Aminopeptidase Inhibitory Properties and Analgesic Activity of (2*S*,3*R*)-3,7-Diamino-2-hydroxy-heptanoic Acid Containing Tripeptide Analogues of the *N*-Terminal Tripeptide of Probestin

Rosario Herranz[a]*, Soledad Vinuesa[a], Concepción Pérez[a], M. Teresa García-López[a], María L. De Ceballos[b], Francisco M. Murillo[b], and Joaquín del Río[b]

[a] Instituto de Química Médica, C.S.I.C. Juan de la Cierva, 3. 28006 Madrid, Spain

[b] Instituto Cajal, C.S.I.C. Doctor Arce, 37. 28002 Madrid, Spain

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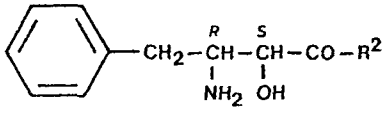
(2*S*,3*R*)-3,7-Diamino-2-hydroxy-heptanoyl-Leu-Pro-OH [(2*S*,3*R*)-DAHHA-Leu-Pro-OH, **4**], analogue of the *N*-terminal tripeptide of probestin, has been synthesized, and tested as inhibitor of AP-B, Leu-AP, AP-M, and enkephalin-degrading APs, and as analgesic. In order to establish structure-activity relationships the dipeptide (2*S*,3*R*)-DAHHA-Pro-OH (**5**) and the tripeptide (2*S*,3*R*)-DAHHA-Ala-Pro-OH (**6**) were also prepared. Compounds **4** and **6** were potent and selective inhibitors of enkephalin-degrading APs and showed a prolonged antinociceptive effect.

Hemmung von Aminopeptidasen und analgetische Wirkung von (2*S*, 3*R*)-3,7-Diamino-2-hydroxy-heptansäure enthaltenden Tripeptidanaloga des *N*-terminalen Tripeptids von Probestin

(2*S*,3*R*)-3,7-Diamino-2-hydroxy-heptanoyl-Leu-Pro-OH [(2*S*,3*R*)-DAHHA-Leu-Pro-OH, **4**], ein Analoges des *N*-terminalen Tripeptids von Probestin, wurde synthetisiert und sowohl als Hemmstoff von AP-B, Leu-AP, AP-M und von Enkephalin-abbauenden APs und auch als Analgetikum geprüft. Zur Untersuchung von Struktur-Aktivität-Beziehungen wurden ebenfalls das Dipeptid (2*S*,3*R*)-DAHHA-Pro-OH (**5**) und das Tripeptid (2*S*,3*R*)-DAHHA-Ala-Pro-OH (**6**) hergestellt. Die Verbindungen **4** und **6** sind starke und selektive Hemmstoffe von Enkephalin-abbauenden APs und zeigen eine verlängerte antinociceptive Wirkung.

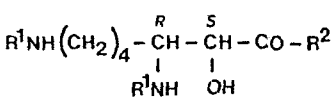
Aminopeptidase B (AP-B, EC.3.4.11.6) and aminopeptidase M (AP-M, EC.3.4.11.2) are two cell surface APs which play a key role in processes of inflamation, immunity, oncogenesis, metastasis, virus infection, and pain[1-4]. For this reason, inhibitors of these enzymes are among the most promising drugs in cancer chemotherapy, modification of immune responses, and pain control. These enzymes are strongly inhibited by the natural dipeptide bestatin (**1**, Ubenimex, (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine, (2*S*,3*R*)-AHPBA-L-Leu][5], which shows *in vivo* activity as antitumor, immunomodifying, and analgesic agent. This last activity is the consequence of the inhibition of the degradation of enkephalins by APs[4]. These neuropeptides are mainly degraded by two membrane-bound APs[6], one of which was characterized and identified as AP-M[7,8]. Recently, *Aoyagi* et al. have reported the isolation of the natural tetrapeptide probestin (**2**)[9,10] which shows selective inhibition of AP-M[9].



1: R^2 = Leu - OH

2: R^2 = Leu - Pro - Pro - OH

In search of enkephalin degrading AP inhibitors, we synthesized compound **3**, an analogue of bestatin, in which the AHPBA residue was replaced by the lysine analogue 3,7-diamino-2-hydroxy-heptanoic acid (DAHHA). This analogue showed selective enkephalin degrading AP inhibition and improved analgesic activity with respect to bestatin[11]. This paper deals with the synthesis, inhibitory properties against AP-B, Leu-AP (EC.3.4.11.1), AP-M and enkephalin-degrading APs, and analgesic activity of the tripeptide **4**, analogue of the *N*-terminal tripeptide of probestin, in which the β-amino-α-hydroxy acid has been replaced by DAHHA. In order to establish structure-activity relationships the dipeptide **5** and the tripeptide **6** were also prepared.



