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Bioorganic & Medicinal Chemistry Letters 15 (2005) 3886–3890
Bioorganic & Medicinal Chemistry Letters

Lipophilic versus hydrogen-bonding effect in P_3 on potency and selectivity of valine aspartyl ketones as caspase 3 inhibitors

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Received 2 March 2005; revised 25 May 2005; accepted 26 May 2005
Available online 14 July 2005

Abstract—Caspase 3 is a cysteinyl protease that mediates apoptotic cell death. Its inhibition may have an important impact in the treatment of several degenerative diseases. The P_1 aspartic acid residue is a required element of recognition for this enzyme that was maintained constant along with the adjacent natural valine as the P_2 group. The thiobenzylmethylketone warhead on the aspartate was conveniently handled through solid-phase synthesis allowing modification in the P_3 region that eventually led to simpler derivatives with increased potency against caspase 3. The key to such an effect is the introduction of hydroxyl group alpha to the P_3 carbonyl.

The human caspases constitute an ensemble of at least 13 cysteinyl-aspartate-specific proteinases that are key elements in the molecular pathways resulting in the apoptosis of cells.[1] Caspases 1, 4, and 5 mediate cytokine maturation and are linked to the inflammatory phenomenon. Caspases 2, 3, and 7 are the major effectors of cell death. Caspases 6, 8, 9, and 10 are activators of the effector caspases 2, 3, and 7. Finally, caspase 14 is a heratinocyte-specific caspase. Caspase 3 in particular appears to be a pivotal player in neuronal apoptosis. Peptidic inhibitors of caspase 3 have shown some efficacy in models such as stroke, traumatic brain/spinal cord injury, hypoxic brain damage, and cardiac ischemia/reperfusion injury.[2] The P_1 aspartic acid residue is a required element of recognition of the enzyme, and its scissile amide bond can be replaced with a ketone to afford reversible inhibitors. Here, we report dipeptidic aspartyl ketones as potent, selective, and cell-penetrable inhibitors of caspase 3.

We recently disclosed that an α-methylthiobenzyl aspartyl ketone warhead was a suitable electrophile, replacing with advantage over the aldehyde warhead in a peptide structure.[3] The ketone itself, in conjunction with the aspartic acid residue, was a capricious functionality whose synthesis required tedious protection–deprotection sequences or convoluted routes. We therefore developed a very convenient method to rapidly access a variety of P_3 derivatives in that series using solid-phase chemistry.

Hence, commercial Fmoc-aspartic acid β-*t*-butyl ester **1** (Scheme 1) was activated using the mixed anhydride method[4] and was treated with diazomethane. The resulting α-diazoketone was reacted with 48% aqueous HBr at low temperature to yield the corresponding α-bromoketone **2**. At this point, the α-bromoketone **2** was attached to a Merrifield resin that had previously been elaborated to unveil hydrasemicarbazone **3**.[5] The bromide was displaced with benzyl mercaptan to give thioether **4** followed by removal of the Fmoc group. The free amine was then coupled to FmocValine to yield intermediate **5**. Again, the Fmoc was removed using piperidine to liberate the amine of the polymer bound valine

Keywords: Caspase 3 inhibitors ketones hydrogen bond.
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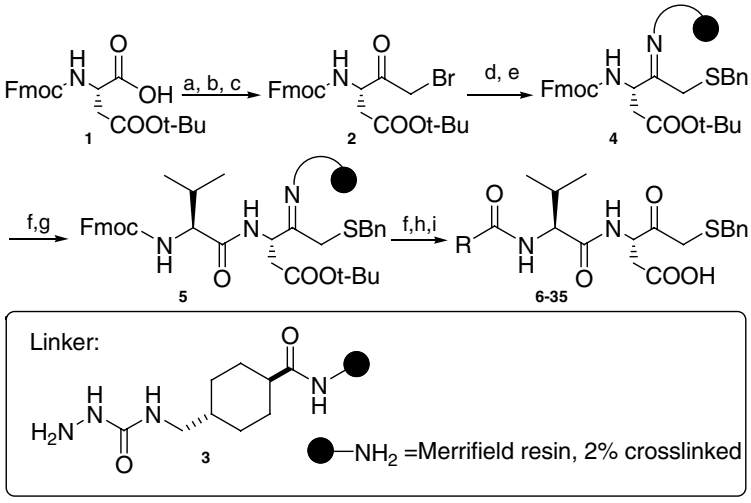
doi:10.1016/j.bmcl.2005.05.116



