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Dipeptidyl aspartyl fluoromethylketones as potent caspase inhibitors: peptidomimetic replacement of the P_2 α-amino acid by a α-hydroxy acid

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Abstract—As a continuation of our SAR studies of dipeptidyl aspartyl-fmk as caspase inhibitors, we explored the replacement of the P_2 α-amino acid by a peptidomimetic α-hydroxy acid. These α-carbamoyl-alkylcarbonyl-aspartyl fluoromethylketones were found to be potent caspase inhibitors, and the SAR of these compounds is similar to the corresponding dipeptidyl aspartyl-fmk. MX1153, (*S*)-3-methyl-2-(phenylcarbamoyl)butanoyl-Asp-fmk, is identified as a potent broad-spectrum caspase inhibitor, and is selective for caspases versus other proteases. MX1153 also has good activity in the cell apoptosis protection assays and is active in the mouse liver apoptosis model.

Caspases is a group of cysteine proteases with strict substrate specificity for aspartic acid as the P_1 amino acid.[1] Caspases can be divided into two groups. One group, represented by caspase-1, plays an important function in cytokine maturation.[2] The other group, including caspase-3, -8, and -9, plays a critical role in apoptosis by cleaving numerous important proteins.[3] Excessive apoptosis is known to be involved with various diseases, including acute diseases such as brain ischemia[4] and myocardial infarction,[5] as well as chronic diseases such as Huntington's disease and Alzheimer's disease.[6] Due to the important function of caspases in both inflammation and apoptosis, the discovery and development of caspase inhibitors could result in novel anti-inflammatory and anti-apoptotic drugs for the treatment of a variety of diseases.[7]

Many caspase inhibitors have been designed and synthesized based on substrate specificity of caspases. These include peptide based inhibitors,[8–10] and peptidomimetic-based inhibitors,[11] as well as non-peptide inhibitors discovered through screening of compound libraries.[12] Some of these are selective for specific caspases, while others are broad-spectrum caspase inhibitors. Two caspase inhibitors have advanced into clinical trials. VX-740 (Pralnacasan) is a peptidomimetic, reversible, and selective caspase-1 inhibitor, and was in clinical trial for the potential treatment of inflammatory diseases.[13] IDUN-6556 is a dipeptide based, irreversible, and broad-spectrum caspase inhibitor, and is in clinical trail for liver diseases.[14,15]

We have reported the discovery of MX1013 (Cbz-Val-Asp-fmk) as a potent and broad-spectrum caspase inhibitor, with potent in vivo activities in several animal models of apoptosis.[16–18] The crystal structures of caspase-1, -3, -7, and -8 in complex with tetrapeptide based inhibitors have been determined by X-ray crystallography,[19] and no hydrogen bonding between the P_2 NH of the inhibitors with the enzymes was found. Therefore the P_2 nitrogen contributes little to the binding of the inhibitors to the caspases. To maintain the preferred dipeptide scaffold of MX1013 and to reduce the peptide characteristics of the inhibitor, we elected to explore the replacement of the P_2 α-amino acid by a α-hydroxy acid (Chart 1). Herein we report the synthesis and biological evaluation of a group of α-hydroxy acid derivatives, α-carbamoyl-alkylcarbonyl-Asp-fmk, as caspase inhibitors.

(*S*)-2-(Phenylcarbamoyl)propionyl-Asp-fmk (**5a**) was prepared similar to the dipeptide-fmk inhibitors as reported previously.[17] (*S*)-(−)-2-(phenylcarbamoyl)propionic acid (**1a**) was coupled with amine **2**[20] to give amide **3a**.

Keywords: Caspase inhibitor; Apoptosis.
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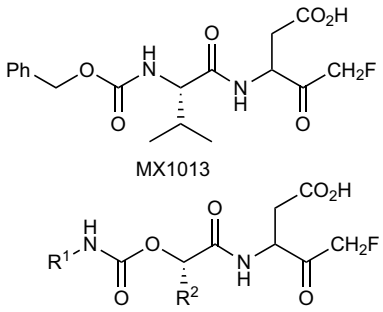
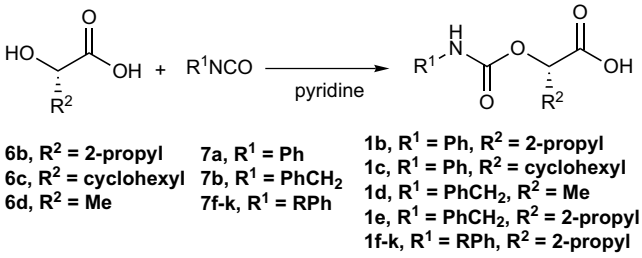


Chart 1.