3: R^1 = H ; R^2 = Leu - OH

4: R^1 = H ; R^2 = Leu - Pro - OH

5: R^1 = H ; R^2 = Pro - OH

6: R^1 = H ; R^2 = Ala - Pro - OH

7: R^1 = Z ; R^2 = Leu - OH

8: R^1 = Z ; R^2 = Leu - Pro - OBn

9: R^1 = Z ; R^2 = OH

10: R^1 = Z ; R^2 = Pro - OBn

11: R^1 = Z ; R^2 = Ala - Pro - OMe

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Results and Discussion

Chemistry

The tripeptide **4** was obtained by coupling of the dipeptide (2*S*,3*R*)-3,7-bis(Z)-DAHHA-Leu (**7**)[11] with the benzyl ester of L-Pro, by the dicyclohexylcarbodiimide (DCC) method, in the presence of 1-hydroxybenzotriazole, followed by removal of the protecting groups in **8**, by hydrogenolysis over 10% Pd/C. The protected dipeptide **10** and the tripeptide **11** were obtained by coupling of the (4:1) diastereoisomeric mixture of (2*S*,3*R*)-3,7-bis(Z)-DAHHA (**9**) and (2*R*,3*R*)-3,7-bis(Z)-DAHHA[11] with the benzyl ester of L-Pro or the methyl ester of the dipeptide L-Ala-L-Pro, respectively, followed by chromatographic isolation of the major (2*S*,3*R*)-isomers **10** and **11**. Removal of the protecting groups, by hydrogenolysis of **10**, and by saponification and hydrogenolysis of **11**, yielded the deprotected compounds **5** and **6**.

Biological Data

AP Inhibition

The inhibitory potency of the peptides **4**, **5**, and **6** against AP-B, Leu-AP, AP-M and the enkephalin-degrading APs are listed in Table 1. For comparison, (2*S*,3*R*)-DAHHA-Leu-OH (**3**) bestatin (**1**) and the reported data for probestin (**2**)[9] have been included. The introduction of the Pro residue in the dipeptide **3**, to give **4**, led to an increase in the inhibitory potency against the different APs tested. This increase was especially significant in the case of the enkephalin-degrading APs. However, removal of the Leu residue of **4**, to give the dipeptide **5**, or its replacement by Ala, to give the tripeptide **6**, led to an important decrease in potency, with the exception of the inhibition of AP-M by **6**. These data show the importance of the Leu and Pro residue for the binding of these compounds to the APs mentioned.

Table 1: Inhibitory potency of compounds **4**,**5** and **6** on AP-B, Leu-AP, AP-M and enkephalin-degrading APs

Compound	AP-B[a] IC_{50} (μM)	Leu-AP[a] IC_{50} (μM)	AP-M[a] IC_{50} (μM)	Enkephalin-degrading APs[a] IC_{50} (μM)
4	43	19	6.51	0.0083
5	101	749	250	65
6	192	72	0.98	0.36
(2S,3R)-DAHHA-Leu-OH[b] (**3**)	481	-	201	2.01
Bestatin (**1**)	6	0.032	19	0.30
Probestin (**2**)	73.7	0.17	0.059	-

[a] Values are the mean of 4-5 experiments with 3-5 different concentrations of inhibitor. S.E. were less than 10% of the mean. [b] Ref. 11. [c] ref. 9.

Table 2: Analgesic response to the dipeptides **4-6**

Compound	Dose μg/mouse icv	% Change in reaction time (min)[a] 5	30	60	90	120
Saline	-	14±8	-2±10	-15±7	-10±9	-3±6
4	5	18±9	-7±6	9±7	0±10	2±7
	25	60±17*	85±15*	99±17*	98±20*	49±14
	50	81±22*	169±31*	133±21*	121±25*	79±16*
	100	119±23*	174±25*	218±32*	218±30*	143±27*
5	5	21±6	32±10	28±12	31±12	-
	25	35±8	40±10	54±13*	10±4	-
6	5	16±8	1±5	2±7	-	-
	25	25±8	50±13*	75±19*	18±12	-
(2S,3R)-DAHHA-Leu-OH[b] (**3**)	5	62±20*	21±15	23±15	-	-
Bestatin (**1**)	25	26±9	3±8	-3±8	-	-
	100	10±8	42±12*	59±16*	81±30*	55±18*

[a] Results are the mean ± S.E. obtained with groups of 10-12 mice. (*) Significant change ($P < 0.05$ or better, Student's t test). [b] Ref. 11.

