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Design and Synthesis of Inhibitors Incorporating β-Amino Acids of Metalloendopeptidase EC 3.4.24.15

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Abstract: Endopeptidase EC 3.4.24.15 (EP 24.15) is a thermolysin-like metalloendopeptidase which is expressed widely throughout the body, with the highest concentrations in the brain, pituitary and testis. While the precise role of EP 24.15 remains unknown, it is thought to participate in the regulated metabolism of a number of specific neuropeptides. Of the limited number of inhibitors described for EP 24.15, *N*-[1-(*R*,*S*)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-amino benzoate (CFP) is the most widely studied. CFP is a potent and specific inhibitor, but is unstable *in vivo* due to its cleavage between the alanine and tyrosine residues by the enzyme neprilysin (EP 24.11). The cpp-Ala-Ala *N*-terminal product of this cleavage is a potent inhibitor of angiotensin converting enzyme, which further limits the use of CFP *in vivo*. To generate specific inhibitors of EP 24.15 that are resistant to *in vivo* proteolysis by EP 24.11, β-amino acids have been incorporated into the structure of CFP. We have prepared racemic mixtures of β-amino acids containing proteogenic side chains, which are 9-fluorenylmethoxycarbonyl (Fmoc)-protected, and several analogues of CFP containing β-amino acids have been synthesized by solid phase peptide synthesis. The results of stability and inhibitory studies of these new analogues show that the incorporation of β-amino acids adjacent to the scissile bond can indeed stabilize the peptides against cleavage by EP 24.11 and still inhibit EP 24.15. The results obtained in these studies demonstrate the potential of these amino acids in the synthesis of peptidomimetics and in the design of new stable and specific therapeutics.

Keywords: β-amino acid; endopeptidase inhibitors; metalloendopeptidase EC 3.4.24.15; neprilysin

INTRODUCTION

Thimet oligopeptidase (EC 3.4.24.15, EP24.15) is a zinc containing metalloendopeptidase that is widely distributed throughout the body [1]. While the three dimensional structure is unknown, the active site structure, which features the His-Glu-X-X-His (HEXXH) motif, places EP 24.15 in the thermolysin-like metalloendopeptidase family [2]. *In vitro*, EP 24.15 cleaves bradykinin, gonadotrophin releasing hormone, neurotensin, dynorphin A 1-8, somatostatin and substance P [3,4]. However, the precise role of EP 24.15 *in vivo* is still to be characterized, although it has been implicated in the metabolism of vasoactive peptides and neuropeptides [5]. The inability to characterize the physiological role of EP 24.15 is due in part to the lack of stable and specific inhibitors. One potent inhibitor of EP 24.15 is the compound carboxyphenylpropyl-Ala-Ala-Tyr-*p*-aminobenzoate (CFP) with a K_i of 16 nM [6] as shown in Figure 1. However, *in vivo* this inhibitor is cleaved by the endopeptidase EC 3.4.24.11, to give the product cpp-Ala-Ala which is a potent inhibitor of angiotensin converting enzyme which, thus, limits the use of CFP for *in vivo* studies [7,8]. Modification of this inhibitor by the incorporation of aminoisobutyric acid has yielded an inhibitor which

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is resistant to proteolytic cleavage by EP 24.11 both *in vivo* and *in vitro* with a K_i of 22 nM [9].

More recently, the incorporation of β-amino acids into peptide sequences has emerged as an alternative approach to stabilizing peptides against proteolytic attack [10]. However, studies published so far have not yet demonstrated whether proteolytic-resistant peptides containing β-amino acids are still able to bind to proteolytic enzymes. This must be clearly established if β-amino acid containing peptides are to be considered as potential lead compounds in the development of enzyme inhibitors for therapeutic applications. In the present study, we have developed new analogues of CFP containing β-amino acids and which are stable to proteolysis by EP 24.11. In particular, the *C*-terminal *p*-aminobenzoic acid has been replaced with a β-glycine and the alanine residue at the scissile bond has been replaced by β-alanine. While the IC_{50}s were in the μM range, all peptides containing a β-amino acid at the scissile bond were resistant to cleavage by EP 24.11, clearly demonstrating the potential of β-amino acids in the design of novel peptidomimetics.

