Analgesic Dipeptide Derivatives. Part 8.[1] 3-Amino-2-hydroxy-4-[2-(*o*-nitrophenylthio)indol-3-yl]butanoic Acid [AH(Nps)IBA]-Containing Dipeptide Analogues of the Analgesic Compound H-Trp(Nps)-Lys-OMe

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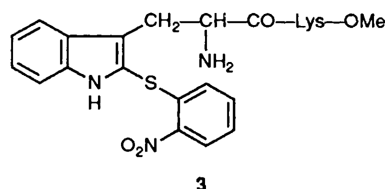
A series of diastereoisomeric dipeptides, analogues of the analgesic compound H-Trp(Nps)-Lys-OMe, containing 3-amino-2-hydroxy-4-[2-(*o*-nitrophenylthio)indol-3-yl]butanoic acid [AH(Nps)IBA] and Lys or Leu has been synthesized. These compounds were tested as aminopeptidase-M and -B (AP-M and AP-B) inhibitors and as analgesics. The AH(Nps)IBA-Leu dipeptides, independently of their stereochemistry, were poor inhibitors of AP-M and AP-B, with IC_{50}-values in the 10^{-4} mol dm^{-3} range, while the AH(Nps)IBA-Lys derivatives were poor AP-B inhibitors, with IC_{50}-values also in the 10^{-4} mol dm^{-3} range, and did not inhibit AP-M up to 10^{-3}. All the AH(Nps)IBA-Lys derivatives induced a significant dose-related analgesic activity at 1–5 μg per mouse, which was dependent on the stereochemistry, while no analgesia was observed with the corresponding Leu-containing analogues. There is no relationship between the antinociceptive effects and the AP-M inhibitory potencies of this series of compounds, indicating that the inhibition of enkephalin-degrading AP-M is not an important factor for the mode of action of this series of analgesic dipeptides.

In previous papers[2,3a] it was reported that the synthetic dipeptide H-Lys-Trp(Nps)-OH (Nps = *o*-nitrophenylthio) **1** and its methyl ester **2** exhibited naloxone-reversible analgesia in mice, comparable with that of the enkephalin analogue *D*-Ala-Met-enkephalinamide (DAME) with regard to both maximum effect and the time-course of analgesia. Studies to establish the structural requirements for the antinociceptive effect of compounds **1** and **2** showed the need for a basic amino acid,[2,4] the importance of the Nps moiety,[2,3] and the dependence of the analgesic activity on the absolute configuration of each amino acid.[5] The analgesic activity was found to be independent of the order of the amino acid sequence,[5] since the dipeptide H-Trp(Nps)-Lys-OMe **3** showed an antinociceptive effect similar to that of its isomer **2**. Studies on the mechanism of action of compound **1** appear to indicate that these Trp(Nps)-containing dipeptides do not act directly on opioid receptors, but their antinociceptive effects could possibly be explained by a mixture of a moderate enkephalin-degrading aminopeptidase (AP) inhibition and Met-enkephalin-releasing properties.[2] We considered that a structural modification able to increase the AP inhibitory potency of these dipeptide derivatives could help to clarify the participation of this inhibition in the observed analgesic effect and, therefore, in its mode of action. With this aim, the Trp(Nps) residue of compound **3** has now been replaced by its corresponding α-hydroxy-substituted homologue, 3-amino-2-hydroxy-4-[2-(*o*-nitrophenylthio)indol-3-yl]-butanoic acid [AH(Nps)IBA]. In these compounds the α-hydroxy group could mimic the tetrahedral intermediate formed during the substrate's hydrolysis by APs. This concept has been applied to explain the potent inhibition of APs by the natural compound (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine [(2*S*,3*R*)-AHPBA-Leu, bestatin **4**],[6] which protects the endogenous enkephalins, released from K^+-depolarized brain slices, from degradation by this type of enzyme.[7] This paper deals with the synthesis, inhibitory properties against AP-M (EC 3.4.11.2) (the main membrane-bound AP which degrades enkephalins),[8] and the antinociceptive activity of a series of stereoisomeric dipeptide derivatives **5**, in which the absolute configuration of each asymetric centre has been varied systematically. For comparative purposes the bestatin analogues **6**, in which the AHPBA residue has been replaced by AH(Nps)IBA, have also been synthesized and included in the biological assays. Since bestatin is also a well known AP-B (EC 3.4.11.6) inhibitor, inhibition which is related to its immunomodifier activity,[9] dipeptides **5** and **6** were also evaluated against this enzyme. All biological data are compared with those of the parent compounds **3** and bestatin **4**.



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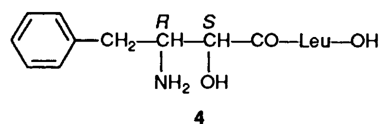

