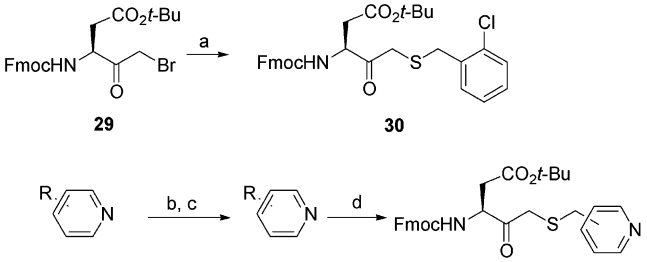
Synthesis of Warhead Groups. The thiomethyl ketone derivatives were prepared as shown in Scheme 6. *N*-Fmoc-Asp(O*t*-Bu)-CH_2Br **29**, synthesized in three steps from literature methods,[20] was reacted with the

Scheme 5. Synthesis of 3,5-Disubstituted Thiophene Linker[a]



[a] Reagents and conditions: (a) $NaBH_4$, THF. (b) TBSCl, imidazole, CH_2Cl_2. (c) *n*-BuLi, THF, −78 °C and then $ClCO_2Et$. (d) TBAF, HOAc, THF. (e) CBr_4, PPh_3, THF. (f) NaN_3, DMF, 50 °C. (g) 10% Pd/C, H_2, EtOH. (h) Aqueous LiOH, dioxane. (i) Fmoc-OSu, $NaHCO_3$.

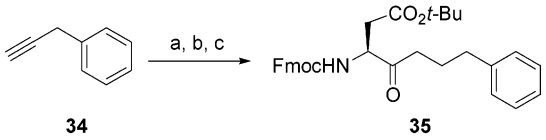
Scheme 6. Synthesis of Thiomethyl Ketone Warheads[a]



31a R = 3-$ClCH_2$- **32a** R = 3-$HSCH_2$- **33a** (3-pyridyl)
31b R = 4-$ClCH_2$- **32b** R = 4-$HSCH_2$- **33b** (4-pyridyl)

[a] Reagents and conditions: (a) 2-Chlorobenzenemethanethiol, THF. (b) Thiourea, EtOH. (c) Aqueous NaOH. (d) Compound **29**, DIEA, THF.

Scheme 7. Synthesis of Phenylpropyl Ketone Warheads[a]



[a] Reagents and conditions: (a) *n*-BuLi, THF, −78 °C and then *N*-Fmoc-Asp(O*t*-Bu)-CHO. (b) 10% Pd/C, H_2, EtOH. (c) Dess−Martin periodinane, CH_2Cl_2.

appropriately substituted 2-chlorobenzene methanethiol or pyridylmethanethiols[30] **32a**,**b** to furnish thiomethyl ketones **30** or **33a**,**b**, respectively, which were attached to support **43** for analogue synthesis.

The phenylpropyl ketone intermediate **35** was synthesized in a three-step sequence as shown in Scheme 7. The anion of 3-phenyl-1-propyne **34** was reacted with *N*-Fmoc-Asp(O*t*-Bu)-CHO,[31] hydrogenated over Pd(0), and oxidized with Dess−Martin periodinane to afford **35**. Efforts to generate the phenylpropyl ketone through the addition of phenylpropyl Grignard to *N*-Fmoc-Asp-(O*t*-Bu)-CHO resulted in no reaction. The fully functionalized analogue was prepared by attaching compound **35** to the support through the semicarbazide linker **43** followed by standard analogue synthesis.

The keto-oxazole and keto-benzoxazole analogues **41b** and **42b** were prepared in solution as shown in Scheme 8. The elaborated thiophenecarboxylic acid **40** was prepared starting from commercially available **36**. Esterification with diazomethane followed by bromination with NBS provided bromide **37**. Treatment with azide followed by zinc reduction and acylation with 5-chlorosulfonyl-2-hydroxybenzoic acid provided **38**. It was necessary at this point to differentially protect the salicylic acid as the *tert*-butyl ester. Standard conditions using EDC/*tert*-butyl alcohol or $H_2SO_4/MgSO_4$[32] yielded no product. However, treatment with dimethylformamide (DMF) di-*tert*-butyl acetal[31] gave the desired product **39** in modest yields. Saponification of **39** yielded the elaborated thiophenecarboxylic acid **40**, which was coupled with the appropriate amino alcohols **41a** and **42a**[34] using standard EDC and HOBt coupling conditions. Dess−Martin periodinane oxidation of the secondary alcohol followed by hydrolysis of the *t*-butyl ester afforded the keto-oxazole and keto-benzoxazole analogues **41b** and **42b**, respectively.

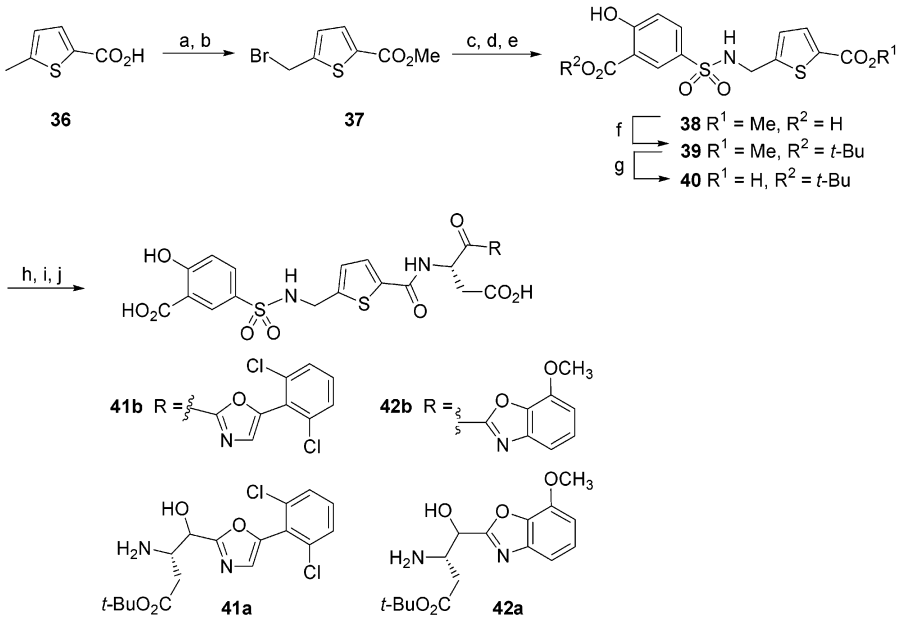
Synthesis of Aldehyde and Thiomethyl Ketone Analogues. All compounds bearing either the aldehyde warhead (Tables 1 and 3) or the methyl ketone warhead (Tables 3−6) were prepared as shown in Scheme 9. Standard Fmoc deprotection and amine acylation protocols were used with the two methods differing only in the conditions for cleavage of the final product. In the case of the aldehyde warhead, the aldehyde was released from support after treatment with a cocktail of tetrahydrofuran (THF)/HOAc/CH_3CHO/trifluoroacetic acid (TFA) followed by hydrolysis of the *tert*-butyl ester with TFA/CH_2Cl_2/H_2O. The ketone analogues were released from support and hydrolyzed in a one step treatment with TFA/H_2O.

Analogues **47a**−**c** were prepared as shown in Scheme 10. Following removal of the Fmoc group, resin **44a** was acylated with either linker **21** or **22**. Removal of the second Fmoc group followed by treatment with 5-formyl-2-hydroxy-benzoic acid and $NaBH(OAc)_3$ provided the reductive amination products. Treatment with TFA provided **47a**,**c**. Analogue **47b** was prepared in the same manner as **47a** except the reductive amination step was followed by acylation with acetic anhydride.

Results and Discussion

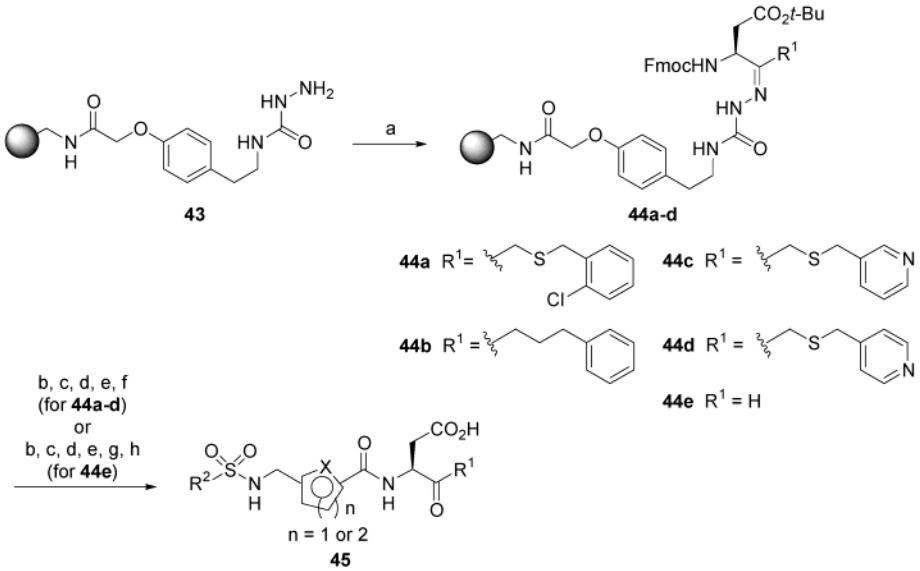
As previously reported, a novel salicylic acid fragment that binds in the S_4 subsite of caspase-3 was identified by extended tethering.[18] Incorporation of the salicylic acid fragment onto an acyclic linker containing the requisite Asp-CHO warhead at P_1 provided compound **2**, which possessed a K_i value of 2.8 μM for caspase-3 inhibition (Figure 1). Upon examination of the X-ray crystal structure of the tethered hit **1**[18] bound in the caspase-3 active site, we reasoned that conformational restriction of the flexible acyclic linker and, thus, the relative positions of the salicylic acid and sulfonamide groups in compound **2** could provide improvements in binding affinity. To this end, compound **3** was synthesized in which a phenyl ring replaces the four carbon acyclic linker of compound **2**. This substructure was selected in order to maintain the distance between the salicylic acid and the aspartic acid warhead while

Scheme 8. Synthesis of Keto-Oxazole Analogues[a]



[a] Reagents and conditions: (a) $TMSCH_2N_2$. (b) NBS, $(PhCO_2)_2$. (c) NaN_3, AcCN. (d) Zn, HOAc. (e) 5-Chlorosulfonyl-2-hydroxybenzoic acid, CH_2Cl_2. (f) DMF di-*tert*-butyl acetal, 90 °C. (g) Aqueous LiOH, dioxane. (h) Compounds **41a** or **42a**, EDC, NMM, HOBt, DMF. (i) Dess−Martin periodinane, CH_2Cl_2. (j) TFA, CH_2Cl_2.

Scheme 9. Solid-Phase Synthesis of Carbonyl Analogues[a]

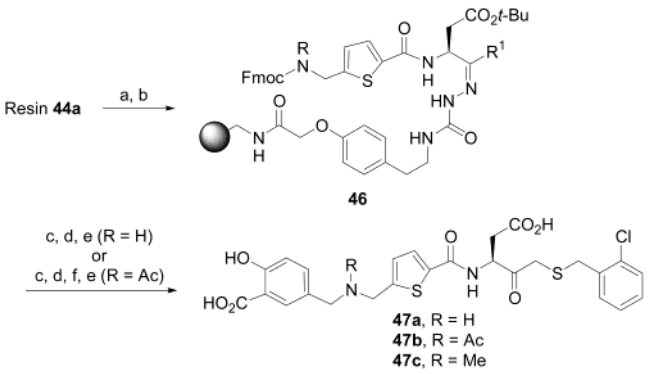


[a] Reagents and conditions: (a) Compound **30** or **33a** or **33b** or **35**, HOAc, THF. (b) Piperidine, DMF. (c) *N*-Fmoc-heterocyclic acid, PyBOP, DIPEA, DMF. (d) Piperidine, DMF. (e) R_2SO_2Cl, DIPEA, CH_2Cl_2. (f) 9:1 TFA/H_2O. (g) 5:1:1:0.25 THF/HOAc/CH_3CHO/TFA. (h) 1:1:0.1 TFA/CH_2Cl_2/H_2O.

simultaneously providing overall rigidification of the molecule. Compound **3** exhibited 14-fold increase in inhibitory potency as compared to **2** with K_i values of 0.20 and 2.8 μM, respectively. Molecular modeling studies of **3**, working from the crystal structure of the initial tethering hit, suggested that this nonpeptidic structure was capable of replicating many of the key hydrogen-bonding interactions formed by peptidic inhibitors. However, this inhibitor appeared unable to preserve a common hydrogen-bonding interaction normally formed between the main chain N−H of Arg207 and the main chain carbonyl of the P_3 residue (Figure 2). Thus, we reasoned that this conserved hydrogen-bonding interaction might be restored through incorporation of a hydrogen bond-accepting moiety in the phenyl ring of **3**. Replacement of the phenyl ring with a 2,5-disubstituted pyridyl ring provided compound **4**, which exhibited a 4-fold improvement in potency over **3** with a K_i = 0.05 μM. The fluoromethyl ketone analogue of **4** was prepared to facilitate cocrystallization for X-ray crystallography. Through this structure of **4**, we were able to confirm the formation of a hydrogen bond interaction of the pyridyl ring with Arg207 (Figure 3). With compound **4** as a suitable starting point, we then proceeded with our optimization strategy.

Structure−Activity Strategy. With the goal of improving in vitro potency and obtaining good cellular activity of compound **4**, we systematically examined three regions of the molecule: the salicylic acid group, the aspartyl aldehyde warhead, and the linker portion

Scheme 10[a]



[a] Reagents and conditions: (a) Piperidine, DMF. (b) Compound **21** or **22**, PyBOP, DIPEA. (c) Piperidine, DMF. (d) 5-Formyl-2-hydroxy-benzoic acid, $NaBH(OAc)_3$. (e) 9:1 TFA/H_2O. (f) Ac_2O, DIPEA.

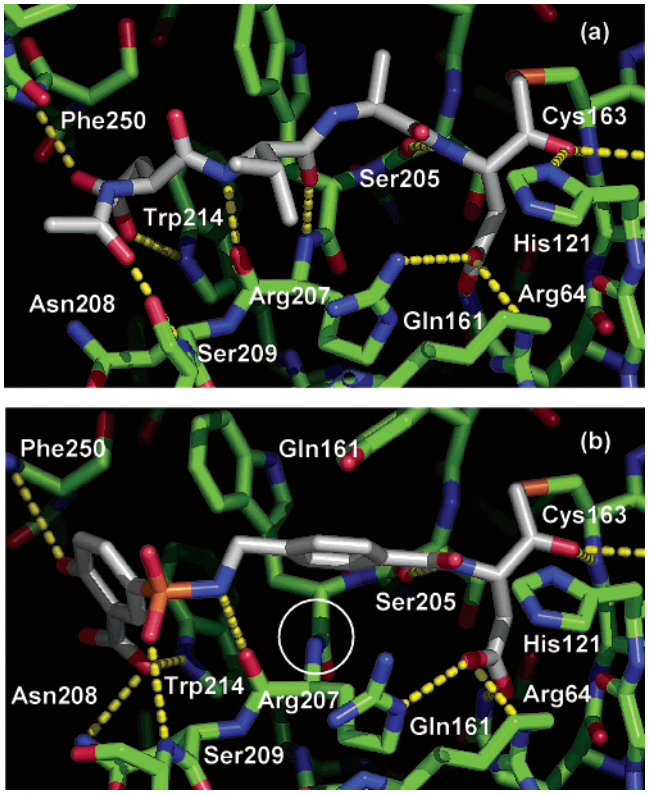


Figure 2. Crystal structures of irreversible fluoromethyl ketone analogues of (a) peptide AcDVAD and (b) compound **3**. In panel b, note the absence of a hydrogen-bonding group capable of interacting with the main chain N−H of Arg207.

