Synthesis and Inhibitory Activities against Aminopeptidase B and Enkephalin-Degrading Enzymes of Ketomethylene Dipeptide Analogues of Arphamenines

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Three ketomethylene pseudodideptide analogues [(*S*)Lysψ(COCH$_2$)(*R* and *S*)Phe (**14** or **15** and **15** or **14**) and (*S*)Lysψ(COCH$_2$)(ξTrp (**19**)] of natural arphamenine A [(*S*)Argψ(COCH$_2$(*R*,*S*)Phe (**1**)] were easily prepared by a route involving two successive main reactions: a malonic ester alkylation with Z-protected lysine iodomethyl ketone and the introduction of a benzyl or (indol-3-yl)methyl moiety in position 2 of the resulting 4-ketodiester. The isomer of **1** with reversed sequence, (*S*)Pheψ(COCH$_2$)(*R*,*S*)Arg (**22**) was synthesized by guanidylation and subsequent deprotection of Z-(*S*)Pheψ(COCH$_2$)(*R*,*S*)Orn. The inhibitory effects of compounds **14**, **15**, **19**, and **22**, and the related ketomethylene dipeptides (*S*)Alaψ(COCH$_2$)(*R*,*S*)Phe (**3**), (*S*)Pheψ(COCH$_2$)(*R*,*S*)X [X=Ala (**4**), Orn (**5**)] and (*S*)Trpψ(COCH$_2$) (*R*,*S*)Y [Y=Orn (**6**), Lys (**7**), Arg (**8**)] on aminopeptidase B (AP-B), and enkephalin-degrading enzymes [aminopeptidase N (APN) and neutral endopeptidase (NEP)] were compared with that of the model compound **1**.

Synthese von Ketomethylen-Dipeptid Analogen von Arphameninen und deren Hemmwirkung gegenüber Aminopeptidase B und "enkephalin-degrading" Enzymen

Drei Ketomethylen-Pseudodipeptid Analoga [(*S*)Lysψ(COCH$_2$)(*R* und *S*)Phe (**14** oder **15** und **15** oder **14**) und (*S*)Lysψ(COCH$_2$)(ξTrp (**19**)] von natürlichem Arphamenin A [(*S*)Arg(COCH$_2$)(*R*,*S*)Phe (**1**)] werden auf einfache Weise über eine Folge von zwei Hauptreaktionen hergestellt: eine Malonester-Alkylierung mit Z-geschütztem Lysin-iodmethylketon und anschließende Einführung eines Benzyl- oder (Indol-3-yl)methylrestes in die 2-Stellung des erhaltenen 4-Ketodiesters. Das Isomer von **1** mit Umkehrsequenz, (*S*)Pheψ(COCH$_2$)(*R*,*S*)Arg (**22**) wurde durch Guanidylierung und anschließende Entfernung der Schutzgruppe von Z-(*S*)Pheψ(COCH$_2$)(*R*,*S*)Orn hergestellt. Der Hemmeffekt der Verbindungen **14**, **15**, **19** und **22** und der entspr. Ketomethylendipeptide (*S*)Alaψ(COCH$_2$)(*R*,*S*)Phe (**3**), (*S*)Pheψ (COCH$_2$) (*R*,*S*)X [X=Ala (**4**), Orn (**5**)] und (*S*)Trpψ(COCH$_2$)(*R*,*S*)Y [Y=Orn (**6**), Lys (**7**), Arg (**8**)] gegenüber Aminopeptidase B (AP-B) und "enkephalin-degrading" Enzym wurde mit dem Effekt der Modell-Verbindung **1** verglichen.

In the course of a screening for aminopeptidase B (AP-B) inhibitors of microbial origin, *Umezawa* et al.[1,2] found that 5(*S*)-amino-2(*R*)-benzyl-8-guanidino-4-oxooctanoic acid [(*S*)Argψ(COCH$_2$)(*R*)Phe,[3] arphamenine A (**1**)] and 5(*S*)-amino-2(*R*)-(4-hydroxybenzyl)-8-guanidino-4-oxooctanoic acid [(*S*)Argψ(COCH$_2$)(*R*)Tyr, arphamenine B (**2**)], the first naturally occurring carba analogues of peptides, are selective and potent inhibitors of this enzyme. Subsequently, it was reported that compound **1** also inhibits all the enkephalin-degrading enzymes from bovine small intestine (IC_{50} NEP*) = 10^{-4} M, IC_{50} APN*) = 10^{-4} M)[4]. The structure of arphamenines, particularly the presence of a ketomethylene group instead of the scissile

H-(S)Argψ(COCH$_2$)(R)Phe-OH (1)

H-(S)Argψ(COCH$_2$)(R)Tyr-OH (2)