MATERIALS AND METHODS

Chemicals and Solvents

All solvents were analytical reagent (AR) grade except acetonitrile which was high pressure liquid chromatography (HPLC) grade and dimethylformamide (DMF) which was peptide synthesis grade. Dichloromethane, methanol and DMF were stored over 4 Å sieves. Petroleum ether (petrol) was boiling range 60–80 °C and distilled prior to use. Tetrahydrofuran (THF) was distilled from sodium and benzophenone prior to use. Water was distilled and deionized in the Milli-Q system (Millipore, Bedford, MA, USA). Benzylamine, ethyl-2-oxo-4 phenyl butyrate, methyl crotonate, methyl methacrylate, methyl propiolate, palladium hydroxide on carbon (Perlman's catalyst), sodium carbonate, sodium cyanoborohydride, sodium hydride, sodium sulphate and triethylamine were obtained from Aldrich Chemicals (Milford, MA, USA). Hydrochloric acid was obtained from BDH Chemicals (Poole, UK). Deuterium oxide and deuterochloroform were obtained from Cambridge Isotope Laboratories (Cambridge, UK). Hydrogen and nitrogen were obtained from BOC gases (Sydney, Australia). Chloroform was obtained from Mallinkrodt (Kentucky, USA). Dichloromethane, diethyl ether, ethyl acetate, glacial acetic acid and petroleum spirit 60–80 °C (petrol) were obtained from Selby-Biolab (Melbourne, Australia). 4-*N*-*N*-Dimethylaminopyridine was obtained from Sigma Chemical Co. (St Louis, MO, USA). Acetonitrile, methanol and sodium hydroxide were obtained from Ajax Chemicals (Melbourne, Australia). Dioxan was obtained from Matheson, Coleman and Bell (Norwood, OH, USA). ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a 300 MHz Varian Mercury 300 spectrometer. All ^{1}H NMR spectra were recorded in deuterochloroform, unless stated otherwise, using tetramethylsilane (TMS) as an internal standard. ^{13}C spectra were recorded at 75 MHz on the Varian Mercury 300 NMR spectrometer with proton decoupling at 300 MHz. All ^{13}C spectra were recorded in deuterochloroform, unless stated otherwise, with TMS as an internal standard.

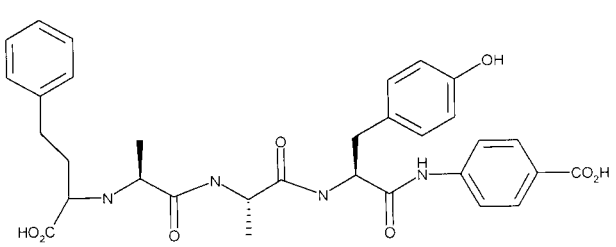

Figure 1 The structure of EP 24.15 inhibitor cpp-Ala-Ala-Tyr-*p*Ab (CFP).

Synthesis of Fmoc-β3Ala

Methyl N-benzyl-2-aminobutyrate (1). A solution of methyl crotonate (10 g, 99.9 mmol) and benzylamine (10.5 ml, 99.9 mmol) in methanol (190 ml) was stirred at room temperature for 48 h. The solvent was then removed to yield a yellow oil (19.1 g, 92.3%). ^{1}H NMR (300 MHz, $CDCl_3$) δ7.26-7.24 (m, 5 H), 3.86–3.73 (*q*, $J = 11.4$ Hz, 2 H), 3.67 (s, 3 H), 3.19–3.13 (m, $J = 6$ Hz, 1 H), 2.54–2.36 (m, $J = 20$ Hz, 2 H), 1.17–1.15 (*d*, $J = 6$ Hz, 3 H).

Methyl 2-aminobutyrate (2). A solution of methyl *N*-benzyl-2-aminobutyrate (1) (10 g, 48 mmol) was stirred with $Pd(OH)_2/C$ (4.6 g) in a 1% solution of acetic acid in a 1:1 mixture of methanol and water (273 ml) under an atmosphere of hydrogen. The solution was stirred overnight and then filtered through celite, which was then washed with methanol. The solvent was removed to yield a yellow oil. ^{1}H NMR (300 MHz, D_2O) δ3.65–3.56 (m, 1 H), 3.58 (s, 3 H), 2.63–2.59 (*dd*, $J = 2$ Hz, 3.5 Hz, 2 H), 1.20–1.18 (*d*, $J = 6.7$ Hz, 3 H).

