Eur J Med Chem (1991) 26, 435-442

Analogues of substance P containing an α-hydroxy, β-amino acid: synthesis and biological activity

A Ewenson[1], R Laufer[2], M Chorev[3]*, Z Selinger[2], C Gilon[1]

[1]*Departments of Organic,* [2]*Biological and* [3]*Pharmaceutical Chemistry*
The Hebrew University of Jerusalem, Jerusalem 91120, Israel

(Received 21 April 1989; accepted 15 November 1990)

Summary — Several bestatin-like analogues of pGlu[6]-Phe[7]-Phe[8]-Gly[9]-Leu[10]-Met[11]-NH_2, a C-terminal hexapeptide derived from substance P (SP), an endogenous mammalian neuropeptide, were prepared. In this sequence [pGlu[6]]SP(6–11), Phe[7] or Phe[8] were substituted by the amino acid. (R,S)-α-hydroxy, (S)-β-amino, 4-phenylbutyric acid (AHPA). Several key diastereomeric products were resolved by selective precipitation from the reaction mixture and the resolved components were characterized by chromatography. FAB-mass spectrometry and amino acid analysis. The inhibitory potency of these peptides on SP degradation in rat diencephalon preparations was assayed. It was found that one of the resolved diastereomeric analogues, [pGlu[6], AHPA[8]]SP(6–11) (**9a**) was a potent inhibitor of SP degradation with an IC_{50} of 20 μM. The analogues prepared in this study were devoid of spasmogenic activity on the guinea-pig ileum assay.

Résumé — Analogues de la substance P contenant un α-hydroxy, β-aminoacide: synthèse et activité biologique. *Quelques analogues ressemblant à la bestatine – analogues de la séquence de l'hexapeptide C-terminal de la substance P (SP) et de fragments de cette séquence ont été préparés. Dans ces composés, Phe[7] ou Phe[8] de SP ont été remplacés par l'acide aminé AHPA [acide α-(R,S)- hydroxy, β-(S)-amino, 4-phénylbutyrique]. Quelques produits diastéréoisomères ont été résolus par la précipitation sélective des mélanges réactionnels et les composés obtenus ainsi ont été caractérisés par chromatographie, spectrométrie de masse et par analyse d'acides aminés. L'inhibition par ces peptides de la dégradation de SP par des préparations de diencéphale de rat a été étudiée. L'un des analogues [pGlu[6], AHPA[8]]SP(6–11) (**9a**) est un inhibiteur efficace de la dégradation de SP (IC_{50} = 20 μM).*

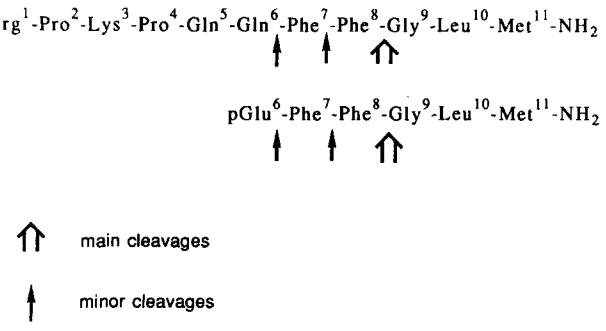
substance P analogues / substance P degradation inhibitors / peptide backbone modifications / α-hydroxy-β-amino acids / metallopeptidase inhibitors / bestatin-like analogues

Introduction

The last few years have been marked by a growing interest in the biological activities of substance P (SP). This undecapeptide is a putative neurotransmitter in the central and peripheral nervous systems [1]. It elicits a variety of biological actions [2] and it has been assigned a possible role in the transmission of pain stimuli together with an interactive relationship with the enkephalins [3, 4]. The implications of a neurotransmitter role for SP have triggered an increasing interest in its metabolic degradation as a potential means of inactivation. Indeed, rapid inactivation of the peptide in various tissues has been observed and has hampered the study of its functions, modes of action and possible therapeutic uses [5]. It has been claimed that this inactivation is caused mainly by enzymatic cleavage of the peptide; furthermore, specific SP-degrading enzymes have been described. Major and minor degradation sites of SP are shown in scheme 1 [6, 7].

To this end, the design of specific inhibitors of SP degradation could provide a means to circumvent the problems posed by metabolic inactivation of this neuropeptide. It could also yield an alternative approach to obtaining protracted activity as attempted by the design of metabolically stable analogues. Such an approach has been successfully applied to the design and synthesis of potent inhibitors of enzymes such as renin [8], angiotensin converting enzyme (ACE) [9–11], enkephalinase [12] and thermolysin [13].

*Correspondence and reprints



Scheme 1. Degradation patterns of SP and [pGlu^6] SP_{6-11} in rat diencephalon preparations [6].

SP is a potent vasodilator, hypotensive and natriuretic agent [2], and has been implicated in the pathogenesis of severe constipation [14] and familial dysautonomia [15]. Inhibitors of SP degradation might thus prove to be valuable tools for study of the pathophysiology and possibly the treatment of some human diseases.