H-(S)Alaψ(COCH$_2$)(R,S)Phe-OH (3)

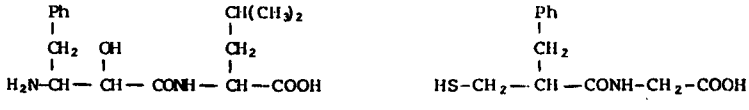
H-(S)Pheψ(COCH$_2$)(R,S)Ala-OH (4)

H-(S)Pheψ(COCH$_2$)(R,S)Orn-OH (5)

H-(S)Trpψ(COCH$_2$)(R,S)Orn-OH (6)

H-(S)Trpψ(COCH$_2$)(R,S)Lys-OH (7)

H-(S)Trpψ(COCH$_2$)(R,S)Arg-OH (8)



Bestatin Thiorphan

Scheme 1

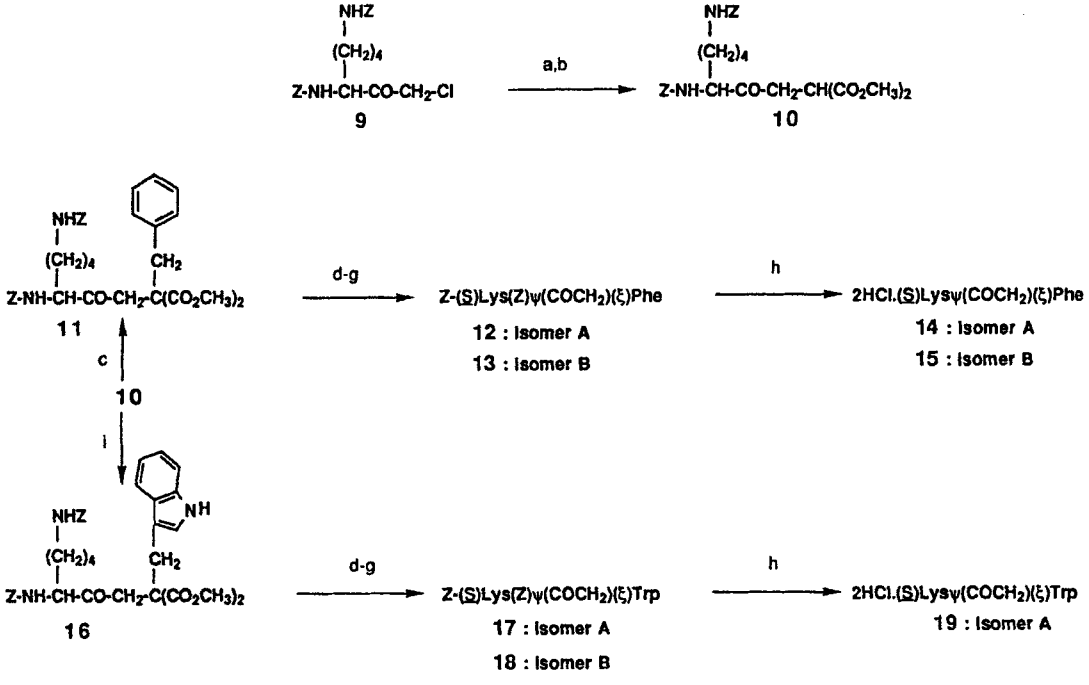
*) NEP: Neutral endopeptidase (EC 24.11)
APN: Aminopeptidase N

  0365-6233/92/0101-0003 $ 3.5 + .25/0

peptide bond, is rather intriguing and unrelated to other well known inhibitors of aminopeptidases or neutral endopeptidase (EC 24.11, NEP), such as bestatin[5], amastatin[6] or thiorphan[7]. A structure-modification study of arphamenines showed the necessity of the free amino and carboxyl groups for activity[8].

For these reasons, we considered of interest to compare the inhibiting properties of other ketomethylene dipeptides with those of arphamenines. With this aim, we recently developed a facile route to this type of pseudopeptides which was applied to the synthesis of (*S*)Alaψ(COCH$_2$)(*R*,*S*)Phe (**3**)[9], (*S*)Pheψ(COCH$_2$) (*R*,*S*)X [X=Ala (**4**)[9], Orn (**5**)[10]] and (*S*)Trpψ(COCH$_2$) (*R*,*S*)Y [Y=Orn (**6**), Lys (**7**), Arg (**8**)][10]. Now, we have extended this versatile route to the preparation of various arphamenines analogues with one basic amino acid (Arg, Lys) and the other one being aromatic (Phe, Trp), including the isomer of **1** with reversed sequence. This paper deals with the synthesis and the effect of these ketomethylene dipeptides on AP-B and enkephalin-degrading enzymes [aminopeptidase N (APN) and NEP]. Since compounds **3-8** are structurally related to the pseudodipeptides here reported, they have also been assayed and the inhibitory activities of all these ketomethylene dipeptides are compared with those of **1**.

