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Synthesis and biological activity of homoarginine-containing opioid peptides

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Abstract: Two tris-alkoxycarbonyl homoarginine derivatives, Boc-Har{ω,ω'-[Z(2Br)]$_2$}-OH and Boc-Har{ω,ω'-[Z(2Cl)]$_2$}-OH, were prepared by guanidinylation of Boc-Lys-OH, and used for the synthesis of neo-endorphins and dynorphins. The results were compared with that obtained in the synthesis in which Boc-Lys(Fmoc)-OH was incorporated into the peptide chain, and after removing Fmoc protection, the resulting peptide-resin was guanidinylated with N,N'-[Z(2Br)]$_2$- or N,N'-[Z(2Cl)]$_2$-*S*-methylisourea. The peptides were tested in the guinea-pig ileum (GPI) and mouse vas deferens (MVD) assays. The results indicated that replacement of Arg by Har may be a good avenue for the design of biologically active peptides with increased resistance to degradation by trypsin-like enzymes. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: dynorphin A; dynorphin-32; guanidinylation; homoarginine; α-neoendorphin; β-neoendorphin; opioid peptides

INTRODUCTION

Bioactive opioid peptides are formed as a result of processing large protein precursors. A survey of various precursors suggests that pairs of basic amino acids are primary sites of proteolytic cleavage. Since the rate of hydrolysis at each pair is different, several peptides possessing unique biological activities still contain one or more pairs of basic amino acids within the sequence. These peptides could be precursors of smaller biologically active peptides or could be transformed into inactive peptides.

We recently demonstrated that replacement of Arg-Lys in GH-RH(1–29)-NH_2 with Har-Har produces a highly active analogue that is completely resistant to trypsin [1]. The aim of the present work was twofold: (i) to elaborate a convenient method for the synthesis of homoarginine-containing peptides and (ii) to obtain opioid peptides in which arginine is substituted by homoarginine and compare their biological activity with those of the corresponding native peptides. For this reason, we selected neo-endorphins [2,3], dynorphins [4,5] and human preproenkephalin(100–111) [6].

MATERIALS AND METHODS

N,N'-[Z(2Cl)]$_2$-*S*-methylisothiourea and N,N'-[Z(2Br)]$_2$-*S*-methylisothiourea were prepared as described earlier [7]. Boc-Lys-OH was purchased from IRIS Biotech, Boc-Thr(Bzl)-PAM-resin was a Novabiochem product and Boc-Lys[Z(2Cl)]-PAM-resin was a Sigma product.

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Preparation of Boc-Har{ω,ω'-(Z(2Cl))$_2$}-OH

A solution of $NaHCO_3$ (0.630 g, 7.5 mmol)) and N^{α}-Boc-Lys-OH (1.53 g, 6.25 mmol) in 20 ml of dioxane: water (1 : 1) was stirred for 5 min, and N,N'-[Z(2Cl)]$_2$-*S*-methylisothiourea (2.13 g, 5 mmol) was added. The mixture was stirred for 48 h at 40 °C. The solvent was removed under reduced pressure and the crystalline residue was dissolved in ethyl acetate (400 ml). The solution was washed with aqueous 10% solution of citric acid (2 × 50 ml) and water (3 × 30 ml), dried with $MgSO_4$ and the solvent was evaporated to give a white powder. The product was purified by recrystallization from methanol: 2.7 g (86% calculated for the guanidinylation reagent); m.p. 104–105 °C; $[\alpha]_D^{20} = +1.5$ (c = 3, MeOH); Anal. calcd for $C_{28}H_{34}N_4O_8Cl_2$: C, 53.77; H, 5.48; N, 8.96; Cl, 11.33. Found: C, 53.88; H, 5.43; N, 8.94; Cl, 11.30. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.44 (s, 9H), 1.58–1.86 (m, 8H), 3.43 (m, 2H), 4.28 (m, 1H), 5.25 (s, 2H), 5.30 (s, 2H), 7.21–7.48 (m, 8H), 8.38 (s, 1H), 11.75 (s, 1H). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 22.57, 28.31, 28.52, 40.86, 53.18, 64.38, 65.40, 80.21, 126.82, 127.05, 128.97, 129.04, 129.31, 129.69, 130.07, 132.29, 132.90, 133.75, 134.43, 153.69, 155.70, 156.10, 163.42, 176.36.

Preparation of Boc-Har{ω,ω'-(Z(2Br))$_2$}-OH

This compound was prepared according to the procedure described above using N,N'-[Z(2Br)]$_2$-*S*-methylisothiourea (2.58 g, 5 mmol). Yield 79%; m.p. 62–65 °C; $[\alpha]_D^{20} = +6.6°$ (c = 1, $CHCl_3$); Anal. Calcd for $C_{28}H_{34}N_4O_8Br_2$: C, 47.07; H, 4.80; N, 7.84. Found: C, 47.35; H, 4.97; N, 7.70. ^{1}H NMR ($CDCl_3$, 200 MHz): 1.44 (s, 9H), 1.48–1.86 (m, 4H), 3.42 (m, 2H), 4.30 (m, 1H), 5.22 (s, 2H), 5.28 (s, 2H), 7.10–7.61 (m, 8H), 8.39 (t, 1H), 11.85 (s, 1H). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 22.57, 28.32, 28.52, 31.93, 40.87, 53.16, 66.63, 67.61, 80.25, 122.55, 123.45, 127.46, 127.67, 128.00, 128.90, 129.20, 130.05, 130.27, 132.56, 132.79, 132.97, 133.94, 136.04, 153.65, 156.11, 163.37, 176.05.