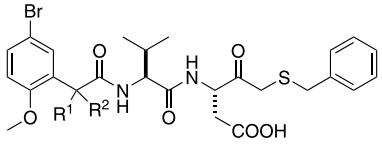
Scheme 1. Synthesis of dipeptidic aspartylthiobenzylmethylketones. Reagents and conditions: (a) *i*-BuOCOCLI, NMM, −10 °C, CH_2CL_2; (b) CH_2N_2, Et_2O, 0 °C; (c) HBr/AcOh, −78 °C; (d) linker-NH_2 **3**, AcOH, THF; (e) $PhCH_2SH$, DMF, $(iPr)_2NEt$; (f) 20% piperidine/DMF; (g) FMOCVal-H, HATU, DMF, $(iPr)_2NEt$; (h) RCOOH (see **6**–**35**), HATU, DMF, $(iPr)_2NEt$; (i) 9:1 TFA/H_2O.

aspartylthiobenzylmethylketone. Amide formation with a variety of carboxylic acids followed by the treatment of the resin with wet trifluoroacetic acid afforded dipeptides **6**–**35** on 10–20 mg scale, generally in good to excellent purity.

One of the hits against caspase 3[6] that was obtained from coupling commercially available reagents was a 2,5-substituted phenylacetic acid (compound **6**, Table 1). At that stage, we decided to introduce substitution into the open aliphatic position using enolate alkylation chemistry (Scheme 2). Generally, 5-bromo-2-methoxyphenylacetic acid methyl ester underwent deprotonation when added into a cold solution of lithium diisopropyl amide via a cannula. The resulting nucleophile was quenched with an excess of an alkyl halide. The ester was then purified by flash chromatography on silica gel and hydrolyzed to afford the acid that was used for solid-phase chemistry. This resulted in a mixture of diastereomers that were separated only when they were distinct by flash chromatography.

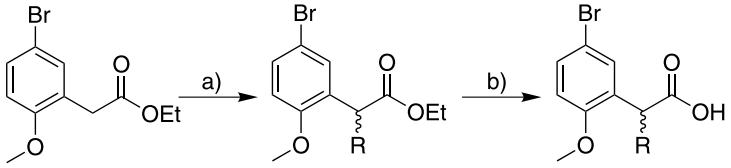
Introduction of a methyl group (compound **7**) at this benzylic position was deleterious to the potency on caspase 3. Attempt to maximize lipophilic interactions with the enzyme by increasing the size of the substituents resulted in decreased caspase 3 activity (compounds **8** and **9**). This was especially true when introducing sterically more demanding groups (compounds **10** and **11**). However, potency was restored with the narrower substituents as demonstrated by the allyl (**12**) and propargyl groups (**13** and **14**). In the latter case, potency was modulated by the stereochemistry of that group (compare **13** versus **14**), although the stereochemistry was not established at the benzylic position. Overall, compound **14** only matched the potency of the unsubstituted parent

Table 1. Substituted 5-bromo-2-methoxyphenylacetic acid analogs in P_3



Compound	R^1	R^2	Enzyme IC_{50} (nm)			
			C-1[a]	C-3[a]	C-7[a]	C-8[a]
6	H	H	3040	18	284	1885
7	H	Me	>10,000	176	3690	5380
8	H	*n*-Pr	>10,000	274	5300	4505
9	H	Benzyl	5810	181	4200	5830
10	H	*i*-Pr	>10,000	9445	>10,000	1320
11	Cyclopropyl		>10,000	323	>10,000	>10,000
12	H	Allyl	6505	80	2215	1625
13	H	Propargyl	>10,000	341	3625	4470
14	Propargyl	H	1030	17	139	92

[a] Recombinant human caspases 1, 3, 7, and 8. See Ref. 6.