2-Aminobutyrate (3). The oil (2) was dissolved in a 50% HCl solution and stirred at 70 °C overnight. The solvent was then removed to yield a yellow oil. ^{1}H NMR (300 MHz, D_2O) δ3.57 (m, 1 H), 2.61–2.58 (m, $J = 2$ Hz, 4 Hz, 2 H), 1.21–1.18 (*d*, $J = 6$ Hz, 3 H).

Fmoc 2-aminobutanoic acid (4). Acid (3) was dissolved in a 1:1 mixture of dioxan/10% Na_2CO_3 (300 ml) and 9-fluorenylmethoxycarbonyl-chloride (Fmoc-Cl) (12.4 g, 0.048 mmol) was slowly added. After stirring overnight, water (100 ml) was added and the resulting solution was extracted with ether (2 × 200 ml). The aqueous phase was then acidified with 10% citric acid and the resultant precipitate was filtered and washed with water. The precipitate was allowed to air dry yielding a white powder (5.119 g, 31.5% overall). The Fmoc-amino acid was then used directly for peptide synthesis with no further purification. It could be recrystallized from ethyl acetate/petrol to yield white crystals. M_p 158–160 °C. ^{1}H NMR (300 MHz, $CDCL_3$) δ7.75 (*d*, $J = 7.5$ Hz, 2 H), 7.58 (*d*, $J = 6.9$ Hz, 2 H), 7.37 (*t*, $J = 7.5$ Hz, 2 H), 7.29 (*t*, $J = 7.5$ Hz, 2 H), 5.20 (brs, 1 H), 4.37 (br, 2 H), 4.19 (*t*, $J = 6.3$ Hz, 1 H), 4.15–4.07 (m, 1 H), 2.57 (br, 2 H). ^{13}C NMR (75 MHz, $CDCl_3$) δ143.8 C, 141.3 C, 127.7 CH, 127.1 CH, 125.0 CH, 120.0 CH, 66.9 CH_2, 62.5 CH_2, 47.4 CH, electrospray MS: 326.1.

Synthesis of Fmoc-β2Ala

Methyl N-benzyl-3-amino-2-methylpropionate (5). A solution of methyl methacrylate (10 g, 99.9 mmol) and benzylamine (10.5 ml, 99.9 mmol) in methanol (140 ml) was stirred at room temperature for 48 h. The solvent was then removed to yield an orange oil (14.0 g, 68%). The product was purified by chromatography on a 3-cm diameter column packed with silica (Merck LiChroprep Si 60, 25–40 μm, Merck, Darmstadt, Germany) and eluted with a 1:1 mixture of petrol/ethyl acetate. The solvent from the pooled fractions was removed to yield an orange oil (6.96 g, 33%). ^{1}H NMR (300 MHz, $CDCL_3$) δ7.32 (s, 2 H), 7.30 (s, 2 H), 7.24 (m, 1 H), 3.78 (s, 2 H), 3.67 (s, 3 H), 2.88 (m, 1 H), 2.67 (m, 2 H), 1.61 (Br, 1 H), 1.18 (*d*, $J = 7$ Hz, 3 H). ^{13}C NMR (75 MHz, $CDCl_3$) δ171.5, 135.8, 123.8, 123.4, 122.3, 49.3, 47.7, 47.1, 35.7, 10.9.

Methyl 3-amino-2-methylpropionate (6). A solution of methyl *N*-benzyl-3-amino-2-methyl-propionate (5) (2 g, 9.66 mmol) was stirred with $Pd(OH)_2$/C (0.9 g) in a 1% solution of acetic acid in a 1:1 mixture of methanol and water (50 ml) under an atmosphere of hydrogen. The solution was stirred overnight and then filtered through celite, which was washed with methanol. The solvent was removed to yield a pale red oil (1.92 g). ^{1}H NMR (300 MHz, DMSO-d_6) δ3.62 (s, 3 H), 3.03–2.90 (br, 1 H), 2.8–2.7 (br, 2 H), 1.15–1.10 (*d*, $J = 7$ Hz, 3 H). ^{13}C NMR (75 MHz, DMSO-d_6) δ170.6, 47.7, 37.8, 19.5, 10.6.