Antinociceptive Activity

The antinociceptive effect in mice of the peptides **4**, **5**, and **6** in the tail-flick test, given by the icv route are listed in Table 2. For comparison, (2*S*,3*R*)-DAHHA-Leu-OH and bestatin have been included. Compounds **4-6** showed a dose-related antinociceptive effect, higher than bestatin, and with the antinociceptive peak at 60-90 min. The introduction of the Pro residue in (2*S*,3*R*)-DAHHA-Leu-OH (**3**) led to an increase in the effective dose for **4**, but this compound had a more prolonged response and its antinociceptive peak was shifted from 5 to 60-90 min. The order in antinociceptive effect (**4** > **6** > **5**) is the same as in the enkephalin-degrading APs inhibitory potency, suggesting that their analgesic activity is related with the inhibition of these APs.

In conclusion, the tripeptides **4** and **6** show a prolonged antinociceptive effect and are potent and selective inhibitors of enkephalin-degrading APs. These compounds could be useful tools in the study and characterization of these APs.

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Experimental Part

Elemental analyses: Heraeus CHN-O-RAPID instrument.- ^{1}H-NMR spectra: Varian XL-300 (300 MHz), TMS int. stand.- Analytical TLC: Aluminium sheets coated with a 0.2 mm layer silica gel (60 F_{254}, Merck).- Column chromatography: silica gel (60, 230-400 mesh, Merck). Bestatin, Tyr, Tyr-Gly-Gly, Lys-NA, Leu-NA, and Fast Garnet GBC: Sigma (UK).- ^{3}H-Leu-enkephalin: Amersham International (UK).- *Dulbecco's* modified *Eagle's* medium and 10% fetal calf serum: Flow Labs (UK).- Enzymatic bioassays: performed as described (L cell surface-associated AP-B[1], AP-M[12], Leu-AP[13], enkephalin-degrading APs[14]). Antinociceptive assays: male ICR swiss albino mice weighing 20-25 g.

(2S,3R)-Bis(Z)-DAHHA-Leu-Pro-OBn (**8**)

1-Hydroxybenzotriazole (3.2 mmol) and triethylamine (2.7 mmol) were added to a solution of the dipeptide (2*S*,3*R*)-bis(*Z*)-DAHHA-Leu-OH (**7**) (2.7 mmol) and L-Pro benzyl ester hydrochloride (3.2 mmol) in dry THF (15 ml) at 0°C. After 30 min of stirring at 0°C, DCC (2.7 mmol) dissolved in dry CH_2Cl_2 (15 ml) was added, and stirring was continued at room temp. for 24 h. After removal of the solvents under reduced pressure, the residue was purified by flash chromatography, using hexane-ethyl acetate mixture as eluant, obtaining **8** as a foam (1.67 g, 83%): ^{1}H-NMR [$(CD_3)_2CO$]: δ (ppm) = 0.82 and 0.88 [2d, 6H, J = 6 Hz, $(CH_3)_2$ Leu], 1.10-2.10 (m, 13 H, CH_2-CH Leu, β- and γ-CH_2 Pro, 4-, 5-, and 6-CH_2 DAHHA), 2.83 (m, 2H, 7-CH_2, DAHHA), 3.60-3.90 (m, 2H, δ-CH_2 Pro), 3.98 (m, 1H, 3-H DAHHA), 4.10 (dd, 1H, J_1 = 2.5 Hz, J_2 = 6 Hz, 2-H, DAHHA), 4.53 (m, 1H, α-H Pro), 4.83 (m, 1H, α-H Leu), 5.04 (m, 2H, CO_2-CH_2-Ph), 5.05 (s, 2H, CH_2Z), 5.13 (m, 2H, CH_2Z), 5.26 (d, 1H, J = 6 Hz, OH), 6.15 (d, 1H, J = 9.5 Hz, 3-NH-Z), 6.36 (s, 1H, 7-NH-Z), 7.51 (d, 1H, J = 9 Hz, NH Leu), 7.20-7.60 (m, 15 H, Z and Bn Ph).- $C_{41}H_{53}N_4O_9$ (745.8): Calcd. C 66.0 H 7.16 N 7.5 Found C 66.0 H 7.19 N 7.9.