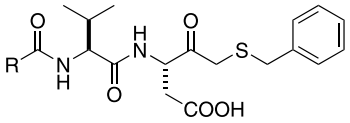
Scheme 2. Alkylation of 5-bromo-2-methoxyphenylacetic acid. Reagents and conditions: (a) i—LDA, −78 °C, 30 min and ii—RX, 2–5 equiv., −78 °C to room temperature; (b) $LiOH{\cdot}H_2O$, MeOH–THF–H_2O, room temperature.

6. Furthermore, the selectivity profile against other caspases generally worsened with these modifications. These modifications also resulted in an increase in the molecular weight as well as more metabolically labile sites. We thus turned our attention to a different subclass of phenylacetic acids: the mandelic derivatives.

The (*R*) mandelic acid derivative **16** (Table 2) showed a 20-fold increase in potency when compared with the unsubstituted parent **15**. This large gain in potency from a hydroxyl group suggests a hydrogen bonding interaction with a not well-solvated[7] backbone carbonyl group in the enzyme S_3 pocket. As in the case with alkyl groups, potency varied with stereochemistry but only slightly within a pair of diastereoisomers (compounds **16** versus **17** and **18** versus **19**). Noteworthy is that the preferred stereochemistry depended on the exact nature of the substituent when compared across pairs (pair **16** and **17** compared to pair **18** and **19**). This suggested that a powerful hydrogen bond was overriding beneficial lipophilic interactions. In fact, when the hydroxyl group was capped with a methyl group (compound **20**), a considerable loss in potency was observed, in agreement with a hydrogen bond formation with the enzyme backbone although undesirable steric effects cannot be ruled out. To reduce the potential for metabolism at the benzylic position, this position was quaternized with a methyl group (compound **21**). This racemic derivative was almost as potent as the optically pure mandelate **16**. This was unexpected since alpha substitution in the phenylacetic acid derivatives showed a marked decrease in potency. This observation constituted a second hint for the existence of a different binding mode driven by a hydrogen bond in the hydroxyl series. Achiral disubstituted α-hydroxy-acids were then incorporated via solid-phase chemistry. Clearly, less sterically demanding groups afforded more potent derivatives as demonstrated by the sequence of compounds culminating in a cyclopropyl derivative (compounds **22**–**25**). Interestingly, the bistrifluoromethyl alcohol **26** showed a marked increase in potency, despite being larger than the gem-dimethyl **24**. This can be rationalized by invoking a stronger hydrogen bond due to increased acidity of the bistrifluoromethyl alcohol **26**. Overall, the introduction of a hydroxyl group was beneficial to the selectivity profile over other caspases. The most potent mandelate derivatives still offered the tantalizing possibility to gain further potency via substitution on the phenyl ring.

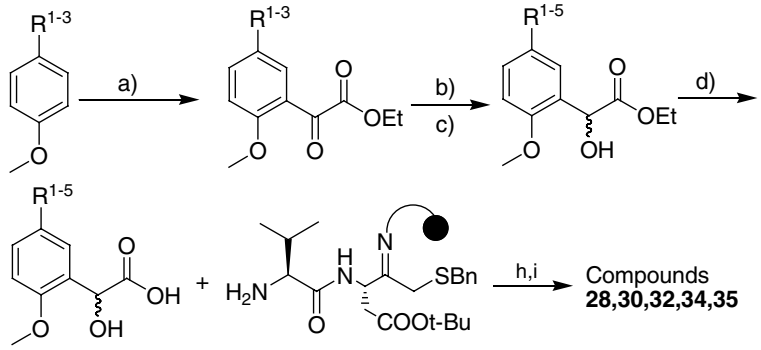
To access racemic 2,5-disubstituted mandelic acid (Scheme 3), Friedel–Craft reactions were performed on 4-substituted anisole derivatives with ethyl oxalyl chloride in the presence of aluminum trichloride. The resulting keto esters were reduced with sodium borohydride to obtain a racemic mixture of α-alkoxy esters. To introduce electron-poor heteroaromatic groups on the phenyl ring, palladium-catalyzed elaboration of the halide produced the desired derivatives uneventfully.[8,9] After alkaline hydrolysis of the α-hydroxyl ester, the acid was coupled with the amine on solid phase using HATU in