3-Amino-2-methylpropionic acid (7). Ester (6) was dissolved in a 6 M HCl solution (25 ml) and stirred at 60 °C overnight. The solvent was then removed to yield a cream coloured solid (1.81 g). ^{1}H NMR (300 MHz, D_2O) δ3.22–3.03 (m, $J = 8.6$, 4.8 Hz, 2 H), 2.90–2.78 (m, $J = 4.9$ Hz, 1 H), 1.22–1.19 (*d*, $J = 7.3$ Hz, 3 H). ^{13}C NMR (75 MHz, D_2O) δ172.6, 36.9, 32.5, 9.64.

Fmoc 3-amino-2-methylpropionic acid (8). The oil (7) was dissolved in a 1:1 mixture of dioxan/10% Na_2CO_3 (60 ml) and Fmoc-Cl (5.0 g, 0.019 mol) was slowly added. After stirring overnight water (50 ml) was added and the resulting solution was extracted with ether (2 × 100 ml). The aqueous phase was then acidified with 10% citric acid and the resultant precipitate was filtered and washed with water. The white powder was allowed to air dry to yield a white powder (1.14 g, 36% overall). The Fmoc-amino acid was then used directly for peptide synthesis with no further purification. It could be recrystallized from ethyl acetate/petrol to yield white crystals. M_p 162–164 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ7.75–7.72 (*d*, $J = 7.5$ Hz, 2 H), 7.57–7.54 (*d*, $J = 7.3$ Hz, 2 H), 7.37 (*t*, $J = 7$ Hz, 2 H), 7.28 (*t*, $J = 7$ Hz, 2 H), 4.53–4.41 (m, 2 H), 4.26–4.15 (m, 1 H), 3.45–3.05 (m, 2 H), 2.79–2.28 (m, 1 H), 1.21–0.98 (*dd*, $J = 7$ Hz, 3 H). ^{13}C NMR (75 MHz, $CDCL_3$) δ180.1 CO, 143.6 C, 141.0 C, 127.5 CH, 126.8 CH, 124.8 CH, 119.8 CH, 66.7 CH_2, 47.1 CH, 43.1 CH, 39.8 CH_2, 14.6 CH_3.

Peptide Synthesis

O-Benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluroniumhexafluorophosphate (HBTU), di-*tert*-butyl dicarbonate, diisopropylcarbodiimide (DIC), diisopropylethylamine (DIPEA), DMF, Fmoc-Cl, Fmoc-tyrosine, Fmoc-alanine and Fmoc β-glycine (Fmoc–3-aminopropionic acid), 1-hydroxybenzotriazole (HOBt), piperidine and trifluoroacetic acid (TFA) were obtained from Auspep (Melbourne, Australia).

The coupling of Fmoc-β-Gly onto hexamethylphosphoric triamide TentaGel (HMP)-TG resin (Novabiochem, Laufelfingen, Switzerland) as follows: 1 g (0.15 mmol/g loading) of resin was swollen for 10 min in DMF (30 ml) with occasional stirring. The Fmoc-β-Alanine (466 mg, 1.5 mmol) and 4-dimethylaminopyridine (DMAP) (18.3 mg,

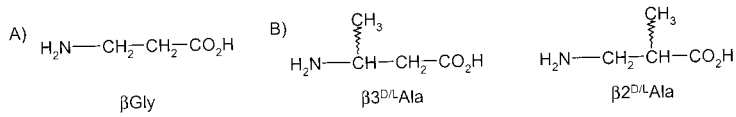

Figure 2 Structures of β-amino acids used in this study.

0.15 mmol) were dissolved in a small quantity of DMF (5 ml). This mixture was then added to the solution containing the resin and DIC (189 mg, 234 μl, 1.5 mmol) was added and the solution was mixed by nitrogen bubbling. After the reaction was left overnight the mixture was transferred to a synthesis vessel and the resin was washed with DMF (5 × 40 ml), methanol (3 × 40 ml) and ether (3 × 40 ml) and allowed to dry under vacuum. Both α- and β-amino acids were then coupled using standard Fmoc-based protocols.