protected amino acid halomethyl ketone and the introduction of a suitable substituent in position 2 of the resulting 4-ketodiester (Scheme 2). Thus, conversion of the L-lysine chloromethyl ketone **9**, obtained from the corresponding diazoketone, to the iodomethyl ketone, followed by *in situ* reaction with the monosodium derivative of dimethyl malonate afforded the 4-ketodiester **10**. Treatment of the sodium derivative of **10** with benzyl bromide and methiodide of gramine, *in situ* generated from gramine and methyl iodide, led to the 2-benzyl and 2-(3-methylindole) substituted derivatives **11** and **16**, respectively. Alkaline hydrolysis of these 2-substituted 4-ketodiesters and subsequent decarboxylation gave the expected Z-protected pseudodipeptides, namely Z-(*S*)Lys(Z)ψ(COCH$_2$)(*R*,*S*)Phe and Z-(*S*)Lys(Z)ψ(COCH$_2$) (*R*,*S*)Trp in which the C-terminal amino acids are fully racemic, due to the lack of stereoselectivity during decarboxylation. As reported[9,10], the asymmetric centre of the starting amino acid derivative is not affected in this synthetic route. The 1:1 mixtures of diastereomers were chromatographically separated, in each case, to provide the Phe- and Trp-containing pseudodipeptides **12** and **13**, and **17** and **18**, respectively. Hydrogenolysis of **12**, **13**, and **17** in 6N HCl/MeOH (1:20), using 10% Pd/C to remove the Z groups, gave the fully de-



(a) NaI, DME; (b) $^{+}$Na^{-}CH(CO$_2$CH$_3$)$_2$; (c) NaOMe, BrCH$_2$C$_6$H$_5$; (d) 6N NaOH; (e) HCl; (f) Dioxane, 100°C; (g) chromatographic separation; (h) H$_2$/Pd-C, HCl/MeOH (1:20); (i) NaOMe, methiodide of gramine

Scheme 2

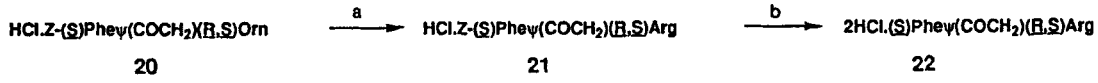
Results

Chemistry

The Lys-containing compounds **14**, **15**, and **19** were prepared following the reported procedure[9,10] involving two successive main steps: a malonic ester alkylation with the Z-blocked compounds **14**, **15**, and **19**. However, in the case of **18** a similar deprotection reaction led to a complex mixture of unstable compounds which were not identified. Very small differences were found between the ^{1}H-NMR spectra of the separated isomers **14** and **15**. Therefore, the absolute configuration of the C-terminal phenylalanine or tryptophan in **14** and **15** or **19** could not be assigned. In the course of our

work, H-Lysψ($COCH_2$) (*R,S*)Phe-OH was prepared from Boc-Lys(Boc) bromomethyl ketone and dibenzyl benzylmalonate[11]. Contrastingly to our present results, poor yields in the alkylation of Z-protected bromomethyl ketones with malonyl derivatives were obtained and no separation of the diastereomers were reported.

intramolecular reaction between the δ-amino and keto groups, since deprotection of this amino group occurred during the alkylation of **23** with the methiodide of gramine, as shown by a ^{1}H-NMR spectrum. A similar removal of one Z group from the guanidino function of Z-Arg(Z_2)-OH has been previously observed in alkaline medium[15].



20 **21** **22**

(a) 1-amidino-3,5-dimethylpyrazole nitrate, DMF; (b) H_2/Pd-C, HCl/MeOH (1:20)

Scheme 3

Compound **22**, which corresponds to the isomer of arphamenine A with reversed sequence, was prepared by guanidylation of the ornithine analogue **20**[10], using 1-amidino-3,5-dimethylpyrazole nitrate (ADMP) by a method similar to that of *Klausner* et al.[12], and subsequent hydrogenolysis of the Z group (Scheme 3). Neither **22** nor the protected derivative **21** could be separated chromatographically; therefore, **22** is probably a mixture of diastereomers in the same ratio as the starting compound **20** (1:1).