(2S,3R)-Bis(Z)-DAHHA-Pro-OBn (**10**)

1-Hydrodroybenzotriazole (3.2 mmol) and triethylamine (2.7 mmol) were added to a solution of (4:1) diastereomeric mixture of (2*S*,3*R*)- and (2*R*,3*R*)-bis(Z)-DAHHA-OH (**9**)[11] (2.7 mmol) and L-Pro benzyl ester hydrochloride (3.2 mmol) in dry TFA (15 ml) at 0°C. After 30 min of stirring at 0°C, DCC (2.7 mmol) dissolved in dry CH_2Cl_2 (15 ml) was added. Then, processing as **8** provided the title compound as a foam (1.02 g, 60%): ^{1}H-NMR [$(CD_3)_2CO$]: δ (ppm) = 1.10-1.55 (m, 6-H, 4-, 5-, and 6-CH_2 DAHHA), 1.62-1.98 (m, 3H, β-H and γ-CH_2 Pro), 2.16 (m, 1H, β-H Pro), 2.91 (m, 2H, 7-CH_2 DAHHA), 3.43 (m, 1H, δ-H Pro), 3.72 (m, 2H, δ-H Pro and 3-H DAHHA), 4.15 (dd, 1H, J_1 = 3 Hz, J_2 = 6 Hz, 2-H, DAHHA), 4.35 (m, 1H, α-H Pro), 4.98 (s, 2H, CH_2Z), 5.04 (s, 2H, CH_2Z), 5.04-5.16 (m, 2H, CO_2-CH_2-Ph), 5.21 (d, 1H, J = 6 Hz, OH), 6.15 (d, 1H, J = 9 Hz, 3-NH-Z), 6.74 (s, 1H, 7-NH-Z), 7.10-7.50 (m, 15 H, Z and Bn Ph).- $C_{35}H_{41}N_3O_8$ (631): Calcd. C 66.5 H 6.54 N 6.6 Found C 66.2 H 6.42 N 6.4.

(2S,3R)Bis(Z)-DAHHA-Ala-Pro-OMe (**11**)

1-Hydroxybenzotriazole (3.2 mmol) and triethylamine (2.7 mmol) were added to a solution of a (4:1) diastereomeric mixture of (2*R*,3*R*)- and (2*S*,3*R*)-bis(Z)-DAHHA-OH (**9**)[11] (2.7 mmol) and L-Ala-L-Pro methyl ester hydrochloride (3.2 mmol) in dry TFA (15 ml) at 0°C. After 30 min of stirring at 0°C, DCC (2.7 mmol) dissolved in dry CH_2Cl_2 (15 ml) was added. Then, processing as **8** afforded **11** as a foam (1.07 g, 63%): ^{1}H-NMR [$(CD_3)_2CO$]: δ (ppm) = 1.22 (d, 3H, J = 7 Hz, CH_3 Ala), 1.30-1.70 (m, 6H, 4-, 5-, and 6-CH_2 DAHHA), 1.80-2.10 (m, 3H, β-H and γ-CH_2 Pro), 2.20-2.30 (m, 1H, β-H Pro), 3.15 (m, 2H, 7-CH_2 DAHHA), 3.60-3.70 (m, 2H, δ-CH_2 Pro), 3.66 (s, 3H, OCH_3), 4.01 (m, 1H, 3-H DAHHA), 4.10 (dd, 1H, J_1 = 3 Hz, J_2 = 6 Hz, 2-H DAHHA), 4.43 (m, 1H, α-H Pro), 4.65 (q, 1H, J = 7 Hz, α-H Ala), 5.05 (s, 4H, CH_2 Z), 5.21 (d, 1H, J = 6 Hz, OH), 6.10 (d, 1H, 3-NH-Z), 6.40 (s, 1H, 7-NH-Z), 7.30-7.40 (m, 10 H, Z Ph), 7.62 (d, 1H, NH Ala).- $C_{32}H_{42}N_4O_9$(627): Calcd. C 61.3 H 6.71 N 8.9 Found C 61.5 H 6.57 N 8.8.

General Procedure for Deprotection of Compounds 8 *and* **10**

A solution of the protected (2*S*,3*R*)-bis(Z)-DAHHA-containing peptides **8** or **10** (1 mmol) in a 0.1 N HCl solution in MeOH (15 ml) was hydrogenated over 10% Pd/C (47 mg) at 2 atm H_2 and room temp. for 1 h. The catalyst was filtered off, washed with MeOH (10 ml) and the solvent was removed *in vacuo*. The residue was purified by flash chromatography, using $CHCl_3$-MeOH (3/1 v/v), obtaining **4** and **5** as foams in 80% yield.