(Figure 4). An immediate goal was to remove one of the two negative charges present in the molecule to facilitate cell membrane permeability. Specifically, we wanted to determine whether the carboxylic acid group of the salicylic acid could be replaced with a neutral surrogate capable of forming an equivalent set of hydrogen-bond interactions. We also investigated alternative warheads containing activated ketones as a means of avoiding the potential metabolic liability of the starting aldehyde. The use of activated ketones with cysteine proteases is well-precedented[35] and can permit formation of additional binding interactions in the S_1' site of the enzyme. Finally, the linker portion of the molecule was examined to see if improvements in potency could be

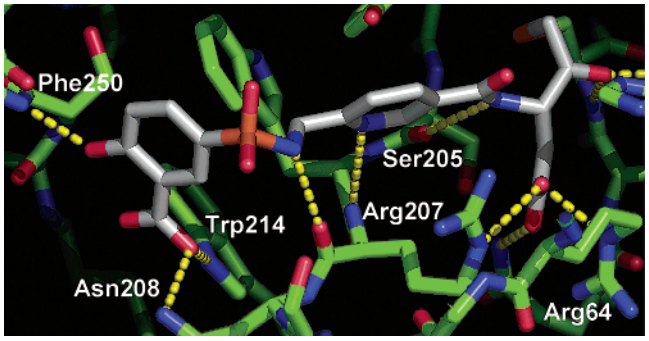


Figure 3. X-ray cocrystal structure of an irreversible derivative of compound **4** bound within the active site of caspase-3. Hydrogen-bonding interaction of pyridyl nitrogen with Arg207 and other key interactions is shown by dashed yellow lines.

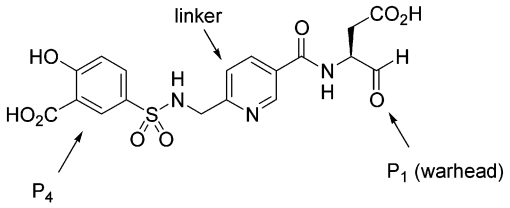


Figure 4. Optimization strategy of pyridine analogue **4**.

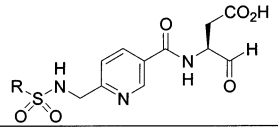
obtained through incorporation of various heterocycles. Such linking functionality has the potential to form various hydrogen-bond interactions in the active site directing appropriate substituents into the S_2 and/or S_3 subsites of the enzyme.

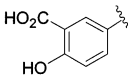
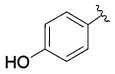
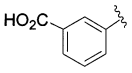
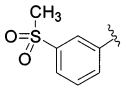
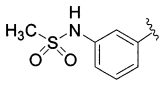
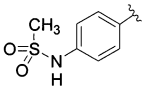
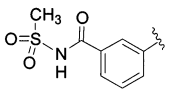
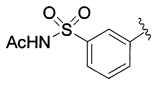
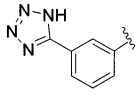
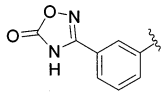
P_4 Salicylic Acid Group Modifications of Compound 4. Truncations of the salicylic acid group were examined to identify which aspects of this fragment were critical for enzyme binding (Table 1). Removal of the 4-hydroxyl group (**50**) resulted in a modest decrease in caspase-3 inhibitory activity, K_i = 0.16 μM. In contrast, binding affinity was significantly decreased when the carboxylic acid at the 3-position was removed (**49**), K_i = 2.3 μM. Molecular modeling studies based on the crystal structure of the tethered fragment suggest that hydrogen bonds are likely formed between this important carboxylate and the indole of Trp214 and perhaps the side chain of Asn208 but not the main chain of Phe250, as would normally be the case for aspartyl P_4 substituents.

To address possible cell membrane permeability issues, nonacidic pharmacophores such as the methyl sulfone (**51**) and methyl sulfonamide (**52**) were selected for their potential ability to maintain the important hydrogen-bond interaction to Trp214. These compounds however resulted in a loss of inhibitory activity with K_i values of 1.7 and 1.9 μM for compounds **51** and **52**, respectively. It appears that the only P_4 substituents that can maintain moderate caspase-3 inhibitory activity (within 10-fold of **4**) are those possessing acidic functionality. Namely, incorporation of known carboxylic acid bioisosteres such as an acylsulfonamide (**55**), tetrazole (**56**), or a 5-oxo-1,2,4-oxadiazole moiety (**57**) gave compounds with K_i values of 0.47, 0.44, and 0.40 μM, respectively. Interestingly, the reverse acylsulfonamide (**54**) showed only weak activity with K_i = 1.4 μM.

Aspartyl Aldehyde Warhead Analogues of Compound 4. On the basis of evidence of a hydrophobic binding pocket in the S_1' subsite of caspases, several

Table 1. Caspase-3 Inhibition by Substituted P_4 Aryl Sulfonamides on a Pyridine Scaffold

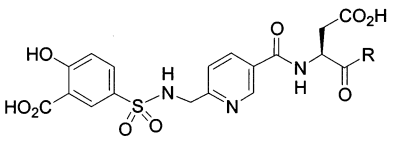


Cmpd	R	K_i (μM)
4		0.05
48		1.7
49		2.3
50		0.16
51		1.7
52		1.9
53		1.3
54		1.4
55		0.47
56		0.44
57		0.40

ketone warheads were examined. Incorporation of a heteroatom or an electron deficient group at the β'-atom is one strategy used to enhance the potency of cysteine protease inhibitors.[35−37] Replacing the aldehyde portion of the warhead of **4** with a methyl thiomethyl ketone (**58**) provided a compound with a $K_i = 0.19$ μM, which is 5-fold less active than the corresponding aldehyde **4** (Table 2). However, incorporating the more lipophilic 2-chlorobenzyl thiomethyl ketone warhead (**59**) resulted in comparable activity ($K_i = 0.03$ μM) relative to **4**.

Keto-oxazoles and keto-benzoxazoles have also been reported as suitable P_1 warheads in cysteine protease inhibitors where the oxazole nitrogen can hydrogen bond to a histidine side chain in the putative oxyanion hole of the enzyme. On the basis of work by Vertex in the area of ICE (caspase-1) inhibitors,[34] 5-phenyl benzoxazole (**60**) and benzoxazole (**61**) warheads were incorporated into our lead compound with the hope that we could potentially pick up similar hydrogen-bonding

Table 2. Caspase-3 Inhibition by P_1 Aspartyl Ketone Derivatives on a Pyridine Scaffold



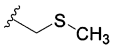
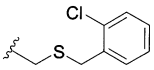
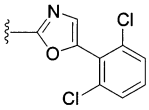
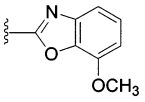
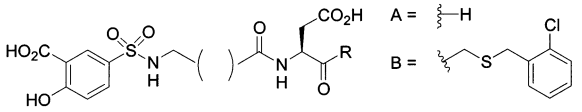
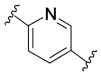
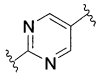
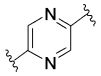
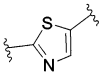
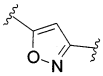
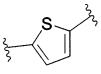
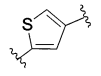
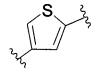
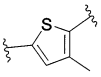
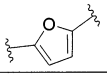
Cmpd	R	K_i (μM)
4		0.05
58		0.19
59		0.03
60		0.45
61		0.41

Table 3. Caspase-3 Inhibition by Heterocyclic Derivatives: P_1 Aspartyl Aldehyde vs Aryl Thiomethyl Ketone



Cmpd	( )	R	K_i (μM)
4		A	0.05
59		B	0.03
62a		A	0.02
62b		B	0.02
63a		A	0.48
63b		B	0.09
64a		A	0.07
64b		B	0.03
65a		A	0.16
65b		B	0.06
66a		A	0.12
66b		B	0.02
67a		A	2.5
67b		B	0.27
68a		A	4.0
68b		B	0.21
69a		A	0.29
69b		B	0.04
70a		A	12.5
70b		B	4.0

interactions in the caspase-3 oxyanion hole. Unfortunately, both compounds **60** and **61** exhibited a 10-fold decrease in activity with K_i values of 0.45 and 0.41 μM,

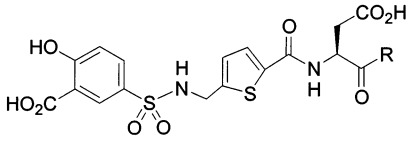
respectively. The loss of inhibitor potency observed with these large rigid groups may reflect the more occluded nature of the caspase-3 active site, which is considerably more narrow and deep than that of ICE.

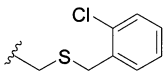
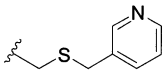
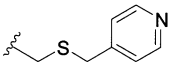
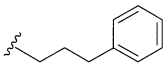
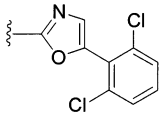
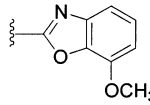
Linker Replacement with Heterocycles. We examined the replacement of the pyridyl linker in compound **59** with a variety of heterocycles in the context of the 2-chlorobenzyl thiomethyl ketone/aldehyde warhead and the salicylic acid group at P_4 (Table 3). The pyrimidine analogue **62b** showed comparable activity to the corresponding pyridyl analogue **59**, $K_i = 0.02$ and 0.03 μM, respectively, while pyrazine derivative **63b** was 3-fold less active (0.09 μM). The pyridyl linker of **59** could also be successfully replaced with various five-membered heterocycles. The corresponding 2,5-thiazole (**64b**), 2,5-thiophene (**66b**), and 3,5-isoxazole linkers (**65b**) all exhibited excellent in vitro potencies against caspase-3 with K_i values of 0.03, 0.02, and 0.06 μM, respectively. Among the various thiophene analogues **66**−**68a**,**b**, inhibitory activity seemed to be strongly dependent on the substitution pattern around the thiophene ring. Both the 3-aminomethyl-5-carboxy (**68b**) and 2-aminomethyl-4-carboxy (**67b**) analogues were significantly less potent than the corresponding 2-aminomethyl-5-carboxy analogue **66b**. A methyl group introduced at the 4-position (**69b**) resulted in comparable activity to **66b**, K_i values of 0.04 and 0.02 μM, respectively. We speculated that substitution at the 4-position with an appropriate binding element could allow us to pick up additional binding affinity in the S_2 subsite of the enzyme. By analogy, introduction of a methyl group at the 3-position of **66b** resulted in a completely inactive compound (in the context of the aldehyde warhead; data not shown).

Interestingly, the same substitution pattern on the furan linker (**70a**,**b**) resulted in compounds with substantial decreases in potency. This suggests that inhibitor activity in this series can be quite sensitive to the relative positioning of the groups occupying the S_1 and S_4 pockets, given the relatively small differences in distances and bond angles that distinguish substituents around furan and the various thiophenes. From the results seen in Table 3, we selected **66b** for further structure−activity relationship (SAR) studies for reasons of excellent in vitro potency, synthetic accessibility, and the potential to incorporate and direct substituents at the 4-position of the thiophene ring for binding into the S_2 subsite of the enzyme.

P_1 Warhead Studies on Thiophene Analogues. An evaluation of P_1 warheads similar to that performed on the pyridyl linker was conducted on thiophene analogues **66a**,**b** (Table 4). In general, SAR of the thiophene analogues paralleled those of the pyridyl analogues except in one significant way. There was a 6-fold difference in inhibitory activity observed between the aldehyde warhead (**66a**) and the 2-chlorobenzyl thiomethyl ketone (**66b**), $K_i = 0.12$ vs 0.02 μM, respectively, which was not observed in the corresponding pyridyl compounds **4** and **59** or the pyrimidine derivatives **62a**,**b** (and the other five-membered heterocycles analogues shown in Table 3). As stated earlier, it was proposed (and supported by crystallography) that the gain in potency from **3** to **4** arose from the hydrogen-bond interaction of the basic amine of the pyridyl

Table 4. Caspase-3 Inhibition by P_1 Aspartyl Ketones on a Thiophene Scaffold



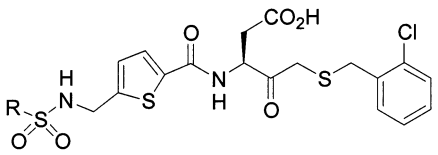
Cmpd	R	K_i (μM)
66a		0.12
66b		0.02
71		0.02
72		0.03
73		0.24
41b		0.38
42b		0.29

nitrogen and the side chain of Arg207 in the S_3 subsite, and the same argument can presumably be made for the pyrimidine analogues **62a**,**b**. However, in the case of thiophene analogues (**66**−**68a**,**b**), no such hydrogen bond could be formed, so the high affinity binding of certain members of this series must reflect other improvements relative to **3**. Specifically, the highest affinity variants in this set may be due to a subtle repositioning of the key contacts, such as those made by the S_4 salicylic acid group.

Thiomethyl ketones bearing a pyridyl substituent (**71**) and (**72**) were synthesized in order to explore the possibility of productive polar interactions with residues such as Glu123 in the S_1' subsite. Both compounds exhibited excellent in vitro potency but showed no improvement over **66b**. It is interesting to note that the relatively unactivated phenylpropyl ketone warhead (**73**) exhibited only a 10-fold decrease in activity as compared to the parent compound **66b**.

P_4 Modifications on Compound 66b. Compound **66b** was selected as a compound for further analogue studies around the salicylic acid moiety analogous with that performed on compound **4**. Similar truncations of the salicylic acid group were examined (Table 5), and the results found are essentially the same as those for the pyridyl linker. Namely, acidic functionality at the 3-position is critical for good caspase-3 inhibitory activ-

Table 5. Caspase-3 Inhibition by Substituted P_4 Aryl: Sulfonamides on a Thiophene Scaffold



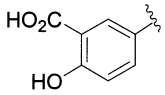
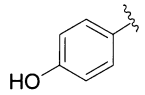
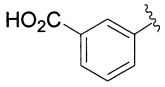
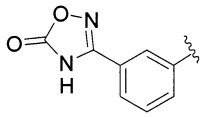
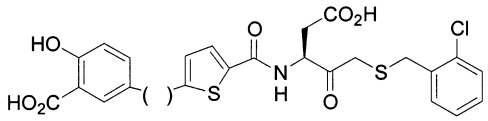
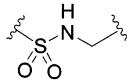
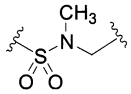
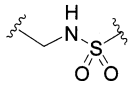
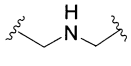
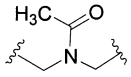
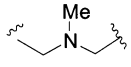
Cmpd	R	K_i (μM)
66b		0.02
74		2.1
75		2.4
76		0.40
77		0.31

Table 6. Effects on Caspase-3 Inhibition by P_3 Sulfonamide-Modified Thiophene Derivatives



Cmpd	( )	K_i (μM)
66b		0.02
78		0.14
79		1.9
47a		0.80
47b		0.14
47c		0.09

ity. Additional analogues in this series were not pursued based on the lack of success on the pyridyl scaffold.

One obstacle in identifying suitable surrogates for the salicylic acid group was the small number of commercially available sulfonyl chlorides as well as the lack of suitable precursors for chlorosulfonylation chemistry.

Table 7. Crystallographic Data and Refinement Parameters

data set	space group	cell constants α, β, γ (Å)	resolution (Å)	R_{cryst} (%)	R_{free} (%)
3[a]	*C*2	123.5, 71.0, 95.9 $\beta = 136.4°$	1.7	15.1	18.3
4[a]	*C*2	124.0, 71.6, 95.8 $\beta = 136.2°$	2.0	19.2	23.9
66b	*I*222	71.2, 84.3, 95.5	1.55	15.7	18.8

[a] Crystallization was performed with the irreversible derivatives of these analogues.

