Inhibitors of Metalloendopeptidase EC 3.4.24.15 and EC 3.4.24.16 Stabilized against Proteolysis by the Incorporation of β-Amino Acids

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ABSTRACT: The enzyme EC 3.4.24.15 (EP 24.15) is a zinc metalloendopeptidase whose precise function in vivo remains unknown but is thought to participate in the regulated metabolism of a number of specific neuropeptides. The lack of stable and selective inhibitors has hindered the determination of the exact function of EP 24.15. Of the limited number of EP 24.15 inhibitors that have been developed, N-[1-(*R*,*S*)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate (CFP) is the most widely studied. CFP is a potent and specific inhibitor, but it is unstable in vivo due to cleavage between the alanine and tyrosine residues by the enzyme neprilysin (EP 24.11). This cleavage by EP 24.11 generates a potent inhibitor of angiotensin converting enzyme, thereby limiting the use of CFP for in vivo studies. To develop specific inhibitors of EP 24.15 that are resistant to in vitro and potentially in vivo proteolysis by EP 24.11, this study incorporated β-amino acids replacing the Ala−Tyr scissile α-amino acids of CFP. Both C2 and C3 substituted β-amino acids were synthesized and substituted at the EP 24.11 scissile Ala−Tyr bond. Significant EP 24.15 inhibitory activity was observed with some of the β-amino acid containing analogues. Moreover, binding to EP 24.11 was eliminated, thus rendering all analogues containing β-amino acids resistant to degradation by EP 24.11. Selective inhibition of either EP 24.15 or EP 24.16 was also observed with some analogues. The results demonstrated the use of β-amino acids in the design of inhibitors of EP 24.15 and EP 24.16 with K_i's in the low micromolar range. At the same time, these analogues were resistant to cleavage by the related metalloendopeptidase EP 24.11, in contrast to the α-amino acid based parent peptide. This study has therefore clearly shown the potential of β-amino acids in the design of stable enzyme inhibitors and their use in generating molecules with selectivity between closely related enzymes.

Peptides are an important class of signaling molecules present in virtually every biological system. Bioactive peptides are initially synthesized as larger precurser proteins, from which the active moiety is excised through cleavage by specific peptidases. Other peptidases regulate the levels of peptide by mediating their degradation. The thermolysin-like zinc metalloendopeptidases are a family of mammalian peptidases which are critical for the metabolism of numerous peptides, particularly in the cardiovascular system and central nervous system (*1*). The membrane-bound members of this family, such as angiotensin-converting enzyme (ACE), neprilysin (EP 24.11), and endothelin converting enzyme have been extensively studied, and their role in peptide turnover is well-established (*1*). Less well-known are the soluble metallopeptidases, particularly thimet oligopeptidase (endopeptidase EC 3.4.24.15 (EP 24.15)) and neurolysin (endopeptidase EC 3.4.24.16 (EP 24.16)). EP 24.15 (*2*) was originally isolated from rat brain extracts (*3*) and has subsequently been found in high levels in the pituitary and testis and in lower levels throughout the body (*4*, *5*). The active site of EP 24.15 contains a zinc ion which coordinates to the characteristic HEXXH active site motif at residues 473−477 (*6*), in which the two histidine residues together with a second glutamic acid at position 497 directly coordinate the zinc ion while glutamic acid 474 binds a catalytic water molecule (*7*).

The activity of EP 24.15 is thiol-dependent (*8*), and the enzyme is able to cleave a wide variety of peptide substrates, including dynorphin A_{1-8}, β-neoendorphin, bradykinin, neurotensin (*9*), lysylbradykinin (*10*), and gonadotropin-releasing hormone (GnRH) (*11*). It has been shown that substrate specificity is dependent on substrate conformation to fulfill secondary binding requirements such as hydrogen bonding (*12*) to enable the binding of specific functional groups to active site subsites (*13*). The role of EP 24.15 in vivo is not clear, although it is thought to be involved in the degradation of neuropeptides (*14*, *15*) and thus play a role in reproduction and blood pressure regulation. In addition, EP 24.15 has been suggested to have a role in the processing of Aβ protein associated with Alzheimer's disease (*16*, *17*).

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[1] Abbreviations: ACE, angiotensin converting enzyme; CFP, carboxyphenylpropyl-Ala-Ala-Tyr-*p*Ab; cp, carboxyphenylpropyl moiety; EP 24.15, metalloendopeptidase EC 3.4.24.15; EP 24.16, metalloendopeptidase EC 3.4.24.16; EP 24.11, neprilysin/metalloendopeptidase EC 3.4.24.11.

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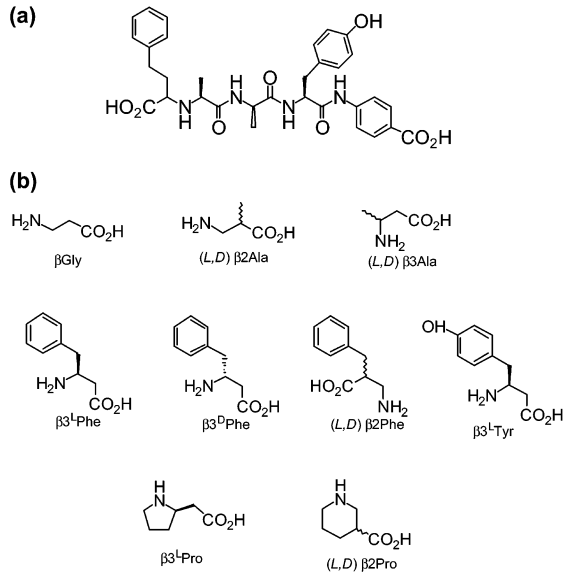

FIGURE 1: Structure of (a) CFP and (b) the structures and nomenclature of β-amino acids incorporated into CFP analogues.