The available knowledge on SP degradation points to the fact that this neuropeptide undergoes degradation by metallodipeptidyl carboxypeptidases such as ACE and by enkephalinase [14, 15] as was also established in rat diencephalon membrane and slice preparations [16]. These enzymes cause about 30% of the total of SP cleavage in the above-mentioned systems. The remaining degradation activity is attributed to a highly specific membrane metalloendopeptidase [7]. The SP cleavage pattern of this enzyme is also shown in scheme 1. This enzyme presents a high substrate preference for SP and analogues such as [pGlu^6]-SP(6–11).

As part of our ongoing effort to prepare potent inhibitors of SP degradation [17], we postulated that the synthesis of analogues of the hexapeptide [pGlu^6]-SP(6–11) containing a metal coordinating function in the region undergoing cleavage by specific enzymes might yield a potent inhibitor of SP-degrading activity.

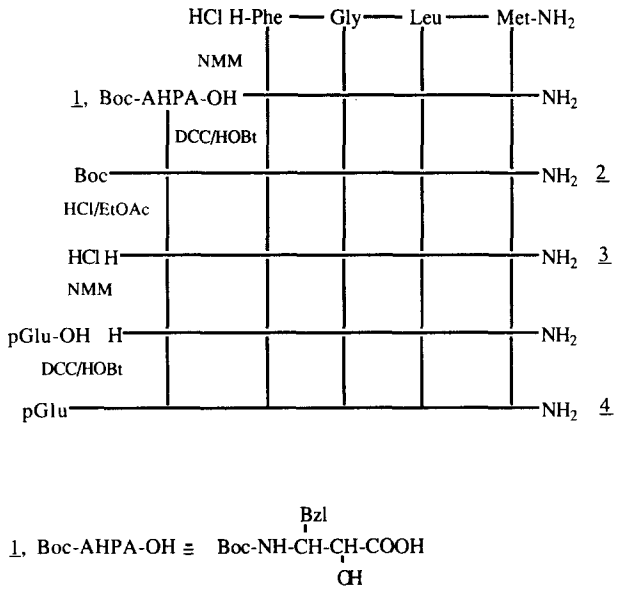
It has been proposed that the inclusion in the substrate molecule of non-degradable moieties resembling the cleavage transition state can result in a compound with enhanced affinity towards the active site [18]. The successful application of this concept was demonstrated in the case of renin inhibitors, in which the substrate was modified to include a statyl moiety (3-hydroxy-4-amino-7 methyl octanoyl, Sta) [19]. In this type of compound the hydroxyl group of Sta replaces an amide carbonyl; the tetrahedral carbon structure of this moiety conceivably mimics the transition state of the proteolytic cleavage process [20]. Bestatin, a compound of microbial origin with the dipeptide structure of AHPA-Leu is a potent inhibitor of the metalloprotease, amino-peptidase B [21]. The uncommon amino acid (2*S*, 3*R*)-3-amino-2-hydroxy-4-phenylbutyric acid (AHPA) contains an α-hydroxyl group apparently bestowing a transition state-like character to bestatin. Its suitably located OH group could likewise turn the AHPA residue into a bidentate metal ligand which could explain the high potency of this inhibitor [22]. In this paper we report the synthesis of several 'bestatin-like' (AHPA containing) SP-analogues. Their inhibitory effects on SP-specific degradation were studied using rat brain preparations.

Chemistry

The amino acid (2*RS*, 3*S*) AHPA was conveniently synthesized by a known procedure [22] and its free amino group was protected by a *t*-butyloxycarbonyl (Boc) group. No attempt to resolve the diastereomers was made at this stage. The peptide synthesis was carried out by using a fragment condensation strategy with 1-hydroxybenzotriazole (HOBt) mediated *N,N*-dicyclohexylcarbodiimide (DCC) coupling in DMF. The Boc amino protecting group was utilised throughout and it was removed by acidolysis. No hydroxyl protection was deemed necessary since it was anticipated that during coupling steps involving AHPA, incoming amines would react preferentially with activated carboxyl groups, due to their higher nucleophilicity. The protected amino acid was thus coupled to the tetrapeptide HCl·H-Phe-Gly-Leu-Met-NH_2, and the pseudopentapeptide **2** (scheme 2) was obtained in adequate yield and purity. As expected, the free hydroxyl group of AHPA did not interfere in the peptide assembly process. The remaining steps leading to the synthesis of the analogue [pGlu^6, AHPA^7]-SP(6–11) (**4**) are summarized in scheme 2.

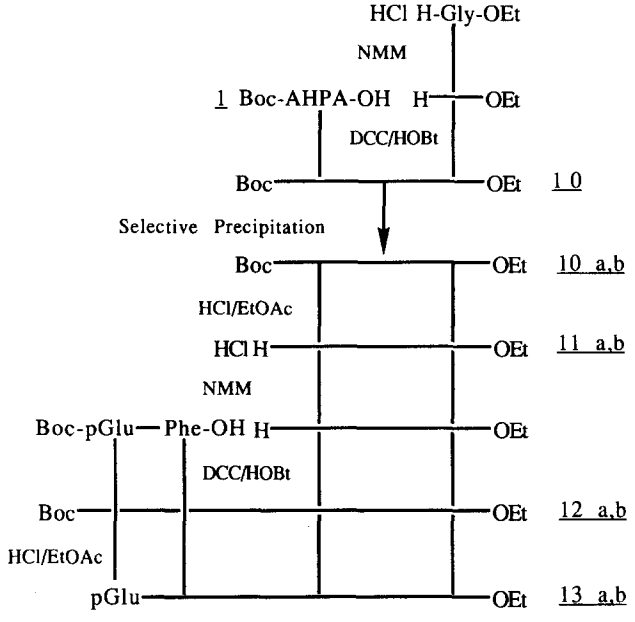
Analytical HPLC of the final pseudohexapeptide **4** showed it to be a mixture of 2 compounds in a ratio of roughly 1:1. As expected, additional analytical data (FAB-MS and amino acid analysis) pointed out that the peaks seen on HPLC corresponded to a mixture of 2 diastereomeric peptides. Compound **4** was obtained from the reaction mixture in adequate purity and no further purification steps were necessary (see table I). Analogue **4** was assayed as an unresolved mixture of stereoisomers.