(2S,3R)-DAHHA-Leu-Pro-OH (**4**) *dihydrochloride*

^{1}H-NMR (DMSO-d_6): δ (ppm) = 0.73 [d, 6H, J = 5 Hz, $(CH_3)_2$CH Leu], 1.10-1.60 (m, 9H, 4-, 5-, and 6-CH_2 DAHHA and CH_2-CH Leu), 1.70-1.90 (m, 3H, β-H and γ-CH_2 Pro), 2.09 (m, 1H, β-H Pro), 2.93 (m, 2H, 7-CH_2 DAHHA), 3.29 (m, 1H, 3-H DAHHA), 3.40-3.70 (m, 2H, δ-CH_2 Pro), 4.08 (d, 1H, $J_{2,3}$ = 4.5 Hz, 2-H DAHHA), 4.24 (m, 1H, α-H Pro), 4.53 (m, 1H, α-H Leu), 7.70-8.20 (br s, 6H, NH_3^+ DAHHA), 8.15 (d, J = 7 Hz, 1H, NH Leu).- $C_{18}H_{34}N_4O_5 \cdot 2$ HCl (425): Calcd. C 47.1 H 7.90 N 12.2 Found C 47.3 H 7.89 N 11.8.

(2S,3R)-DAHHA-Pro-OH (**5**) *dihydrochloride*

^{1}H-NMR (DMSO-d_6): δ (ppm) = 1.10-1.60 (m, 6H, 4-, 5-, and 6-CH_2 DAHHA), 1.70-2.20 (m, 4H, β- and γ-CH_2 Pro), 2.72 (m, 2H, 7-CH_2 DAHHA), 3.19 (m, 1H, 3-H DAHHA), 4.25 (m, 1H, α-H Pro), 4.31 (d, 1H, $J_{2,3}$ = 5 Hz, 2-H DAHHA), 6.80-8.40 (br s, 6H, NH_3^+ DAHHA).- $C_{12}H_{23}N_3O_4 \cdot 2$ HCl $\cdot$ 3 H_2O (346): Calcd. C 36.0 H 7.75 N 10.5 Found C 35.9 H 7.81 N 10.3.

(2S,3R)-DAHHA-Ala-Pro-OH (**6**) *dihydrochloride*

6N NaOH (0.6 ml, 1.2 mmol) was added to a solution of (2*S*,3*R*)-bis(Z)-DAHHA-Ala-Pro-OMe (**11**) (0.63 g, 1 mmol) in a (1:1) dioxane-water mixture (25 ml), and the solution was stirred at room temp. for 2 h. Then, the mixture was concentrated (10 ml), diluted with water (20 ml), and extracted with CH_2Cl_2 (3 x 20 ml). The aqueous phase was acidified to pH 3-4 with Dowex 50 W-X4 resin. The resin was filtered and washed with

ethyl acetate (20 ml). The aqueous phase was extracted with ethyl acetate (3 x 20 ml), and the org. extracts were dried over Na_2SO_4 and evaporated, to give the corresponding carboxylic acid. After hydrogenolysis, to remove the *N*-Z protecting groups, as above, the deprotected tripeptide **6** was obtained as a foam (0.26 g, 75%): ^{1}H-NMR (DMSO-d_6): δ (ppm) = 1.27 (d, 3H, J = 7 Hz, CH_3 Ala), 1.30-1.60 (m, 6H, 4-, 5-, and 6-CH_2 DAHHA), 1.80-2.00 (m, 3H, β-H and γ-CH_2 Pro), 2.10-2.20 (m, 1H, β-H Pro), 2.74 (m, 2H, 7-CH_2, DAHHA), 3.26 (m, 1H, 3-H DAHHA), 3.60 (m, 2H, δ-CH_2 Pro), 4.11 (d, 1H, $J_{2,3}$ = 4 Hz, 2-H DAHHA), 4.29 (m, 1H, α-H Pro), 4.55 (q, 1H, J = 7 Hz, α-H Ala), 7.80-8.20 (br s, 6H, NH_3^+ DAHHA), 8.35 (d, 1H, J = 7 Hz, NH Ala).- $C_{15}H_{28}N_4O_5 \cdot 2$ HCl (417): Calcd. C 43.2 H 7.25 N 13.4 Found C 42.9 H 7.46 N 13.5.

Antinociceptive Activity

Antinociception was evaluated in mice by means of the tail-flick test[15], immersing the tail into water at 52°C, using a cutoff time of 10 sec. The observer was blind to the compound injected. Results were expressed as % change in reaction time *vs.* predrug score (1.9-2.5 sec). A group of mice injected with saline was tested in parallel. Saline administration had no effect on the tail-flick latency at any time postinjection.

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