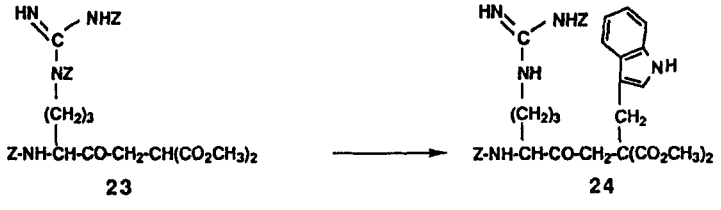
At this point, it should be pointed out that *Umezawa* et al.[1,2,13] reported that natural and synthetic arphamenines always contain a small amount of the (2*S*,5*S*)-diastereomer (*epi*-arphamenines), due to inevitable epimerization at C-2. Although in our case, no epimerization was observed in the pure diastereomers **14**, **15**, and **19**, the ^{1}H-NMR spectrum of commercial arphamenine A, we used for biological comparisons, showed the presence of the *epi*-isomer in 50%. For this reason, we considered **22** as the isomer of arphamenine A with reversed sequence, and our biological work was done using 1:1 mixtures of (2*R*,5*S*)- and (2*S*, 5*S*)-diastereomers in the case of compounds **3-8** and **22**.

Biological Results and Discussion

Ketomethylene dipeptides **3-8**, **14**, **15**, **19**, and **22** were evaluated as inhibitors of AP-B activity, associated with the surface of mouse L cells, using L-lysine-β-naphtylamide (Lys-NA) as substrate[16], and as inhibitors of purified membrane-bound APN and NEP from rat brain[17], with [^{3}H]Leu-enkephalin as substrate, and the results were compared with those of **1**. Bestatin and thiorphan were also included in the aminopeptidases and the NEP assays respectively.

a) *AP-B Inhibition*

As shown in Table 1, compounds **1**, **3-8**, **14**, **15**, **19**, and **22** were completely equipotent in inhibiting AP-B, with IC_{50} values in the 10^{-4} M range. The low inhibitory potency of **1** against the AP-B utilized, as compared to that reported by *Umezawa* et al.[18] against AP-B, associated with the cell surface of mouse spleen ($IC_{50} \sim 10^{-6}$ M), and by *Harberson* and *Rich*[11,19] on purified AP-B from rat liver tissue, is striking. This fact does not seem to be related to a hypothetical



Scheme 4

Attempts to prepare the C-terminal modified analogue of arphamenines (*S*)Argψ($COCH_2$)(*R,S*)Trp from N^{α},N^{δ},N^{ω}-tri-Z-arginine chloromethyl ketone, in a similar manner to that described for **19**, were unsuccessful, as the demethoxycarbonylation of the 2-substituted 4-ketodiester **24** afforded an untractable mixture of unstable compounds which could not be identified. It is known that protection of the guanidino function of arginine with a single Z group does not prevent the intramolecular cyclization to the 2-piperidone derivative during activation and coupling reactions[14]. Therefore, our failure could be due to the formation of unstable compounds, such as cyclic aminals or imines, resulting from the difference in the diastereomeric ratio between the arphamenine A, we used (2*R*,5*S* : 2*S*,5*S* = 1) and that employed by these authors (presence of the (2*S*,3*S*)-isomer, but in an undetermined amount)[1,2,13,18], since the IC_{50}'s of pure diastereomers **14** and **15** were almost identical.

b) *Enkephalin-Degrading Enzymes Inhibition*

As for AP-B inhibition, this series of ketomethylene dipeptides, including compound **1**, were modest inhibitors of APN (Table 1). However, it is interesting to note that the IC_{50} obtained for compound **1** is better than that determined

on APN from bovine intestine (IC_{50} ~ 10^{-4} M)[4]. By contrast, compound **1** did not affect NEP, up to 10^{-3} M (Table 1), although it has been reported to exhibit a certain inhibitory activity on NEP from bovine intestine (IC_{50} ~ 10^{-4} M)[4]. In our assays, NEP was only inhibited, although weakly, by compound **4**, which corresponds to the ketomethylene analogue of Phe-Ala, an inhibitor of NEP with an IC_{50} value in the micromolar range[20]. The 100-fold decrease in inhibitory activity by replacing the amide -CONH-linkage by a ketomethylene -$COCH_2$-group in Phe-Ala supports the existence of a hydrogen bonding interaction between the amide group of the dipeptide and the active site of NEP[20]. As in the case of the dipeptides Phe-X[20], the presence of a charged lateral chain (Orn, Lys, Arg) in **5-8** and **27** leads to an unfavourable effect on NEP inhibition.

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Experimental Part

Chemistry

Mp. (uncorrected): Kofler hot-stage apparatus.- Elemental analyses: Heraeus CHN-O-RAPID instrument.- ^{1}H-NMR spectra: Varian XL-300 spectrometer, TMS int. stand.- Analytical TLC: Aluminium sheets, 0.2 mm layer of silicia gel 60 F_{254} (Merck).- Column chromatography: silica gel 60 (230-400 mesh) (Merck). Compounds were detected with UV light (254 nm) and ninhydrin spray. N^{α},N^{ε}-(Dibenzyloxycarbonyl)-L-lysine and N^{α},N^{δ},N^{ω}-(tribenzyloxycarbonyl)arginine: Bachem (Switzerland).