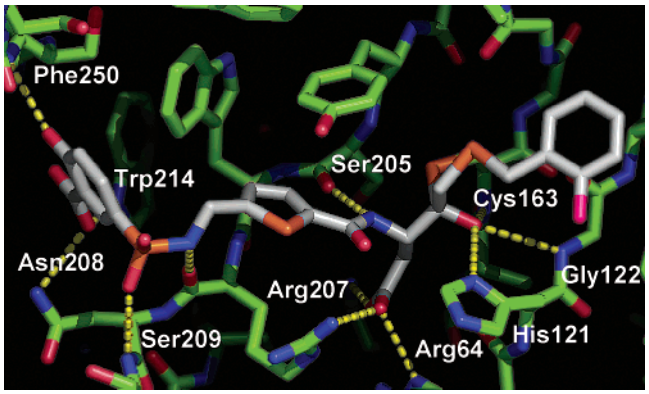


Figure 5. Resolution (1.55 Å) X-ray cocrystal stucture of inhibitor **66b** bound within the active site of caspase-3. Hydrogen-bonding interactions are shown using dashed yellow lines.

We realized that the sulfonamide linkage connecting the salicylic acid group to the rest of the molecule was a potential bottleneck that would hamper our abilities to fully probe this region of the active site. Therefore, we decided to look for replacements of the sulfonamide linkage that would allow us to introduce more readily available building blocks containing the necessary functionality to satisfy the hydrogen-bond interactions observed between the salicylic acid group with the active site residues in the S_4 subsite. Several alternate linkages were examined, and the results are shown in Table 6. The reverse sulfonamide (**79**) would allow the use of amine building blocks while maintaining the necessary sulfonamide N−H group for hydrogen bonding. However, compound **79** was 100-fold less active as compared to the parent compound **66b**. Replacing the sulfonamide with an amine linkage was also examined. These types of linkages would allow the introduction of a wide variety of potential P_4 groups by reductive amination reactions utilizing amine and/or aldehyde building blocks. The secondary amine (**47a**) displayed 40-fold less activity than the parent compound, but the tertiary amine (**47c**) and the corresponding acetamide (**47b**) appeared to be well-tolerated with K_i values of 0.09 and 0.14 μM, respectively. Molecular modeling of **47c** suggested that the tertiary amine, when protonated, could make similar hydrogen-bond interactions as that of the sulfonamide N−H in **66b** in the enzyme active site. The relative importance of this hydrogen-bond interaction to overall binding affinity is evident in compound **78** where *N*-methylation results in a 7-fold decrease in caspase-3 inhibitory activity (K_i = 0.14 μM). Together, these results suggest an alternative strategy for introducing pharmacophores at S_4. Through reductive amination chemistry utilizing amines or aldehydes building blocks onto the corresponding aldehyde or amine link-

Table 8. Inhibitor Selectivity among Various Caspases

	K_i (μM)							
compd	casp-1	casp-2	casp-3	casp-4	casp-5	casp-6	casp-7	casp-8
3	0.16	9.0	0.20	21	12	1.0	0.47	0.15
4	3.9	0.96	0.05	92	22	1.4	0.13	0.88
47c	12	30	0.09	96	98	22	0.19	4.0
59	1.5	11	0.03	38	11	0.12	0.09	0.81
62b	25	30	0.02	22	32	0.19	0.09	28
64b	8.3	16	0.03	13	23	2.3	0.09	19
66a	1.9	21	0.12	38	24	0.54	0.12	0.05
66b	3.2	64	0.02	>100	63	9.7	0.13	0.33
69b	2.7	37	0.04	27	36	2.4	0.94	0.71

ers, a large and diverse set of potential binding elements can be evaluated.

Crystal Structure of 66b Bound to Caspase-3. The 1.55 Å resolution X-ray cocrystal structure of inhibitor **66b** bound within the active site of caspase-3 was determined (Figure 5, Table 7). As observed in other inhibitor/caspase-3 structures, the S_1 subsite of the enzyme is occupied by the Asp side chain of the inhibitor, which is held between the Arg64 (2.8 Å), Arg207 (2.8 Å), and Gln161 (2.9 Å) residues. As predicted, the salicylic acid group makes many specific interactions in the S_4 subsite. The 3-carboxyl group interacts with Trp214 (2.9 Å) and Asn208 (3.1 Å), and the 4-hydroxyl interacts with the main chain N−H of Phe250. The sulfonamide N−H and one of the oxygens closely interact with the main chain oxygen of Arg207 (2.9 Å) and the main chain N−H of Ser209 (3.1 Å), respectively. Finally, the main chain N−H of the P_1 aspartyl group forms a hydrogen bond with the carbonyl of Ser205 (3.1 Å). The S_1' subsite is occupied by the 2-chlorobenzyl ring of the thiomethyl ketone warhead, which does not appear to be making any specific interactions with the enzyme. This high resolution structure reveals the remarkable manner in which this caspase inhibitor, discovered through tethering technology, is able to effectively mimic seven of the eight hydrogen-bonding interactions seen in peptide inhibitors, such as AcDVAD-CHO.[39]

Selectivity Studies. The nine most active compounds were screened for selectivity studies against caspase-1−8. In general, these compounds are very poor inhibitors of caspase-1, -2, -4, and -5, indicating that our compounds are highly specific for inhibiting the apoptotic caspases (3 and 6−8) and not the inflammatory caspases (1 and 4). As shown in Table 8, compound **3,** which possesses the simple phenyl linker, has only moderate selectivity between the caspases; however, inhibitors that incorporate a heterocyclic linker show 200−3000-fold selectivity for caspase-3 over caspase-1 (and others relative to compound **3**). In only two cases were compounds relatively active against caspase-6 (compounds **59** and **62b**). Even though caspase-3 and caspase-7 have almost identical cleavage specificities,[9] we surprisingly found that some compounds showed selectivity for caspase-3, e.g., compounds **59** and **62b** show ∼3−5-fold selectivity. These compounds therefore represent some of the most specific nonpeptidic compounds for caspase-3 reported to date, with the only other reported compounds that showed specificity being the isatin series reported by SmithKline Beecham (∼4-fold selectivity for caspase-3 vs caspase-7).[39]

Conclusions

In this paper, we have described the design, synthesis, and in vitro activities of a series of potent and selective nonpeptidic small-molecule inhibitors of caspase-3 that originated from an initial hit identified from our extended tethering technology. By using information obtained from the X-ray crystal structure of the tethering hit in conjunction with molecular modeling studies, inhibitor **4** was identified as a potent and reversible inhibitor of caspase-3. With compound **4** as a starting point for our medicinal chemistry efforts, we were able to identify additional compounds with improved inhibitory potency and selectivity for caspase-3. Evaluation of analogues of the salicylic acid group of **4** established that compounds possessing a carboxylic acid bioisostere (i.e., tetrazole, acylsulfonamide, or a 5-oxo-1,2,4-oxadiazole) exhibited good caspase-3 inhibitory activity. This result is not surprising since it is known that an aspartic acid is the preferred residue for the S_4 pocket of caspase-3. In addition, we examined several substituted methyl ketones as potential aldehyde replacements for the Asp-CHO warhead. Although this was not looked at extensively, the 2-chlorobenzyl thiomethyl ketone warhead appeared to give the most potent inhibitory activity relative to the corresponding aldehyde. The linker portion of the molecules was extensively examined. It was shown that various heterocycles such as pyrimidine, thiazole, thiophene, and isoxazole could be used effectively as replacements for the pyridine ring of **4**. Interestingly, in the thiophene series of inhibitors **66a**,**b**−**68a**,**b**, the substitution pattern around the thiophene was critical for inhibitory activity. Further optimization of this series resulted in the discovery that a tertiary amine is an effective replacement for the sulfonamide group, yielding compound **47c** (K_i = 90 nM). Finally, a 1.55 Å high resolution X-ray cocrystal structure of **66b** bound within the active site of caspase-3 was determined. Examination of this structure confirmed that the key binding interactions of inhibitor **66b** with active site residues are indeed made and are consistent with the observed SAR.

In summary, the application of extended tethering to caspase-3 yielded a novel, weak binding fragment (a salicylic acid), which exhibited appreciable binding interactions with the S_4 pocket of the active site. Exploitation of this weak binding fragment eventually led to the discovery of a series of potent, selective, and reversible nonpeptidic inhibitors of caspase-3.

Experimental Section

General Methods. Unless otherwise noted, all reactions were conducted under a nitrogen atmosphere. All commercially available starting materials and solvents were reagent grade or better and used without further purification. Solutions containing products were dried over anhydrous magnesium sulfate ($MgSO_4$) or sodium sulfate (Na_2SO_4) followed by vacuum filtration. Flash column chromatography was carried out using Merck Kieselgel 60 silica gel (230−400 mesh). Solid-phase reactions were conducted on aminomethylated polystyrene HL (100−200 mesh) from Novabiochem in Alltech 8 or 25 mL Extract-Clean reservoirs fitted with the corresponding frits and caps with agitation supplied by an orbital shaker table and a Supelco Vacuum Manifold used for resin washing. Preparative high-performance liquid chromatography (HPLC) purification was carried out on a Gilson HPLC fitted with a Waters Nova-Pak C-18 (25 mm × 100 mm) column eluting at

25 mL/min with a gradient of 10−100% acetonitrile in water (0.1% TFA) over 10 min and holding at 100% for 3 min. Fractions were pooled and lyophilized to provide final products as the free acid or TFA salt as indicated. ^{1}H NMR spectroscopy was determined on a 400 MHz Bruker spectrometer with chemical shifts reported in units of parts per million (ppm). High-resolution mass spectra (HRMS) were determined on a Applied Biosystems QStar Pulsar-i. Elemental analyses were carried out by Robertson Microlit Laboratories Inc., Madison, NJ.

General Procedure for Chlorosulfonylation of Substituted Anilines 5−9. An appropriately substituted aniline (5.0 mmol) was dissolved in concentrated HCl (5 mL) and water (2 mL) followed by the addition of glacial acetic acid (5 mL) to give a yellow solution, which was cooled to −10 °C. A solution of $NaNO_2$ (6.0 mmol) in water (3 mL) was added dropwise while maintaining the temperature below −5 °C. The resulting mixture was stirred at −5 °C for 15 min and added to a cooled (5 °C) solution of glacial acetic acid (10 mL) containing CuCl (1.25 mmol) that was previously saturated with SO_2 gas for 45 min. After vigorous gas evolution, the resulting green mixture was stirred for 1−2 h while allowing the reaction to warm to room temperature. Excess SO_2 and acetic acid were removed under reduced pressure, and the desired sulfonyl chloride was isolated by either precipitation from water or extraction with ethyl ether and washing with 1 N HCl, water, and brine and drying. Using this method, compounds **5**−**9** were obtained and of sufficient purity for use in the next step without further purification.

3-Methanesulfonylamino-benzenesulfonyl Chloride (5). Compound **5** was prepared from *N*-(3-amino-phenyl)methanesulfonamide as an oil (120 mg, 15%).

4-Methanesulfonylamino-benzenesulfonyl Chloride (6). Compound **6** was prepared from *N*-(4-amino-phenyl)methanesulfonamide as an oil (200 mg, 25%).

3-Methanesulfonylaminocarbonyl-benzenesulfonyl Chloride (7). To a mixture of 3-*tert*-butoxycarbonylaminobenzoic acid (1.19 g, 5.00 mmol), methanesulfonamide (0.62 g, 6.50 mmol), and *N*,*N*-(dimethylamino)pyridine (DMAP) (0.79 g, 6.50 mmol) in CH_2Cl_2 (17 mL) was added EDC (1.25 g, 6.50 mmol). The reaction mixture was stirred at room temperature for 24 h, concentrated in vacuo, and purified by flash column chromatography (40% ethyl acetate in hexanes with 1% acetic acid) to afford the acylsulfonamide as a white solid (1.5 g, 95%). ^{1}H NMR ($CDCl_3$): δ 7.94 (s, 1H), 7.54 (bd, 2H), 7.40 (m, 1H), 6.80 (bs, 1H), 3.42 (s, 3H), 1.53 (s, 9H). This material was treated with 4 N HCl in dioxane for 2 h at room temperature, concentrated in vacuo, and subjected to previously described chlorosulfonylation conditions to provide **7** as a white solid (376 mg, 63%). ^{1}H NMR ($CDCl_3$): δ 8.51 (s, 1H), 8.22 (d, J = 7.4 Hz, 1H), 8.16 (d, J = 7.5 Hz, 1H), 7.69 (t, J = 7.7 Hz, 1H), 3.30 (s, 3H).

3-Acetylsulfamoyl-benzenesulfonyl Chloride (8). Compound **8** was prepared from *N*-acetyl-3-amino-benzenesulfonamide as a yellow powder (458 mg, 51%). ^{1}H NMR (DMSO-d_6): δ 12.12 (s, 1H), 8.09 (s, 1H), 7.85 (m, 2H), 7.58 (t, J = 7.8 Hz, 1H), 1.92 (s, 3H). EI-MS m/z: 320.0 $(M + Na)^+$.

3-(1*H*-Tetrazol-5-yl)benzenesulfonyl Chloride (9). Compound **9** was prepared from 3-(1*H*-tetrazol-5-yl)aniline as a pale yellow solid (832 mg, 68%). ^{1}H NMR (DMSO): δ 10.5 (s, 1H), 8.32 (s, 1H), 7.99 (d, J = 7.7 Hz, 1H), 7.78 (d, J = 7.8 Hz, 1H), 7.55 (t, J = 7.7 Hz, 1H). EI-MS m/z: 245.0 $(M + H)^+$.

3-(5-Oxo-4, 5-dihydro[1,2,4]oxadiazol-3-yl)benzenesulfonyl Chloride (12). To a solution of **10** (6.54 g, 30.0 mmol) in ethanol (60 mL) was added NH_2OH (3.6 mL, 60 mmol of a 50% w/w solution in water). The reaction was refluxed at 80 °C for 3 h and concentrated to dryness to afford the *N*-hydroxy amidine as a yellow solid (7.5 g, 99%) of sufficient purity for use in the next step without further purification. 2-Ethylhexyl chloroformate (2.9 mL, 15 mmol) was added dropwise to a mixture of this material (2.51 g, 10.0 mmol) and pyridine (1.21 mL, 15.0 mmol) in THF (20 mL) at 0 °C. After it was stirred for 30 min, the reaction mixture was diluted with water and extracted with ethyl acetate. The organic layer was washed with water and brine, dried, and concentrated in vacuo to give a residue that was dissolved in xylene (50 mL). The solution was heated under reflux for 3 h, concentrated under reduced pressure, and partitioned between water and ethyl acetate. The organic layer was separated, washed with water and brine, and dried. Concentration in vacuo followed by purification of the residue by flash column chromatography (10% ethyl acetate to 50% ethyl acetate in hexanes) afforded the 5-oxo-1,2,4-oxadiazole derivative as an off-white powder (1.95 g, 70%). ^{1}H NMR (DMSO-d_6): δ 12.90 (s, 1H), 9.63 (s, 1H), 8.05 (s, 1H), 7.54 (d, 1H), 7.43 (t, 1H), 7.35 (d, 1H), 1.47 (s, 9H). This material (831 mg, 3.0 mmol) was dissolved in anhydrous 4 N HCl in dioxane (6 mL) and stirred for 3 h at room temperature. The solution was concentrated to dryness and of sufficient purity for use in the next step without further purification. This intermediate was subjected to previously described chlorosulfonylation conditions to provide compound **12** as a yellow solid (159 mg, 61%). ^{1}H NMR (DMSO-d_6): δ 13.04 (s, 1H), 8.10 (s, 1H), 7.85 (d, J = 7.5 Hz, 1H), 7.75 (d, J = 7.5 Hz, 1H), 7.54 (t, J = 7.7 Hz, 1H).