The inability to characterize the physiological role of EP 24.15 is in part due to a lack of stable and specific inhibitors. However, the development of EP 24.15-specific inhibitors is complicated by the similarity of the EP 24.15 active site with the other zinc metalloendopeptidases, particularly the closely related enzyme, neurolysin (EP 24.16). Of the inhibitors developed to date, few have overcome this problem of selectivity (*18*−*20*). *N*-Carboxymethyl peptide based inhibitors which utilize a carboxylic acid functional group to coordinate with the zinc ion (*21*) are also potent inhibitors of EP 24.15. For example, the commonly used analogue cpp-Ala-Ala-Tyr-*p*Ab (CFP) (Figure 1a) has a K_i of 16nM (*18*, *22*). The proline-substituted analogue, cpp-Ala-Pro-Tyr-*p*Ab, is twice as potent as CFP, with a K_i of 7nM (*23*). However, CFP also inhibits EP 24.16, although somewhat less potently (K_i of 600nM) and is rapidly metabolized by the membrane bound metalloendopeptidase neprilysin (EP 24.11). One of the products of this cleavage at the Ala−Tyr bond, cpp-Ala-Ala, was found to be a potent inhibitor of ACE (*24*, *25*), thus limiting the use of the inhibitor in vivo.

Previous attempts to stabilize this scissile bond include the substitution of Asp, Val, Ser, or Leu as well as the incorporation of D-Ala to reduce the susceptibility of the Ala−Tyr bond that is cleaved by EP 24.11 (*22*). Although significantly more stable to cleavage by EP 24.11, these changes also led to a reduction in EP 24.15 inhibitory activity. Recently, an analogue of CFP containing a reduced bond modification was found to be stable against degradation by EP 24.11, but it was 1000-fold less potent than CFP in inhibiting EP 24.15 (*26*). In the same study, an analogue incorporating aminoisobutyric acid in place of Ala at the N-terminal side of the scissile bond was also resistant to EP 24.11 degradation yet remained a potent inhibitor of EP 24.15 with a K_i of 23nM. This analogue was a poor inhibitor of ACE, EP 24.11, and endothelin-converting enzyme but, like the parent compound CFP, was also a potent inhibitor of EP 24.16, thereby reducing its usefulness as an EP 24.15 in vivo probe (*26*, *27*).

β-Amino acids have recently reemerged as a potential peptidomimetic tool in the design of metabolically stable bioactive peptides (*28*−*34*). We recently reported the synthesis of a selection of CFP analogues incorporating β-amino acids which retained inhibitory activity, demonstrating that β-amino acid-containing peptides are able to bind to target proteolytic enzymes (*30*). In the present study, we report the synthesis and inhibitory activity of a wide range of CFP analogues containing β-amino acids substituted at either the N- or C-terminal side of the scissile bond in order to stabilize this bond against cleavage by EP 24.11. In addition, comparison of the inhibition of EP 24.15 and the closely related enzyme EP 24.16 was also evaluated for all analogues. The results indicate that specificity of binding to closely related enzymes can also be manipulated through the use of β-amino acids.

EXPERIMENTAL PROCEDURES

Chemicals and Solvents. All solvents were AR grade except acetonitrile, which was HPLC-grade and *N*,*N*-dimethylformamide (DMF), which was peptide synthesis-grade. Dichloromethane (DCM), methanol, and DMF were stored over 4 Å sieves. Water was distilled and deionized in the Milli-Q system (Millipore, Bedford, MA). *O*-Benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluroniumhexafluorophosphate (HBTU), di-*tert*-butyl dicarbonate, diisopropylcarbodiimide (DIC), diisopropylethylamine (DIPEA), dimethylformamide (DMF), 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl), 1-hydroxybenzotriazole (HOBt), piperidine and trifluoroacetic acid (TFA), and all Fmoc amino acids were obtained from Auspep (Melbourne, Australia).

Recombinant rat EP 24.15 was provided by Dr. M. J. Glucksman (Fishberg Center, Mt Sinai School of Medicine, New York). Recombinant EP 24.16 was provided by Dr E. S. Ferro (University of São Paulo, Brazil). Recombinant EP 24.11 was provided by Dr Glenn Dale, Hoffmann-La Roche, Basel, Switzerland. CFP was synthesized by Dr J. Boublik and L. Lakat at the Baker Institute. The quenched fluorescent substrate (QFS) and Leu-enkephalin were obtained from AUSPEP (Parkville, Victoria, Australia). Racemic β2Phe and enantiomerically pure $\beta3^{D}$Phe, $\beta3^{L}$Phe, $\beta3^{L}$Tyr, and $\beta3^{L}$Pro were synthesized by Dr M. Harte and Dr S. Bond (School of Chemistry, Monash University, Melbourne, Australia).