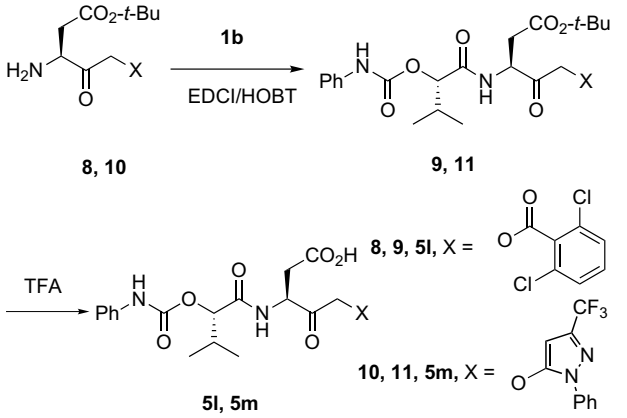
Oxidation of compound **3a** by Dess–Martin reagent produced the corresponding ketone **4a**. The *t*-Bu ester was then cleaved by TFA to give the free acid **5a** (Scheme 1). Other α-carbamoyl-alkylcarbonyl-Asp-fmk **5b–k** were prepared similarly from the carbamoyl protected α-hydroxy acids **1b–k**.

The carbamoyl protected α-hydroxy acids **1b–k** were prepared by reaction of the corresponding α-hydroxy acid with the corresponding phenyl or benzyl isocyanate (Scheme 2). Reaction of (*S*)-(+)-2-hydroxy-3-methylbutyric acid (**6b**) with phenyl isocyanate (**7a**) in pyridine produced (*S*)-3-methyl-2-(phenylcarbamoyl)butanoic acid (**1b**), and (*S*)-2-cyclohexyl-2-(phenylcarbamoyl)acetic acid (**1c**) was produced by reaction of (*S*)-(+)-hexahydromandelic acid (**6c**) with phenyl isocyanate. (*S*)-2-(Benzylcarbamoyl)propionic acid (**1d**) was prepared by reaction of L-(+)-lactic acid (**6d**) with benzyl isocyanate (**7b**), and (*S*)-2-(benzylcarbamoyl)-3-methylbutanoic acid (**1e**) was prepared similarly from reaction of (*S*)-(+)-2-hydroxy-3-methylbutyric acid (**6b**) with **7b**. Substituted (*S*)-3-methyl-2-(phenylcarbamoyl)butanoic acids **1f–k** were prepared similarly by reaction of (*S*)-(+)-2-hydroxy-3-methylbutyric acid (**6b**) with the corresponding commercial available substituted phenyl isocyanates **7f–k**.

(*S*)-3-Methyl-2-(phenylcarbamoyl)butanoyl-Asp-CH_2DCB (2,6-dichlorobenzoyloxy) (**5l**) was prepared as shown in Scheme 3. Coupling of NH_2-Asp(O-*t*-Bu)-CH_2DCB[21] (**8**) with (*S*)-3-methyl-2-(phenylcarbamoyl)butanoic acid



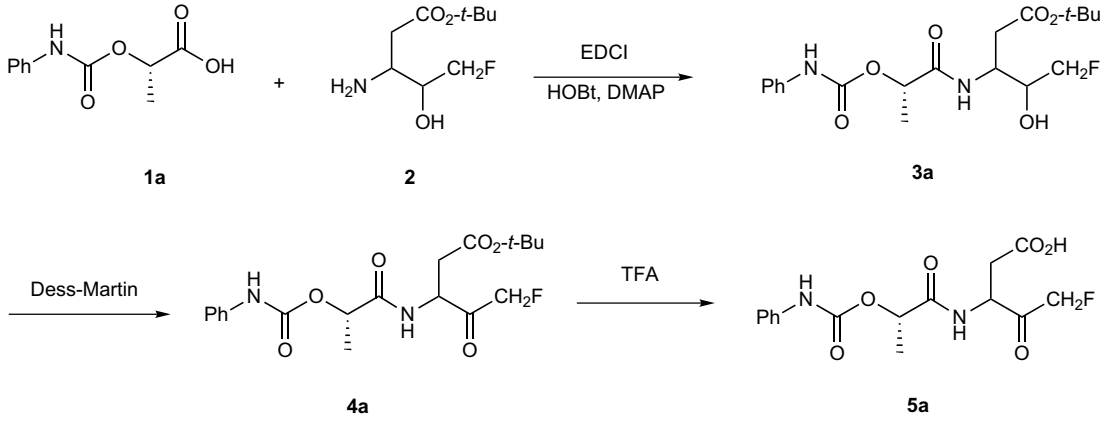
Scheme 2.