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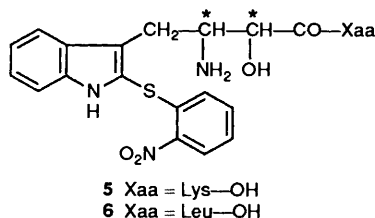
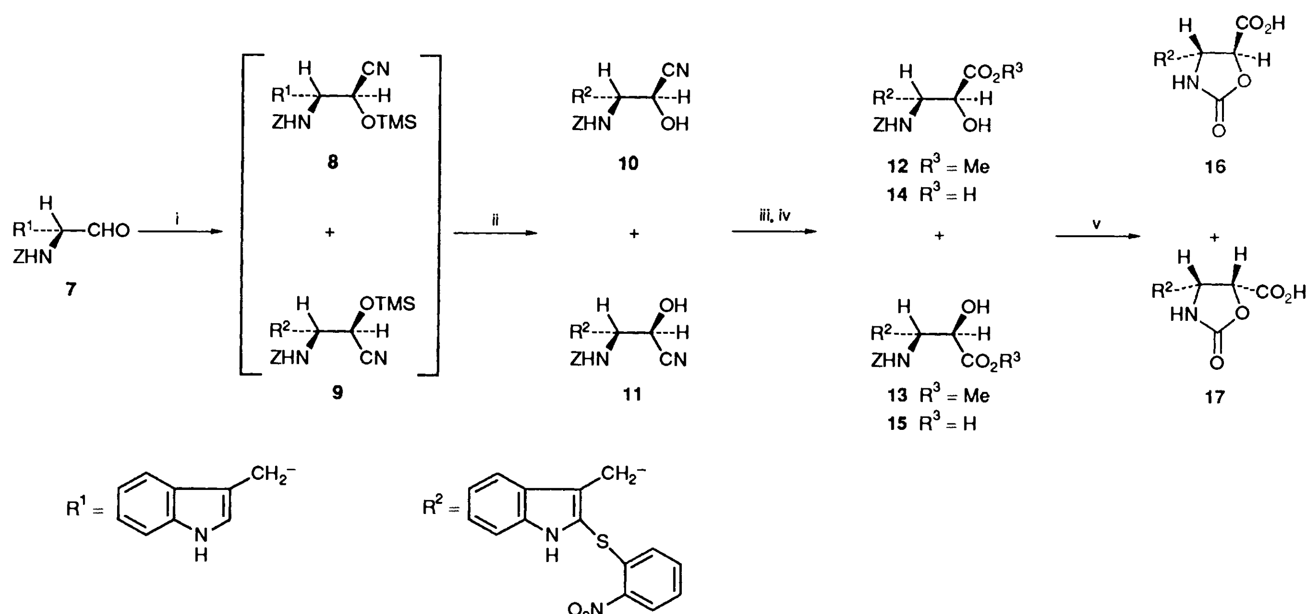


5 Xaa = Lys—OH
6 Xaa = Leu—OH

Results and Discussion

Chemistry.—As indicated in Scheme 1, the *N*-protected AH(Nps)IBA **14** and **15** were prepared following our method



Scheme 1 *Reagents:* i, TMSCN; ii, Nps-Cl, H^+; iii, HCl, MeOH; iv, water; v, NaOH, MeOH

for the stereoselective synthesis of (2*S*,3*R*)- and (2*R*,3*S*)-3-amino-2-hydroxy acids recently reported.[10] Thus, reaction of *N*-Z-D-tryptophanal **7**, freshly prepared[11] by the method of Fehrentz and Castro,[12] with trimethylsilyl cyanide (TMSCN) in dry dichloromethane yielded a (4:1) mixture of the corresponding *threo* and *erythro* *o*-(trimethylsilyl) cyanohydrins **8** and **9**. These cyanohydrins were sulphenylated *in situ*, by treatment with a solution of *o*-nitrobenzenesulphenyl chloride (Nps-Cl) in dry 1 mol dm^{-3} HCl in MeOH,[4,13] to give a (4:1) mixture of the *o*-nitrophenyl thio(Nps)cyanohydrins **10** and **11**. This mixture could not be separated by chromatography using different elution systems. The UV spectrum of the **10** + **11** mixture showed two absorption maxima, at 358 nm (ε 3.346 × 10^3) and 279 nm (ε 1.246 × 10^4), characteristic of Trp(Nps)-derivatives.[4,13b] The mixture of cyanohydrins **10** + **11** was transformed into a (4:1) mixture of the 2-hydroxy methyl esters **12** + **13** by treatment with dry methanolic hydrogen chloride, followed by *in situ* hydrolysis of the imidate hydrochloride intermediates. The ratio of diastereoisomers was determined by measurement of the integrals of the two singlets, corresponding to the methyl ester groups, in the ^1H NMR spectrum of the mixture. Attempts to transform directly the mixture of *O*-trimethylsilyl cyanohydrins **8** + **9** into the corresponding Nps-unsubstituted methyl esters gave a complex and intractable mixture, due to the instability of tryptophan derivatives in strongly acidic media.[14] On the other hand, attempts to transform the mixture of cyanohydrins **8** + **9** into the corresponding imidates by treatment with sodium methoxide led to the starting aldehyde **7**.

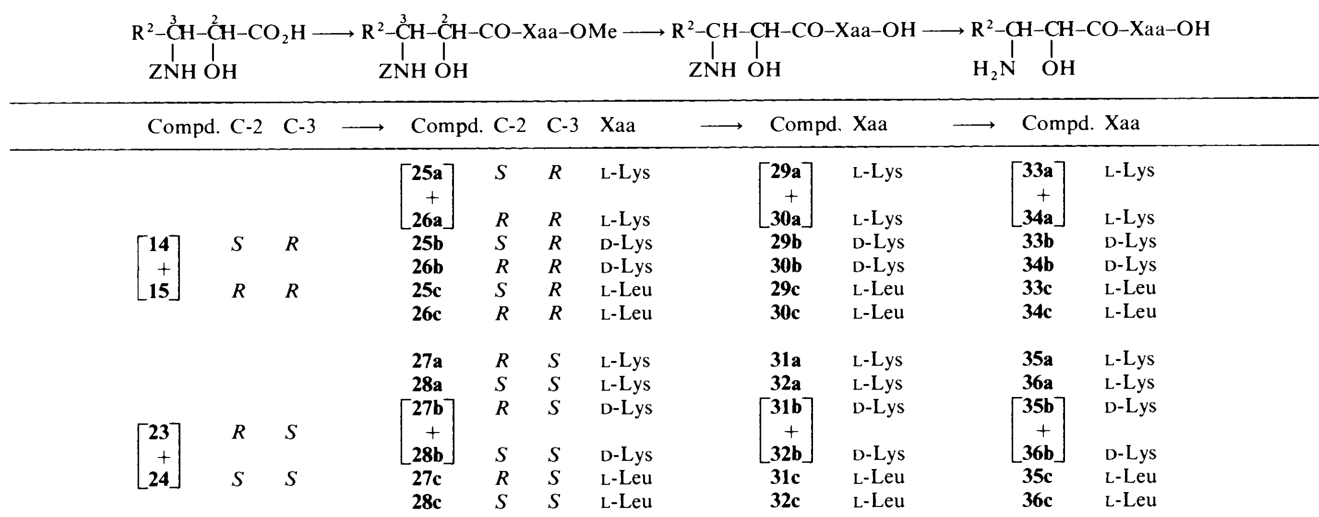
The mixture of methyl esters **12** + **13** was saponified to yield a (4:1) mixture of acids **14** + **15**, which could not be separated. The C-2 configuration of these compounds was established on the basis of the ^1H NMR spectrum of the mixture of the corresponding oxazolidin-2-ones[15] **16** + **17**. The major oxazolidinone **16** had a H^4,H^5 *trans* disposition, as indicated by its *J*-value of 4.5 Hz, while the minor isomer **17** had a $J_{4,5}$-value of 9 Hz, consistent with a *cis* disposition. Similarly, a (4:1) mixture of (2*R*,3*S*)- (**23**) and (2*S*,3*S*)- (**24**) enantiomers of compounds **14** and **15**, respectively, was obtained from *N*-Z-L-tryptophanal **18**.