Peptide synthesis. Peptide synthesis and purification protocols were followed using hydroxymethylphenyl Tenta Gel (HMP-TG) resin (Novabiochem, Laufelfingen, Switzerland). Peptides were manually synthesized using a Biorad 10 mL PolyPrep chromatography column with vacuum applied to remove solvent and excess reagents. Standard solid-phase synthesis protocols were used for the coupling, deprotection and cleavage of all peptides.

The general procedure for the first amino acid coupling was as follows: 1 g (0.15 mmol/g loading) of resin was swollen in DMF (30 mL). Fmoc−βGly (466 mg, 1.5 mmol) and DMAP (18.3 mg, 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, 234μL, 1.5 mmol). The reaction vessel was closed, 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, methanol, and ether and dried under vacuum.

The general procedure for Fmoc deprotection was as follows: to the resin was added a solution of 20% piperidine

in DMF (30 mL), and the solution was occasionally mixed for 15 min. After deprotection the solvent was drained, and the resin was washed with DMF.

The general procedure for amino acid coupling was as follows: equimolar amounts of the Fmoc−amino acid, HBTU, and HOBt at four molar equivalents to the resin loading were dissolved in DMF (30 mL). On complete dissolution, DIPEA (0.25 mL), was added, and the mixture stirred briefly. The activated amino acid solution was then added to the resin and mixed occasionally for 2−3 h. The extent of coupling was tested using the 2,4,6-trinitrobenzene sulfonic acid, TNBSA test (*35*) using a 0.1 M TNBSA solution in DMF from Fluka (Buchs, Switzerland). If the coupling was incomplete, a double coupling was carried out using the above quantities of reagents. On complete coupling the resin was washed with DMF.

The peptides were completed by the N-terminal addition of 1-carboxy-3-phenylpropane via a reductive alkylation of 2-oxo-4-phenylbutanoic acid to the free N-terminal amine (*30*). In brief, ethyl 2-oxo-4-phenylbutanoate (2.0 g, 1.83 mL, 9.6 mmol) was hydrolyzed by stirring in a solution of 1 M NaOH (25 mL) overnight at room temperature. After reaction, the solution was neutralized with 6M HCl, the product was extracted with diethyl ether (3 × 40 mL), and the solvent was removed yielding solid 2-oxo-4-phenylbutanoic acid, which was used in the reductive alkylation without any further purification. To a solution of peptide-bound resin in DMF (10 mL) were added 2-oxo-4-phenylbutanoic acid (267 mg, 1.5 mmol) and acetic acid (200 μL). The mixture was stirred for 5 min, after which $NaCNBH_3$ (150 mg, 3 mmol) was added. The solution was stirred overnight with a magnetic stirrer at room temperature. The resin was filtered and washed with DMF, acetic acid, DCM, methanol, and ether, after which it was dried in a desiccator overnight.

On completion of peptide synthesis, the resin was washed with DMF (5 × 30 mL), methanol (5 × 30 mL), and ether (5 × 30 mL) and dried under vacuum. The peptides were cleaved with 95% TFA (2 mL), and the resin was filtered and washed with TFA (1 mL). The TFA was then removed and the residue redissolved in a 1:1 mixture of acetonitrile/water and lyophilised. The peptides were then purified by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Waters Assoc. (Millford, MA) liquid chromatography system. The HPLC separation of the synthetic peptides was performed using a semipreparative Brownlee C-18 column (100 × 10 mm I. D.) and a gradient from 0% to 60% acetonitrile containing 0.1%TFA over 60 min. The purity of the peptide was confirmed with analytical HPLC using a Zorbax 300 SB-C18 reversed-phase column (4.6 mm × 15 cm) with particles 5μm in diameter and 300 Å average pore size. The standard analytical gradient conditions used were 0−85% of 0.1% TFA in a 60:40% mixture of acetonitrile:water. The peptides were characterized by LC-MS using a Hewlett-Packard 1100 series LC with an on-line mass spectrometric detector (Agilent Technologies, Palo Alto, CA). All peptides had the predicted molecular mass.

EP 24.15 and EP 24.16 Inhibition Assays. All peptide analogues were 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. Identical conditions and reagents were used for the screen of inhibitory activity against recombinant EP 24.16, with the exception that dithiothreitol (DTT) was not added.

In a 96-well microtiter plate, 25μL of a peptide stock was added to 50ng (in 10 μL) of EP 24.15 to give final concentrations of 10 μM, 1 μM, 100 nM, and 10 nM when the volume was made up to 230 μL with Tris-buffered saline (TBS: 125 mM NaCl, 25 mM Tris, 0.1 mM DTT, pH of 7.4). The 96-well plate was preincubated at 37 °C in the oven of the fluorometric plate reader (Fmax, Molecular Devices) for 30 min. After the preincubation period 20 μL of 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 320 nm and emission detected at 420 nm. The percentage inhibition was calculated by comparison of the observed fluorescence with the control containing no inhibitor and the IC_{50} determined from the plot of % inhibition against peptide concentration.