The preparation of the second analogue [pGlu^6, HAPA^8]SP(6–11) (**9**), proceeded along similar lines to the synthesis of compound **4**, and again no protection of the hydroxyl group was needed. In contrast with the case of compound **4**, this diastereomeric mixture lent itself to easy resolution by fractional precipi-



Scheme 2.

tation. It was found that upon work up of the reaction mixture at the end of the coupling step between pGlu and the pseudopentapeptide **8**, the addition of aqueous bicarbonate and brine to the DMF solution caused one of the diastereomers (**9a**, corresponding to the late RP-HPLC peak) to precipitate out, slightly contaminated by the second, the less retained, diastereomer. The second diastereomer (**9b**, early RP-HPLC peak) was isolated from the DMF/bicarbonate/brine solution after cold storage. The residual contaminants in both resolved peptides were eliminated by repeated recrystallization from EtOAc/petroleum ether. The respective structures were confirmed by elemental analysis, FAB-MS and amino acid analysis. The synthesis of the tetrapeptide analogue **13** was accomplished by the pathway outlined in scheme 3.



Scheme 3.

Table I. Characterization of AHPA containing-SP analogues.

Compound	*Amino acid analysis*	*FAB MS[M+H]+*	*HPLC-k'*	$[\alpha]_D^{25}$
4	Met:1; Leu:1; Gly:1 Phe:1; AHPA:1; Glx:1	755	1.72, 1.98[1]	
9a	Met:1; Leu:1; Gly:1	755	5.49[2]	
				– 40° (C = 6.5, MeOH)
	Phe:1; AHPA:1; Glx:1			
9b	Met:1; Leu:1; Gly:1	755	5.41[2]	
				– 7.14° (C = 9.6, MeOH)
	Phe:1; AHPA:1; Glx:1			
13a	Gly:1; AHPA:1; Phe:1; Glx:1	539	1.26[3]	
13b	Gly:1; AHPA:1; Phe:1; Glx:1	539	1.67[3]	

[1]Equal size peaks, attributed to diastereomers; 70% MeOH, RP18. [2]2 to 60% MeCN over 20 min; RP8. [3]70% MeOH, RP18.

In this case we could resolve the mixture of protected pseudopeptides **10a, b** by selectively precipitating them from the reaction mixture. The satisfactory separation of the diastereomers **10a** and **10b** was demonstrated by comparing their HPLC elution profiles, proton NMR spectra and FAB-MS results. The separation of these diastereomeric dipeptide building blocks made possible the parallel syntheses of the pseudotetrapeptide analogues **13a** and **13b**. This was accomplished by the usual fragment condensation methods, in which 2 partially protected dipeptides **11a** and **11b** were coupled and the products **13a** and **13b**, respectively, which were sufficiently pure, were characterized by mass spectrometry and amino acid analysis. The analytical data of all final products described here is summarized in table I.

Pharmacology

A fast screening assay allowed the determination of inhibitory potencies of the analogues synthesized in this work [23]. Briefly, the compounds to be assayed were incubated with rat diencephalon membrane preparation in the presence of [N_α-(^{125}I)desaminotyrosyl]SP(6–11) ([^{125}I-BH]SP(6–11)) and the degradation rate of the radioiodinated substrate was monitored. This was accomplished by ion-exchange chromatographic separation of the intact substrate from its proteolytic fragments, and quantitation of the remaining substrate associated radioactivity. The results of this assay were summarized in table II.

It was established that compound **9a** was a potent inhibitor of SP-degradation, whereas the other diastereomer **9b** showed a noticeably lower affinity towards the enzyme.

Table II. Inhibition of degradation of [^{125}I-BH]SP(6–11) in rat diencephalon membranes by SP-analogues containing AHPA[a].

Compound	*Structure*	*IC_{50}[μM][b]*
4	[pGlu^6, AHPA^7]SP(6–11)	na
9a	[pGlu^6, AHPA^8]SP(6–11)[c]	20 ± 2
9b	[pGlu^6, AHPA^8]SP(6–11)[d]	100 ± 10
13a	pGlu-Phe-AHPA-Gly-OEt[c]	na
13b	pGlu-Phe-AHPA-Gly-OEt[d]	100 ± 20

[a]For details of bioassay see experimental section. [b]Values are mean ± SEM from at least 3 experiments. [c]Diastereomer corresponding to slow HPLC peak. [d]Diastereomer corresponding to fast HPLC peak. na No inhibition up to 200 μM.