Scheme 3.

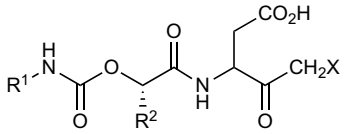
(**1b**) produced the amide **9**, and the *t*-Bu ester was removed by TFA to produce the acid **5l**. (*S*)-3-Methyl-2-(phenylcarbamoyl)butanoyl-Asp-CH_2PTP (1-phenyl-3-(trifluoromethyl)pyrazol-5-yloxy) (**5m**) was prepared similarly as shown in Scheme 3, by coupling of NH_2-Asp(O-*t*-Bu)-CH_2PTP[22] (**10**) with acid **1b** to produce amide **11**, followed by removal of the *t*-Bu ester.

The activity of these compounds to inhibit human recombinant caspase-3 was determined using a standard fluorometric assay,[16,23] and the results are summarized in Table 1. Compound **5a**, with a P_2 side chain equivalent to Ala, had an IC_{50} value of 66 nM against caspase-3. Compound **5b**, with a P_2 side chain equivalent



Scheme 1.

Table 1. Caspase-3 inhibiting activity and cell apoptosis protection activity of α-carbamoyl-alkylcarbonyl-aspartyl-CH_2X



Entry	R^1	R^2	X	IC_{50}[a] (nM)	50% cell protection[e] (nM)
5a	Ph	Me	F	66	1400
5b	Ph	2-Pr	F	17	200
5c	Ph	Cyclohexyl	F	50	ND[f]
5d	$PhCH_2$	Me	F	70	ND
5e	$PhCH_2$	2-Pr	F	20	ND
5f	3-F–Ph	2-Pr	F	6	ND
5g	4-F–Ph	2-Pr	F	19	ND
5h	3,4-DiF–Ph	2-Pr	F	14	ND
5i	2,4-DiCl–Ph	2-Pr	F	14	100
5j	2,5-DiCl–Ph	2-Pr	F	5	ND
5k	4-PhOPh	2-Pr	F	12	ND
5l	Ph	2-Pr	DCB[b]	20	1000
5m	Ph	2-Pr	PTP[c]	70	1100
MX1013		NA[d]		30	250

[a] IC_{50} is determined as described in Ref. 16.
[b] DCB, 2,6-dichlorobenzoyloxy.
[c] PTP, 1-phenyl-3-(trifluoromethyl)pyrazol-5-yloxy.
[d] NA, not applied.
[e] Concentration of inhibitor that provided 50% of cell protection is determined as described in Ref. 16.
[f] Not determined.

to Val, which is preferred for caspase-3, had an IC_{50} value of 17 nM, suggested that replacement of the P_2 α-amino acid by a α-hydroxy acid did not change the preference of the P_2 side chain. Compound **5c**, with a cyclohexyl group as the P_2 side chain, was about 3-fold less potent than **5b**, similar to what is observed from the dipeptide-fmk that valine is preferred over cyclohexylglycine as the P_2 amino acid for caspase-3 inhibitors.[17] Compound **5e** was >3-fold more potent than **5d**, further supporting the above conclusion that the preference of the P_2 side chain was maintained with replacement of the P_2 NH by an O. Interestingly, compound **5e**, with a structure almost identical to MX1013 except the reversal of the carbamate group, has similar potency as MX1013 as a caspase-3 inhibitor. These results are in agreement with the crystal structure of caspase-3, which shows that the P_2 backbone nitrogen is not important for interaction with the enzyme.