6-Bromomethyl-nicotinic Acid Methyl Ester (14). To a solution of **13** (5.0 g, 33 mmol) in benzene (110 mL) were added *N*-bromosuccinimide (5.3 g, 30 mmol), benzoyl peroxide (0.801 g, 3.31 mmol), and acetic acid (2 mL). After it was heated at reflux for 9 h, the solution was diluted with ethyl acetate and washed with 1 N HCl. The organic layer was separated, dried, and concentrated in vacuo to give a dark residue. Purification by flash column chromatography (10% ethyl acetate in hexanes) afforded **14** (2.88 g, 38%). ^{1}H NMR ($CDCl_3$): δ 9.17 (s, 1H), 8.31 (dd, J = 8.1, 2.1 Hz, 1H), 7.53 (d, J = 8.1 Hz, 1H), 4.60 (s, 2H), 3.96 (s, 3H). EI-MS m/z: 232.1 $(M + H)^+$.

6-Aminomethyl-nicotinic Acid Methyl Ester (15). A solution of **14** (2.86 g, 12.8 mmol) and sodium azide (1.09 g, 16.7 mmol) in DMF (64 mL) was heated at 70 °C for 30 min. The reaction mixture was diluted with ethyl acetate and washed with saturated $NaHCO_3$. The organic layer was separated, dried, and concentrated in vacuo to afford the azide in 69% yield. A solution of this material (1.66 g, 8.60 mmol) and 10% palladium on carbon (200 mg) in absolute ethanol (20 mL) was stirred under a 20 psi hydrogen atmosphere for 20 min. The reaction mixture was filtered through a pad of Celite and washed with CH_2Cl_2. The filtrate was concentrated in vacuo to afford **15** as an orange oil of sufficient purity for use in the next step without further purification. EI-MS m/z: 167.1 $(M + H)^+$.

6-[(9*H*-Fluoren-9-ylmethoxycarbonylamino)methyl]-nicotinic Acid Hydrochloride (16). A solution of **15** (770 mg, 4.63 mmol) in dioxane (15 mL) and 1 M LiOH (12 mL) was stirred at room temperature for 5 h, acidified with 1 N HCl, and concentrated to dryness to afford the amino acid as a yellow solid. The crude solid was dissolved in dioxane (20 mL) and water (20 mL) followed by the addition of $NaHCO_3$ (7.80 g, 46.3 mmol) and Fmoc-OSu (1.72 g, 5.10 mmol). After it was stirred at room temperature for 22 h, the reaction was concentrated to dryness, diluted with water, and acidified to pH 1 with 2 N HCl to give the desired product as an off-white solid, which was collected on a sintered glass funnel, washed with water, and dried to provide **16** as an off-white powder (1.67 g, 88%). ^{1}H NMR (CD_3OD): δ 7.79 (d, J = 7.4 Hz, 2H), 7.65 (d, J = 7.7 Hz, 2H), 7.34−7.57 (m, 3H), 7.30 (t, J = 7.4 Hz, 2H), 7.15 (d, J = 7.7 Hz, 1H), 4.40 (d, J = 6.6 Hz, 2H), 4.31 (s, 2H), 4.20−4.22 (m, 1H). EI-MS m/z: 375.0 $(M + H)^+$.

5-Formyl-thiophene-2-carboxylic Acid *tert*-Butyl Ester (18). To a solution of **17** (18.0 g, 94.3 mmol) and 2,3-dimethylbutene (22 mL, 189 mmol) in *tert*-butyl alcohol (470 mL) was added a solution of $NaClO_2$ (10.6 g, 118 mmol) and NaH_2PO_4 (14.1 g, 118 mmol) in water (94 mL). After it was stirred at room temperature for 12 h, the organic solvent was removed under reduced pressure and the remaining slurry was basified with 1 N NaOH (200 mL). The aqueous layer was washed with several portions of hexanes followed by acidification with 1 N HCl and extraction of the desired compound into ether. The organic layer was dried and concentrated in vacuo

to afford the desired product of sufficient purity for use in the next step without further purification. ES-MS *m/z*: 208.9 $(M + H)^+$. To a solution of this material in CH_2Cl_2 (316 mL) was added *tert*-butyl alcohol (116 mL, 1.30 mol), EDC (24.3 g, 12.7 mmol), and DMAP (7.8 g, 63.3 mmol). After it was stirred for 3 h at room temperature, the solution was diluted with CH_2Cl_2 and washed with 1 N HCl. The organic layer was dried, concentrated in vacuo, and purified by flash column chromatography (30% ethyl acetate in hexanes) to afford compound **18** (12.5 g, 50% for two steps). EI-MS *m/z*: 206.9 $(M - t\text{-Bu})^+$.

5-Formyl-thiophene-2-carboxylic Acid *tert*-Butyl Ester (19). To a solution of **18** (7.5 g, 28.5 mmol) in ether (60 mL) at −78 °C was added *n*-butyllithium (32.1 mL, 25.7 mmol, 0.8 M in hexanes) dropwise. After it was stirred for 1 h at −78 °C, a solution of 1-formylpiperidine (4.7 mL, 42.8 mmol) in ether (10 mL) was added dropwise. After it was stirred for an additional 30 min at −78 °C, the reaction was quenched with 1 N HCl and allowed to warm to room temperature. The solution was diluted with ether, washed with 1 N HCl, dried, concentrated in vacuo, and purified by flash column chromatography (30% ethyl acetate in hexanes) to afford compound **19** (3.0 g, 50%). EI-MS *m/z*: 157.1 $(M + H)^+$.

5-Aminomethyl-thiophene-2-carboxylic Acid *tert*-Butyl Ester (20). To a solution of **19** (3.0 g, 14.1 mmol) in MeOH (15 mL) was added aqueous NH_2OH (1.3 mL, 2.1 mmol, 16.3 M in water). After it was stirred for 1 h at room temperature, the solvent was removed under reduced pressure to provide the crude oxime of sufficient purity for use in the next step without further purification. ES-MS *m/z*: 172.1 $(M + H)^+$. To a solution of this material in acetic acid (28 mL) at 0 °C was added zinc dust (3.6 g, 56.4 mmol). The heterogeneous mixture was warmed to room temperature and stirred for an additional 3 h. The reaction was filtered through a pad of Celite and concentrated in vacuo to provide the crude amine of sufficient purity for use in the next step without further purification. EI-MS *m/z*: 141.1 $(M - t\text{-Bu} - NH_2)^+$.

5-[(9*H*-Fluoren-9-ylmethoxycarbonylamino)methyl]-thiophene-2-carboxylic Acid (21). To a solution of amine **20** (14.1 mmol) in dioxane (30 mL) and water (30 mL) was added $NaHCO_3$ (5.9 g, 70.5 mmol) and Fmoc-OSu (5.2 g, 15.5 mmol). After it was stirred at room temperature for 3 h, the heterogeneous mixture was diluted with ether and washed with 1 N HCl. The organic layer was dried, concentrated in vacuo, and purified by flash column chromatography (30% ethyl acetate in hexanes) to provide the Fmoc-protected *tert*-butyl ester (4.62 g, 75%). EI-MS *m/z*: 458.0 $(M + Na)^+$. A solution of this material in TFA/CH_2Cl_2/water (1:4:0.1, 30 mL) was stirred at room temperature for 2 h and evaporated to dryness to afford **21**, which was of sufficient purity for use in the next step without further purification. ^{1}H NMR (CD_3OD): δ 7.78 (d, J = 7.4 Hz, 2H), 7.60−7.65 (m, 3H), 7.38 (t, J = 7.3 Hz, 2H), 7.29 (t, J = 7.3 Hz, 2H), 6.95 (s, 1H), 4.45 (s, 2H), 4.40 (d, J = 6.8 Hz, 2H), 4.21 (t, J = 6.7 Hz, 1H). EI-MS *m/z*: 402.0 $(M + Na)^+$.

5-[(9*H*-Fluoren-9-ylmethoxycarbonylamino)methyl]-thiophene-2-carboxylic Acid (22).[40] To a solution of **21** (0.500 g, 1.3 mmol) in TFA/$CHCl_3$ (1:1, 15 mL) was added 37% formaldehyde (1.2 mL, 14 mmol) in water. After it was stirred at room temperature for 30 min, triethylsilane (2.9 mL, 18 mmol) was added and the solution was stirred for an additional 30 min. The solvent was concentrated in vacuo, and the crude product was purified by flash column chromatography (100% ethyl acetate) to afford **22** 0.510 g, 100%). EI-MS *m/z*: 416.2 $(M + Na)^+$.

(4-Bromo-thiophen-2-ylmethoxy)-*tert*-butyl-dimethyl-silane (24). To a solution of **23** (7.1 g, 37.2 mmol) in THF (80 mL) was added $NaBH_4$ (1.5 g, 40.9 mmol) in portions. After it was stirred for 30 min, saturated $NaHCO_3$ was slowly added to quench the reaction. The reaction mixture was diluted with ether, washed with saturated $NaHCO_3$, dried, and concentrated in vacuo to provide the alcohol, which was of sufficient purity for use in the next step without further purification. To a solution of this material (37.1 mmol) in CH_2Cl_2 (70 mL) was added TBSCl (6.1 g, 40.8 mmol) and imidazole (2.8 g, 40.8 mmol). After it was stirred for 30 min at room temperature, the solution was diluted with CH_2Cl_2, washed with 1 N HCl, dried, and concentrated in vacuo. Purification by flash column chromatography (100% hexanes to 10% ethyl acetate in hexanes) afforded **24** (10.6 g, 92% for two steps).

5-(*tert*-Butyl-dimethyl-silanyloxymethyl)thiophene-3-carboxylic Acid Ethyl Ester (25). To a solution of **24** (10.6 g, 34.3 mmol) in THF (60 mL) at −78 °C was added *n*-butyllithium (23.6 mL, 37.8 mmol, 1.6 M in hexanes) dropwise. After it was stirred at −78 °C for 1 h, the reaction solution was added dropwise to a solution of ethyl chloroformate (4.3 mL, 44.7 mmol) in THF (80 mL) at −78 °C via cannula. After it was stirred for 1 h, the reaction was quenched with 1 N HCl and warmed to room temperature. The organic layer was separated, washed with 1 N HCl, and dried. Concentration in vacuo followed by purification by flash column chromatography (10% ethyl acetate in hexanes) afforded **25** (10.1 g, 97%).

5-Bromomethyl-thiophene-3-carboxylic Acid Ethyl Ester (26). To a solution of **25** (10.1 g, 33.6 mmol) in THF (70 mL) was added acetic acid (12 mL) and tetrabutylammonium fluoride (TBAF) (36.9 mL, 36.9 mmol of a 1.0 M solution in THF). After it was stirred for 12 h, the solution was diluted with ether, washed with saturated $NaHCO_3$, and dried. Concentration in vacuo followed by flash column chromatography (30% ethyl acetate in hexanes) afforded the desired alcohol. EI-MS *m/z*: 187.0 $(M + H)^+$. To a solution of the alcohol (3.0 g, 16.1 mmol) in THF (80 mL) was added CBr_4 (5.9 g, 17.7 mmol) and triphenylphosphine (4.6 g, 17.7 mmol). After it was stirred for 1 h, the suspension was filtered, concentrated in vacuo, and purified by flash column chromatography (20% ethyl acetate in hexanes) to provide bromide **26** (4.0 g, 100%). EI-MS *m/z*: 250.9 $(M + H)^+$.

5-Aminomethyl-thiophene-3-carboxylic Acid Ethyl Ester (27). A solution of **26** (4.0 g, 16.1 mmol) and sodium azide (1.2 g, 17.7 mmol) in DMF (30 mL) was stirred at 50 °C for 30 min. The reaction mixture was diluted with ether, washed with water, dried, and concentrated in vacuo to provide 1.9 g (56%) of the azide, which was of sufficient purity for use in the next step without further purification. EI-MS *m/z*: 212.9 $(M + H)^+$. A solution of this material (1.9 g, 9.0 mmol) and 10% palladium on carbon (100 mg) in absolute ethanol (30 mL) was hydrogenated at 30 psi for 2 h. The suspension was filtered through a pad of Celite and concentrated in vacuo to provide amine **27 (**900 mg, 52%), which was of sufficient purity for use in the next step without further purification. EI-MS *m/z*: 169.1 $(M - NH_2)^+$.

5-[(9*H*-Fluoren-9-ylmethoxycarbonylamino)methyl]-thiophene-3-carboxylic Acid (28). To a solution of **27** (0.9 g, 4.9 mmol) in dioxane (10 mL) was added 1 M LiOH (10 mL). The reaction was stirred for 1 h at room temperature, neutralized with 1 N HCl, and concentrated in vacuo to provide the amino acid of sufficient purity for use in the next step without further purification. To a solution of this material in dioxane (5 mL) and water (5 mL) was added $NaHCO_3$ (2.1 g, 24.5 mmol) and Fmoc-OSu (1.8 g, 5.4 mmol). After it was stirred for 3 h at room temperature, the solution was diluted with ether, washed with 1 N HCl, dried, and concentrated in vacuo. Purification of the residue by flash column chromatography (5% methanol in ethyl acetate) afforded **28**. ^{1}H NMR ($CDCl_3$): δ 8.04 (s, 1H), 7.78 (d, J = 7.4 Hz, 3H), 7.63 (d, J = 7.4 Hz, 2H), 7.28−7.39 (m, 4H), 4.43 (d, J = 4.8 Hz, 2H), 4.36 (J = 6.9 Hz, 2H), 4.20 (t, J = 6.8 Hz, 1H). EI-MS *m/z*: 402.3 $(M)^+$.

5-(2-Chloro-benzylsulfanyl)-3-(9*H*-fluoren-9-ylmethoxycarbonylamino)-4-oxo-pentanoic Acid *tert*-Butyl Ester (30). To a solution of Fmoc-Asp(O*t*-Bu)-CH_2Br[20] (20.3 g, 48.4 mmol) in THF (150 mL) was added 2-chlorobenzenemethanethiol (6.9 mL, 53.2 mmol) and diisopropylethylamine (9.3 mL, 53.2 mmol). After 1 h, the solution was diluted with ethyl acetate and washed with saturated $NaHCO_3$. The organic layer was dried and concentrated in vacuo to provide **29** (24.1 g, 100%), which was used without further purification. ^{1}H

NMR ($CDCl_3$): δ 7.75 (d, J = 7.4 Hz, 1H), 7.57 (d, J = 7.3 Hz, 1H), 7.28−7.41 (m, 8H), 3.78−3.83 (m, 5H). ES-MS *m*/*z*: 498.2 $(M + H)^+$.

3-(9*H*-Fluoren-9-ylmethoxycarbonylamino)-4-oxo-5-(pyridin-3-ylmethylsulfanyl)pentanoic Acid *tert*-Butyl Ester (33a). 3-Picolyl mercaptan (**32a**) was prepared from 3-picolyl chloride hydrochloride (**31a**) by literature methods.[30] The corresponding thiomethyl ketone was prepared using the same method as **30** except for using 3-picolyl mercaptan as a reagent instead of 2-chlorobenzenemethanethiol. ES-MS *m*/*z*: 533 $(M + H)^+$.

3-(9*H*-Fluoren-9-ylmethoxycarbonylamino)-4-oxo-5-(pyridin-4-ylmethylsulfanyl)pentanoic Acid *tert*-Butyl Ester (33b). 4-Picolyl mercaptan (**32b**) was prepared from 4-picolyl chloride hydrochloride (**31b**) by literature methods.[30] The corresponding thiomethyl ketone was prepared using the same method as **30** except for using 4-picolyl mercaptan as a reagent instead of 2-chlorobenzenemethanethiol. ES-MS *m*/*z*: 533 $(M + H)^+$.