N^{α},N^{ε}-(Dibenzyloxycarbonyl)-L-lysine chloromethyl ketone (**9**)

N-Methylmorpholine (3.3 mL, 30 mmol) and isobutyl chloroformate (4 mL, 35 mmol) were added to a cooled solution (0°C) of N^{α},N^{ε}-(dibenzyloxycarbonyl)-L-lysine (12.4 g, 30 mmol) in dry THF (50 mL). The mixture was stirred at 0°C for 30 min and then filtered. An ethereal solution of CH_2N_2, prepared from nitrosomethylurea (3.6 g, 35 mmol), was added to the filtrate and the mixture was stirred for 15 min at 0°C, concentrated to a small volume and then, dry HCl was bubbled into the solution until N_2 evolution ceased. Solvents were removed *in vacuo* and the residue purified on a silica gel column eluting with EtOAc/hexane (1:3) to provide 10.3 g (77%) of pure **9** as a white solid; mp. 76°C.- ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.20-1.90 (m, 6H, Lys β-, γ-, and δ-CH_2), 3.13 (m, 2H, Lys ε-CH_2), 4.20 (s, 2H, CH_2Cl), 4.53 (m, 1H, Lys α-CH), 5.03 (s, 4H, benzyl CH_2), 7.30 (s, 10H, benzyl C_6H_5).

Methyl N^5,N^9-(dibenzyloxycarbonyl)-5(S)-9-diamino-2-methoxycarbonyl-4-oxononanoate (**10**)

A mixture of **9** (4.5 g, 10 mmol) and NaI (1.5 g, 10 mmol) in 1,2-dimethoxyethane (30 mL) was stirred at room temp. for 15 min, followed by the addition of the sodium salt of dimethyl malonate (1.7 g, 11 mmol), freshly prepared from the corresponding diester and sodium methoxide, in 1,2-dimethoxyethane (10 mL). Stirring was continued at that temp. for 1 h, the solvents were removed, and the residue was extracted with $CHCl_3$ (50 mL) and washed with H_2O (50 mL). The org. extract was dried (Na_2SO_4) and evaporated leaving a residue which was purified by column chromatography eluting with EtOAc/hexane (1:2) to yield **10** as a syrup (4.0 g, 74%).- ^{1}H-NMR ($CDCl_3$): δ (ppm) = 1.20-1.90 (m, 6H, H-6, H-7, H-8), 3.10 (m, 4H, H-3, H-9), 3.33 (m, 1H, H-5), 3.70 (s, 6H, 2 CO_2CH_3), 3.86 (t, 1H, H-2, $J_{2,3}$ = 7 Hz), 7.26 (s, 10H, benzyl. C_6H_5).

Methyl N^5,N^9-(dibenzyloxycarbonyl)-5(S)-9-diamino-2-benzyl-2-methoxycarbonyl-4-oxononanoate (**11**)

To a stirred solution of **10** (27 g, 5 mmol) and freshly prepared sodium methoxide (5.5 mmol) in 1,2-dimethoxyethane (30 mL) was added benzyl bromide (1 g, 6 mmol). After 1 h of stirring at room temp., solvents were removed leaving a residue which was purified on a silica gel column wiht EtOAc/hexane (1:1) to give **11** as a syrup (2.7 g, 85%).- ^{1}H-NMR ($CDCl_3$): δ (ppm) = 1.30-1.90 (m, 6H, H-6, H-7, H-8), 3.14 (m, 2H, H-9), 3.30 (m, 2H, H-3), 3.40 (m, 2H, CH_2 benzyl), 3.70 (s, 6H, 2 CO_2CH_3), 4.26 (m, 1H, H-5), 7.25 (m, 5H, Ph), 7.30 (s, 10H, benzyl. C_6H_5).

Methyl N^5,N^9-(dibenzyloxycarbonyl)-5(S)-9-diamino-2-[(indol-3-yl) methyl]-2-methoxycarbonyl-4-oxononanoate (**16**)

A cooled solution (0°C) of **10** (2.7 g, 5 mmol) and freshly prepared sodium methoxide (5.5 mmol) in 1,2-dimethoxyethane (30 mL) was treated with gramine (0.9 g, 5 mmol) and methyl iodide (1.42 g, 10 mmol). After 1 h of stirring at 0°C, the mixture was filtered and the filtrate was evaporated to give a residue which was purified by silica gel chromatography using EtOAc/hexane (2:3). A syrup was obtained (2.7 g, 81%).- ^{1}H-NMR ($CDCl_3$): δ (ppm) = 1.10-1.65 (m, 6H, H-6, H-7, H-8), 3.10 (m, 4H, H-3, H-9), 3.60 (m, 2H, CH_2-indole), 3.70 (s, 6H, 2 CO_2CH_3), 4.20 (m, 1H, H-5), 6.80-7.30 (m, 5H, indole), 7.33 (s, 10H, benzyl. C_6H_5).