Reductive Alkylation

The *N*-terminal addition of 1-carboxy-3-phenylpropyl moiety to each peptide was achieved via a reductive alkylation of 2-oxo-4-phenyl butyrate to the free *N*-terminal amine [11]. Ethyl-2-oxo-4-phenylbutanoate (2.0 g, 1.83 ml, 9.6 mmol) was hydrolysed by stirring in a solution of methanol (35 ml) and 1 M NaOH (25 ml) for 5 h at room temperature. After reaction the solution was neutralized with 6 M HCl and the solvent removed to yield a white solid. The solid 2-oxo-4-phenyl-butanoic acid was recovered by washing with DMF (10 ml) to give a 6.7 M stock solution. To a solution of peptide-bound resin in DMF (10 ml), 2-oxo-4-phenylbutanoic acid (250 μl of the 6.7 M solution) and acetic acid (200 μl) were added. The mixture was stirred for 5 min after which $NaCNBH_4$ (150 mg) was added. The solution was gently stirred overnight with a magnetic stirrer at room temperature. After reaction, the resin was filtered and washed with DMF (10 × 30 ml) followed by acetic acid (1 × 30 ml), DCM (6 × 30 ml), methanol (3 × 30 ml) and ether (3 × 30 ml) after which the resin was dried in a desicator overnight.

In vitro Inhibition and Binding Studies

Inhibition of EP 24.15 by CFP analogues. Each peptide analogue was screened for inhibitory activity against the cleavage of the synthetic quenched fluorescent substrate (QFS), 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Pro-D-Lys(2,4-dinitrophenyl) by recombinant (rat testes) EP 24.15 [4]. Using a 96 well plate 25 μl of a peptide dilution to give final concentrations of 10 μM, 1 μM, 100 nM and 10 nM was added to 10 μl of 24.15 to give 50 ng per well and the volume made up to 230 μl with 195 μl TBS containing 0.1 mM dithiothreitol (DTT). Controls with either no inhibitor or no enzyme were included in the assay. The 96 well plate was pre-incubated at 37 °C in the oven of a fluorometric plate reader (F_{max}, Molecular Devices, Sunnyvale, CA, USA) for 30 min. After the pre-incubation period 20 μl of the 0.05 mg/ml QFS solution was added to each well. The plate was incubated for a further 30 min after which the fluorescence was read directly by the plate reader with an excitation wavelength of 314 nm and emission detected at 418 nm. The IC_{50} of the peptide was determined by calculating the percentage inhibition by comparison with the control containing no inhibitor and the IC_{50} determined from the plot of % inhibition against peptide concentration.

Inhibitor Degradation by Kidney Membranes. The stability of the inhibitors to degradation by EP 24.11 were assayed by incubating 5 μg per time point of each inhibitor with 30 μg of rabbit kidney membrane proteins in 400 μl of TBS buffer. Rabbit kidney membranes were used since they are a rich source of EP 24.11 and were prepared as previously described [4]. The peptide and protein extract were incubated at 37 °C and 50 μl aliquots were removed at times 0, 30 min, 1, 2, 4, 6 and 24 h, diluted in 200 μl of methanol/1% TFA. The samples were dried on a centrifugal vacuum evaporator prior to HPLC analysis using a Hewlett–Packard 1100 series LC with on-line mass spectrometric detector (Agilent Technologies, Melbourne, Australia). Samples were loaded onto a Zorbax Eclipse C18 column (at 50 °C) in 1.8% acetonitrile/0.1% TFA/0.02% acetic acid at 0.1 ml/min, and eluted with a 60 min linear gradient to 60% acetonitrile/0.1% TFA. Degradation fragments were identified following mass spectral analysis using Hewlett–Packard ChemStation deconvolution software.

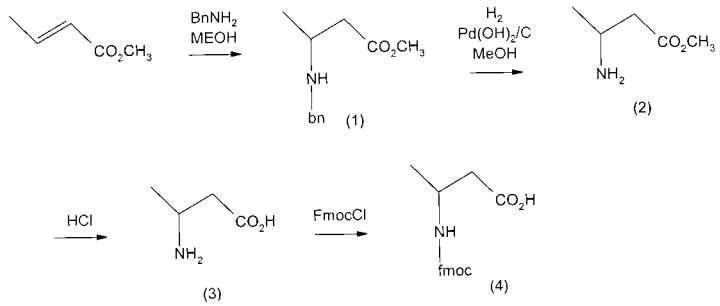

Figure 3 Synthetic scheme for the preparation of β3-Ala.

RESULTS

Synthesis of β-amino acids

The structures of the three β-amino acids used in this study are shown in Figure 2. The synthesis of the two β-alanine analogues (Figure 2(B)) was performed via the conjugate addition of an amine to α,β-unsaturated esters [12] which provided rapid access to the Fmoc-β-amino acids. This method resulted in the formation of all four possible enantiomers of β-alanine in four steps from readily available starting materials which then allowed the rapid screening of a range of stereoisomers of peptides for activity.