3-(9*H*-Fluoren-9-ylmethoxycarbonylamino)-4-oxo-7-phenyl-heptanoic Acid *tert*-Butyl Ester (35). To a solution of **34** (1.8 mL, 14.5 mmol) in anhydrous THF (10 mL) at −78 °C was added *n*-butyllithium (9.08 mL, 14.5 mmol, 1.6 M in hexanes) dropwise over a 10 min period. After it was stirred for 15 min, a solution of *N*-Fmoc-Asp(O*t*-Bu)-CHO (2.61 g, 6.6 mmol) in anhydrous THF (20 mL) was added dropwise over a 15 min period. The solution was stirred at −78 °C for an additional 40 min, quenched with water, and warmed to room temperature. The reaction mixture was diluted with ether, washed with 1 N HCl, dried, and concentrated in vacuo. Purification by flash column chromatography (30% ethyl acetate to 40% ethyl acetate in hexanes) provided the alcohol (890 mg, 27%). A solution of this material (890 mg, 3.91 mmol) and 10% palladium on carbon (125 mg) in absolute ethanol (15 mL) was hydrogenated at atmospheric pressure for 1 h. The suspension was filtered through a pad of Celite and washed with ethanol. The combined filtrates were concentrated in vacuo to provide the alcohol in quantitative yield. To a solution of this material (700 mg, 1.36 mmol) in CH_2Cl_2 (7 mL) was added Dess−Martin periodinane (865 mg, 2.04 mmol) in one portion at room temperature. After it was stirred for 30 min, the solid was filtered and washed with CH_2Cl_2. The combined filtrates were concentrated in vacuo and purified by flash column chromatography (25% ethyl acetate in hexanes) to afford **35** (325 mg, 47%). ^{1}H NMR ($CDCl_3$): δ 7.77 (d, J = 7.6 Hz, 2H), 7.59 (d, J = 7.1 Hz, 2H), 7.41 (t, J = 7.5 Hz, 2H), 7.31 (t, J = 7.1 Hz, 2H), 7.26 (m, 2H), 7.17 (t, J = 7.2 Hz, 3H), 5.87 (d, J = 7.8 Hz, 1H), 4.43 (m, 2H), 4.22 (t, J = 7.0 Hz, 1H), 2.88 (m, 1H), 2.70 (dd, J = 17.1, 4.7 Hz, 1H), 2.58 (m, 4H), 1.93 (m, 2H), 1.42 (s, 9H).

5-Bromomethyl-thiophene-2-carboxylic Acid Methyl Ester (37). To a solution of 5-methylthiophene-2-carboxylic acid **36** (20.0 g, 141 mmol) in benzene (120 mL) and methanol (14 mL) at 0 °C was dropwise added 2.0 M trimethylsilyldiazomethane in hexanes (80 mL, 160 mmol). The reaction was warmed to room temperature, and the solvent was concentrated in vacuo. Distillation under reduced pressure ($BP_{0.5}$ = 88−93 °C) provided the methyl ester as a colorless oil (17.95 g, 82%). ^{1}H NMR ($CDCl_3$): δ 7.61 (d, J = 3.7 Hz, 1H), 6.76 (d, J = 3.6 Hz, 1H), 3.85 (s, 3H), 2.52 (s, 3H). To a solution of the methyl ester (11.4 g, 72.9 mmol) in CCl_4 (200 mL) was added NBS (14.3 g, 80.3 mmol) and 2,2′-azobisisobutyronitrile (AIBN) (91 mg, 0.55 mmol). The solution was heated to reflux for 2 h, at which point ^{1}H NMR of a filtered aliquot indicated a 1:1 ratio of starting material to product. Another portion of AIBN (81 mg, 0.49 mmol) was added, and the solution was refluxed for another 2 h. The reaction was then cooled to room temperature, filtered, and concentrated in vacuo to provide **37** as an orange oil (18.8 g, 110%) that contained ≅10% of the 5-dibromomethyl analogue. ^{1}H NMR ($CDCl_3$): δ 7.63 (d, J = 3.8 Hz, 1H), 7.09 (d, J = 3.8 Hz, 1H), 4.67 (s, 2H), 3.88 (s, 3H).

5-[(3-*tert*-Butoxycarbonyl-4-hydroxy-benzenesulfonylamino)methyl]thiophene-2-carboxylic Acid Methyl Ester (39). To a solution of **37** (18.76 g) in acetonitrile (41 mL) was added NaN_3 (9.39 g, 144 mmol) followed by stirring at room temperature for 3 days. The reaction was diluted with CH_2Cl_2, washed with water, dried, and concentrated in vacuo to provide the azide (14.1 g, 98%), which was used without further purification. ES-MS *m*/*z*: 198 $(M + H)^+$. To a solution of the azide (7.48 g, 38.0 mmol) in acetic acid (75 mL) was added zinc dust (15.1 g, 232 mmol) portionwise. After it was stirred for 2 h, the reaction was filtered through a pad of Celite and washed with acetic acid. The combined filtrates were concentrated in vacuo to provide the crude amine (14.4 g). To a solution of the amine (14.4 g) in CH_2Cl_2 (70 mL) was added NMM (12.5 mL, 124 mmol) followed by 5-chlorosulfonyl-2-hydroxybenzoic acid (8.66 g, 36.7 mmol) and stirred for 2 h. The solution was diluted with CH_2Cl_2, washed with 1 N HCl, dried, and concentrated in vacuo to give the crude **38**, which was used directly in the next step. A solution of this material was suspended in toluene (60 mL) and heated to 90 °C. DMF di-*tert*-butyl acetal[28] (35 mL, 146.0 mmol) was dropwise added to the hot solution. After it was stirred at 90 °C for 1.5 h, the solution was diluted with CH_2Cl_2, washed with 1 N HCl, dried, and concentrated in vacuo. Flash chromatography over silica gel (25% ethyl acetate in hexanes) provided **39** (4.19 g, 26% after three steps). ^{1}H NMR indicated that the product comprised a 1:1 mixture of the *tert*-butyl ester and the *O*-*tert*-butyl phenol. ES-MS *m*/*z*: 450 $(M + Na)^+$.

5-[(3-*tert*-Butoxycarbonyl-4-hydroxy-benzenesulfonylamino)methyl]thiophene-2-carboxylic Acid (40). To a solution of **39** (4.19 g, 9.84 mmol) in dioxane (40 mL) was added 1 M LiOH (40 mL). After it was stirred at room temperature for 45 min, the solution was diluted with ether, washed with 1 N HCl, dried, and concentrated in vacuo to give **40** (4.73 g, 117%) that still contained some residual dioxane. ^{1}H NMR ($CDCl_3$): δ 11.63 (s, 0.5H), 8.26 (d, J = 2.4 Hz, 0.5H), 8.04 (d, J = 2.4 Hz, 0.5H), 7.88 (dd, J = 8.7, 2.4 Hz, 0.5H), 7.80 (dd, J = 8.8, 2.5 Hz, 0.5H), 7.66 (d, J = 3.8 Hz, 0.5H), 7.64 (d, J = 3.8 Hz, 0.5H), 7.17 (d, J = 8.8 Hz, 0.5H), 7.06 (d, J = 8.8 Hz, 0.5H), 6.92 (d, J = 4.7 Hz, 0.5H), 6.92 (d, J = 4.2 Hz, 0.5H), 4.95 (t, J = 6.4 Hz, 0.5H), 4.92 (t, J = 6.3 Hz, 0.5H), 4.39 (d, J = 6.3 Hz, 0.5H), 3.70 (s, 3H), 1.64 (s, 4.5H), 1.49 (s, 4.5H).

5-[(5-{1-Carboxymethyl-2-[5-(2,6-dichloro-phenyl)-oxazol-2-yl]-2-oxo-ethylcarbamoyl}thiophen-2-ylmethyl)-sulfamoyl]-2-hydroxy-benzoic Acid (41b). The phenyloxazole amino alcohol **41a** was prepared according to literature procedure.[34] To a solution of **41a** (0.138 mmol) and **40** (54.9 mg, 0.142 mmol) in DMF (1.4 mL) was added 1-hydroxybenzotriazole (HOBt) (20.3 mg, 0.150 mmol) and EDC (26.9 mg, 0.140 mmol). After it was stirred at room temperature for 1 h, the solution was diluted with ether and washed with water. The organic layer was separated, dried, concentrated in vacuo, and purified by flash column chromatography (50% ethyl acetate in hexanes) to afford the coupled product (80 mg, 74%), which was dissolved in CH_2Cl_2 (1.5 mL) and treated with Dess−Martin periodinane (49.4 mg, 0.116 mmol). The reaction was stirred for 2 h, washed with 1 N HCl, dried, and concentrated in vacuo to provide the ketone (106 mg), which was stirred at room temperature in TFA/CH_2Cl_2 (1:1, 2 mL) for 1.5 h. Volatiles were removed under reduced pressure, and the residue purified by preparative HPLC to provide **41b** (30 mg, 30% for two steps). EI-MS *m*/*z*: 668.0 $(M + H)^+$. HRMS (TOF): exact mass calcd for $C_{26}H_{20}N_3O_{10}S_2Cl_2$ $(M + H)^+$, 667.9961; found, 667.9995.

5-({5-[1-Carboxymethyl-2-(7-methyl-benzoxazol-2-yl)-2-oxo-ethylcarbamoyl]thiophen-2-ylmethyl}sulfamoyl)-2-hydroxy-benzoic Acid (42b). The benzoxazole amino alcohol **42a** was prepared according to literature procedure.[34] Compound **42b** was prepared from **42a** and **40** in the same manner as described for **41b**. ^{1}H NMR (acetone-d_6): δ 8.35 (d, J = 1.8 Hz, 1H), 8.06 (d, J = 7.9 Hz, 1H), 7.96 (d, J = 10.8 Hz, 1H), 7.88 (d, J = 8.3 Hz, 1H), 7.20−7.60 (m, 3H), 7.20 (d, J = 8.1 Hz, 1H), 7.11 (d, J = 9.0 Hz, 1H), 6.94 (d, J = 4.5 Hz, 1H), 5.86 (m, 1H), 4.38 (m, 3H), 4.05 (s, 3H), 3.30 (dd, J = 17.6, 6.0 Hz, 1H), 3.14 (dd, J = 17.6, 7.4 Hz, 1H). ES-MS *m*/*z*: 604 (M

+ H)$^+$. HRMS (TOF): exact mass calcd for $C_{25}H_{22}N_3O_{11}S_2$ (M + H)$^+$, 604.0690; found, 604.0719.

***N*-{1-[5-(2,6-Dichloro-phenyl)oxazole-2-carbonyl]-propyl}-6-[(4-hydroxy-3-methyl-benzenesulfonylamino)-methyl]nicotinamide (60).** The advanced nicotinate intermediate was prepared in the same manner as **40** except using 6-methylnicotinic acid as a reagent instead of **36**. Compound **60** was prepared using this advanced nicotinate intermediate and **41a**[34] (21 mg, 14%) in the same manner as described for **41b**. ^{1}H NMR (acetone-d_6): δ 8.94 (s, 1H), 8.48 (d, J = 7.4 Hz, 1H), 8.36 (d, J = 2.4 Hz, 1H), 8.20 (d, J = 8.0 Hz, 1H), 8.00 (dd, J = 9.0, 2.5 Hz, 1H), 7.55−7.80 (m, 5H), 7.52 (d, J = 7.5 Hz, 1H), 7.15 (m, 1H), 7.11 (d, J = 8.6 Hz, 1H), 5.84 (m, 1H), 4.33 (m, 3H), 3.28 (dd, J = 16.7, 5.5 Hz, 1H), 3.13 (dd, J = 16.5, 7.3 Hz, 1H). ES-MS m/z: 663 (M + H)$^+$. HRMS (TOF): exact mass calcd for $C_{27}H_{21}N_4O_{10}SCl_2$ (M + H)$^+$, 663.0349; found, 663.0381.

6-[(4-Hydroxy-3-methyl-benzenesulfonylamino)methyl]-*N*-[1-(7-methoxy-benzooxazole-2-carbonyl)propyl]nicotinamide (61). The advanced nicotinate intermediate was prepared in the same manner as **40** except using 6-methylnicotinic acid as a reagent instead of **36**. Compound **61** was prepared using this advanced nicotinate intermediate and **42a**[32] in the same manner as described for **42b**. ES-MS m/z: 599 (M + H)$^+$. HRMS (TOF): exact mass calcd for $C_{26}H_{23}N_4O_{11}S$ (M + H)$^+$, 599.1089; found, 599.1078.

Preparation of Resin 44a−e. Resin **43**[20] (63 g, 0.51 mmol/g) was suspended in THF (150 mL) followed by addition of acetic acid (1.0 mL, 17.5 mmol) and the appropriate ketone (48.4 mmol). After it was stirred for 12 h, the resin was filtered, washed successively with CH_2Cl_2 (3 × 200 mL) and ether (3 × 200 mL), and dried in vacuo to provide resin **44a−d**. Resin **44e** was prepared by literature procedures.[19]

General Method for Solid-Phase Synthesis of Aldehyde and Ketone Analogues. Resin **44a−e** (300 mg, 0.06 mmol) was suspended in 20% piperidine in DMF (3 mL) followed by gentle agitation for 20 min. After it was filtered, the resin was washed successively with DMF (3 × 5 mL) and CH_2Cl_2 (3 × 5 mL). The resin was then resuspended in DMF (3 mL) followed by treatment with DIPEA (63 μL, 0.36 mmol), the desired *N*-Fmoc-protected heterocyclic acid (0.12 mmol), and PyBOP (93 mg, 0.18 mmol). After it was agitated at room temperature for 12 h, the solution was drained and the resin was washed successively with DMF (3 × 5 mL) and CH_2Cl_2 (3 × 5 mL). This resin was then treated with 20% piperidine in DMF (3 mL) and agitated for 20 min. The resin was drained and washed successively with DMF (3 × 5 mL) and CH_2Cl_2 (3 × 5 mL). To the resin were then added CH_2Cl_2 (3 mL), the desired benzene sulfonyl chloride (0.12 mmol), and DIPEA (0.063 mL, 0.36 mmol). The suspension was agitated at room temperature for 12 h followed by filtration and washing with DMF (3 × 5 mL) and CH_2Cl_2 (3 × 5 mL). The resin was then cleaved under the appropriate cleavage conditions to provide the fully functionalized analogue **45**.

General Methods for Cleavage of Aldehyde Analogues from Resin. To derivatized aldehyde resin (300 mg, 0.06 mmol) was added THF/CH_3CHO/HOAc/TFA (5:1:1:0.250, 3 mL). After it was agitated at room temperature for 3 h, the resin was filtered and washed with CH_2Cl_2 (2 × 2 mL). The filtrate and washings were combined, and the solvent was removed under reduced pressure to provide a residue, which was treated with CH_2Cl_2/H_2O/TFA (1:1:0.1, 2 mL) at room temperature for 30 min. The solvent was removed under reduced pressure, and the product was purified by preparative reverse-phase HPLC.

General Methods for Cleavage of Ketone Analogues from Resin. To derivatized ketone resin (300 mg, 0.06 mmol) was added TFA/H_2O (9:1, 3 mL). After it was agitated at room temperature for 15 min, the resin was filtered and washed with CH_2Cl_2 (2 × 2 mL). The filtrate and washings were combined, and the solvent was removed under reduced pressure to provide a residue, which was purified by preparative reverse-phase HPLC.

(*S*)-5-{[5-(1-Carboxymethyl-2-oxo-ethylcarbamoyl)-pyridin-2-ylmethyl]sulfamoyl}-2-hydroxy-benzoic Acid Trifluoroacetate (4). Compound **4** was prepared from resin **44e** (5.4 mg, 6%). ^{1}H NMR (CD_3OD): δ 8.94 (s, 1H), 8.45 (d, J = 8.3 Hz, 1H), 8.32 (s, 1H), 7.92 (d, J = 8.7 Hz, 1H), 7.78 (d, J = 2.4 Hz, 1H), 7.10 (m, 1H), 4.70−4.73 (m, 1H), 4.69 (m, 1H), 4.34 (s, 2H), 2.75−2.82 (m, 1H), 2.60−2.68 (m, 1H). EI-MS m/z: 452.1 (M + H)$^+$. HRMS (TOF): exact mass calcd for $C_{18}H_{18}N_3O_9S$ (M + H)$^+$, 452.0758; found, 452.0778.