General Procedures

Method A: Saponification and Decarboxylation

A solution of the 2-substituted diester (4 mmol) in MeOH (40 mL) was treated with 6N NaOH (2 mL) and the mixture was stirred at room temp. for 3 h. After evaporation of the MeOH, the remaining aqueous mixture was diluted with H_2O (30 mL), acidified with conc. HCl to pH 3, and extracted with EtOAc (100 mL). The extract was dried (Na_2SO_4) and evaporated, and the residue was dissolved in dioxane (30 mL) and heated under reflux for 4 h. Removal of the solvent provided diastereomeric pseudodipeptides which were separated as specified in each case.

Method B: Removal of the Benzyloxycarbonyl Protecting Group

A solution of the Z protected pseudodipeptide (2.2 mmol) in MeOH (100 mL) containing 6N HCl (0.5 mL) was hydrogenated at 30 psi and room temp., in the presence of 10% Pd/C (1.2 g) for 8 h. The catalyst was removed by filtration, and the filtrate was evaporated to dryness to leave the crude deprotected pseudodipeptide which was purified by flash chromatography using $CHCl_3$/MeOH (3:1).

Z-(S)Lys(Z)ψ($COCH_2$)(ξ)Phe (**12**, *isomer A and* **13**, *isomer B*)

These compounds were prepared from **11** according to method A and separated by flash chromatography using $CHCl_3$/MeOH (50:1). The first eluted diastereomer was **12**, designated as isomer A, and the last eluted one, **13**, as isomer B.

12: Yield 48%; foam.- ^{1}H-NMR (Me_2SO-d_6+TFA): δ (ppm) = 1.20-1.50 (m, 4H, Lys γ- and δ-CH_2), 1.63 (m, 2H, Lys β-CH_2), 2.45-2.96 (m, 7H, Lys ε-CH_2, Phe α-CH, Phe β-CH_2, $COCH_2$), 3.95 (m, 1H, Lys α-CH), 4.99 and 5.01 (2s, 4H, 2 benzyl CH_2), 7.13-7.38 (m, 15H, 2 benzyl C_6H_5, C_6H_5).

13: Yield 43%; foam.- ^{1}H-NMR (Me_2SO-d_6+TFA): δ (ppm) = 1.20-1.50 (m, 4H, Lys γ- and δ-CH_2), 1.56 (m, 2H, Lys β-CH_2), 2.47-2.96 (m, 7H, Lys ε-CH_2, Phe α-CH, Phe β-CH_2, $COCH_2$), 3.89 (m, 1H, Lys α-CH), 5.00 and 5.02 (2s, 4H, 2 benzyl CH_2), 7.13-7.38 (m, 15H, 2 benzyl C_6H_5, C_6H_5).

Z-(S)Lys(Z)ψ($COCH_2$)(ξ)Trp (**17**, *isomer A and* **18**, *isomer B*)

These compounds were prepared from **16** according to method A and separated by flash chromatography using $CHCl_3$/MeOH (100:1). The first eluted diastereomer was **17**, designated as isomer A, and the last eluted one **18**, isomer B.

17: Yield 40%; foam.- ^{1}H-NMR (Me_2SO-d_6+TFA): δ (ppm) = 1.25-1.45 (m, 4H, Lys γ- and δ-CH_2), 1.67 (m, 2H, Lys β-CH_2), 2.49-3.08 (m, 7H, Lys ε-CH_2, Trp α-CH, Trp β-CH_2, $COCH_2$), 4.00 (m, 1H, Lys α-CH), 5.03 (s, 4H, 2 benzyl CH_2), 7.00-7.42 (m, 15H, 2 benzyl C_6H_5, indole).

18: Yield 42%; foam.- ^{1}H-NMR (Me_2SO-d_6+TFA): δ (ppm) = 1.23-1.45 (m, 4H, Lys γ- and δ-CH_2), 1.58 (m, 2H, Lys β-CH_2), 2.48-3.05 (m, 7H, Lys ε-CH_2, Trp α-CH, Trp β-CH_2, $COCH_2$), 3.88 (m, 1H, Lys α-CH), 5.01 (s, 4H, 2 benzyl CH_2), 6.99-7.40 (m, 15 H, 2 benzyl C_6H_5, idole).