The synthesis of the racemic β-C3 methylated β-alanine (β3Ala) involved the conjugate addition of benzylamine to methyl crotonate in methanol as shown in Figure 3. After the reaction was complete, work up yielded a yellow oil which was mostly compound (1) in Scheme 1 as characterized by NMR and no further purification was necessary. Deprotection of the amino and carboxylic acid groups was achieved via hydrogenation and acid hydrolysis [13]. After work-up, an NMR spectrum of the resultant yellow oil showed that while the debenzylation of the amine had gone to completion, the methyl ester remained unhydrolysed. The oil was then treated with 50% HCl overnight at 70 °C which resulted in the complete hydrolysis of the ester to give (3) which was then Fmoc-protected. The Fmoc amino acid (4) was obtained as a white powder and used for peptide synthesis without further purification.

The synthesis of the C2 substituted β-alanine analogue (β2Ala) is shown in Figure 4 and was initiated with the conjugate addition of benzylamine to methyl methacrylate. The conjugate addition was again performed at room temperature in methanol and was complete after overnight reaction to give (5). The hydrogenation of (5) was carried out as described for the synthesis of β3-Ala and the reaction was complete overnight. Again the methyl ester (6) remained which was removed in 6 M HCl at 60 °C overnight. The resultant white solid was the pure amino acid (7) which was then Fmoc-protected and the white powder (8) was obtained ready for use in peptide synthesis without any further purification.

Peptide Synthesis and Reductive Alkylation of the *N*-terminal Residue

Table 1 lists the sequences of the peptides synthesized in this study. The attachment of the *C*-terminal β-Gly residue to the resin and the subsequent coupling of both α- and β-amino acids followed standard Fmoc-based coupling and deprotection techniques. All coupling and deprotections involving the racemic β-amino acids proceeded without any apparent difficulty and only single couplings were necessary. The carboxyphenylpropyl (cpp) group was incorporated into the peptide by reductive alkylation of the peptide with 2-oxo-3-phenylpropyl carboxylic acid as shown in Figure 5 [11]. This reaction was performed after the second alanine was deprotected and before the cleavage of the peptide from the solid phase resin. The HMP-TG resin was used for peptide synthesis due to its higher stability toward cyanoborohydride, which is used in the reductive alkylation.

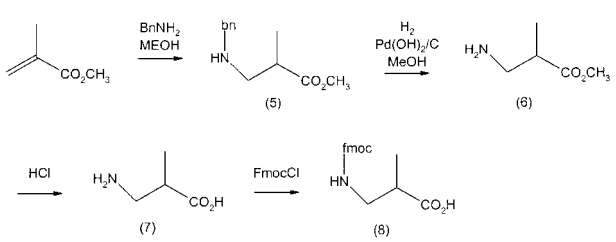

Figure 4 Synthetic scheme for the preparation of β2-Ala.

Table 1 Amino Acid Sequence, EP 24.15 Inhibitory Activity and Extent of Degradation of CFP-Peptides

Code	Sequence	Calc	Obs	IC_{50}	Extent of degradation		
		M_r	M_r	(μM)	30 min	6 h	24 h
CFP-1	cfp-A-A-Y-pAb	604	604.3	0.06	XX	–	–
CFP-2	cfp-A-A-Y-βG	556	556.2	0.12	XX	–	–
CFP-3	cfp-A-βG-Y-βG	556	556.3	5.6	O	O	O
CFP-4	cfp-A-β2A-Y-βG	570	570.2	6.3	O	O	O
CFP-5	cfp-A-β3A-Y-βG	570	570.3	>300	O	O	O

O–no degradation; XX–80–100% degradation.