(*S*)-3-{[6-(Benzenesulfonylamino-methyl)pyridine-3-carbonyl]amino}-4-oxo-butyric Acid Trifluoroacetate (48). Compound **48** was prepared from resin **44e** (8.4 mg, 28%). ^{1}H NMR (CD_3OD): δ 8.90 (s, 1H), 8.30 (dt, J = 8.2, 2.1 Hz, 1H), 7.80−7.90 (m, 2H), 7.59−7.67 (m, 2H), 7.5−7.6 (m, 2H), 4.70 (d, J = 11.1, 4.2 Hz, 1H), 4.47−4.56 (m, 1H), 4.31 (s, 2H), 2.74−2.84 (m, 1H), 2.60−2.70 (m, 1H).

(*S*)-3-({6-[(4-Hydroxy-benzenesulfonylamino)methyl]-pyridine-3-carbonyl}amino)-4-oxo-butyric Acid Trifluoroacetate (49). Compound **49** was prepared from resin **44e** (9.3 mg, 30%). ^{1}H NMR (CD_3OD): δ 8.90 (s, 1H), 8.30 (dt, J = 8.3, 2.0 Hz, 1H), 7.67 (m, 2H), 7.60 (d, J = 8.2 Hz, 1H), (m, 2H), 4.70 (dd, J = 11.1, 4.1 Hz, 1H), 4.46−4.56 (m, 1H), 4.23 (s, 2H), 2.60−2.70 (m, 1H), 2.74−2.84 (m, 2H).

(*S*)-3-{[5-(1-Carboxymethyl-2-oxo-ethylcarbamoyl)-pyridin-2-ylmethyl]sulfamoyl}benzoic Acid Trifluoroacetate (50). Compound **50** was prepared from resin **44e** (3.6 mg, 11%). ^{1}H NMR (CD_3OD): δ 8.84 (s, 1H), 8.44 (s, 1H), 8.15−8.24 (m, 2H), 8.05 (d, J = 7.8 Hz, 1H), 7.65 (t, J = 7.9 Hz, 1H), 7.58 (d, J = 8.3 Hz, 1H), 4.70 (dd, J = 11.3, 4.1 Hz, 1H), 4.47−4.56 (m, 1H), 4.34 (s, 2H), 2.74−2.84 (m, 1H), 2.60−2.70 (m, 1H).

(*S*)-3-({6-[(3-Methanesulfonyl-benzenesulfonylamino)-methyl]pyridine-3-carbonyl}amino)-4-oxo-butyric Acid Trifluoroacetate (51). Compound **51** was prepared from resin **44e** (6.7 mg, 19%). ^{1}H NMR (CD_3OD): δ 8.82 (s, 1H), 8.30 (s, 1H), 8.06−8.20 (m, 3H), 7.80 (t, J = 7.9 Hz, 1H), 7.50 (d, J = 8.1 Hz, 1H), 4.70 (dd, J = 10.6, 4.1 Hz, 1H), 4.46−4.56 (m, 1H), 4.35 (s, 2H), 3.15 (s, 3H), 2.73−2.84 (m, 1H), 2.60−2.70 (m, 1H). EI-MS m/z: 470.0 (M + H)$^+$.

(*S*)-3-({6-[(4-Methanesulfonylamino-benzenesulfonylamino)methyl]pyridine-3-carbonyl}amino)-4-oxo-butyric Acid Trifluoroacetate (52). Compound **52** was prepared from resin **44e** and **5** (10.7 mg, 30%). ^{1}H NMR (CD_3OD): δ 8.86 (s, 1H), 8.20 (dt, J = 8.3, 1.7 Hz, 1H), 7.90 (d, J = 8.6 Hz, 2H), 7.60 (d, J = 8.2 Hz, 1H), 7.30 (d, J = 8.4 Hz, 2H), 4.70 (dd, J = 11.4, 4.0 Hz, 1H), 4.46−4.56 (m, 1H), 4.30 (s, 2H), 3.00 (s, 3H), 2.74−2.83 (m, 1H), 2.59−2.70 (m, 1H).

(*S*)-3-({6-[(3-Methanesulfonylamino-benzenesulfonylamino)methyl]pyridine-3-carbonyl}amino)-4-oxo-butyric Acid Trifluoroacetate (53). Compound **53** was prepared from resin **44e** and **6** (5.8 mg, 16%). ^{1}H NMR (CD_3OD): δ 8.89 (s, 1H), 8.30 (dt, J = 8.3, 1.8 Hz, 1H), 7.70 (s, 1H), 7.65 (d, J = 8.4 Hz, 1H), 7.57 (m, 1H), 7.50 (t, J = 7.8 Hz, 1H), 7.41 (m, 1H), 4.70 (dd, J = 11.4, 4.1 Hz, 1H), 4.47−4.57 (m, 1H), 4.35 (s, 2H), 2.96 (s, 3H), 2.73−2.83 (m, 1H), 2.59−2.69 (m, 1H).

(*S*)-3-({6-[(3-Methanesulfonylaminocarbonyl-benzenesulfonylamino)methyl]-pyridine-3-carbonyl}amino)-4-oxo-butyric Acid Trifluoroacetate (54). Compound **54** was prepared from resin **44e** and **7** (14.0 mg, 37%). ^{1}H NMR (CD_3OD): δ 8.90 (s, 1H), 8.30 (m, 2H), 8.00−8.13 (m, 2H), 7.59−7.72 (m, 2H), 4.70 (dd, J = 11.4, 4.1 Hz, 1H), 4.47−4.56 (m, 1H), 4.40 (s, 2H), 3.40 (s, 3H), 2.74−2.83 (m, 1H), 2.60−2.70 (m, 1H). EI-MS m/z: 513.0 (M + H)$^+$.

(*S*)-3-({6-[(3-Acetylsulfamoyl-benzenesulfonylamino)-methyl]pyridine-3-carbonyl}amino)-4-oxo-butyric Acid Trifluoroacetate (55). Compound **55** was prepared from resin **44e** and **8** (10.0 mg, 27%). ^{1}H NMR (CD_3OD): δ 8.80 (s, 1H), 8.40 (m, 1H), 8.25 (dt, J = 8.2, 2.0 Hz, 1H), 8.17 (d, J = 7.9 Hz, 1H), 8.05 (d, J = 7.9 Hz, 1H), 7.72 (t, J = 7.9 Hz, 1H), 7.58 (dd, J = 8.2, 2.6 Hz, 1H), 4.70 (dd, J = 10.1, 4.0 Hz, 1H), 4.47−4.56 (m, 1H), 4.38 (s, 2H), 2.73−2.83 (m, 1H), 2.59−2.69 (m, 1H), 2.0 (s, 3H). EI-MS m/z: 513.0 (M + H)$^+$. HRMS (TOF): exact mass calcd for $C_{19}H_{21}N_4O_9S_2$ (M + H)$^+$, 513.0744; found, 513.0753.

(*S*)-4-Oxo-3-[(6-{[3-(1*H*-tetrazol-5-yl)benzenesulfon-

ylamino]methyl}pyridine-3-carbonyl)amino]butyric Acid Trifluoroacetate (56). Compound **56** was prepared from resin **44e** and **9** (8.7 mg, 25%). ^{1}H NMR (CD_3OD) δ 8.87 (s, 1H), 8.5 (s, 1H), 8.20−8.35 (m, 2H), 8.05 (d, J = 7.8 Hz, 1H), 7.77 (t, J = 7.8 Hz, 1H), 7.68 (d, J = 8.1 Hz, 1H), 4.70 (dd, J = 12.0, 4.1 Hz, 1H), 4.45−4.55 (m, 1H), 4.40 (s, 2H), 2.73−2.83 (m, 1H), 2.59−2.69 (m, 1H). EI-MS m/z: 460.0 $(M + H)^+$. HRMS (TOF): exact mass calcd for $C_{18}H_{18}N_7O_6S$ $(M + H)^+$, 460.1033; found, 460.1037.

(*S*)-4-Oxo-3-[(6-{[3-(5-oxo-4,5-dihydro[1,2,4]oxadiazol-3-yl)benzenesulfonylamino]methyl}pyridine-3-carbonyl)amino]butyric Acid Trifluoroacetate (57). Compound **57** was prepared from resin **44e** and **12** (11.0 mg, 31%). ^{1}H NMR (CD_3OD): δ 8.87 (s, 1H), 8.18−8.30 (m, 2H), 7.95−8.10 (m, 2H), 7.73 (t d, J = 7.9, 1.7 Hz, 1H), 7.61 (dd, J = 8.2, 3.5 Hz, 1H), 4.70 (dd, J = 11.4, 4.0 Hz, 1H), 4.47−4.57 (m, 1H), 4.37 (s, 2H), 2.74−2.83 (m, 1H), 2.59−2.69 (m, 1H). EI-MS m/z: 476.0 $(M + H)^+$.

(*S*)-5-{[5-(1-Carboxymethyl-3-methylsulfanyl-2-oxo-propylcarbamoyl)pyridin-2-ylmethyl]sulfamoyl}-2-hydroxy-benzoic Acid (58). (4.3 mg, 14%). ^{1}H NMR (CD_3OD): δ 8.88 (d, J = 1.7 Hz, 1H), 8.23−8.27 (m, 2H), 7.88 (dd, J = 8.8, 2.4 Hz, 1H), 7.59 (d, J = 8.2 Hz, 1H), 7.04 (J = 8.8 Hz, 1H), 5.20 (t, J = 6.4 Hz, 1H), 4.30 (s, 3H), 3.51 (d, J = 14.7 Hz, 1H), 3.40 (d, J = 14.7 Hz, 1H), 3.03 (dd, J = 16.9, 6.2 Hz, 1H), 2.80 (dd, J = 16.9, 6.9 Hz, 1H), 2.66 (s, 2H). EI-MS m/z: 512 $(M + H)^+$.

(*S*)-5-({5-[1-Carboxymethyl-3-(2-chloro-benzylsulfanyl)-2-oxo-propylcarbamoyl]pyridin-2-ylmethyl}sulfamoyl)-2-hydroxy-benzoic Acid (59). Compound **59** was prepared from resin **44a** (14.4 mg, 33%). ^{1}H NMR ($CDCl_3$): δ 8.86 (s, 1H), 8.27 (s, 1H), 8.20 (d, J = 6.1 Hz, 1H), 7.88 (d, J = 6.9 Hz, 1H), 7.56 (d, J = 7.6 Hz, 1H), 7.22−7.40 (m, 4H), 7.03−7.05 (m, 1H), 5.18 (m, 1H), 4.29 (s, 2H), 3.83 (s, 2H), 3.44−3.48 (m, 2H), 2.81−3.02 (m, 2H). EI-MS m/z: 622.0 $(M + H)^+$. HRMS (TOF): exact mass calcd for $C_{26}H_{25}N_3O_9S_2Cl$ $(M + H)^+$, 622.0715; found, 622.0719.

(*S*)-5-{[5-(1-Carboxymethyl-2-oxo-ethylcarbamoyl)-pyrimidin-2-ylmethyl]sulfamoyl}-2-hydroxy-benzoic Acid (62a). Compound **62a** was prepared from resin **44e** (1.1 mg, 4%). ^{1}H NMR ($CDCl_3$): δ 8.95 (d, J = 2.8 Hz, 2H), 8.25 (dd, J = 6.3, 2.4 Hz, 1H), 7.84−7.88 (m, 1H), 6.99 (dd, J = 8.8, 3.9 Hz, 1H), 4.68 (dd, J = 9.1, 4.0 Hz, 1H), 4.47−4.51 (m, 1H), 4.40 (d, J = 1.7 Hz, 2H), 2.62−2.77 (m, 2H). EI-MS m/z: 453.0 $(M + H)^+$. HRMS (TOF): exact mass calcd for $C_{17}H_{17}N_4O_9S$ $(M + H)^+$, 453.0710; found, 453.0700.

(*S*)-5-({5-[1-Carboxymethyl-3-(2-chloro-benzylsulfanyl)-2-oxo-propylcarbamoyl]pyrimidin-2-ylmethyl}sulfamoyl)-2-hydroxy-benzoic Acid (62b). Compound **62b** was prepared from resin **44a** (6.4 mg, 17%). EI-MS m/z: 623.0 $(M + H)^+$. HRMS (TOF): exact mass calcd for $C_{25}H_{24}N_4O_9S_2Cl$ $(M + H)^+$, 623.0667; found, 623.0685.

(*S*)-5-{[5-(1-Carboxymethyl-2-oxo-ethylcarbamoyl)-pyrazin-2-ylmethyl]sulfamoyl}-2-hydroxy-benzoic Acid (63a). Compound **63a** was prepared from resin **44e** (3.2 mg, 12%). ^{1}H NMR ($CDCl_3$): δ 9.04 (s, 1H), 8.58 (d, J = 4.9 Hz, 1H), 8.24 (m, 1H), 7.87 (d, J = 8.8 Hz, 1H), 7.02 (d, J = 6.9 Hz, 1H), 4.72 (t, J = 5.4 Hz, 1H), 4.50 (m, 1H), 4.35 (s, 2H), 2.65−2.74 (m, 2H). EI-MS m/z: 453.0 $(M + H)^+$.

(*S*)-5-({5-[1-Carboxymethyl-3-(2-chloro-benzylsulfanyl)-2-oxo-propylcarbamoyl]pyrazin-2-ylmethyl}sulfamoyl)-2-hydroxy-benzoic Acid (63b). Compound **63b** was prepared from resin **44a** (0.8 mg, 2%). ^{1}H NMR ($CDCl_3$): δ 7.45 (d, J = 1.3 Hz, 1H), 6.99 (s, 1H), 6.63 (d, J = 1H), 6.26 (dd, J = 8.8, 2.4 Hz, 1H), 5.74−5.80 (m, 2H), 5.60−5.63 (m, 2H), 5.42 (d, J = 8.8 Hz, IH), 3.62−3.63 (m, 1H), 2.78 (s, 2H), 2.22 (s, 2H), 1.89 (d, J = 15.4 Hz, 1H), 1.85 (d, J = 15.1 Hz, 1H), 1.41 (dd, J = 16.8, 5.6 Hz, 1H), 1.33 (dd, J = 16.8, 5.5 Hz, 1H). EI-MS m/z: 623.0 $(M + H)^+$.

(*S*)-5-{[5-(1-Carboxymethyl-2-oxo-ethylcarbamoyl)-thiazol-2-ylmethyl]sulfamoyl}-2-hydroxy-benzoic Acid (64a). Compound **64a** was prepared from resin **44e** (5.5 mg, 20%). ^{1}H NMR (CD_3OD): δ 8.28 (d, J = 2.6 Hz, 1H), 7.85−7.88 (m, 1H), 7.45 (dd, J = 5.6, 3.9 Hz, 1H), 7.03 (dd, J = 8.9, 3.2 Hz, 1H), 6.84 (d, J = 2.6 Hz, 1H), 4.65 (dd, J = 11.3, 4.1 Hz, 1H), 4.41 (m, 1H), 4.27 (d, J = 1.3 Hz, 2H), 2.60−2.77 (m, 2H). EI-MS m/z: 457.0 $(M + H)^+$. HRMS (TOF): exact mass calcd for $C_{16}H_{16}N_3O_9S_2$ $(M + H)^+$, 458.0322; found, 458.0330.