Coupling of the (4:1) mixtures **14** + **15** or **23** + **24** with the methyl esters of L-Lys, D-Lys or L-Leu, respectively, *via* the dicyclohexylcarbodiimide (DCC) method in the presence of 1-hydroxybenzotriazole,[16] yielded the corresponding mixtures of protected dipeptides **25** + **26** and **27** + **28** indicated in Scheme 2. These mixtures were separated by flash chromatography, with the exception of the mixtures **25a** + **26a** and **27b** + **28b**. Saponification of products **25**–**28** yielded acids **29**–**32**, which after removal of the *N*-Z protecting group, by treatment with trimethylsilyl iodide (TMSI), gave the fully deprotected dipeptides **33**–**36**. As in the case of all Trp(Nps)-containing dipeptides previously reported,[1,3,5] a significant shielding of the Nps 6-H (δ 6.85), with respect to the theoretically calculated δ-value (δ 7.58), was observed.

Biological Data.—*AP-M and AP-B Inhibition.* The new synthetic dipeptide analogues **33**–**36** were tested as inhibitors of AP-M and AP-B. As shown in Table 1, none of the new Lys-containing analogues (**33a** + **34a**, **33b**, **34b**, **35a**, **36a** and **35b** + **36b**) inhibited AP-M in concentrations up to 10^{-3} mol dm^{-3}, while the parent dipeptide **3** and the Leu-containing derivatives **33c**–**36c** were poor inhibitors, with an IC_{50}-value in the 10^{-4} mol dm^{-3} range. Concerning AP-B inhibition, all the AH(Nps)IBA-containing dipeptides were also weak inhibitors, with IC_{50}-values in the 10^{-4} mol dm^{-3} range. The similarity in the IC_{50}-values on both enzymes for all the diastereoisomers, here reported, shows that, in contrast to what happens with bestatin **4**, the influence of the absolute configuration of the *N*-terminal β-amino-α-hydroxy acid moiety is very low.

Antinociceptive Activity.—The antinociceptive effect (in mice) of the dipeptide derivatives **33**–**36**, in the tail-flick test, given by the intracerebroventricular (icv) route, are listed in Table 2. All the AH(Nps)IBA-Lys derivatives (**33a** + **34a**, **33b**, **34b**, **35a**, **36a** and **35b** + **36b**) induced dose-related analgesic activity at 1–10 μg $mouse^{-1}$, which was dependent on the stereochemistry, while no analgesia was observed with the corresponding Leu-containing analogues **33c**–**36c** after the highest dose tested (5 μg $mouse^{-1}$). These facts reflect the need for a basic amino acid, and the influence of the absolute configuration of each amino acid on the antinociceptive effects, previously observed in this series of dipeptide derivatives.[2,4,5] Although the AH(Nps)IBA-Lys dipeptides were approximately 2–20-times less potent than the

Scheme 2 Dipeptide synthesis



R^2-$\overset{3}{C}$H(ZNH)-$\overset{2}{C}$H(OH)-CO_2H ⟶ R^2-$\overset{3}{C}$H(ZNH)-$\overset{2}{C}$H(OH)-CO-Xaa-OMe ⟶ R^2-CH(ZNH)-CH(OH)-CO-Xaa-OH ⟶ R^2-CH(H_2N)-CH(OH)-CO-Xaa-OH

Compd.	C-2	C-3	⟶	Compd.	C-2	C-3	Xaa	⟶	Compd.	Xaa	⟶	Compd.	Xaa
				25a	*S*	*R*	L-Lys		**29a**	L-Lys		**33a**	L-Lys
				+					+			+	
				26a	*R*	*R*	L-Lys		**30a**	L-Lys		**34a**	L-Lys
14	*S*	*R*		**25b**	*S*	*R*	D-Lys		**29b**	D-Lys		**33b**	D-Lys
+				**26b**	*R*	*R*	D-Lys		**30b**	D-Lys		**34b**	D-Lys
15	*R*	*R*		**25c**	*S*	*R*	L-Leu		**29c**	L-Leu		**33c**	L-Leu
				26c	*R*	*R*	L-Leu		**30c**	L-Leu		**34c**	L-Leu
				27a	*R*	*S*	L-Lys		**31a**	L-Lys		**35a**	L-Lys
				28a	*S*	*S*	L-Lys		**32a**	L-Lys		**36a**	L-Lys
				27b	*R*	*S*	D-Lys		**31b**	D-Lys		**35b**	D-Lys
23	*R*	*S*		+					+			+	
+				**28b**	*S*	*S*	D-Lys		**32b**	D-Lys		**36b**	D-Lys
24	*S*	*S*		**27c**	*R*	*S*	L-Leu		**31c**	L-Leu		**35c**	L-Leu
				28c	*S*	*S*	L-Leu		**32c**	L-Leu		**36c**	L-Leu

Table 1 Inhibitory potency of the AH(Nps)IBA-containing dipeptides **33**–**36** on AP–M and AP–B

Compound	AP–M[a] IC_{50} (10^{-6} mol dm^{-3})	AP–B[a] IC_{50} (10^{-6} mol dm^{-3})
33a + **34a**	> 1000	997
33b	> 1000	802
34b	> 1000	622
33c	250	431
34c	456	389
35a	> 1000	923
36a	> 1000	985
35b + **36b**	> 1000	712
35c	506	523
36c	492	562
3	536	> 1000
Bestatin **4**	19.4	6