Endopeptidase 24.11 Stability Assay. Soluble and membrane extracts of rabbit kidney were prepared as previously described (*36*), and the stability assays were performed with the membrane extract. The peptides (40 μg) were incubated with 30 μg of the rabbit kidney membrane extract in 400 μL of TBS buffer. The solution was incubated at 37 °C, and 50μL aliquots equivalent to 5μg of the peptide were removed at times 0, 30 min, 1, 2, 4, 6, and 24 h. The reaction was stopped by diluting the aliquot in 200 μL of methanol/1% TFA. Following centrifugation in a tabletop microfuge to precipitate the membranes (14 000 rpm, 10 min), the supernatant was dried by vacuum centrifugation (Speed-Vac, Savant) and analyzed by HPLC on a Hewlett-Packard (HP) 1100 series HPLC (Agilent technologies, Palo Alto, USA). Samples were injected onto a Zorbax C18 column in 0.1% TFA and eluted over a 30 min linear gradient with a 0.1% TFA in 60:40 acetonitrile:water mixture.

Endopeptidase 24.11 Inhibition Assay. Each peptide was screened for inhibitory activity against the cleavage of the synthetic peptide Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) by recombinant EP 24.11. A 10 μM solution of each peptide was incubated with 20 ng of EP 24.11 in the presence of 0.1 mM $ZnCl_2$ in 100 μL of TBS buffer. The solution was incubated at 37 °C for 15 min, after which 24 μg of Leu−enkephalin was added. At 0, 1, and 2 h time points, a 20 μL aliquot was taken and added to 200 μL of a 80% methanol/0.1% TFA solution and then dried by vacuum centrifugation. Samples were then analyzed as above. The extent of inhibition was estimated by analysis of the substrate peak area in comparison with the zero time aliquot.

RESULTS

Inhibitor Design and Synthesis. On the basis that CFP is cleaved by EP 24.11 at the Ala−Tyr scissile bond, a series of analogues was synthesized in which the amino acids at either the N- or C-terminal side of the scissile bond were substituted by β-amino acids in order to stabilize this bond. The structures of each of the β-amino acids used are shown in Figure 1b.

Alanine at the N-terminal side of the scissile bond was replaced by either β2Ala, β3Ala, βGly, Pro, β2Pro, or β3Pro. On the C-terminal side of the scissile bond, tyrosine was

Table 1: Amino Acid Sequence, Retention Time, EP 24.15 and EP 24.16 Inhibitory Constants, and % Degradation by EP 24.11 of β-Amino Acid Analogues of CFP[a]

peptide number	peptide sequence	retention time (min)	IC_{50} (μM) EP 24.15	IC_{50} (μM) EP 24.16	IC_{50} 24.15/ IC_{50} 24.16	% degraded by EP24.11
(CFP)	cpp-A-A-Y-*p*Ab	16.0	0.06	0.12	0.5	100
			Group 1			
(1)	cpp-A-A-Y-βG	14.9	0.12	0.18	0.71	100
(2)	cpp-A-β2A-Y-βG	15.0	6.3	6.3	1	0
(3a)	cpp-A-β3A-Y-βG	12.7	NI	88	*b*	0
(3b)	cpp-A-β3A-Y-βG	13.2	NI	61	*b*	0
(4)	cpp-A-βG-Y-βG	12.4	5.6	4.8	1.2	0
(5)	cpp-A-A-β3^{L}Y-βG	12.6	25.1	14	1.8	0
			Group 2			
(6)	cpp-A-A-F-βG	14.8	3.6	1.6	2.3	100
(7a)	cpp-A-β2A-F-βG	15.1	2.8	1.8	1.6	0
(7b)	cpp-A-β2A-F-βG	16.1	8.9	7.9	1.1	0
(7c)	cpp-A-β2A-F-βG	16.6	NI	62	*b*	0
(8)	cpp-A-A-β2F-βG	15.0	40	2.2	18.2	0
(9)	cpp-A-A-β3^{L}F-βG	15.9	NI	50	*b*	0
(10)	cpp-A-A-β3^{D}F-βG	15.1−16.6	NI	85	*b*	0
			Group 3			
(11)	cpp-A-β2A-β3^{L}F-βG	15.5−16.8	NI	NI	−	0
(11a)	cpp-A-β2A-β3^{L}F-βG	16.2	63.1	NI	*b*	0
(12)	cpp-β2A-β3^{L}F-βG	16.4−17.9	NI	NI	−	0
(13)	cpp-A-β2A-β3^{D}F-βG	15.7−16.6	NI	NI	−	0
(13a)	cpp-A-β2A-β3^{D}F-βG	15.8	79.4	41	1.9	0
(14)	cpp-β2A-β3^{D}F-βG	16.8−17.6	NI	NI	−	0
(15)	cpp-A-β2A-β2F-βG	15.1−15.7	NI	NI	−	0
(15a)	cpp-A-β2A-β2F-βG	15.5	158	NI	*b*	0
(16)	cpp-β2A-β2F-βG	16.3−17.1	NI	NI	−	0
			Group 4			
(17)	cpp-A-P-Y-βG	15.4	0.66	0.10	6.6	0
(18)	cpp-A-β3^{L}P-Y-βG	15.9	6.9	25	0.3	0
(19)	cpp-A-β2P-Y-βG	15.8	5.6	1.8	3.1	0
(20)	cpp-A-P-F-βG	15.5	0.79	0.17	4.7	0
(21)	cpp-A-β3^{L}P-F-βG	15.7	6.3	10	0.6	0
(22)	cpp-A-β2P-F-βG	15.7	10	1.8	5.6	0