(*S*)-5-({5-[1-Carboxymethyl-3-(2-chloro-benzylsulfanyl)-2-oxo-propylcarbamoyl]thiazol-2-ylmethyl}sulfamoyl)-2-hydroxy-benzoic Acid (64b). Compound **64b** was prepared from resin **44a** (1.7 mg, 5%). ^{1}H NMR (CD_3OD): δ 8.31 (d, J = 2.6 Hz, 1H), 8.14 (s, 1H), 7.90 (d, J = 6.7 Hz, 1H), 7.36−7.37 (m, 2H), 7.21−7.23 (m, 2H), 7.06 (d, J = 8.8 Hz, 1H), 5.12 (t, J = 8.0 Hz, 1H), 4.40 (d, J = 6.0 Hz, 2H), 3.92 (s, 2H), 3.41−3.49 (m, 2H), 2.72−2.99 (m, 2H). EI-MS m/z: 628.0 $(M + H)^+$.

(*S*)-5-{[3-(1-Carboxymethyl-2-oxo-ethylcarbamoyl)isoxazol-5-ylmethyl]sulfamoyl}-2-hydroxy-benzoic Acid (65a). Compound **65a** was prepared from resin **44e** (3.1 mg, 12%). ^{1}H NMR (CD_3OD): δ 8.28 (t, J = 2.7 Hz, 1H), 7.85 (dt, J = 8.2, 2.0 Hz, 1H), 7.02 (dd, J = 8.8, 2.8 Hz, 1H), 6.48 (d, J = 4.4 Hz, 1H), 4.65 (dd, J = 7.6, 4.3 Hz, 1H), 4.39−4.48 (m, 1H), 4.30 (s, 2H), 2.55−2.76 (m, 2H). HRMS (TOF): exact mass calcd for $C_{24}H_{23}N_3O_{10}S_2Cl$ $(M + H)^+$, 612.0507; found, 612.0535.

(*S*)-5-({3-[1-Carboxymethyl-3-(2-chloro-benzylsulfanyl)-2-oxo-propylcarbamoyl]isoxazol-5-ylmethyl}sulfamoyl)-2-hydroxy-benzoic Acid (65b). Compound **65b** was prepared from resin **44a** (8.5 mg, 23%). ^{1}H NMR (CD_3OD): δ 8.25 (d, J = 2.4 Hz, 1H), 7.85 (dd, J = 8.8, 2.4 Hz, 1H), 7.30−7.40 (m, 2H), 7.15−7.25 (m, 2H), 7.00 (d, J = 8.8 Hz, 1H), 6.51 (s, 1H), 5.15 (t, J = 6.0 Hz, 1H), 4.30 (s, 2H), 3.79 (d, J = 3.1 Hz, 2H), 3.40 (q, J = 15.3 Hz, 1H), 2.70−3.05 (m, 2H). EI-MS m/z: 612.0 $(M + H)^+$.

(*S*)-5-{[5-(1-Carboxymethyl-2-oxo-ethylcarbamoyl)-thiophen-2-ylmethyl]sulfamoyl}-2-hydroxy-benzoic Acid (66a). Compound **66a** was prepared from resin **44e** (3.2 mg, 12%). ^{1}H NMR (CD_3OD): δ 8.23 (s, 1H), 7.80 (d, J = 8.8 Hz, 1H), 7.39 (t, J = 3.9 Hz, 1H), 6.97 (dd, J = 8.7, 2.6 Hz, 1H), 6.78 (d, J = 3.6 Hz, 1H), 4.58 (dd, J = 11.2, 4.1 Hz, 1H), 4.35−4.37 (m, 1H), 4.23 (s, 2H), 2.58−2.81 (m, 2H). EI-MS m/z: 457.0 $(M + H)^+$. HRMS (TOF): exact mass calcd for $C_{17}H_{17}N_2O_9S_2$ $(M + H)^+$, 457.0370; found, 457.0386.

(*S*)-5-({5-[1-Carboxymethyl-3-(2-chloro-benzylsulfanyl)-2-oxo-propylcarbamoyl]thiophen-2-ylmethyl}sulfamoyl)-2-hydroxy-benzoic Acid (66b). Compound **66b** was prepared from resin **44a** (5.1 mg, 14%). ^{1}H NMR (CD_3OD): δ 8.26 (d, J = 2.2 Hz, 1H), 7.85 (dd, J = 8.8, 2.3 Hz, 1H), 7.45 (d, J = 3.7 Hz, 1H), 7.33−7.37 (m, 2H), 7.16−7.22 (m, 2H), 7.00 (d, J = 8.8 Hz, 1H), 6.86 (d, J = 3.6 Hz, 1H), 5.09 (t, J = 6.3 Hz, 1H), 4.30 (s, 2H), 3.78 (d, J = 3.9 Hz, 2H), 3.44 (d, J = 15.3 Hz, 1H), 3.36 (d, J = 15.3, 1H), 3.00 (dd, J = 16.9, 6.5 Hz, 1H), 2.75 (dd, J = 16.8, 6.5 Hz, 1H). EI-MS m/z: 627.0 $(M + H)^+$. Anal. Calcd for $C_{25}H_{23}N_2O_9S_3Cl$: C, 47.88; H, 3.70; N, 4.23. Found: C, 47.67; H, 3.61; N, 4.23.

(*S*)-5-{[4-(1-Carboxymethyl-2-oxo-ethylcarbamoyl)-thiophen-2-ylmethyl]sulfamoyl}-2-hydroxy-benzoic Acid (67a). Compound **67a** was prepared from resin **44e** (15.6 mg, 57%). ^{1}H NMR (CD_3OD): δ 8.28 (d, J = 2.3 Hz, 1H), 7.84−7.88 (m, 2H), 7.23 (d, J = 5.1 Hz, 1H), 7.03 (dd, J = 8.8, 2.4 Hz, 1H), 4.66 (dd, J = 13.3, 4.0 Hz, 1H), 4.40−4.47 (m, 1H), 4.26 (s, 2H), 2.69−2.77 (m, 1H), 2.57−2.64 (m, 1H). EI-MS m/z: 457.0 $(M + H)^+$.

(*S*)-5-({5-[1-Carboxymethyl-3-(2-chloro-benzylsulfanyl)-2-oxo-propylcarbamoyl]thiophen-3-ylmethyl}sulfamoyl)-2-hydroxy-benzoic Acid (67b). Compound **67b** was prepared from resin **44a** (5.2 mg, 14%). ^{1}H NMR (CD_3OD): δ 8.27 (d, J = 2.3 Hz, 1H), 7.91 (d, J = 1.2 Hz, 1H), 7.85 (dd, J = 8.8, 2.4 Hz, 1H), 7.34−7.38 (m, 2H), 7.18−7.23 (m, 3H), 7.01 (d, J = 8.8 Hz, 1H), 5.09 (t, J = 6.4 Hz, 1H), 4.28 (s, 2H), 3.81 (d, J = 3.6 Hz, 2H), 3.44 (d, J = 15.2 Hz, 1H), 3.36 (d, J = 15.2 Hz, 1H), 3.00 (dd, J = 16.8, 6.4 Hz, 1H), 2.75 (dd, J = 16.8, 6.5 Hz, 1H). EI-MS m/z: 627.0 $(M + H)^+$.

(*S*)-5-{[5-(1-Carboxymethyl-2-oxo-ethylcarbamoyl)-thiophen-3-ylmethyl]sulfamoyl}-2-hydroxy-benzoic Acid (68a). Compound **68a** was prepared from resin **44e** (17.6 mg, 64%). ^{1}H NMR (CD_3OD): δ 8.26 (s, 1H), 7.86 (d, J = 8.8 Hz, 1H), 7.51 (d, J = 7.0 Hz, 1H), 7.34 (s, 1H), 7.04 (dd, J = 8.8,

2.6 Hz, 1H), 4.67 (dd, J = 12.4, 4.1 Hz, 1H), 4.43−4.44 (m, 1H), 4.06 (s, 2H), 2.64−2.72 (m, 2H). EI-MS m/z: 457.0 $(M + H)^+$.

(*S*)-4-[(4-Hydroxy-3-methyl-benzenesulfonylamino)-methyl]thiophene-2-carboxylic Acid [3-(2-Chloro-benzylsulfanyl)-1-ethyl-2-oxo-propyl]amide (68b). Compound **68a** was prepared from resin **44a** (4.5 mg, 12%). ^{1}H NMR (CD_3OD): δ 8.28 (d, J = 2.3 Hz, 1H), 7.84 (dd, J = 8.8 2.4 Hz, 1H), 7.47 (s, 1H), 7.34−7.38 (m, 2H), 7.20−7.23 (m, 2H), 6.99 (d, J = 8.9 Hz, 1H), 5.09 (t, J = 6.3 Hz, 1H), 4.26 (s, 2H), 3.81 (s, 2H), 3.42−3.47 (m, 2H), 2.99 (dd, J = 16.9, 6.4 Hz, 1H), 2.73 (dd, J = 16.7, 6.5 Hz, 1H). EI-MS m/z: 707.0 $(M + 79)^+$.

(*S*)-5-{[5-(1-Carboxymethyl-2-oxo-ethylcarbamoyl)-4-methyl-thiophen-2-ylmethyl]sulfamoyl}-2-hydroxy-benzoic Acid (69a). Compound **69a** was prepared from resin **44e** (11.9 mg, 42%). ^{1}H NMR (CD_3OD): δ 8.24 (s, 1H), 7.84 (dd, J = 8.8, 1.0 Hz, 1H), 7.03 (d, J = 8.8 Hz, 1H), 6.62 (s, 1H), 4.68 (dd, J = 9.9, 4.1 Hz, 1H), 4.37 (m, 1H), 4.23 (s, 2H), 2.65−2.68 (m, 2H), 2.31 (s, 3H). EI-MS m/z: 471.0 $(M + H)^+$. HRMS (TOF): exact mass calcd for $C_{18}H_{19}N_2O_9S_2$ $(M + H)^+$, 471.0526; found, 471.0544.

(*S*)-5-({5-[1-Carboxymethyl-3-(2-chloro-benzylsulfanyl)-2-oxo-propylcarbamoyl]-4-methyl-thiophen-2-ylmethyl}-sulfamoyl)-2-hydroxy-benzoic Acid (69b). Compound **69b** was prepared from resin **44a** (3.8 mg, 10%). ^{1}H NMR (CD_3OD): δ 6.70 (s, 1H), 6.30 (dd, J = 8.8, 2.2 Hz, 1H), 5.82−5.87 (m, 2H), 5.68 (m, 2H), 5.48 (d, J = 8.8 Hz, 1H), 5.13 (s, 1H), 3.51 (t, Hz, 1H), 1.77 (s, 3H), 1.44 (dd, J = 16.9, 5.8 Hz, 1H)), 1.29 (dd, J = 16.8, 5.8 Hz, 1H). EI-MS m/z: 641.0 $(M + H)^+$. HRMS (TOF): exact mass calcd for $C_{26}H_{26}N_2O_9S_3Cl$ $(M + H)^+$, 641.0483; found, 641.0490.

(*S*)-5-{[5-(1-Carboxymethyl-2-oxo-ethylcarbamoyl)-furan-2-ylmethyl]sulfamoyl}-2-hydroxy-benzoic Acid (70a). Compound **70a** was prepared from resin **44e** (2.5 mg, 9%). ^{1}H NMR (CD_3OD): δ 8.23 (s, 1H), 7.83 (d, J = 9.1 Hz, 1H), 7.02 (d, J = 8.8 Hz, 1H), 6.91 (s, 1H), 6.22 (s, 1H), 4.68−4.74 (m, 1H), 4.43−4.49 (m, 1H), 4.21 (s, 2H), 2.60−2.74 (m, 2H). EI-MS m/z: 441.0 $(M + H)^+$.

(*S*)-5-({5-[1-Carboxymethyl-3-(2-chloro-benzylsulfanyl)-2-oxo-propylcarbamoyl]furan-2-ylmethyl}sulfamoyl)-2-hydroxy-benzoic Acid (70b). Compound **70b** was prepared from resin **44a** (5.5 mg, 14%). ^{1}H NMR (CD_3OD): δ 8.20 (d, J = 2.0 Hz, 1H), 7.82 (dd, J = 8.8, 2.0 Hz, 1H), 7.33−7.38 (m, 2H), 7.19−7.21 (m, 2H), 6.99 (d, J = 8.8 Hz, 1H), 6.92 (d, J = 3.4 Hz, 1H), 6.27 (d, J = 3.3 Hz, 1H), 5.11 (t, J = 6.2 Hz, 1H), 4.18 (s, 2H), 3.80 (d, J = 2.8 Hz, 2H), 3.44 (d, J = 15.2 Hz, 1H), 3.36 (d, J = 15.3 Hz, 1H), 2.98 (dd, J = 16.9, 6.2 Hz, 1H), 2.79 (d, J = 16.9, 6.2 Hz, 1H). EI-MS m/z: 611.0 $(M + H)^+$.

(*S*)-5-({5-[1-Carboxymethyl-2-oxo-3-(pyridin-3-ylmethylsulfanyl)propylcarbamoyl]thiophen-2-ylmethyl}-sulfamoyl)-2-hydroxy-benzoic Acid (71). Compound **71** was prepared from resin **44c** (7.3 mg, 20%). ^{1}H NMR (CD_3OD): δ 8.81 (br s, 1H), 8.69 (br s, 1H), 8.52 (br s, 1H), 8.27 (d, J = 2.0 Hz, 1H), 7.93 (br s, 1H), 7.85 (dd, J = 8.8, 2.3 Hz, 1H), 7.45 (d, J = 3.8 Hz, 1H), 7.02 (d, J = 8.8 Hz, 1H), 6.89 (d, J = 3.7 Hz, 1H), 4.98 (m, 1H), 4.30 (s, 2H), 3.95 (s, 2H), 3.51 (d, J = 15.3 Hz, 1H), 3.41 (d, J = 15.3 Hz, 1H), 3.00 (dd, J = 17.0, 7.0 Hz, 1H), 2.72 (dd, J = 17.0, 5.9 Hz, 1H). EI-MS m/z: 594 $(M + H)^+$.

(*S*)-5-({5-[1-Carboxymethyl-2-oxo-3-(pyridin-4-ylmethylsulfanyl)propylcarbamoyl]thiophen-2-ylmethyl}-sulfamoyl)-2-hydroxy-benzoic Acid (72). Compound **72** was prepared from resin **44d**. ^{1}H NMR (CD_3OD): δ 8.71 (br s, 2H), 8.27 (d, J = 2.2 Hz, 1H), 8.0 (br s, 2H), 7.85 (dd, J = 8.8, 2.2 Hz, 1H), 7.46 (d, J = 3.7 Hz, 1H), 7.02 (d, J = 8.8 Hz, 1H), 6.89 (d, J = 3.8 Hz, 1H), 4.98 (m, 1H), 4.30 (s, 2H), 3.99 (s, 2H), 3.52 (d, J = 15.1 Hz, 1H), 3.43 (d, J = 15.1 Hz, 1H), 3.00 (dd, J = 16.7, 6.7 Hz, 1H), 2.72 (dd, J = 16.7, 6.0 Hz, 1H). EI-MS m/z: 594 $(M + H)^+$.

(*S*)-5-({5-[1-Carboxymethyl-5-(2-chloro-phenyl)-2-oxo-pentylcarbamoyl]thiophen-2-ylmethyl}sulfamoyl)-2-hydroxy-benzoic Acid (73). Compound **73** was prepared from resin **44b**. ^{1}H NMR (CD_3OD): δ 8.27 (d, J = 2.5 Hz, 1H), 7.85 (dd, J = 8.8, 2.4 Hz, 1H), 7.44 (m, 1H), 7.20 (m, 2H), 7.13 (m, 3H), 7.02 (d, J = 8.9 Hz, 1H), 6.88 (d, J = 3.81 Hz, 1H), 4.80 (t, J = 6.5 Hz, 1H), 4.31 (s, 2H), 2.96 (dd, J = 16.8, 6.6 Hz, 1H), 2.67 (m, 1H), 2.58 (m, 4H), 1.89 (m, 2H).