[a] Values are the mean of 4–5 experiments with 3–5 different concentrations of inhibitor. Standard errors were less than 10% of the mean.

model compound **3**, all the new analogues were less toxic and had a longer effect. For example, the peak antinociceptive effect of compound **3** was observed at 5 min following injection, and the effect was completely extinguished at 60 min. However, administration of compounds **33b**, **34b**, **36a** and **35b** + **36b** resulted in prolonged analgesia, still observed at 60 min after injection. Compounds **33a** + **34a** (5 μg $mouse^{-1}$) and **35a** (10 μg $mouse^{-1}$) had an even longer effect, which was observed even 2 h after injection. In all cases the analgesia was almost completely blocked by previous administration of naloxone, 5 mg kg^{-1}, given 15 min before the icv injection. There was no relationship found between antinociceptive effect and AP–M inhibition, since the AH(Nps)IBA–Lys dipeptides were analgesic, but did not inhibit AP–M, while the AH(Nps)IBA–Leu derivatives, which inhibited AP–M weakly, did not have any antinociceptive effect. This fact indicates that the inhibition of enkephalin-degrading AP is not an important factor for the mode of action of this series of analgesic compounds.

The most important aspect emerging from these biological data is that there appears to be no relationship between the antinociceptive effect and the AP–M inhibition by this series of dipeptides.

Experimental

General.— M.p.s were taken on a Reichert-Jung Kofler micro hot stage apparatus, and are uncorrected. Elemental analyses were obtained using a Heraeus CHN-O-RAPID instrument. ^1H NMR spectra were recorded with a Varian XL-300 spectrometer (300 MHz), with Me_4Si as internal standard. *J*-Values are given in Hz. UV absorption spectra were taken with a Perkin-Elmer 550 SE spectrophotometer, with MeOH as sample solvent. Analytical TLC was performed on aluminium sheets coated with a 0.2 mm layer of silica gel 60 F_{254}, obtained from Merck. Silica gel 60 (230–400 mesh; Merck) was used for flash chromatography.

General Procedure for the Synthesis of (4:1) *Mixtures of* threo- *and* erythro-3-(*Benzyloxycarbonylamino*)-2-*hydroxy*-4-[2-(o-*nitrophenylthio*)*indol*-3-*yl*]*butyronitrile* **10** + **11** *and* **19** + **20**.—TMSCN (1.07 g, 10.8 mmol) was added to a solution of *N*-Z-D- or -L-tryptophanal[17] (2.90 g, 9 mmol), freshly prepared in 90% yield from the corresponding *N*-Z-Trp-OH by the method of Fehrentz and Castro,[12] in dry dichloromethane (60 cm^3), and the solution was stirred at room temperature for 5 days. The reaction mixture was then evaporated, and the crude mixture of *threo* and *erythro* *O*-(trimethylsilyl) cyanohydrins was dissolved in a solution of 0.3 mol dm^{-3} HCl in MeOH (60 cm^3), to which Nps-Cl (1.78 g, 9.4 mmol) was added. After being stirred for 2 h at room temperature the mixture was evaporated and the residue was dissolved in dichloromethane (100 cm^3). The solution was washed successively with aq. $NaHCO_3$ (20 cm^3), water (20 cm^3) and brine (20 cm^3), dried over dry Na_2SO_4, and evaporated. The residue was purified by flash chromatography [(4:1)hexane–ethyl acetate], to give a (4:1) mixture **10** + **11** or **19** + **20**, each as a yellow solid (3.16 g, 70%), m.p. 150–156 °C (from benzene–hexane).

(4:1) *Mixture of* (2S,3R)- *and* (2R,3R)-3-(*benzyloxycarbonylamino*)-2-*hydroxy*-4-[2-(o-*nitrophenylthio*)*indol*-3-*yl*]*butyronitrile* **10** + **11** (Found: C, 61.9; H, 4.6; N, 11.0; S, 6.2. $C_{26}H_{22}N_4O_5S$ requires C, 62.15; H, 4.4; N, 11.15; S, 6.4%); λ_{max}(MeOH)/nm 279 (ε 1.246 × 10^4) and 358 (ε 3.346 × 10^3); δ_H [300 MHz; $(CD_3)_2CO$] 3.23 (1 H, m, CH_2-Indol), 3.92 (1 H, m, CH_2-Indol), 4.20 (1 H, m, 3-H), 4.64 [0.2 H, d, $J_{2,3}$ 0.8, 2-H (**11**)], 4.70 [0.8 H, d, $J_{2,3}$ 3.5, 2-H (**10**)], 4.93 (2 H, m, OCH_2), 5.97 (1 H, m, OH), 5.56 (1 H, m, N*H*Z), 6.85 [1 H, d, *J* 7.8, 6″-H (Nps)], 7.12–7.49 [9 H, m, Ph (Z), 5′-H, 6′-H and 7′-H (Indol) and 4″-H (Nps)], 7.83 [0.2 H, d, *J* 8, 4′-H (Indol **11**)], 7.87 [0.8 H, d, *J* 8, 4′-H (Indol **10**)], 8.23 [1 H, d, *J* 8, 3″-H (Nps)] and 10.62 [1 H, s, NH (Indol)].

(4:1) *Mixture of* (2R,3S)- *and* (2S,3S)-3-(*benzyloxycarbonylamino*)-2-*hydroxy*-4-[2-(o-*nitrophenylthio*)*indol*-3-*yl*]*butyro*-