Table 2. Potency and selectivity from SAR around the OH group



Compound	R	Enzyme IC_{50} (nm)			
		C-1[a]	C-3[a]	C-7[a]	C-8[a]
15		21,200	746	8360	22,050
16		4160	41	1050	1150
17		4060	100	10,860	3650
18		>10,000	330	>10,000	7270
19		3015	118	3405	7340
20		>10,000	425	>10,000	9175
21		18,900	85	2760	8000
22		>10,000	1295	>10,000	>10,000
23		>10,000	2020	>10,000	9580
24		18,400	467	10,860	13,800
25		9590	160	5095	12,300
26		>10,000	146	>10,000	>10,000

[a] Recombinant human caspases 1,3,7, and 8. See Ref. 6.



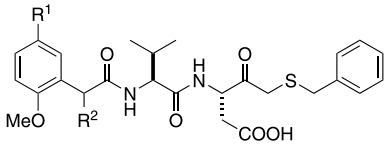
Scheme 3. Preparation of substituted methoxymandelic acid. Reagents and conditions: (a) Ethyloxayl chloride, $ALCL_3$, −10 °C to 25 °C, $(CH_2CL)_2$; (b) $NaBH_4$, MeOH, 0 °C; (c) R^{1-3} = OMe, Br, and I: anisoles were commercially available; R^4 = oxadiazole: see Ref. 9; R^5 = acetyl; see Ref. 8; (d) $LiOH{\cdot}H_2O$, MeOH–THF–H_2O, room temperature; (h) HATU, DMF, $(iPr)_2NEt$; (i) 9:1 TFA/H_2O.

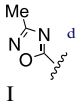
DMF. Under these conditions, the hydroxyl group was inert and no ester formation was detected. After acidic cleavage from the resin and purification on silica gel, the elaborated α-benzylthioketones were obtained in 10–20 mg amounts.

The SAR around the phenyl ring in the mandelic acid series paralleled the SAR for the corresponding phenylacetic acid derivatives as shown by their relative rank order of potencies (see pairs in Table 3). However, as the substitution on the ring fit better into the lipophilic part of the enzymatic pocket (compounds **28**–**35**), the potency advantage initially provided by the introduction of a hydroxyl group (Table 2) diminished and eventually vanished. It was thus concluded that the geometry of binding allowing optimal lipophilic interactions was not attainable while maintaining a strong hydrogen bond and vice versa. In the optimization process, we discovered that a simple iodo substituent could replace the oxadiazole on the phenyl ring to afford the most potent compound (**35**) in the NT2 whole cell assay.[10] Moreover, on top of being easy to synthesize, the iodo derivative offers a great handle to develop a cell-permeable radioactive tool.[11]

In summary, the discovery and exploration of a hydrogen-bonding effect allowed us to determine that two distinct and mutually exclusive binding modes existed in the P_3 region. The strong gain in potency upon hydrogen-bond formation allowed for the reduction of the molecular weight of our inhibitors, diminished the number of potentially metabolically labile sites and increased their selectivity against other caspases (e.g., compound **26**). With the simpler derivatives **15**–**26**, the potency of these inhibitors bottomed out at 146 nM. In the case where the lipophilic interactions were maximized by optimal substitution around the phenyl ring (**27**–**34**), the presence of the hydroxyl group diminished the selectivity of these derivatives. Future in vivo evaluation of each class of compounds should give different options for their development.

Table 3. Hydroxyl group effect in optimally substituted phenyl analogs



Compound	R^1	R^2	IC_{50} (nm)				
			C-1[a]	C-3[a]	C-7[a]	C-8[a]	NT2[b]
27	OMe	H	7070	53	3240	7910	5360
28	OMe	OH	4490	29	545	975	945
29	Br	H	3050	18	285	1890	—
30	Br	OH	1540	5	91	104	710
31	AC[c]	H	6190	13	555	3880	1225
32	AC[c]	OH	4850	16	290	635	1590
33		H	5450	9	97	3290	415
34		OH	2460	12	63	280	310
35	I	OH	675	16	135	98	224

[a] Recombinant human caspases 1, 3, 7, and 8. See Ref. 6.
[b] NT2 whole cell assay. See Ref. 10.
[c] Acetyl.
[d] 3-Methyl-1,2,4-oxadiazole. See Ref. 9.

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