[a] NI = no inhibition. [b] Analogues which showed inhibition of only EP 24.15 or EP 24.16.

replaced by either β3Tyr, β2Phe, or β3Phe. The residue βGly was obtained as commercially available β-alanine and, for the purposes of this project, is referred to as β-Gly in reference to the glycine-like side chain. Racemic mixtures of both β2Ala and β3Ala were synthesized via amine−enolate conjugate addition as previously reported (*30*). Racemic β2Phe was synthesized by conjugate addition and β2Pro was obtained via saponification of commercially available (±)-nipecotic ethyl ester. Enantiomerically pure β3^{D}Phe, β3^{L}Phe, and β3^{L}Pro were synthesized as previously described (*37*). All β-amino acids were Fmoc-protected as reported previously (*30*). The sequences of all peptides synthesized are listed in Table 1.

All peptides were synthesized via standard solid-phase peptide synthesis methodology, and the cpp N-terminal moiety was added via reductive alkylation as previously described (*30*). As no stereoselectivity was obtained in the cpp coupling, all peptides were generated as diastereomeric mixtures. Although some HPLC resolution of these isomers was generally observed on an analytical scale, the preparative resolution of these isomers was not possible, and the peptides were therefore assayed as diastereomeric mixtures. There were some exceptions to this, and these peptides are labeled as Xa and Xb where X is the peptide number; however, the configuration of these compounds remains unknown.

The peptides were divided into four groups, depending on the sequence of amino acids in relation to CFP. The first group of peptides, shown in Table 1, was based on the Tyr-containing parent sequence (CFP_{Tyr}) and incorporated βAla or βGly substitutions on the N-terminal side of the scissile bond or a β3^{L}Tyr residue on the C-terminal side of the scissile bond. The second group was based on the Phe-substituted sequence cpp-AAF-*p*Ab, (CFP_{Phe}) and incorporated either β2Ala on the N-terminal side of the scissile bond or substituted β2Phe, β3^{L}Phe, or β3^{D}Phe for Phe. The third group incorporated β-amino acid substitutions at both sides of the scissile bond. The fourth group of peptides substituted proline and its C2 and C3 β-amino acid equivalents for alanine in both CFP_{Tyr} and CFP_{Phe} sequences.

Inhibition of EP 24.15 and EP 24.16. As shown in Table 1, the structures of the group 1 peptides are based on the control sequence cpp-Ala-Ala-Tyr-βGly and were synthesized to examine the effect of a single β-amino acid substitution on peptides containing the tyrosine side chain. The IC_{50} values for all peptides are listed in Table 1. The results of the tyrosine-based CFP analogues incorporating β-amino acids at the scissile bond and βGly at the C-terminus also includes the data previously published (*30*) for comparative purposes. Peptide 1 is the α-amino acid control where the *p*Ab residue of the parent compound was replaced by a βGly residue. The activity of this peptide was 2-fold lower than that of CFP, with an IC_{50} of 0.12 μM. Peptide 2, incorporating β2Ala at the scissile bond, although less potent than the control 1, showed significant activity, with an IC_{50}

of 6.3 μM. In contrast, peptide 3 incorporating β3Ala was inactive, and no inhibitory activity was observed with either of the enantiomers 3a and 3b. The removal of the methyl side chain by the substitution of βGly in peptide 4 showed similar potency to peptide 2, with an IC_{50} of 5.6 μM. Peptide 5, incorporating $\beta3^{L}$Tyr substitution on the C-terminal side of the scissile bond, showed only moderate activity and was around 200 times less potent than the α-amino acid control peptide 1. Hence analogues 2, 4, and 5 inhibited EP 24.16 and EP 24.15 to a similar extent. However, some selectivity was obtained with the β3Ala substitutions which demonstrated high micromolar inhibition of EP 24.16 but failed to inhibit EP 24.15.

The group 2 peptides are phenylalanine-based CFP analogues with the parent control sequence cpp-Ala-Ala-Phe-βGly (peptide 6). These peptides incorporate either a β2Ala residue on the N-terminal side of the scissile bond or a β2Phe or either enantiomer of β3Phe on the C-terminal side of the scissile bond. The control peptide 6 had an IC_{50} of 3.6 μM, which was 30-fold less active than that of the tyrosine substituted counterpart 1 and 60-fold less active than that of CFP. This result was in contrast to the smaller differences reported between the original CFP peptides where a phenylalanine substitution for tyrosine resulted in a 2-fold loss of activity (*23*, *38*). Peptide 7, incorporating β2Ala at the N-terminal side of the scissile bond, was resolved into three isomers by HPLC, although the actual configuration of each isomer remains unknown. The earliest eluting peptide 7a was the most potent, with an IC_{50} value of 2.8 μM, similar to that observed with the α-amino acid control 6. The second isomer, peptide 7b, was 3.2-fold less active than peptide 7a. Most significantly the last eluting isomer 7c was completely inactive. Generally, similar levels of inhibition of EP 24.16 were observed for all analogues with the exception of 7c, which was in the high micromolar range for EP 24.16 but did not inhibit EP 24.15.