Table 2 Antinociceptive effect of the AH(Nps)IBA-containing dipeptides **33–36**

Compound	Dose (μg mouse⁻¹ icv)	% Change in reaction time (min)[a]			
		5	30	60	120
33a + **34a**	1	33 ± 10*	2 ± 11	8 ± 11	
	5	62 ± 10*	104 ± 12*	106 ± 15*	65 ± 12*
	10	237 ± 30*	267 ± 20*	121 ± 21*	*b*
33b	1	10 ± 7	−11 ± 6	−2 ± 5	
	5	99 ± 30*	118 ± 22*	73 ± 19*	30 ± 14
	10	121 ± 21*	78 ± 10*	40 ± 13*	8 ± 13
34b	1	24 ± 13	21 ± 10	28 ± 9	
	5	71 ± 30*	87 ± 21*	54 ± 16*	13 ± 9
	10	124 ± 25*	98 ± 17*	72 ± 12*	8 ± 13
33c	5	21 ± 6	5 ± 7	2 ± 11	
34c	5	15 ± 8	6 ± 4	−5 ± 12	
35a	1	33 ± 5*	62 ± 18*	53 ± 10*	23 ± 6
	5	69 ± 14*	53 ± 13*	50 ± 19*	30 ± 8
	10	149 ± 30*	117 ± 21*	109 ± 22*	72 ± 11*
36a	1	132 ± 21*	57 ± 15*	50 ± 12*	14 ± 7
	5	*b*	*b*	*b*	
35b + **36b**	1	152 ± 10*	82 ± 15*	46 ± 12*	25 ± 5
	5	*b*	*b*	*b*	
35c	5	29 ± 13	22 ± 12	15 ± 11	
36c	5	10 ± 15	6 ± 11	−2 ± 9	
3[c]	0.5	138 ± 15*	76 ± 8*	33 ± 5	
	1	*b*	*b*	*b*	

[a] Results are the means ± standard error obtained with groups of 10–12 mice. [b] Signs of strong neurotoxicity. [c] Ref. 5. * Significant change ($p < 0.05$ or better, Student's t test).

nitrile **19** + **20** (Found: C, 62.0; H, 4.65; N, 10.9; S, 6.3%). The spectroscopic data were identical with those of the (4:1) mixture of their enantiomers **10** + **11**.

General Procedure for the Synthesis of (4:1) *Mixtures of* (2S,3R)- *and* (2R,3R)-N-*Z-AH(Nps)IBA* (**12** + **13**) *and* (2R,3S)- *and* (2S,3S)-N-*Z-AH(Nps)IBA* (**21** + **22**) *Methyl Esters*.—The (4:1) mixture of cyanohydrins **10** + **11** or **19** + **20** (3.01 g, 6 mmol) was dissolved in a dry, cooled (0 °C), (3:1) Et_2O–MeOH mixture (70 cm^3), previously saturated with HCl. This solution was stirred at below 5 °C for 24 h and was then treated with ice–water (15 cm^3) while the temperature was kept below 10 °C, and the mixture was stirred and kept for 24–48 h until the disappearance of the imidate intermediate was detected by TLC [(5:1) $CHCl_3$–MeOH]. The reaction mixture was concentrated (10 cm^3), and extracted with dichloromethane (3 × 50 cm^3). The combined extracts were washed successively with water (30 cm^3) and brine (30 cm^3), dried over Na_2SO_4, and evaporated. The residue was purified by flash chromatography with (3:1) hexane–ethyl acetate mixtures as eluents, to give the corresponding (4:1) mixtures of methyl esters of *N*-Z-AH(Nps)IBA as foams (80%).

(4:1) *Mixture of* (2S,3R)- *and* (2R,3R)-N-*Z-AH(Nps)IBA Methyl Esters* **12** + **13** (Found: C, 60.7; H, 4.8; N, 7.9; S, 6.2. $C_{27}H_{25}N_3O_7S$ requires C, 60.6; H, 4.7; N, 7.85; S, 6.0%); δ_H [300 MHz; $(CD_3)_2CO$] 2.98 (1 H, m, CH_2-Indol), 3.12 (1 H, m, CH_2-Indol), 3.44 [0.6 H, s, Me (**13**)], 3.47 [2.4 H, s, Me (**12**)], 4.02 [0.8 H, dd, $J_{2,3}$ 3, 2-H (**12**)], 4.10 [0.2 H, dd, $J_{2,3}$ 1, 2-H (**13**)], 4.14 (1 H, m, 3-H), 4.8 (2 H, s, CH_2Z), 5.42 (0.8 H, d, J 8, OH (**12**)], 5.85 [0.2 H, d, J 8, OH (**13**)], 6.87 [0.2 H, d, J 9, NHZ (**13**)], 6.99 [0.8 H, d, J 9, NH-Z (**12**)], 6.68 [1 H, d, J 8, 6″-H (Nps)], 7.07 [1 H, m, 6′-H (Indol)], 7.11 [1 H, m, 5′-H (Indol)], 7.20–7.40 [7 H, m, Ph, 7′-H (Indol), 4″-H(Nps)], 7.51 [1 H, m, 5″-H (Nps)], 7.64 [1 H, d, J 8, 4′-H (Indol)], 10.56 [0.8 H, br s, NH (Indol) (**12**)] and 10.66 [0.2 H, br s, NH (Indol) (**13**)].

(4:1) *Mixture of* (2R,3S)- *and* (2S,3S)-N-*Z-AH(Nps)IBA Methyl Esters* **21** + **22** (Found: C, 60.75; H, 4.7; N, 7.7; S, 6.1%). The spectroscopic data were identical with those of the (4:1) mixture of their enantiomers **12** + **13**.

General Procedure for the Synthesis of (4:1) *Mixture of* (2S,3R)- *and* (2R,3R)-N-*Z-AH(Nps)IBA* **14** + **15** *and* (2R,3S)- *and* (2S,3S)-N-*Z-AH(Nps)IBA* **23** + **24**.—The (4:1) mixture of methyl esters **12** + **13** or **21** + **22** (2.14 g, 4 mmol) was added to a solution of NaOH (0.19 g, 4.8 mmol) in (1:1) dioxane–water (120 cm^3). After being stirred for 2 h at room temperature the reaction mixture was concentrated (25 cm^3), washed with dichloromethane (3 × 25 cm^3), and the aq. phase was acidified to pH 3–4 with Dowex 50W-X4 resin. The resin was filtered off and washed with dichloromethane (20 cm^3). The aq. phase was extracted with dichloromethane (3 × 50 cm^3), and the combined organic phases were dried over Na_2SO_4 and evaporated to dryness, to give (quantitatively) the corresponding (4:1) mixture of *N*-Z-AH(Nps)IBA **14** + **15** or **23** + **24**.