The fact that tetrapeptide analogues **13a, b** were virtually devoid of any inhibitory activity suggest that residues in positions **10** and **11** — namely Leu and Met — contribute significantly to the recognition process by the active site. It has been postulated in the past that a rather large portion of the substrate such as the entire C-terminal pentapeptide is needed for recognition by the SP-degrading peptidase [5]. Conceivably, tetrapeptide analogues **13a, b** do not fulfill most of the structural prerequisites needed for proper anchoring onto the active site.

Comparison of the inhibition potencies of the hexapeptide analogues show that one diastereomeric form, namely **9a**, provides the correct anchoring to the active site which requires 2 consecutive hydrophobic residues next to the metal coordinating moiety. Furthermore, it appears that a precise positioning of the different metal binding elements is mandatory to attain an enhanced degree of inhibition, as in the case of **9a** (see scheme 4). In a recent report, Harbeson and Rich [24] presented evidence for the binding mode of bestatin type inhibitors to the active site metal ion of arginine aminopeptidase, a metalloprotease. In this model the adjacent hydroxyl and carbonyl groups of AHPA form the prongs of the bidentate ligand by which the inhibitor attaches itself to the enzyme. The inhibitory potency of **9a** may thus be attributed to its ability to coordinate the active site metal as a bidentate ligand, in which both the hydroxyl and amide carbonyl groups of AHPA participate in the metal binding complex. The opposite stereochemistry of the hydroxyl carbon in diastereomer **9b** could lead to the unavailability of this last group for coordination with the metal, thus explaining the reduced potency of this analogue.

The pseudohexapeptides **4** and **9a, b** were tested for biological activity in the guinea-pig ileum assay. All of them were devoid of biological activity, thus implying that the addition of a carbon atom to the peptide backbone disrupts conformational features essential for receptor recognition. Some analogues in which the Phe^7-Phe^8 bond was modified have shown high biological activity and enhanced selectivity towards different tachykinin receptor subtypes [25]. In analogue **4**, the backbone in the peptide bond region Phe^7-Phe^8 was extended by one carbon atom bearing the α-OH function, thus forming an additional chiral center. This particular modification which contributes to increased flexibility and possesses a potential hydrogen bonding function, has a deleterious effect on the biological activity. The fact that analogues **9a** and **9b** are devoid of agonistic properties, indicates a limit of latitude in the modification of the Phe^8-Gly^9 bond. This bond has been found to tolerate a relatively large number of structure modifications without greatly impairing activity [17], as can be seen in table III.

Table III. Biological activity of pseudopeptide analogues of [pGlu^6]SP(6–11)[a].

Analogue[b]	*EC_{50}(nM)*[c]	*Relative potency*	*Ref*
[pGlu^6]SP(6–11)	1.5 ± 0.2	1	
[pGlu^6, Phe$^8\psi$(CO-CH$_2$)Gly^9]SP(6–11)	2.0 ± 0.3	0.75	[17]
[pGlu^6, Phe$^8\psi$(E)-(CH=CH)Gly^9]SP(6–11)	6 ± 1	0.25	[26]
[pGlu^6, Phe$^8\psi$(NH-CO)Gly^9]SP(6–11)	7 ± 1	0.21	[25]
[pGlu^6, Phe$^8\psi$(CH$_2$-O)Gly^9]SP(6–11)	6 ± 1	0.25	[25]
[pGlu^6, Sar^9]SP(6–11)	2.0 ± 0.25	0.75	[7]
[pGlu^6, AHPA^8]SP(6–11) **9a**	600 ± 100	0.0025	This work
9b	700 ± 100	0.0021	This work
[pGlu^6, AHPA^7]SP(6–11) (**4**)	850 ± 150	0.0018	This work

[a]Established by the guinea-pig ileum assay [29]. [b]The symbol ψ(-)Gly denotes the type of modification (in brackets) which replaces the amide bond. [c]Each result represents at least 3 independent experiments.

Interestingly, Blumberg and Teichberg reported that only replacement in Boc-SP(6–11) of Gly^9 with Sar or Ala retained potency. Replacement of Gly with D-Ala [27] caused a loss of activity. This observation points again to the fact that the Phe^8-Gly^9 region plays a pivotal role in the interaction of SP with its receptors.

Conclusion

The use of solution phase techniques with minimal protection of functional groups, allowed the straightforward synthesis of several analogues of SP containing the α-hydroxy, β-amino, 4-phenylbutyric acid (AHPA) in place of phenylalanine. The fact that some key diastereomeric products had markedly different solubility properties greatly facilitated their resolution from their diastereomeric mixtures by selective precipitation resulting in simplified purification procedures. Substitution by the amino acid AHPA of residues in a region of the neuropeptide SP which undergoes proteolytic inactivation furnished a compound (**9a**) which inhibited the degradation of a radioactive SP-related substrate. We postulate that this is due to the appropriate geometry found in this particular analogue in which the hydroxyl group of AHPA and its amide carbonyl group may form a bidentate ligand which binds a metal atom in the active site of a SP degrading protease (fig 1). In addition, the tetrahedral α-hydroxyl carbon of AHPA could mimic a substrate proteolysis transition state intermediate, thus enhancing its binding affinity to the enzyme.