Biological Activity

Table 1 lists the sequences of the CFP-related peptides used in this study. The *p*-aminobenzoate (*p*Ab) moiety at the *C*-terminus was included in the original design of the parent peptide [6] for a number of reasons. First, it protected the inhibitor from cleavage by carboxypeptidases and second, it acted as a chromophore in the enzymatic assays. Third, it provided a hydrophobic group and a carboxyl group, which were both thought to be important for binding to EP 24.15. All previous reports of the synthesis of CFP involved the use of either solution phase or *t*-Boc-based solid phase synthesis techniques. Since the incorporation of the β-amino acids into peptides involved Fmoc chemistry, our aim was to develop a Fmoc-based protocol for the total synthesis of the CFP-analogues. While this required the initial coupling of the *p*Ab group to the resin, significant difficulties were encountered in the attachment of Fmoc-protected *p*Ab to the resin. In order to explore the possible role of β-amino acids as stabilizers of peptide bonds against proteolytic cleavage at the *C*-terminus, it was decided to substitute the *p*Ab group with a β-glycine (β-Gly) residue. A specific chromophore is not required in the present study since the EP 24.15 assay is now based on a quenched fluorescence substrate. In addition, the β-Gly residue can also protect the peptide from *C*-terminal proteolytic degradation, and it contains an ethyl and carboxyl group to bind to the corresponding sites in the EP 24.15 active site. Furthermore, Fmoc-protected β-Gly can be easily incorporated into a peptide sequence via standard protocols.

Peptide CFP-2 corresponds to the peptide in which the *p*Ab group has been replaced by a β-Gly group. This modification resulted in only a small decrease in the IC_{50} value indicating that the presence of the β-Gly residue does not significantly alter the binding of the parent CFP inhibitor to EP 24.15. In addition, as shown in Table 1, the extent of degradation of CFP-2 in the presence of kidney membranes, is similar to that observed for the parent peptide CFP-1, further demonstrating that the substitution of β-Gly for the *p*Ab group does not result in any significant changes to the biological activity of this peptide.

The next modification was the substitution of a β-amino acid at the scissile bond of CFP-2 to stabilize the compound against proteolytic cleavage by EP 24.11. In the first instance, β-Gly was substituted for the Ala residue at the scissile bond to give CFP-3. This resulted in a 50-fold decrease in IC_{50}, but was still in the μM range. Most significantly, however, there was no evidence of proteolytic cleavage of CFP-3 upon incubation with kidney membranes (a rich source of EP 24.11) after 24 h (see Table 1). This is in contrast to the total degradation of CFP-1 after 30 min. Thus, while CFP-3 still binds to EP 24.15, the presence of the β-Gly at the scissile bond has prevented cleavage of the peptide by EP 24.11.

The alanine residue at the scissile bond was then replaced by either a βC2-Ala or βC3-Ala to give CFP-4 and CFP-5 respectively. CFP-4 exhibited a similar IC_{50} to CFP-3, demonstrating that the addition of a methyl group at the 2-position has little effect on the binding of the peptide to EP 24.15. However, the inhibitory activity against EP 24.15 was abolished completely with CFP-5 demonstrating that, while a methyl group can be tolerated at the 2 position, the presence of the methyl group at the 3 position prevents the binding of CFP-5 to the

EP 24.15 active site. CFP-5 was also not degraded by EP 24.11.

DISCUSSION

The mechanism of peptide cleavage by zinc-metalloendopeptidases involves the activation of a zinc coordinated water molecule, which then acts as the nucleophile in the proteolytic attack of the peptide substrate [14]. The main strategy adopted in the design of inhibitors of metalloendopeptidases has been to incorporate a zinc-binding motif, such as phosphono-, sulfydryl-, hydroxamic acid or carboxyl- groups, which will bind to the catalytic zinc [15]. Enzyme specificity between metalloendopeptidases is then engineered through variation in the chemical moieties that flank the zinc-coordinating group that can be accommodated in the substrate binding pockets. In the case of EP 24.15, a number of inhibitors have been designed with a carboxyphenlypropyl group [6,9], a thiol [16] or a phosphinic group [17] as the zinc-coordinating group. Approaches to stabilizing the scissile bond between alanine and tyrosine have also been reported. Reduction of the carbonyl group at the scissile bond to a methylene group resulted in a stable compound but inhibitory activity was lost while the incorporation of aminoisobutyric acid was active and was also resistant to proteolytic cleavage [9].

In the present study, we have demonstrated the potential of β-amino acids to stabilize peptide bonds against proteolytic attack at two sites in the EP 24.15 inhibitor CFP. First, the use of β-Gly at the *C*-terminus in place of *p*Ab (CFP-2) led to an inhibitor that was equally potent against EP 24.15 as the parent CFP-1. This result indicates that the alkyl and carboxyl moieties of β-Gly are sufficient for recognition by EP 24.15. CFP-2 was also cleaved by EP 24.11 in an identical manner to CFP-1, further demonstrating the similar structural properties of CFP-1 and CFP-2. The β-Gly residue was therefore subsequently included as the *C*-terminal residue in place of the *p*Ab group, to protect all peptides against possible cleavage by carboxypeptidases.