(4:1) *Mixture of* (2S,3R)- *and* (2R,3R)-N-*Z-AH(Nps)IBA* **14** + **15** (Found: C, 59.6; H, 4.7; N, 7.8; S, 5.95. $C_{26}H_{23}N_3O_7S$ requires C, 59.9; H, 4.4; N, 8.1; S, 6.1%); δ_H [300 MHz; $(CD_3)_2CO$] 2.97 (1 H, m, CH_2-Indol), 3.30 (1 H, m, CH_2-Indol) 3.92 [0.8 H, d, $J_{2,3}$ 3, 2-H (**14**)], 4.04 [1 H, m, $J_{2,3}$ 1.5, 2-H (**15**)], 4.21 (1 H, m, 3-H), 4.78 [0.4 H. s, CH_2Z (**15**)], 4.89 [1.6 H, s, CH_2Z (**14**)], 6.70 [1 H, d, J 8, 6″-H (Nps)], 6.90–7.50 [10 H, m, Ph, 5′-, 6′- and 7′-H (Indol), 4″- and 5″-H (Nps)], 7.79 [1 H, m, 4′-H (Indol)] and 8.27 [1 H, dd, J 1 and 8, 3″-H (Nps)].

(4:1) *Mixture of* (2R,3S)- *and* (2S,3S)-N-*Z-AH(Nps)IBA* **23** + **24** (Found: C, 59.8; H, 4.6; N, 7.9; S, 6.9%). The spectroscopic data were identical with those of the (4:1) mixture of their enantiomers **14** + **15**.

(4:1) *Mixture of* (4R,5S)- *and* (4R,5R)-4-{[2-(o-*Nitrophenylthio)indol-3-yl]methyl*}-2-*oxooxazolidine-5-carboxylic Acids* **16** + **17**.—The (4:1) mixture of methyl esters **12** + **13** (0.54 g, 1 mmol) was added to a solution of NaOH (0.08 g, 2 mmol) in MeOH (20 cm^3). After being stirred at room temperature for 2 h the reaction mixture was evaporated to dryness. The residue was taken up in water (20 cm^3), washed with dichloromethane (2 × 20 cm^3), and acidified to pH 3–4 with Dowex 50W-X4 resin. The resin was filtered off and washed with ethyl acetate (20 cm^3). The aq. phase was extracted with ethyl acetate (2 × 30 cm^3), and the combined organic phases were dried over

Table 3 Analytical and spectroscopic data of *N*-Z-AH(Nps)IBA-containing dipeptides **25**–**32**

			Found (%) (Required)				Relevant ^{1}H NMR data (δ)^a							
							AH(Nps)IBA					Xaa-OR		
Compd.	Yield (%)	Formula	C	H	N	S	2-H	3-H	$J_{2,3}$/Hz	4-H	6″-H (Nps)	α-H	NH	OMe
25a + **26a**	70	$C_{42}H_{44}N_5O_{10}S$	61.9	5.7	8.4	3.6	3.94	4.25	3	2.94	6.71	4.25	8.03	3.57
			(62.2)	(5.4)	(8.6)	(3.95)	4.07	4.25	3	2.94, 3.13	6.66	4.25	7.99	3.53
25b	60	$C_{42}H_{44}N_5O_{10}S$	61.9	5.7	8.6	4.2	3.96	4.15	3	2.92, 3.12	6.72	4.15	8.12	3.55
			(62.2)	(5.4)	(8.6)	(3.95)								
26b	15	$C_{42}H_{44}N_5O_{10}S$	62.5	5.8	8.5	4.3	4.21	4.42	3	3.01, 3.10	6.90	4.42	8.15	3.75
			(62.2)	(5.4)	(8.6)	(3.95)								
25c	62	$C_{33}H_{36}N_4O_8S$	61.4	5.8	8.8	4.9	3.94	4.15	3	2.91, 3.12	6.74	4.35	8.01	3.57
			(61.1)	(5.6)	(8.6)	(4.9)								
26c	17	$C_{33}H_{36}N_4O_8S$	60.95	5.4	8.35	4.9	4.03	4.32	3	2.91, 3.15	6.68	4.32	8.05	3.60
			(61.1)	(5.6)	(8.6)	(4.9)								
27a	59	$C_{42}H_{44}N_5O_{10}S$	61.9	5.6	8.6	3.9	3.96	4.15	3	2.92, 3.12	6.72	4.15	8.12	3.55
			(62.2)	(5.4)	(8.6)	(3.95)								
28a	16	$C_{42}H_{44}N_5O_{10}S$	61.85	5.6	8.5	4.3	4.21	4.42	3	3.01, 3.10	6.90	4.42	8.15	3.75
			(62.2)	(5.4)	(8.6)	(3.95)								
27b + **28b**	76	$C_{42}H_{44}N_5O_{10}S$	61.9	5.6	8.5	4.2	3.94	4.25	3	2.94	6.71	4.25	8.03	3.57
			(62.2)	(5.4)	(8.6)	(3.95)	4.07	4.25	3	2.94, 3.13	6.66	4.25	7.99	3.53
27c	63	$C_{33}H_{36}N_4O_8S$	61.3	5.9	8.4	5.05	3.96	4.12	4	3.00	6.74	4.24	8.14	3.53
			(61.1)	(5.6)	(8.6)	(4.9)								
28c	17	$C_{33}H_{36}N_4O_8S$	61.3	5.9	8.9	5.1	4.03	4.32	3	2.80, 2.95	6.63	4.32	8.04	3.61
			(61.1)	(5.6)	(8.6)	(4.9)								
29a + **30a**	85	$C_{41}H_{42}N_5O_{10}S$	61.7	5.5	8.45	3.9	3.94	4.25	2	2.75, 3.08	6.67	4.15	7.76	
			(61.8)	(5.3)	(8.8)	(4.0)	4.07	4.15	1	2.75, 3.08	6.67	4.15	7.76	
29b	75	$C_{41}H_{42}N_5O_{10}S$	61.6	5.4	8.8	4.4	3.98	4.15	2	2.90, 3.15	6.74	4.15	7.92	
			(61.8)	(5.3)	(8.8)	(4.0)								
30b	80	$C_{41}H_{42}N_5O_{10}S$	61.9	5.6	8.4	4.15	4.07	4.26	1	2.88	6.73	4.26	7.77	
			(61.8)	(5.3)	(8.8)	(4.0)								
29c	90	$C_{32}H_{34}N_4O_8S$	54.5	5.6	9.6	4.85	3.94	4.16	1	2.90–3.10	6.72	4.30	7.80	
			(54.8)	(5.3)	(10.0)	(5.0)								
30c	95	$C_{32}H_{32}N_4O_8S$	54.6	5.4	9.7	4.8	4.07	4.20	2	2.90, 3.10	6.73	4.32	7.80	
			(54.8)	(5.3)	(10.0)	(5.0)								
31a	99	$C_{41}H_{42}N_5O_{10}S$	61.6	5.4	8.5	4.4	3.98	4.15	2	2.90, 3.15	6.74	4.15	7.92	
			(61.8)	(5.3)	(8.8)	(4.0)								
32a	99	$C_{41}H_{42}N_5O_{10}S$	61.8	5.2	8.6	4.3	4.07	4.26	1	2.88	6.73	4.26	7.77	
			(61.8)	(5.3)	(8.8)	(4.0)								
31b + **32b**	99	$C_{41}H_{42}N_5O_{10}S$	61.8	5.1	8.4	4.4	3.94	4.15	2	2.75, 3.08	6.67	4.15	7.76	
			(61.8)	(5.3)	(8.8)	(4.0)	4.07	4.15	1	2.75, 3.08	6.67	4.15	7.76	
31c	90	$C_{32}H_{34}N_4O_8S$	55.1	5.0	9.9	4.6	3.95	4.10	2	2.90–3.20	6.72	4.20	7.82	
			(54.8)	(5.3)	(10.0)	(5.0)								
32c	89	$C_{32}H_{34}N_4O_8S$	55.1	5.0	10.0	4.6	4.08	4.33	2	2.75, 2.98	6.62	4.33	7.75	
			(54.8)	(5.3)	(10.0)	(5.0)								