Experimental protocols

Melting points were measured on a Thomas–Hoover capillary melting point apparatus and are uncorrected. TLC was performed on silica gel plastic sheets by Macherey Nagel Co, Polygram Sil NH-8/UV 254 or RP18 precoated glass plates F 254 from Merck. Elution systems were: (A) CH_2Cl_2:MeOH, 9:1; (B) CH_2Cl_2:MeOH, 1:1; (C) *n*-BuOH:pyridine:HOAc (10%, aq) 5:3:1; (D) MeOH:H_2O, 7:3; (for RP18 plates); (E) MeCN,H_2O, 7:3 (for RP18 plates). Visualization was carried out by the following means: (I) exposure to UV light at 254 nm; (II) ninhydrin 0.1% spray in EtOH; (III) fluorescamine 0.1% spray in acetone and exposure to UV light at 365 nm; (IV) a) *t*-Bu-OCl in cyclohexane 1% spray, b) b1) *n*-Bu_4NI (1% in MeOH); b2) *o*-tolidine (1% in MeOH:H_2O); 1:1 solution of b1 and b2.

HPLC was performed on a Spectra Physics SP 8000 or a Merck–Hitachi 655 LC system using the following columns: Merck Hibar Lichrosorb RP18 (7 μM mean particle size, 0.4 cm x 25 cm) or Lichrospher RP8 (5 μM particle size, 0.4 cm x 12.5 cm).

NMR spectra were recorded on a Bruker WH300 (300 MHz) spectrometer, using TMS as reference. FAB-MS was performed by Dr K Eckart, using a FAB ion source. Elemental analyses were obtained at the Microanalytical Laboratory of the Institute of Chemistry of the Hebrew University.

Amino acid analyses were performed on a LKB 4400 apparatus coupled to a Spectra Physics SP 4100 printer plotter computing integrator, using 4 component sodium buffer systems and a standard 54 min programme. Hydrolysis of samples for amino acid analysis was carried out on 0.5 mg samples with constant boiling HCl solution (0.5 ml) which was degassed and sealed at high vacuum, and heated for 20 h at 120°C. The hydrolysate was dried over KOH pellets at high vacuum and diluted in 0.5 ml of sodium citrate buffer (pH 2.2).

General coupling procedure

In an ice-cooled flask under argon positive pressure, the amine hydrochloride (1 eq), the Boc-protected amino acid or peptide

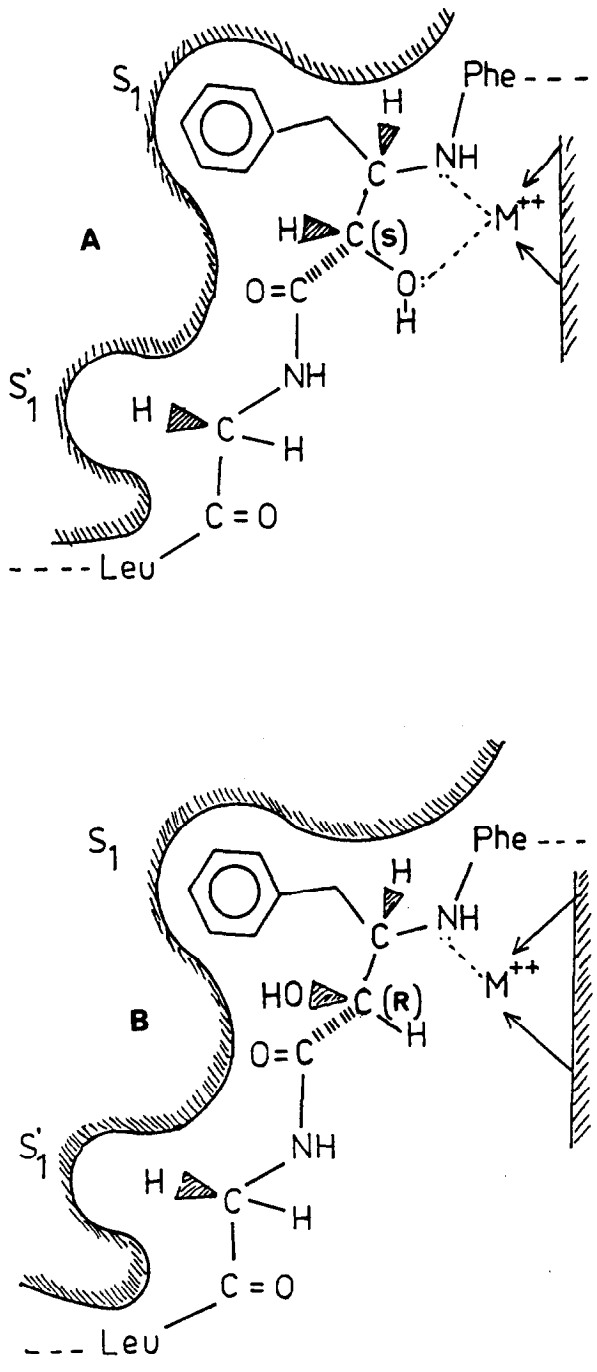


Fig 1. Schematic representation of ligand-putative active-site interactions. Panel A, ligand containing a residue of (2*S*, 3*S*)-α-hydroxy-β-amino-4-phenylbutyric acid provides more efficient interaction with active-site leading to a stronger inhibition. Panel B, ligand containing a residue of (2*R*, 3*S*)–α-hydroxy-β-amino-4-phenyl butyric acid can not provide its α-OH as a ligand to the M^{+2} at the active-site resulting in a less effective inhibition.