Peptides 8−10 included β-phenylalanine substitutions at the C-terminal side of the scissile bond. Peptide 8 was substituted with β2Phe and had an IC_{50} of 40 μM which was 11 times less active than peptide 6, (cpp-A-A-F-βG), whereas peptides 9 and 10 containing $\beta3^{L}$Phe and $\beta3^{D}$Phe, respectively, were inactive. These peptides also showed greater selectivity toward inhibiting EP 24.16 in that 8 was 20 times more potent for EP 24.16 and peptides 9 and 10 showed some inhibition of EP 24.16 but did not inhibit EP 24.15.

The group 3 peptides have the general sequences of either cpp-A-β2Ala-Xxx-βGly or cpp-β2Ala-Xxx-βGly, where Xxx denotes a substitution of either β2Phe or one of the enantiomers of β3Phe or $\beta3^{L}$Tyr. This group of peptides was synthesized to examine the effect of a double substitution of β-amino acids on either side of the scissile bond. Analogues were also synthesized in which the alanine residue adjacent to the cpp residue was removed to decrease the backbone length since the presence of two β-amino acids extends the backbone length by two atoms. These peptides were synthesized as diastereoisomeric mixtures due to the incorporation of racemic β2Ala and cpp. The preparation of peptides 15 and 16 gave even more complex mixtures due to the incorporation of two racemic β-amino acids in addition to cpp. Only partial resolution of these mixtures was achieved, and all were assayed as mixtures of at least two or more diastereoisomers. Since it was observed that most of these fractions were inactive, the results were grouped together and reported in Table 1 as single peptides. For fractions where some activity was observed, these are reported separately as Xa where X is the peptide number, and the retention time of the active fraction is indicated. These peptides were generally inactive with the exception of three peptides 11a, 13a, and 15a, in which only one or two of the isomers present exhibited moderate activity in the high micro-molar range; the most active of these was 11a, with an IC_{50} of 63 μM. This peptide was also the most selective inhibitor for EP 24.15 in this group with no observable inhibition observed against EP 24.16. Removal of the alanine adjacent to the cpp moiety, which reduces the backbone length of the peptides, completely abolished the inhibitory activity of all the purified isomers of these peptides against either EP 24.15 or EP 24.16.

Finally, group 4 peptides consisted of the proline substituted CFP analogues incorporating β2Pro and $\beta3^{L}$Pro at the scissile bond and βGly at the C-terminus. Peptides (17) and (20) are the α-amino acid controls with either a Tyr or Phe on the C-terminal side of the scissile bond. The activity of these peptides against EP 24.15 was similar at 0.66 and 0.79 μM, respectively, 6-fold less than that of 1. This was in contrast to the findings of Knight and Barrett, where Pro was favored over Ala in this position when C-terminally blocked by *p*-aminobenzoic acid (*23*). All of the peptides incorporating β-Pro showed significant activity inhibiting EP 24.15, where peptides 18, 19, and 21 were the most potent, with an IC_{50} value around 6 μM. Overall, the peptides incorporating Pro and β2Pro were more potent inhibitors of EP 24.16, with between 5- and 10-fold increases in potency for EP 24.16 compared with EP 24.15. Peptide 18, incorporating $\beta3^{L}$Pro, was the most selective inhibitor for EP 24.15 in this study, where it was 5 times more active against EP 24.15 than EP 24.16.

Stability of Inhibitors to Cleavage by EP 24.11. The stability of the inhibitors was determined by incubating the peptides in the presence of rabbit kidney membrane proteins, a rich source of EP 24.11, which were prepared as previously described (*22*), and the results are listed in Table 1. The parent compound CFP was readily degraded within 30 min by the kidney membranes consistent with previous results (*22*). Similarly, analogues 1 and 6, containing the βGly substitution at the C-terminus, were also readily degraded within 30 min. The degradation of these peptides can be attributed to EP 24.11 in the kidney membrane extract since it has been previously shown in the presence of known EP 24.11 inhibitors such as phosphoramidon and SCH 39370 ([*N*-[*N*-1-(*S*)-carboxyl-3-phenylpropyl]-(*S*)-phenylalanyl]-(*S*)-isoserine) (*39*) inhibits cleavage of CFP (*22*). In contrast, none of the peptides containing β-amino acid substitutions at the scissile bond were degraded within the assay time. Even after 24 h, there was no evidence of any degradation of the peptide. In addition, this resistance to EP 24.11 degradation was displayed by all diastereoisomers since the peptide profile of the racemic mixtures did not change after 24 h. None of the proline analogues were degraded by EP 24.11 over this time period.

Inhibition of EP 24.11 by CFP Analogues. To gain further insight into their interaction with EP 24.11, we also assayed all peptides as inhibitors of EP 24.11 to determine whether

they bind to the active site. The peptides were incubated in the presence of recombinant EP 24.11 and Leu−enkephalin as a substrate, and aliquots were taken at 0, 1, and 2 h, and the samples were analyzed by HPLC. As a control, the known EP 24.11 inhibitor SCH 39370 (*39*) was also assayed. All peptides were assayed with the exception of CFP and peptides 1 and 6, which were demonstrated to be substrates themselves in the stability assay, and thus would not act as an inhibitor but a competitive substrate. All remaining peptides did not inhibit the degradation of Leu−enkephalin, suggesting they did not bind to the active site of EP 24.11.