We then explored analogs of compound **5b** with fluoro, chloro, or phenoxy substituted on the Ph group, with the goal of increasing the hydrophobicity of the compounds. Due to the presence of the free carboxylic acid, these dipeptide inhibitors in general have relatively low hydrophobicity and *c*Log *P* values. For example, compound **5b** and MX1013 have *c*Log *P* values of 1.43 and 1.60, respectively. As shown in Table 1, these analogs **5f–k** have similar potency as, or are slightly more potent than **5b**, in the caspase-3 assay with IC_{50} values ranged between 5–19 nM. This is similar to what was observed with Cl and F substitutions on the Ph group of Z-V-D-fmk (MX1013),[24] those compounds also are equal or slightly more potent than MX1013 in a caspase-3 assay. Interestingly, compound **5k**, with a phenoxy group substituted in the 4-position of the Ph group, is about as potent as **5b**, indicating that a large group is tolerated in that position.

We also explored the incorporation of large DCB[9,21] and PTP[22] as the leaving groups, which were first successfully used for the preparation of caspase-1 inhibitors. Compound **5l**, with the DCB as the leaving group, was found to have similar potency as that of compound **5b**, in agreement with results obtained from other series of caspase inhibitors that a large group like DCB can be tolerated in the P' side.[9,21] Compound **5m**, with a PTP group, was found to be about 3.5-fold less active than **5l**. It is known that as a caspase-1 inhibitor, Z-Val-Asp-CH_2DCB is about 2-fold more active than Z-Val-Asp-CH_2PTP.[9,22]

Selected compounds were tested in the HeLa cell apoptosis protection assay,[16] which measures the protecting effects of caspase inhibitors against apoptosis induced by TNF-α. The viability of the cells was quantified by calcein AM uptake, and the concentration of inhibitor that provided 50% of cell protection is summarized in Table 1. Compound **5b** was found to protect 50% of the TNF-α treated cells at a concentration of 200 nM, which is similar to that of MX1013. Compound **5a** was 7-times less potent than compound **5b** in the cell protection assay, slightly more than the 4-fold difference observed from the caspase-3 enzyme assay. This is probably due to the larger P_2 side chain group in **5b** (*c*Log *P* = 1.43), which makes the compound more hydrophobic with increased *c*Log *P* value, and thus more cell permeable relative to compound **5a** (*c*Log *P* = 0.51). Compound **5i** (*c*Log *P* = 2.58), with a di-chloro substituted Ph group, and similar caspase-3 enzyme activity as that of **5b**, was found to be 2-fold more potent than **5b** in the cell protection assay, confirming that the increased hydrophobicity can increase cell permeability of the inhibitors, similar to what was observed for analogs of MX1013 with Cl and F substituted on the Ph group.[24] Compound **5l** (*c*Log *P* = 3.96) and **5m** (*c*Log *P* = 6.51), with a large and more hydrophobic groups in the P' side, and very high *c*Log *P* values, were ~5-times less active than **5b** in the cell protection assay.

Compounds **5b** (MX1153) was then tested against other caspases and proteases for selectivity and the results are summarized in Table 2. Compound **5b** was found to have high activity in caspase-1, -3, -6, -7, -8, and -9, with IC_{50} values between 11–50 nM. This is in agreement with the crystal structure of caspase-1, -3, -7, and -8 in complex with tetrapeptide inhibitors that the P_2 backbone nitrogen does not form hydrogen bonding with the enzymes and contributes little to the binding of the inhibitors to the enzymes. Testing against other proteases, compound **5b** was found to be inactive against other cysteine proteases, Calpain-1, Cathepsin B, as well as the serine protease, Factor Xa, at up to the high concentration of 100,000 nM. Therefore **5b** is >5000-fold

Table 2. Inhibiting activity of compound **5b** (MX1153) against different proteases

Enzyme	IC_{50}[a] (nM)
Caspase-1	12
Caspase-3	17
Caspase-6	50
Caspase-7	11
Caspase-8	17
Caspase-9	30
Calpain-1	>100,000
Cathepsin B	>100,000
Factor Xa	>100,000

[a] IC_{50} is determined as described in Ref. 16.