(1 eq) and HOBt (2 eq) were dissolved in DMF. DCC (1 eq) dissolved in the same solvent was cooled and added to the reaction mixture immediately followed by NMM (1 eq). This mixture was usually allowed to reach room temperature and was left stirring overnight. DCU was then filtered off, the filtrate was cooled in an ice bath and a saturated of $KHCO_3$ or $NaHCO_3$ was added (4–6 times the vol of DMF). Then brine was added (about half the amount of DMF), the resulting precipitate was collected on a sintered glass filter, and triturated with water until no more Cl^- was detected with an $AgNO_3$ test. At this point the solid collected was dried overnight over P_2O_5 at high vacuum.

General deprotection procedure

Boc-protected intermediates were dissolved in EtOAc saturated with HCl. The mixture was protected from moisture with a $CaCl_2$ trap and magnetically stirred for 0.5–1 h at room temperature. The residue obtained from the evaporation of the solvent was triturated under ether followed by decantation of the solvent. This was repeated twice more and then the remaining residue was left to dry at high vacuum over KOH pellets.

Materials

Phosphoramidon was purchased from the Peptide Research Institute (Osaka, Japan), catopril was graciously provided by MA Ondetti (Squibb Institute, Princeton, NJ) and [^{125}I-BH]SP(6–11) was prepared according to previously published procedures [23].

3(S)tert-butoxycarbonylamino-2(RS)-4-hydroxyphenyl butyric acid (Boc-AHPA-OH) 1

(2RS; 3S)AHPA-HCl•H_2O was synthesized according to the method of Nishizawa *et al* [22]; 1.82 g (9 mmol) of it were dissolved in dioxan (20 ml), water (10 ml) and NaOH 1 N (10 ml). To this was added Boc_2O (2.21 g, 10 mmol), while cooling in an ice-water bath. After one h the solvents were partially evaporated and the aqueous residue was washed once with EtOAc. Acidification to pH = 2 of the aqueous layer with a saturated solution of $KHSO_4$ was followed by extraction of the product into EtOAc. This was repeated twice more and then the organic layer was washed once with brine dried over $MgSO_4$ (anh), filtered and evaporated. 1.89 g (68.7%) of dry product were obtained. The materials were sufficiently pure for further use. An analytical sample was recrystallized from EtOAc–petroleum ether, yielding material with mp = 82–90°C. TLC R_f: 0.09 (A), 0.62 (B), 0.9 (D). HPLC, k' (MeOH%/$H_2$0%): a) 2.69 (55/49), RP18 (a and b are roughly equal in size. They are attributed to diastereoisomers). Elemental analysis: C, H, N for $C_{15}H_{21}NO_5$•0.5H_2O: FAB-MS: *m/e* 296 $[M+H]^+$.

Boc-AHPA-Phe-Gly-Leu-Met-NH_2 2

Boc-AHPA-OH (0.42 mmol) and HCl•H-Phe-Gly-Leu-Met-NH_2 (0.21 g, 0.42 mmol) reacted according to the general coupling procedure, in DMF (6 ml). The product was isolated from the reaction mixture as a semi-solid mass, weighing 0.19 g (60.8%). TLC, R_f: 0.74 (A), 0.86 (B). FAB-MS: *m/e*: 744 $[M+H]^+$. Amino acid analysis: Met: 1, Leu: 1, Gly:1, Phe: 1, AHPA: 1 (the retention time of AHPA was determined in an independent standard run. It was detected at approximately the same elution time as Lys in the standard amino acid analysis mixture).

pGlu-AHPA-Phe-Gly-Leu-Met-NH_2 ***4***
The amine protecting group of **2** (37.9 mg, 0.056 mmol) was removed by acidolysis by the above described general procedures: the amine hydrochloride **3** thus obtained (34.5 mg, 90% yield) was reacted with pGlu-OH (9.2 mg, 0.07 mmol) following the standard coupling procedure, in DMF (1 ml). The product was isolated by precipitation from the reaction mixture; after drying overnight it weighed 14.8 mg (38.7%), mp = 260–265°C.

Boc-AHPA-Gly-Leu-Met-NH_2 ***5***
A solution of **1** (0.66 g, 2.2 mmol) in DMF (7.5 ml) was mixed with HCl•H-Gly-Leu-Met-NH_2 (0.8 g, 2.2 mmol). This was reacted according to the general coupling procedure and the product was isolated by precipitation from the reaction mixture. After drying it weighed 0.58 g (44%) mp = 115–120°C. TLC R_f: 0.8 (E), 0.19 (D). FAB-MS: *m/e* 597 $[M+H]^+$; 496 $[(M\text{-}Boc)+H]^+$. Amino acid analysis: Met: 1; Leu: 1; Gly: 1; AHPA: 1.