2HCl·(S)Lysψ(COCH$_2$)(ξ)Phe (**14**, *isomer* A)

The title compound was obtained from **12** according to method **B**: yield 90%; foam.- ^{1}H-NMR (D_2O): δ (ppm) = 1.40 (m, 2H, Lys γ-CH_2), 1.70 (m, 2H, Lys δ-CH_2), 1.92 and 2.05 (2m, 2H, Lys β-CH_2), 2.75-3.20 (m, 6H, Lys ε-CH_2, Phe β-CH_2, $COCH_2$), 3.22 (m, 1H, Phe α-CH), 4.25 (m, 1H, Lys α-CH), 7.25-7.41 (m, 5H, C_6H_5).

2HCl·(S)Lysψ(COCH$_2$)(ξ)Phe (**15**, *isomer* **B**)

This compound was obtained from **13** according to method **B**: yield 89%; foam.- ^{1}H-NMR (D_2O): δ (ppm) = 1.41 (m, 2H, Lys γ-CH_2), 1.69 (m, 2H, Lys δ-CH_2), 1.83 and 2.01 (2m, 2H, Lys β-CH_2), 2.84-3.10 (m, 6H, Lys ε-CH_2, Phe β-CH_2, $COCH_2$), 3.23 (m, 1H, Phe α-CH), 4.26 (m, 1H, Lys α-CH), 7.26-7.42 (m, 5H, C_6H_5).

2HCl·(S)Lysψ(COCH$_2$)(ξ)Trp (**19**, *isomer* A)

This compound was prepared from **17** according to method **B**: yield 80%; foam.- ^{1}H-NMR (D_2O): δ (ppm) = 1.18 (m, 2H Lys γ-CH_2), 1.47 (m, 2H, Lys δ-CH_2), 1.72 (m, 2H, Lys β-CH_2), 2.56-3.12 (m, 6H, Lys ε-CH_2, Trp β-CH_2, $COCH_2$), 3.13 (m, 1H, Trp α-CH), 3.97 (m, 1H, Lys α-CH), 7.00-7.55 (m, 5H, indole).

HCl·Z-(S)Pheψ(COCH$_2$)(R,S)Arg (**21**)

A solution of **20**[10] (0.80 g, 1.7 mmol) in dry DMF (10 mL) was added to a solution of 1-amidino-3,5-dimethylpyrazole nitrate (0.41 g, 2.0 mmol) in dry DMF (10 mL), adjusted to pH 8-9 with triethylamine (0.25 mL), and the pH of the mixture was brought to pH 8-9 with more triethylamine (0.50 mL). After 7 d at room temp., the solvent was removed, the residue was dissolved in 2N HCl (10 mL) and washed with EtOAc (30 mL). The aqueous phase was evaporated to dryness, and the residue was purified on a silica gel column ($CHCl_3$/MeOH (3:1)), to give **21** (0.53 g, 64%) as a foam.- ^{1}H-NMR (Me_2SO-d_6+TFA): δ (ppm) = 1.35-1.60 (m, 4H, Arg β- and γ-CH_2), 2.55-3.09 (m, 5H, Arg α-CH, Phe β-CH_2, $COCH_2$), 3.10 (m, 2H, Arg δ-CH_2), 4.26 (m, 1H, Phe α-CH), 5.00 (s, 2H, benzyl CH_2), 7.30 (s, 5H, benzyl C_6H_5), 7.20-7.40 (m, 5H, C_6H_5).

2HCl·(S)Pheψ(COCH$_2$)(R,S)Arg (**22**)

The title compound was prepared from **21** according to method **B**: yield 85%; foam.- ^{1}H-NMR (D_2O): δ (ppm) = 1.50-1.80 (m, 4H, Arg β- and γ-CH_2), 2.75-3.22 (m, 6H, Arg δ-CH_2, Phe β-CH_2, $COCH_2$), 3.46 (m, 1H, Arg α-CH), 4.56 (m, 1H, Phe α-CH), 7.20-7.50 (m, 5H, C_6H_5).

Biological Tests

The following commercial drugs were used: arphamenine A and bestatin (Peptide Institute, Inc., Japan), L-Lys-NA (Sigma, UK), thiorphan (CRB, UK) and ^{3}H-Leu-enkephalin (The Radiochemical Centre, UK).

Mouse L cells were grown in *Dulbecco's* modified *Eagle's* medium and 10% fetal calf serum. L Cells, media and serum were supplied by Flow Labs (UK).

AP-B Assays

Cell surface-associated AP-B activities were determined according to *Aoyagi*[16]. The incubation mixture consisted of 2mM L-Lys-NA (0.25 mL), *Hank's* balanced salt solution (0.65 mL) and distilled water (0.1 mL) with, or without the inhibitor. After 3 min of incubation (37°C), the mixture was added to monolayer cultures of mouse L cells (~ 5 x 10^5 cells), and the incubation was stopped after 30 min by adding the stabilized diazonium salt Garnet GBC (1 mL, 1 mg/mL) in 1 M acetic acid buffer at pH 4.2, containing 10% Tween 20. The mixture was left at room temp. for 15 min, centrifuged and its absorbance was measured at 525 nm.