^{a} In $(CD_3)_2SO$

Na_2SO_4 and evaporated to give the (4:1) mixture **16** + **17** as a foam (80%). This mixture could not be separated (Found: C, 54.9; H, 3.4; N, 10.0; S, 7.8. $C_{19}H_{15}N_3O_6S$ requires C, 55.2; H, 3.6; N, 10.2; S, 7.75); δ_H [300 MHz; $(CD_3)_2SO$] 2.60 [0.2 H, m, CH_2-Indol (**17**)], 3.02 [0.2 H, m, CH_2-Indol (**17**)], 3.11 [1.6 H, m, CH_2-Indol (**16**)], 4.00 [0.8 H, m, 4-H (**16**)], 4.17 [0.2 H, m, 4-H (**17**)], 4.47 [0.2 H, s, NH (**17**)], 4.61 [0.8 H, d, $J_{4,5}$ 4, 5-H (**16**)], 4.97 [0.8 H, s, NH (**16**)], 5.16 [0.2 H, d, $J_{4,5}$ 9, 5-H (**17**)], 6.74 [0.2 H, d, J 8, 6″-H (Nps) (**17**)], 6.78 [0.8 H, d, J 8, 6″-H (Nps) (**16**)], 7.08 [1 H, m, 6′-H (Indol)], 7.21 [1 H, m, 5′-H (Indol)], 7.44 [3 H, m, 7′-H (Indol), 4″-H and 5″-H-(Nps)], 7.68 [1 H, d, J 9, 4′-H (Indol) and 8.26 [1 H, d, J 8, 3″-H (Nps)].

General Procedure for the Synthesis of Protected AH(Nps)-IBA-containing Dipeptides **25a**–**28c**.—To a solution of the (4:1) mixture of *threo*- and *erythro*-*N*-Z-AH(Nps)IBA **14** + **15** or **23** + **24** (1.3 mmol) and L-Lys, D-Lys, or L-Leu methyl ester hydrochlorides (1.6 mmol) in dry tetrahydrofuran (THF) (13 cm^3) at 0 °C were added 1-hydroxybenzotriazole (1.6 mmol) and triethylamine (1.3 mmol). After the mixture had been stirred at 0 °C for 30 min, a solution of DCC (1.3 mmol) in dry dichloromethane (20 cm^3) was added, and the mixture was stirred at room temperature for 24 h. Solvents were removed under reduced pressure, and the residue was purified by flash chromatography with (2:1) hexane–ethyl acetate mixtures as eluent. In this way the corresponding (4:1) mixtures **25** + **26** or **27** + **28** were obtained which, except for mixtures **25a** + **26a** and **27b** + **28b**, were separated by repeated flash chromatography [(4:1) hexane–ethyl acetate]. The analytical and more significant spectroscopic data of the protected dipeptides **25**–**28** are summarized in Table 3.

General Procedure for Saponification of AH(Nps)IBA-containing Dipeptides. Synthesis of compounds **29**–**32**.—The methyl ester of the corresponding AH(Nps)IBA-containing dipeptide, ester **25**–**28** (0.75 mmol), was added to a solution of NaOH (0.033 g, 0.83 mmol) in (1:1) dioxane–water (25 cm^3). After being stirred for 3 h at room temperature the reaction mixture was concentrated (10 cm^3), then was washed with dichloromethane (3 × 10 cm^3), and the aq. phase was acidified

Table 4 Analytical and spectroscopic data of AH(Nps)IBA-containing dipeptides **33**–**36**