Boc-Phe-AHPA-Gly-Leu-Met-NH_2 ***7***
The tetrapeptides (**5** 0.48 g, 0.8 mmol) was deprotected by HCl/EtOAc as usual. The obtained peptide amine hydrochloride **6** (0.34 g, 0.64 mmol) was coupled to Boc-Phe-OH (0.17 g, 0.64 mmol) by using the standard procedure, in DMF (2 ml). The isolated product weighed 0.34 g (71%), mp = 115–118°C. HPLC k': 2.76 (MeOH%/H_2O, 70:30). FAB-MS: *m/e* 745 $[M+H]^+$; 643 $[(M\text{-}Boc)+H]^+$. Amino acid analysis: Met: 1; Leu: 1; Gly: 1; AHPA: 1; Phe: 1.

pGlu-Phe-AHPA-Gly-Leu-Met-NH_2 ***9****;*
separation of diastereomers ***9a*** *and* ***9b***
The N-Boc protecting group of **7** (0.34 g, 0.45 mmol) was cleaved using the general deprotection method. The product **8** (0.2 g, 0.29 mmol) was coupled to pGlu-OH (38 mg, 0.29 mmol), according to the general coupling procedure. The first crop of isolated product **9a** precipitated from the reaction mixture after bicarbonate and brine addition. It was collected, washed with water, dried and recrystallized 3 times from EtOA–petroleum ether. Weight: 0.1 g (48.5%) mp = 128–134°C. Elemental analysis: C, H, N for $C_{37}H_{51}N_7O_8S\cdot H_2O$.

The second diastereomer precipitated from the work-up mixture after extended storage in the cold. It was recrystallized twice from EtOAc–petroleum ether. Weight of dried product, **9b**: 36 mg (16.5%), mp = 195–200°C. Elemental analysis: C, H, N.

Boc-AHPA-Gly-OEt ***10****;*
separation of diastereomers ***10a*** *and* ***10b***
Boc-AHPA-OH (1.0 g, 3.4 mmol) and HCl•H-Gly-OEt (0.47 g, 3.3 mmol) were coupled in DMF (9 ml) by using the general coupling procedure. The product was isolated in 2 crops, by the usual work-up procedure. The first crop, diastereomer **10a**, weighed after collecting, washing and drying, 0.48 g (37%), mp = 105–110°C. TLC R_f: 0.6 (E), 0.41 (D). HPLC k' (MeOH%/$H_2$0%) 2.17 (60/40) RP 18. FAB-MS; *m/e* 381 $[M+H]^+$. NMR ($CDCl_3$) δ ppm: 7.26 (s, C*H*Cl_3), 7.23 (M, C_6H_5-AHPA, partly covered by $CHCl_3$), 5.54 (1H, m, -CO-N*H*-Gly), 4.96 (1H, d, Boc-N*H*-AHPA-), 4.21 (3H, m, Et-CH_2-, CH(O*H*)-AHPA), 4.05 (4H, m, Gly α-CH_2 α-*CH*-(OH)-AHPA, β-C*H*-AHPA), 3.05 (2H, m, τ-CH_2-AHPA), 1.40 (9H, s, Boc), 1.28 (3H, t, Et-CH_3).

The second crop **10b** precipitated during cold storage of the work-up mixture. This too was collected, washed and recrystallized as above. Dry weight: 0.22 g (17%), mp = 107–119°C. TLC R_f: 0.64 (E), 0.33 (D). HPLC k' (MeOH%/$H_2$0%) 2.63 (60/40) RP 18. FAB-MS; *m/e* 381 $[M+H]^+$. NMR ($CDCl_3$) δ ppm: 7.26 (s, C*H*Cl_3), 7.23 (M, C_6H_5-AHPA, partly covered by $CHCl_3$), 5.45 (1H, m, -CO-N*H*-Gly), 5.06 (1H, d, Boc-N*H*-AHPA-), 4.17 (3H, m, Et-CH_2-, CH(O*H*)-AHPA), 4.03 (4H, m, α-CH_2-Gly, β-C*H*-AHPAA, α-C*H*(OH)-AHPA), 3.06 (2H, m, -CH_2-AHPA), 1.38 (9H, s, Boc), 1.27 (5H, t, Et-CH_3).

pGlu-Phe-AHPA-Gly-OEt ***13a***
The protected dipeptide **10a** (0.3 g, 0.78 mmol) reacted according to the standard deprotection procedure to yield **11a** (0.2 g, 0.63 mmol). This was coupled to Boc-pGlu-Phe-OH (0.24 g, 0.63 mmol) following the general coupling procedure, in DMF (2.5 ml). The isolated and dried product **12a** (70 mg, 0.1 mmol) was deprotected as described above yielding **13a**, which was washed with ether and dried weighing 53 mg (90% yield in the final step), mp = 218°C (d).

pGlu-Phe-AHPA-Gly-OEt ***13b***
The separate diastereomer of **13a** was prepared in the same manner as this last compound, starting from **10a** (0.15 g, 0.39 mmol). The isolated final product **13b** weighed 68 mg (85% yield in the final step) mp = 185°C (d).

Biological assays, isolated guinea-pig ileum assay
This was done as previously described [28].

Rat diencephalon membrane preparation
Diencephalons from 7 male albino rats were homogenized in 25 ml of ice-cold Hepes buffer (50 mM, pH 7.4), with 10 strokes of a Teflon-glass homogenizer at 800 rpm, and centrifuged at 100 *g* for 10 min. The supernatant was recentrifuged at 17 000 *g* for a further 10 min. The resulting pellet was suspended with a Dounce Homogenizer in cold buffer and centrifuged again at 17 000 *g* for 10 min. The final pellet was washed with cold buffer and resuspended in 5 ml of buffer (1–3 mg/ml of protein).