DISCUSSION

This study reports the use of β-amino acids to stabilize the scissile bond of a peptide-based inhibitor of EP 24.15 against enzymatic cleavage by endopeptidase EP 24.11. All β-amino acids were readily incorporated into standard solid-phase peptide synthesis techniques, thereby facilitating their routine incorporation into analogues of the peptide, *N*-[1-(*R*,*S*)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate (CFP). This molecule is a potent inhibitor of EP 24.15 but is unstable in vivo due to cleavage between the alanine and tyrosine residues by the enzyme EP 24.11. The incorporation of β-amino acids at the scissile bond was used to stabilize CFP against cleavage by EP 24.11. In addition, the synthesis of peptides incorporating both C2 and C3 substituted β-amino acids provided a wide range of diastereoisomers to explore structure−activity relationships of these analogues.

The C-terminal *p*Ab moiety of the original CFP molecule was replaced in the present study by βGly. Although originally postulated that CFP binds to a hydrophobic pocket (*38*), it was suggested that *p*Ab also provides a negative charge via the carboxylic acid (*23*), as shown schematically in Figure 2. In the present study, the βGly residue may also act as a C-terminal blocking group to resist the action of carboxypeptidases and also provides a negative charge for binding. As previously reported (*30*), the substitution of the C-terminal βGly in peptide (1) resulted in only a 2-fold loss of activity compared to CFP, and a C-terminal βGly was thus incorporated into all CFP analogues.

Overall, the presence of β-amino acid substitutions on the N-terminal side of the Ala−Tyr scissile bond reduced binding to EP 24.15 compared to the α-amino acid control (1) with the relative order β2Ala ≈ βGly > β3Ala. The reduced affinity is likely to be due to the loss in complementary binding between the analogues and the active site subsites as a result of the backbone elongation or increased peptide flexibility. The similar activity of peptides incorporating β2Ala or βGly substitution at the scissile bond suggested that the methyl side chain is not specifically required to bind to an active site subsite. In contrast, the loss of activity with the presence of a β3Ala is probably due to steric hindrance of the methyl group at the C3 position restricting binding of this peptide.

The β-amino acid substitutions on the C-terminal side of the scissile bond consisted of the β-analogues of either the tyrosine residue or the phenylalanine residue. The relative order of potency was found to be β3Tyr > β2Phe > β3Phe, with the β3Phe-containing analogues completely inactive. These results indicate first that the presence of a hydroxyl group is important for binding of these β-amino acid containing analogues possibly mediated through the formation of a hydrogen bond. Second, the position of the phenyl side chain at the C3 position β-amino acid prevents the binding of these analogues to EP 24.15 but not EP 24.16, again demonstrating the subtle differences between these two closely related enzymes.

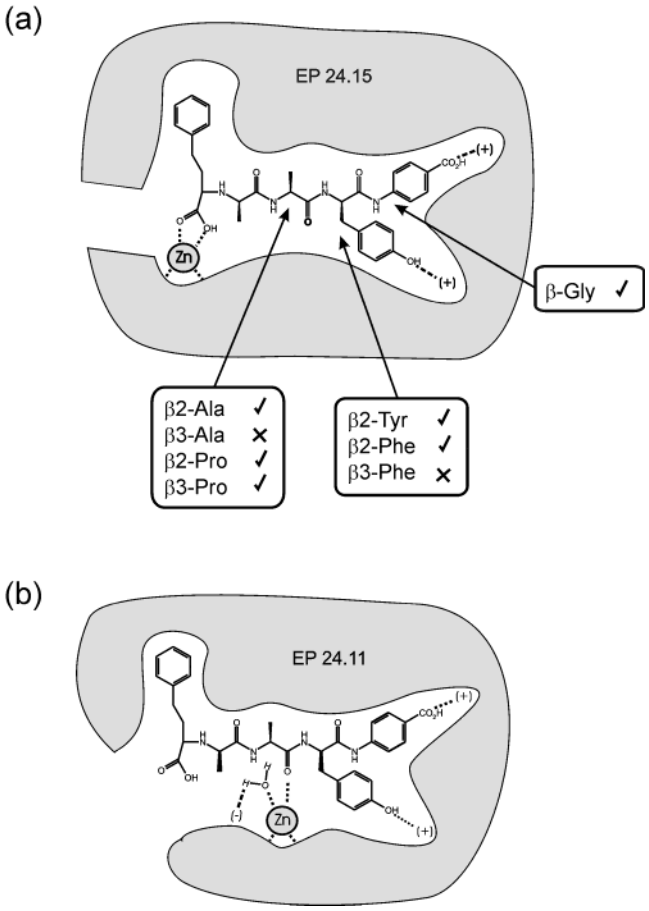

FIGURE 2: (a) Schematic of the binding of CFP to the active site of EP 24.15 and the β-amino acid substitutions tolerated by the enzyme. It is proposed that the that CFP binds to the Zn^{2+} atom through the cpp moiety and the remaining side chains fit into complementary binding sites in the active site. In addition, tyrosine and *p*-aminobenzoate may H-bind to positively charged residues in the active site. The β-amino acid-substitutions that give rise to analogues that are still able to bind to the active site are indicated by a "√", while those amino acid substitutions that result in inactive analogues are indicated by a "×". (b) Schematic of the binding of CFP to the active site of EP 24.11. Since CFP is cleaved by EP 24.11 at the Ala−Tyr bond, it is likely to be coordinated to the zinc ion via the carbonyl of the scissile bond in order for hydrolysis to occur. In contrast to the results with EP 24.15 and EP 24.16, the presence of any β-amino acid on either side of the scissile bond disrupts this interaction and prevents binding at the active site altogether.