The second site of proteolytic cleavage in CFP-1 is the peptide bond between Ala and Tyr. While substitution of the scissile alanine residue by β-Gly or β2-Ala resulted in some loss of inhibitory activity of CFP-3 and CFP-4, both peptides were also totally resistant to proteolytic attack by EP 24.11. This striking observation clearly demonstrates that peptides containing β-amino acids can bind to proteolytic enzymes and, hence, represent potential lead molecules in the design of enzyme inhibitors. It should also be noted that CFP-4 was assayed as a racemic mixture of the β2Ala residues. Given that previous studies in which the introduction of D-amino acids resulted in the abolition of inhibitory activity of CFP [4,17], it is likely that only one isomer is active. In contrast, the presence of the β3-Ala (CFP-5) totally abolished inhibitory activity clearly demonstrating the steric exclusion of even small side chains from this sub-site in the EP 24.15 active site. This peptide was also resistant to cleavage by EP 24.11. While the focus of the present study was the inhibition of EP 24.15, it is possible that the CFP analogues may also bind to EP 24.11. Studies are thus underway to determine if these peptides bind to EP 24.11. A positive result would indicate that the β-amino acid-containing CFP analogues should serve as lead compounds for the design of new inhibitors of EP 24.11.

The likely mechanism for the stabilization of the peptide bond by the β-amino acids involves the displacement of the peptide scissile bond from the active site reflecting the presence of the additional carbon atom in the backbone, which then prevents proteolysis. This is shown schematically in Figure 6 where the zinc-bound water molecule binds to the scissile carbonyl of the α-peptide (upper panel), an interaction that cannot occur with the β-amino acid-containing peptides (lower panel).

In conclusion, substitution of β-analogues of α-amino acids adjacent to the scissile bond in CFP-1

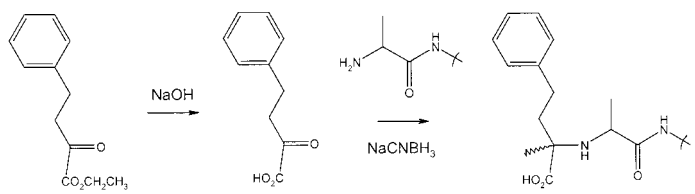

Figure 5 Synthetic scheme for the reductive alkylation of 2-oxo-3-phenylpropyl carboxylic acid at the *N*-terminus of the peptide.

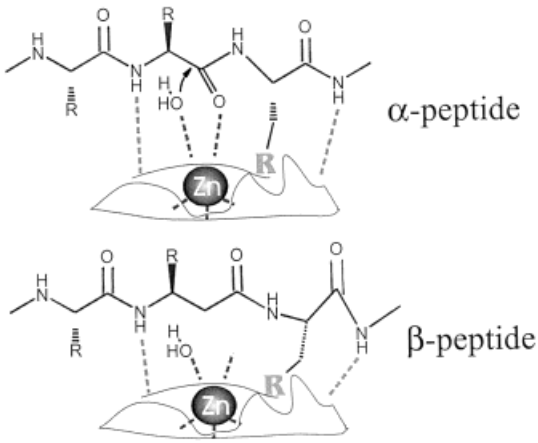

Figure 6 Schematic diagram of the binding of an α-peptide (upper panel) and a β-amino acid-containing peptide (lower panel) to the active site of EP 24.15 demonstrating how a β-peptide binds but is not cleaved by the peptidase.

has stabilized the peptide against proteolysis by EP 24.11 while still maintaining inhibition of EP 24.15. The advantages in the use of β-amino acids to stabilize peptide bonds is the ease of preparation and their ease of incorporation into peptide sequences using standard solid-phase peptide synthesis techniques. Furthermore, there is increased opportunity to maintain or enhance enzyme specificity through the use of naturally occurring substrates into which the appropriate β-amino acid peptide bond stabilization can be engineered. While further modification to the CFP sequence is required to improve the potency of the CFP-related peptides, the results of the present study clearly show that β-amino acid-containing peptides can bind to proteolytic enzymes and demonstrate the potential of β-amino acids in the design of stable, potent and specific enzyme inhibitors.

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