Assay of peptidase acting on the C-terminal sequence of substance P
The degradation of [^{125}I-BH]SP(6–11) by rat diencephalon membrane preparation and its inhibition by the above mentioned compounds was assayed as previously described [16]. High concentrations (10^{-6} M each) of captopril and phosphoramidon, potent inhibitors displayed high affinities toward angiotensin converting enzyme and enkephalinase, respectively [18, 29] were included in the assay in order to detect SP degrading activities distinct from those 2 enzymes. The IC_{50} values of all peptides were determined as described previously [23].

References

1 Nicoll RA, Schenker C, Leeman SE (1980) *Annu Rev Neurosci* 3, 227–268
2 Pernow B (1983) *Pharm Rev* 3, 85–141
3 Henry JL (1982) *Ciba Found Symp* 91, 206–224
4 Laufer R, Wormser U, Friedman ZY, Gilon C, Chorev M, Selinger Z (1985) *Proc Natl Acad Sci USA* 82, 7444–7448
5 Sandberg BEB, Iversen LL (1982) *J Med Chem* 25, 9, 1009–1015
6 Lee CM, Sandberg CM, Hanley MR, Iversen LL (1981) *Eur J Biochem* 114, 315–327
7 Wormser U, Gilon C, Chorev M, Laufer R, Selinger Z (1984) *In: Peptides 1984* (Rangnarsson U, ed) Almquist and Wiksell International, Stockholm, 355–358

8 Evans BE, Rittle KE, Bock MG, Bennet CD, Doi-Pardo RM, Boger J, Poe M, Ulm EH, LaMont BI, Blaine EH, Fannelli GM, Stabilito II, Veber DF (1975) *J Med Chem* 28, 1755–1756

9 Thorsett ED, Harris EE, Aster SD, Peterson ER, Snyder JP, Springer JP, Hirshfield J, Tristram EW, Patchett AA, Ulm EH, Vassil TC (1986) *J Med Chem* 29, 251–260

10 Almquist RG, Jennings-White C, Chao WR, Steeger T, Wheeler K, Rogers J, Mtoma C (1985) *J Med Chem* 28, 1062–1066

11 Matarajan S, Gordon EM, Sabo ED, Godfrey JD, Weller HN, Pluscek J, Rom MB, Cushman DW (1984) *Biochim Biophys Res Commun* 124, 141–147

12 Fournie-Zaluski MC, Bouboutou R, Waksman G, Roques B (1985) *In: Peptides, Structure and Function* (Deber CM, Kopple K, Hruby VJ, eds) Pierce Chem Co, Rockford, IL, 807–810

13 Nishino N, Powers JC (1979) *Biochemistry* 18, 4340–4346

14 Cascieri MA, Bull H, Mumford RA, Patchett AA, Thornberry N, Liang T (1984) *Mol Pharmacol* 25, 287–295

15 Matsas R, Fulcher IS, Kenny AJ, Turner AJ (1983) *Proc Natl Acad Sci USA* 80, 3111–3115

16 Laufer R, Ewenson A, Gilon C, Chorev M, Selinger Z (1985) *Eur J Biochem* 150, 135–140

17 Ewenson A, Laufer R, Chorev M, Selinger Z, Gilon C (1986) *J Med Chem* 29, 295–299

18 Ondetti MA, Cushman DW (1981) *Biopolymers* 20, 2001–2010

19 Boger J, Payene LS, Perlow DS, Lohr NS, Poe M, Blaine EH, Ulm EH, Schorn TW, La Mont BI, Lin TY, Kaway M, Rich DH, Veber DJ (1983) *J Med Chem* 28, 1779–1790

20 Rich DH, Salituro FG, Holladay MW, Schmidt PG (1984) *In: Conformationally Directed Drug Design* (Vida JA, Gordon M, eds) ACS Symp Ser, No 251, 211–237

21 Umezawa H, Aoyagi T, Suda H, Hamada M, Takeuchi T (1976) *J Antibiot* 29, 97–100

22 Nishizawa R, Saino T, Takita T, Suda H, Aoyagi T, Umezawa H (1977) *J Med Chem* 20, 510–515

23 Laufer R, Wormser U, Selinger Z, Chorev M, Gilon C (1984) *J Chromatogr* 301, 415–424

24 Harbeson SL, Rich DH (1988) *Biochemistry* 27, 7301–7310

25 Chorev M, Rubini E, Hart Y, Gilon C, Wormser H, Selinger Z (1986) *Eur J Pharm* 127, 187–196

26 Cox MT, Gormely JJ, Hayward CF, Peter NN (1980) *J Chem Soc Chem Commun* 800–803

27 Blumberg S, Teichberg VI (1981) *Biol Biophy Res Commun* 99, 752–758

28 Wormser U, Chorev M, Gilon C, Laufer R, Friedman Z, Selinger Z (1984) *Biochem Biophys Acta* 798, 28–35

29 Mumford RA, Pierzchala PA, Strauss AW, Zimmermann M (1981) *Proc Natl Acad Sci USA* 78, 6623–6627