(*S*)-3-{[5-(Benzenesulfonylamino-methyl)thiophene-2-carbonyl]amino}-5-(2-chloro-benzylsulfanyl)-4-oxo-pentanoic Acid (74). Compound **74** was prepared from resin **44a** (10.8 mg, 32%). ^{1}H NMR (CD_3OD): δ 7.79 (d, J = 7.4 Hz, 2H), 7.55 (d, J = 7.3 Hz, 1H), 7.49 (d, J = 7.7 Hz, 2H), 7.46 (m, 1H), 7.34 (m, 2H), 7.21 (m, 2H), 6.86 (d, J = 3.8 Hz, 1H), 5.08 (t, J = 6.5 Hz, 1H), 3.82 (d, J = 13.3 Hz, 1H), 3.77 (d, J = 13.3 Hz, 1H), 3.39 (m, 2H), 2.99 (dd, J = 16.9, 6.3 Hz, 1H), 2.74 (dd, J = 16.8, 6.8 Hz, 1H). EI-MS m/z: 567.0 $(M + H)^+$.

(*S*)-5-(2-Chloro-benzylsulfanyl)-3-({5-[(4-hydroxy-benzenesulfonylamino)methyl]thiophene-2-carbonyl}amino)-4-oxo-pentanoic Acid (75). Compound **75** was prepared from resin **44a**. ^{1}H NMR (CD_3OD): δ 7.62 (m, 2H), 7.45 (d, J = 3.81 Hz, 1H), 7.32 (m, 2H), 7.16 (m, 2H), 6.83 (m, 3H), 5.05 (t, J = 6.6 Hz, 1H), 4.19 (s, 2H), 3.76 (m, 2H), 3.37 (m, 2H), 3.26 (m, 2H), 2.96 (dd, J = 16.8, 6.4 Hz, 1H), 2.70 (dd, J = 16.8, 6.6 Hz, 1H).

(*S*)-3-({5-[1-Carboxymethyl-3-(2-chloro-benzylsulfanyl)-2-oxo-propylcarbamoyl]thiophen-2-ylmethyl}sulfamoyl)-benzoic Acid (76). Compound **76** was prepared from resin **44a** (4.3 mg, 12%). ^{1}H NMR (CD_3OD): δ 8.37 (s, 1H), 8.16 (d, J = 7.8 Hz, 1H), 7.97 (d, J = 7.9 Hz, 1H), 7.59 (t, J = 7.8 1H), 7.42 (d, J = 3.8 Hz, 1H), 7.36 (m, 1H), 7.21 (m, 1H), 6.86 (d, 3.8 Hz, 1H), 5.08 (t, J = 6.6 Hz, 1H), 4.33 (s, 2H), 3.80 (d, J = 4.3 Hz, 2H), 3.44 (d, J = 15.2 Hz, 1H), 3.35 (d, J = 15.3 Hz, 1H), 3.00 (dd, J = 16.9, 6.5 Hz, 1H), 2.74 (dd, J = 16.8, 6.7 Hz, 1H). EI-MS m/z: 611.0 $(M + H)^+$.

(*S*)-5-(2-Chloro-benzylsulfanyl)-4-oxo-3-[(5-{[3-(5-oxo-4,5-dihydro[1,2,4]oxadiazol-3-yl)benzenesulfonylamino]-methyl}thiophene-2-carbonyl)amino]pentanoic Acid (77). Compound **77** was prepared from resin **44a** and **11** (3.4 mg, 9%). ^{1}H NMR (CD_3OD): δ 8.16 (s, 1H), 7.98 (d, J = 8.2 Hz, 1H), 7.95 (d, J = 7.9 Hz, 1H), 7.66 (t, J = 7.9 Hz, 1H), 7.42 (d, J = 3.7 Hz, 1H), 7.34−7.38 (m, 2H), 7.20−7.23 (m, 2H), 6.88 (d, J = 3.6 Hz, 1H), 5.08 (t, J = 6.4 Hz, 1H), 4.38 (s, 2H), 3.80 (d, J = 3.7 Hz, 2H), 3.44 (d, J = 15.2 Hz, 1H), 3.37 (d, J = 15.3 Hz, 1H), 2.99 (dd, J = 16.8, 6.4 Hz, 1H), 2.75 (dd, J = 16.9, 6.6 Hz, 1H). EI-MS m/z: 651.0 $(M + H)^+$.

(*S*)-5-({5-[1-Carboxymethyl-3-(2-chloro-benzylsulfanyl)-2-oxo-propylcarbamoyl]thiophen-2-ylmethyl}methyl-sulfamoyl)-2-hydroxy-benzoic Acid (78). Compound **78** was prepared from resin **44a** (5.8 mg, 15%). ^{1}H NMR (CD_3OD): δ 8.28 (s, 1H), 7.89 (d, J = 8.9 Hz, 1H), 7.55 (d, J = 3 Hz, 1H), 7.34−7.36 (m, 2H), 7.19−7.21 (m, 2H), 7.11 (dd, J = 8.8, 2.5 Hz, 1H), 7.01 (s, 1H), 5.10 (t, J = 9.0 Hz, 1H), 4.44 (s, 2H), 3.80 (d, J = 3.5 Hz, 2H), 3.46 (d, J = 15.3 Hz, 1H), 3.38 (d, J = 15.2 Hz, 1H), 2.85−3.04 (m, 1H), 2.76−2.78 (m, 1H), 2.72 (s, 3H). EI-MS m/z: 641.0 $(M + H)^+$.

(*S*)-5-({5-[1-Carboxymethyl-3-(2-chloro-benzylsulfanyl)-2-oxo-propylcarbamoyl]thiophene-2-sulfonylamino}-methyl)-2-hydroxy-benzoic Acid (79). Compound **79** was prepared from resin **44a** (1.0 mg, 3%). ^{1}H NMR (CD_3OD): δ 7.71 (d, J = 1.9 Hz, 1H), 7.60 (d, J = 3.9 Hz, 1H), 7.45 (d, J = 3.9 Hz, 1H), 7.36 (m, 3H), 7.21 (m, 2H), 6.82 (d, J = 8.5 Hz, 1H), 5.15 (t, J = 6.5 Hz, 1H), 4.12 (s, 2H), 3.82 (s, 2H), 3.48 (d, J = 15.3 Hz, 1H), 3.40 (d, J = 15.3 Hz, 1H), 3.16 (m, 1H), 2.86−3.01 (m, 1H), 2.75−2.85 (m, 1H). EI-MS m/z: 627.0 $(M + H)^+$.

(*S*)-5-[({5-[1-Carboxymethyl-3-(2-chloro-benzylsulfanyl)-2-oxo-propylcarbamoyl]thiophen-2-ylmethyl}amino)-methyl]-2-hydroxy-benzoic Acid (47a). Compound **47a** was prepared from resin **44a** (12.2 mg, 35%). ^{1}H NMR (CD_3OD): δ 8.05 (m, 1H), 7.71 (m, 1H), 7.58 (m, 1H), 7.36 (m, 3H), 7.21 (m, 2H), 7.01 (m, 1H), 5.14−5.17 (m, 1H), 4.50 (s, 2H), 4.21 (s, 2H), 3.81 (s, 2H), 3.46 (d, J = 15.6 Hz, 1H), 3.39 (d, J = 15.1 Hz, 1H), 3.02 (dd, J = 17.0, 6.3 Hz, 1H), 2.78 (J = 17.0, 7.2 Hz, 1H). EI-MS m/z: 427.0 $(M + H)^+$.

(*S*)-5-[(Acetyl-{5-[1-carboxymethyl-3-(2-chloro-benzylsulfanyl)-2-oxo-propylcarbamoyl]thiophen-2-ylmethyl}-amino)methyl]-2-hydroxy-benzoic Acid (47b). Compound

47b was prepared from resin **44a** (2.4 mg, 6%). ^{1}H NMR (CD_3-OD; reported as a mixture of rotamers): δ 7.90 (s, 1H), 7.75 (s, 0.5H), 7.69 (d, 0.5H), 7.59 (d, J = 3.6 Hz, 0.5H), 7.50 (d, J = 3.9 Hz, 0.5H), 7.32−7.39 (m, 3H), 7.20−7.22 (m, 2H), 5.08−5.11 (m, 1H), 4.66−4.73 (m, 1H), 3.80 (d, J = 4.7 Hz, 2H), 3.46 (d, J = 15.3 Hz, 1H), 3.38 (d, J = 15.7 Hz, 1H), 2.99−3.04 (m, 1H), 2.72−2.78 (m, 1H), 2.66 (s, 3H), 2.21 (s, 1H). EI-MS m/z: 619.0 $(M + H)^+$. HRMS (TOF): exact mass calcd for $C_{28}H_{28}N_2O_8S_2Cl$ $(M + H)^+$, 619.0970; found, 619.0996.

(*S*)-5-[({5-[1-Carboxymethyl-3-(2-chloro-benzylsulfanyl)-2-oxo-propylcarbamoyl]thiophen-2-ylmethyl}methyl-amino)methyl]-2-hydroxy-benzoic Acid (47c). Compound **47c** was prepared from resin **44a** (5.8 mg, 16%). ^{1}H NMR (CD_3-OD): δ 8.06 (d, J = 2.0 Hz, 1H), 7.74 (d, J = 3.6 Hz, 1H), 7.61 (dd, J = 8.3, 1.8 Hz, 1H), 7.35−7.42 (m, 3H), 7.21−7.23 (m, 2H), 7.05 (d, J = 8.5 Hz, 1H), 5.49 (s, 1H), 5.16 (t, J = 6.4 Hz, 1H), 4.71 (1H), 4.47−4.59 (m, 2H), 4.24−4.29 (m, 1H), 3.81 (d, J = 2.2 Hz, 2H), 3.47 (d, J = 15.2 Hz, 1H), 3.40 (d, J = 15.0 Hz, 1H), 3.03 (dd, J = 16.7, 5.7 Hz, 1H), 2.75−2.83 (m, 1H). EI-MS m/z: 591.0 $(M + H)^+$.

Enzyme Assays. The effectiveness of compounds against the activity of human recombinant caspase-1−8 was measured using fluorometric assays. Caspase-1, -2, and -4−7 were purchased from BIOMOL Research Laboratories Inc. Caspase-3 and -8 were expressed and purified as described.[8,41] Coumarin-based fluorogenic substrates that incorporated optimal peptide recognition motifs for each enzyme were purchased from Alexis Biochemicals. Enzyme was added to test compounds titrated in dimethyl sulfoxide (DMSO) and incubated at room temperature for 30 min. Substrate addition initiated the reaction, bringing the final reaction volume to 50 μL. Preferred 1X buffer solutions were as follows: 25 mM HEPES, pH 7.4, 0.1% CHAPS, 50 mM KCl, and 5 mM β-mercaptoethanol (β-ME) for caspase-1−4 and -6; 100 mM HEPES, pH 7.4, 10% sucrose, 0.1% CHAPS, 200 mM NaCl, and 10 mM DTT for caspase-5; 25 mM HEPES, pH 7.4, 0.1% CHAPS, 50 mM KCl, 5 mM $CaCl_2$, and 5 mM β-ME for caspase-7; 100 mM HEPES, pH 7.4, 10% sucrose, 0.1% CHAPS, and 10 mM DTT for caspase-8. The final concentration of the peptide substrates (and the corresponding enzyme) was 4 μM Ac-Trp-Glu-His-Asp-AFC (3 nM caspase-1), 40 μM Ac-Val-Asp-Val-Ala-Asp-AFC (17 nM caspase-2), 5 μM Ac-Asp-Glu-Val-Asp-AFC (1.6 nM caspase-3), 30 μM Ac-Trp-Glu-His-Asp-AFC (60 nM caspase-4 and 83 nM caspase-5), 7 μM Ac-Val-Glu-Ile-Asp-AFC (2 nM caspase-6), 30 μM Ac-Asp-Glu-Val-Asp-AFC (1 nM caspase-7), and 3 μM Ac-Ile-Glu-Thr-Asp-AFC (10 nM caspase-8). Assays were carried out in black 96 well flat bottom, polystyrene plates (Corning Incorporated Costar 3915). Caspase activity was monitored using a Molecular Devices' Microplate Spectrofluorometer Gemini XS with an excitation wavelength of 365 nm and an emission wavelength of 495 nm. Kinetic data were collected over a 15 min assay run at room temperature. IC_{50} values were calculated through direct fits of the data to a four parameter fit curve using the computer application SOFTmax PRO. $K_{i(apparent)}$ values were calculated using the following equation: $K_{i(app)} = IC_{50}/(1 + [substrate]/K_m)$.

Typically, all compounds with a $K_i < 0.2$ μM were screened at least two times, and usually more, against caspase-3. Because control compounds were included in all assays, we have been able to estimate the accuracy of our in vitro assays. In particular, compound **4** had an average K_i of 0.05 ± 0.017 μM (n = 30), compound **59** had an average K_i of 0.03 ± 0.014 μM (n = 10), compound **66a** had an average K_i of 0.12 ± 0.06 μM (n = 6), and compound **66b** had an average K_i of 0.02 ± 0.007 μM (n = 26). In validating our in-house caspase-3 assay, we initially included control compounds such as the isatin compound,[37] which had a calculated K_i value of 0.039 μM, which is similar to the reported K_i value of 0.015 μM.

Molecular Modeling. Models were built using PyMOL[41] and evaluated using MacroModel[42] with the MMFF[43] force field or using Amber 6.0[43] with an updated version of the Amber force field.[45] For key compounds, 250 ps molecular dynamics simulations with explicit solvation were performed in order to generate hypothetical conformational ensembles of inhibitor in complex with caspase-3. Initial molecular dynamics models were built by placing the proposed compound in the protein active site using crystal structure coordinates[15] surrounded by a 20 Å sphere of intact protein residues, plus sufficient TIP3P waters to fill a rectangular box with a 5 Å buffer. Sodium and chloride ions were introduced as needed to achieve neutrality. Simulations were performed using the particle mesh Ewald electrostatic approximation with an NTP ensemble, an integration step of 1 fs, and with shake constraints applied to bonds involving hydrogen. Each simulation was initiated with 30 ps of solvent relaxation during which all solute atoms were subjected to harmonic positional restraint with a force constant of 1.0 (kcal/mol·Å^2). Thereafter, only solute atoms >15 Å from the compound were restrained.

Crystallization of the Complex of Caspase-3 with Compounds 3, 4, and 66b. Crystals of caspase-3 were grown at 20 °C using the hanging drop vapor diffusion method. Equal volumes of protein solution (5−10 mg/mL of previously modified protein in 10 mM Tris pH 8.5) were mixed with the reservoir solution containing 100 mM sodium citrate, pH 5.9, 4% glycerol, 10−20% PEG6000, and 10 mM DTT. Crystallization of caspase-3 and compound **3** required using the 2,6-dichloroacyloxymethyl ketone of **3**. Crystallization of caspase-3 and compound **4** required using the fluoromethyl ketone of **4**. For the complex between caspase-3 and compound **66b**, a 2-fold excess of **66b** was added to the protein solution prior to setting up the drops.

Small rhombic plates usually appeared after 1−2 weeks. They reached their maximum size of approximately 200 μm × 200 μm × 20 μm after 2 months. Before data collection, crystals were dipped briefly into reservoir solution containing 25% glycerol and then flash-frozen in liquid nitrogen.

Diffraction data for caspase-3 modified with the 2,6-dichloroacyloxymethyl ketone of **3** and the fluoromethyl ketone of **4** were collected at 100 K using a Rigaku (Tokyo) RU-3R generator, an R-axis-IV detector, and processed using D*Trek.[46] Diffraction data for caspase-3 modified with **66b** were collected at beamline 7.1 of the Stanford Synchrotron Radiation Laboratory and processed using programs Mosflm[48] and Scala. The crystals were isomorphous with previously solved structures; therefore, refinement began with rigid body refinement using Refmac.[46] Compound models were constructed in Pymol,[41] and the models were adjusted using program O[49] and refined using program Refmac.[50]

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