selective for caspase-3 versus these three non-caspase proteases. Similar to MX1013[17] and MX1122,[24] compound **5b** is a broad-spectrum caspase inhibitor and is selective for caspases versus other proteases. It is known that the free carboxylic acid in the P_1 aspartic acid of peptide based inhibitors is key for the specificity of caspases versus other proteases. Our data suggest that the non-specificity reported recently for several irreversible caspase inhibitors, including Z-DEVD-fmk, Z-YVAD-fmk, and Z-VAD-fmk,[25] most probably is because the inhibitors used for the assays were methyl esters, and not due to the irreversible fmk group.[26]

Compound **5b** was also tested in a mouse liver apoptosis model[16,27] to determine the in vivo anti-apoptotic efficacy of this novel caspase inhibitor. Mice were injected intravenously with anti-Fas monoclonal antibody, followed 5 min later by IV administration of various doses of compound **5b** formulated in an aqueous vehicle containing 50 mM Tris–HCl. The number of surviving animals in each group was determined at 1, 3, 6, 24 h and 3 days post-injection, and reported as a percentage of the total number of animals in each group (Table 3). Control mice, injected with 8 μg (microgram) of anti-Fas antibody and without treatment, were all dead by the 1 h time point. Compound **5b**, tested at 0.25 mg/kg, was able to protected 100% of the mice from the lethal effects of anti-Fas antibody at the 1 and 3 h time point, but none of the mice were protected at the 6 h time point. At 1 mg/kg, all the animals were protected at the 1 and 3 h time point, and 50% of the mice were protected at the 6 h time point. At 10 mg/kg dose, **5b** protected 100% of the mice at the 1, 3, and 6 h time points, and 83% at 24 h and 3 days time point. These results indicate that the in vivo protecting effects of **5b** is relatively short and a high dose of **5b** is needed to provide long lasting protection. Compound **5i**, which has similar caspase-3 inhibition potency as **5b**, and is about 2-fold more active than **5b** in the cell protection assay, also was tested in the in vivo model. Compound **5i** was slightly more active than **5b** in vivo, providing 100% protection at all time points at a dose of 10 mg/kg, and 66% protection at 6 h time point at a dose of 1 mg/kg. In comparison, MX1013 provided 100% protection in the same model at all time points at a dose of 1 mg/kg.[16] Since **5b** and MX1013 have similar activity in the caspase enzyme assays as well as in the cell protecting assays, the in vivo activity difference suggested that **5b** might have a shorter PK half-life. In fact, compound **5b** was found to have a relatively short plasma $t_{1/2}$ of about 0.2 h.

Table 3. Survival of mice with compound **5b** (MX1153) treatment after induction of apoptosis by anti-Fas antibody

Dose of **5b** (mg/kg)	% Survival ($n = 6$)				
	1 h	3 h	6 h	24 h	3 days
0	100	0	0	0	0
0.25	100	100	0	0	0
1	100	100	50	0	0
10	100	100	100	83	83

Mice were injected intravenously (IV) with 8 μg of anti-Fas antibody (clone JO-2; Pharmingen), followed 5 min later with IV injection of compound **5b** at the indicated doses or by vehicle. Six mice were treated in each group and the number of surviving mice was monitored for up to 3 days, when the experiment was terminated.

In conclusion, based on the crystal structure of caspases bound with tetrapeptide based inhibitors, we have designed and synthesized a group of novel peptidomimetic based caspase inhibitors by replacement of the P_2 α-amino acid with a α-hydroxy acid. These α-carbamoyl-alkylcarbonyl-Asp-fmks were found to have similar SAR and to be as potent as that of dipeptidyl aspartyl-fmk, in agreement with the discovery from the crystal structure of caspases that the P_2 backbone nitrogen of caspase inhibitors does not contribute to the binding with the enzymes. Substitution of chloro and fluoro groups in the phenylcarbamoyl capping group had little effect on their potency as caspase-3 inhibitors. In the cell apoptosis protecting assays, compounds that are more hydrophobic were found to be more active, suggesting that the increased hydrophobicity makes them more cell permeable. Compound **5b** (MX1153) is identified as a potent and broad-spectrum caspase inhibitor. It is selective for caspases versus other proteases and is highly active in the cell apoptosis protection assays. Compound **5b** also was found to be active in the mouse liver apoptosis model.

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