Stereochemistry was also shown to be important, as demonstrated by the different potencies exhibited by peptides 7a, 7b, and 7c. It has been previously shown that the isomers of the cpp chiral center exhibit inhibitory activities that differ by an order of magnitude (*21*). Furthermore, attempts to stabilize CFP through the substitution of D-amino acids resulted in analogues that displayed no activity at all (*22*). It is therefore possible that specific stereoisomers within each peptide mixture are more potent than indicated by the IC_{50} values reported in Table 1.

With the exception of isomers of peptides 11, 13, and 15, the disubstituted peptides, where both the scissile alanine and tyrosine were replaced by their β-analogues, were largely inactive. This loss in binding is presumably due to the elongation of the backbone of these peptides by two atoms, which moves either the cpp or the Tyr/Phe side chains away from their respective binding sites. However, decreasing the backbone length of the disubstituted peptides (relative to CFP) by one atom through removal of the first alanine residue (i.e., peptides 12, 14, and 16) did not restore inhibitory activity. These results further demonstrate how β-amino acids can be used to probe the active site topography of both EP 24.15 and EP 24.16.

It has been shown previously that the incorporation of proline into CFP increases its affinity for EP 24.15 (*23*). In contrast to peptides in groups 1 and 2, where only the C2 β-amino acid substitutions produced active compounds, the β-proline substituted peptides produced active compounds regardless of C2 or C3 substitution. The resultant activity of these compounds is perhaps due to the conformational constraint of these cyclic amino acids restricting the peptides to more favorable conformations and allowing efficient binding to the enzyme active site.

Taken together, the results obtained in this study can be used to develop a β-amino acid pharmacophore for EP 24.15 as depicted in Figure 2a. If it is assumed that CFP binds to the Zn^{2+} atom of EP 24.15 through the cpp moiety, the constituent side chains fit into complementary binding sites in the active site of EP 24.15. The β-amino acid-containing analogues are still able to bind to the active site albeit with reduced affinity due to a disruption of the optimum binding orientation. While further studies are required to demonstrate that the CFP analogues bind directly to the Zn^{2+} atom, previous studies have shown that the affinity of CFP for EP 24.15 is markedly reduced either upon removal of the carboxyl group (*21*) in CFP or mutation of zinc-coordinating amino acid residues in the HEXXH motif (*7*) and a similar mode of binding is likely to occur for the CFP analogues in this study.

The results of the EP 24.11 degradation and inhibition studies conclusively demonstrate that the substitution of β-amino acids has stabilized CFP against cleavage by the kidney membrane-associated enzyme by preventing binding to the EP 24.11 active site. The peptides with the two α-amino acids were cleaved within 30 min, whereas all peptides containing β-amino acid substitutions at the scissile bond remained intact after 24 h. As none of these peptides inhibited the cleavage of Leu−enkephalin by EP 24.11, these β-amino acid modifications have therefore eliminated the affinity of CFP for EP 24.11 while still maintaining affinity for EP 24.15. A plausible model for the orientation of binding of CFP to the active site of EP 24.11 is shown in Figure 2b. Given that CFP is cleaved at the Ala−Tyr bond by EP 24.11, it is likely to be coordinated to the zinc ion via the carbonyl of the scissile bond in order for the hydrolysis to occur. The results of the present study suggest that the presence of a β-amino acid on either side of the scissile bond completely disrupts this interaction.

Of the peptides investigated in this study which inhibited both EP 24.15 and EP 24.16, the highest selectivity observed between the two enzymes was a factor of 18 for peptide 8. However, there were also a number of examples where analogues were only active against either EP 24.15 or EP 24.16. The results of this study with a limited number of β-amino acids suggest that the use of β-amino acids may be very useful in differentiating between the topographies of these two similar active sites. Molecular docking of these analogues with the recently published X-ray crystal structure of EP 24.16 (*40*) should provide insight into the molecular basis of the inhibitory activity of these molecules. Overall, this study has clearly demonstrated that the scissile bond of a peptide-based inhibitor can be stabilized against proteolytic cleavage by the incorporation of β-amino acids.

CONCLUSION

The use of peptidomimetics has emerged as a powerful means for overcoming the limitations inherent in the physical characteristics of peptides, thus improving their therapeutic potential. The present study has demonstrated the potential of β-amino acids as peptidomimetics in the design of stable and selective enzyme inhibitors. In particular, the selectivity between closely related endopeptidases has been manipulated whereby affinity has been maintained for EP 24.15 and/or EP 24.16, while binding to EP 24.11 has been completely eliminated. Further, this has been achieved via changes in stereo- and regiochemistry of the β-amino acids. Together with their ease of incorporation into peptide synthesis strategies, this sets β-amino acids apart from many other classes of peptidomimetics in terms of flexibility in molecular design. In addition, the ability to stabilize molecules against proteolysis may offset the decrease in potency that could result from β-amino acid substitutions thereby improving the efficacy of unstable but highly potent lead molecules. This study has therefore further demonstrated that β-amino acids have great potential as a new platform technology for the design of both peptide and protein mimetics.

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