Compd.	Yield (%)	Formula	Found (%) (Required) C	H	N	S	Relevant ^{1}H NMR data (δ)[a] AH(Nps)IBA 2-H	3-H	4-H	6″-H (Nps)	Xaa α-H
33a + **34a**	74	$C_{25}H_{30}N_5O_6S{\cdot}2H_2O$	53.0 (53.2)	6.4 (6.0)	12.5 (12.4)	5.4 (5.7)	4.34 / 4.04	3.86 / 3.58	2.78–3.00 / 3.10–3.22	6.76 / 6.74	4.21 / 4.21
33b	76	$C_{25}H_{30}N_5O_6S{\cdot}2H_2O$	53.4 (53.2)	6.1 (6.0)	12.6 (12.4)	5.6 (5.7)	4.02	3.60	3.10–3.20	6.72	4.24
34b	82	$C_{25}H_{30}N_5O_6S{\cdot}2H_2O$	53.6 (53.2)	6.3 (6.0)	12.5 (12.4)	5.4 (5.7)	4.33	3.58	3.05	6.73	4.16
33c	50	$C_{24}H_{28}N_4O_6S{\cdot}2H_2O$	53.9 (53.7)	6.3 (6.0)	10.0 (10.4)	5.6 (6.0)	4.08	3.50	2.94, 3.22	6.75	4.30
34c	52	$C_{24}H_{28}N_4O_6S{\cdot}2H_2O$	53.5 (53.7)	6.1 (6.0)	10.0 (10.4)	5.8 (6.0)	4.30	3.52	2.98–3.10	6.74	4.32
35a	45	$C_{25}H_{30}N_5O_6S{\cdot}2H_2O$	53.5 (53.2)	6.0 (6.0)	12.7 (12.4)	6.1 (5.7)	4.02	3.60	3.10–3.20	6.72	4.24
36a	46	$C_{25}H_{30}N_5O_6S{\cdot}2H_2O$	53.0 (53.2)	6.4 (6.0)	12.5 (12.4)	5.85 (5.7)	4.33	3.58	3.05	6.73	4.16
35b + **36b**	70	$C_{25}H_{30}N_5O_6S{\cdot}2H_2O$	53.6 (53.2)	5.9 (6.0)	12.7 (12.4)	5.7 (5.7)	4.34 / 4.04	3.86 / 3.58	2.78–3.00 / 3.10–3.22	6.76 / 6.74	4.21 / 4.21
35c	73	$C_{24}H_{28}N_4O_6S{\cdot}2H_2O$	53.3 (53.7)	6.0 (6.0)	10.3 (10.4)	5.7 (6.0)	4.04	3.50	2.99, 3.18	6.73	4.33
36c	45	$C_{24}H_{28}N_4O_6S{\cdot}2H_2O$	53.2 (53.7)	6.4 (6.0)	9.95 (10.4)	5.8 (6.0)	4.33	3.85	2.92, 2.98	6.67	4.31

[a] In $(CD_3)_2SO$.

to pH 3–4 with Dowex 50W-X4 resin. The resin was filtered off and washed with ethyl acetate (10 cm^3). The aq. phase was extracted with ethyl acetate (3 × 10 cm^3), and the combined organic phases were dried over Na_2SO_4 and evaporated to dryness, to give the corresponding dipeptide **29**–**32** as foams, whose analytical and spectroscopic data are listed in Table 3.

General Procedure for the Removal of the Z Protecting Group in AH(Nps)IBA-containing Dipeptides. Synthesis of compounds **33**–**36**. TMSI (0.12 g, 0.6 mmol) was added to a solution of the corresponding *N*-Z-protected dipeptide **29**–**32** (0.5 mmol) in (2:1) acetone–methanol (15 cm^3). After being stirred for 10 min at room temperature the reaction mixture was evaporated to dryness and the residue was purified by flash chromatography [(4:1) $CHCl_3$–MeOH], to obtain the deprotected dipeptides **33**–**36**, whose analytical and spectroscopic data are listed in Table 4.

Biological Methods.—Materials. The following commercial compounds were used: bestatin **4**, Tyr, Tyr-Gly-Gly, Lys-NA (NA = 2-naphthylamide), Leu-NA and Fast Garnet GBC (Sigma UK), Microsomal (AP-M, EC 3.4.11.2) porcine kidney leucine-aminopeptidase was purchased from Sigma (UK). Mouse L cells were grown in Dulbecco's modified Eagle's medium and 10% foetal calf serum. L Cells' media and serum were supplied by Flow Labs (UK). Male ICR swiss albino mice weighing 20–25 g and male Wistar rats (250–300 g) were used. Animals had free access to water and food. Mice were housed in the behavioural room at least two days before testing, which was done at the same time of day for all animals.

AP-M Assays. This activity was determined by the described method.[6a] A solution of 2 mmol dm^{-3} L-Leu-NA (0.25 cm^3) in 0.1 mol dm^{-3} Tris-HCl buffer (0.5 cm^3) at pH 7.0 was added to distilled water with or without an inhibitor in a series of test tubes in a 37 °C bath. After 3 min the enzyme solution (0.05 cm^3) was added and the solutions were mixed well. Exactly 30 min later the reaction was stopped by addition of a 1 mg cm^{-3} solution of the stabilized diazonium salt Fast Garnet GBC (1 cm^3) in acetic acid buffer at pH 4.2, containing 10% Tween 20. After storage for 15 min at room temperature, absorbancy was read at 525 nm. The reaction was also carried out without enzyme solution, and the result was taken as the control blank.

AP-B Assays. Cell surface-associated AP-B activities were determined following the Aoyagi method.[18] The incubation mixture consisted of 2 mmol dm^{-3} L-Lys-NA (0.25 cm^3), Hank's balanced salt solution (0.65 cm^3), and distilled water (0.1 cm^3) with or without the inhibitor. After incubation (3 min; 37 °C) the mixture was added to monolayer cultures of mouse L cells (~5 × 10^5 cells), and the incubation was stopped after 30 min by addition of the stabilized diazonium salt Fast Garnet GBC (1 cm^3; 1 mg cm^{-3}) in 1 mol dm^{-3} acetic acid buffer at pH 4.2, containing 10% Tween 20. The mixture was left at room temperature for 15 min, then was centrifuged, and its absorbance was measured at 525 nm.

Antinociceptive activity. Antinociception was evaluated in mice by means of the tail-flick test,[19] by immersing the tail into water at 52 °C, using a cut-off time of 10 s. The observer was 'blind' to the compound injected. Results were expressed as percentage change in reaction time *vs.* predrug score (1.9–2.5 s). A group of mice injected with saline was also tested in parallel. Saline administration had no effect on the tail-flick latency at any time postinjection.

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