APN and NEP Assays

A membrane preparation from rat striatum was obtained as described[17]. After a 10 min preincubation to eliminate endogeneous enkephalins, incubations (15 min, 25°C) were started by addition of ^{3}H-Leu-enkephalin (20 nM final concentration) and different concentrations of the tested inhibitors. ^{3}H-Metabolites were separated from intact ^{3}H-Leu-enkephalin by TLC on silica gel using isopropanol/EtOAc/AcOH (2:2:1). The spots, detected by ninhydrin in EtOH solution, were removed, the peptide extracted with 1 mL of MeOH and the radioactivity was determined by liquid scintillation spectrometry.

Table 1: Elemental analyses of compounds **9-22**

			Cald.				Found			
No	Formula	Mw	C	H	Cl	N	C	H	Cl	N
9	$C_{23}H_{27}ClN_2O_5$	446.9	61.8	6.08	7.9	6.3	61.8	6.15	7.7	6.0
10	$C_{28}H_{34}N_2O_9$	542.6	62.0	6.31	-	5.2	61.9	6.03	-	5.3
11	$C_{35}H_{40}N_2O_9$	632.7	66.4	6.37	-	4.4	66.2	6.23	-	4.3
12	$C_{32}H_{36}N_2O_7$	560.6	68.5	6.47	-	5.0	68.3	6.50	-	4.8
13	$C_{32}H_{36}N_2O_7$	560.6	68.5	6.47	-	5.0	68.2	6.38	-	4.7
14	$C_{16}H_{26}Cl_2N_2O_3$	365.3	52.6	7.17	19.4	7.6	52.5	7.33	19.3	7.5
15	$C_{16}H_{26}Cl_2N_2O_3$	365.3	52.6	7.17	19.4	7.6	52.3	7.11	19.4	7.3
16	$C_{37}H_{41}N_3O_9$	671.7	66.1	6.15	-	6.2	65.8	6.30	-	6.1
17	$C_{34}H_{37}N_3O_7$	599.7	68.1	6.21	-	7.0	68.5	6.10	-	6.6
18	$C_{34}H_{37}N_3O_7$	599.7	68.1	6.21	-	7.0	68.5	5.19	-	6.7
19	$C_{18}H_{27}Cl_2N_3O_3$	404.3	53.5	6.73	17.5	10.4	53.7	6.48	17.3	10.1
21	$C_{24}H_{30}ClN_4O_5$	490.0	58.8	6.17	7.2	11.4	58.6	6.21	7.1	11.2
22	$C_{16}H_{26}Cl_2N_4O_3$	393.3	48.8	6.66	18.0	14.2	48.8	6.81	17.8	13.9

Table 2: Inhibitory Potency of Various Ketomethylene Dipeptides on Aminopeptidase (AP-B), Associated with the Surface of Mouse L Cells, and on Enkephalin-Degrading Enzymes (APN and NEP) from Rat Striatum

Compound	no	AP-B IC_{50} x 10^{-4} M[a]	APN IC_{50} x 10^{-4} M[a]	NEP IC_{50} M[a]
(S)AlaΨ($COCH_2$)(R,S)Phe	3	6.5	3.1	$>10^{-3}$
(S)PheΨ($COCH_2$)(R,S)Ala	4	6.9	2.6	1.5×10^{-4}
(S)PheΨ($COCH_2$)(R,S)Orn	5	5.5	1.1	$>10^{-3}$
(S)TrpΨ($COCH_2$)(R,S)Orn	6	6.1	1.9	$>10^{-3}$
(S)TrpΨ($COCH_2$)(R,S)Lys	7	5.6	0.7	$>10^{-3}$
(S)TrpΨ($COCH_2$)(R,S)Arg	8	5.6	2.0	$>10^{-3}$
(S)LysΨ($COCH_2$)(S)Phe (isomer A)	14	5.0	1.7	$>10^{-3}$
(S)LysΨ($COCH_2$)(S)Phe (isomer B)	15	5.3	7.4	$>10^{-3}$
(S)LysΨ($COCH_2$)(S)Trp (isomer A)	19	6.0	2.6	$>10^{-3}$
(S)PheΨ($COCH_2$)(R,S)Arg	22	5.0	5.8	$>10^{-3}$
(S)ArgΨ($COCH_2$)(R,S)Phe (arphamenine A)	1	5.1	0.2	$>10^{-3}$
Bestatin		0.06	0.03	
Thiorphan				10^{-8}

[a] Values are the mean of 4.5 experiments with 3-5 different concentrations of the inhibitor- S. E- were less than 10% of the mean.

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