Synthesis of the peptides

Method 1. Guanidinylation of Lys-containing peptide on the resin. Merrifield resin (0.6 meq/g, 0.5 meq) was used for synthesis of **2**. α-Amino functions were Boc protected, and the side chains were blocked with the following groups: Tyr, Z(2Br) and Lys, Fmoc. The peptide chain assembly was performed according to standard procedures: (a) deprotection with 55% TFA in DCM (1×1 min, 1×20 min); (b) washing with DCM (3×1 min); (c) washing with 30% dioxane in DCM (2×1 min); (d) washing with DCM (3×1 min); (e) neutralization with 5% DIEA (1×1 min, 1×5 min); (f) washing with DCM (6×1 min); (g) coupling of Boc-amino acid (1.5 mmol) in the presence of DIPC (1.5 mmol) in DCM for 2 h; (h) washing with DCM (6×1 min). The resin was washed with DMF (3×1 min) and treated with 50% piperidine in DMF (1×10 min, 1×60 min) to remove the Fmoc group. It was then washed with DMF (3×1 min) and DCM (3×1 min). Guanidinylation was performed by treatment with *N*,*N*′-[Z(2Cl)]$_2$-*S*-methylisothiourea (2.58 g, 5 mmol) in DCM (10 ml) in the presence of 4-(*N*,*N*-dimethylamino)pyridine (0.61 g) for 16 h. The Boc group of the last amino acid was removed by running steps (a)–(f) of the schedule. The peptide-resin was treated with liquid HF in the presence of anisole for 1 h at 0 °C. The yield of crude peptide was 0.424 g. ESI-MS spectra were run to assess the purity of the product using a Finnigan MAT 95S spectrometer. As judged from the height of the signals, the main product contained about 10% of the product/products containing one Lys residue. For the synthesis of **4**, Boc-Lys[(Z(2Cl)]-PAM- resin (0.35 meq/g, 0.1 mmol, 0.286 g) was used. Amino acid derivatives and the procedure for synthesis were as described above with the only exception that Z(2Cl)-protected Lys was used for the incorporation of the Lys residue in position 11. Each coupling step was monitored using the Kaiser test. Fmoc protection was removed as described above. The product (237 mg) was divided into two portions which were transferred to small flasks. One portion was treated with *N*,*N*′-[Z(2Cl)]$_2$-*S*-methylisothiourea, and the second one with *N*,*N*′-[Z(2Br)]$_2$-*S*-methylisothiourea. The reaction was run in 2 ml of DCM: DMF (1 : 1) for 24 h with stirring. Boc protection was removed by treatment with a TFA/DMC solution and the peptides were cleaved from the resin by treatment with HF in the presence of anisole. As judged from the mass spectra of the crude products, the guanidinylation was complete in both experiments. The crude peptides were purified using a Knauer HPLC system with a Vertex column Nucleosil-300 C_{18} (8×200 mm, 5 μm): solvent system A, 0.1% TFA in water; B, 60% MeCN in A; Elution: 2–35% B in 15 min, then 35–40% B in 15 min and 40–100% B in 10 min; Flow rate: 2 ml/min. Fractions were analyzed on a Vertex column Nucleosil-100 C_{18} (4×250 mm, 5 μm) using a gradient of 20–80% in 20 min; flow rate 1 ml/min; detection at 220 nm.

Peptide **2**: r.t. = 10.3. min.; ESI-MS: $[M + 1H]^{1+} = 1009.8$ (calcd 1009.6); $[M + 2H]^{2+} = 505.4$ (calcd 505.3). The crude compound showed also $[M + 1H]^{1+} = 967.6$ and $[M + 2H]^{2+} = 484.3$.

Peptide **4**: r.t. = 14.5 min; ESI-MS: $[M + 2H]^{2+} = 824.0$ (calcd 284.0); $[M + 3H]^{3+} = 549.7$, calcd 549.7.

Method 2. The use of Boc-Har{ω,ω′-(Z(2Cl))$_2$}-OH. The synthesis was carried out using the procedures described above for peptide chain assembly, and Boc-Har{ω, ω'-[Z(2Cl)]$_2$}-OH was used for the incorporation of Har residues. [Har^{6,7,9,19,25,26}] dynorphin-32 (**5**) was synthesized using Boc-Thr(Bzl)-PAM-resin (0.91 meq/g, 015 mmol, 166.5 mg), Boc-Har{ω, ω'-[Z(2Cl)]$_2$}-OH for incorporation of the Har residues, formyl for protection of tryptophan and Boc-Asn-ONp and Boc-Gln-ONp for introducing Asn and Gln, respectively. The couplings were performed in *N*-methyl-2-pyrrolidinone, and HOBt was added. After HF treatment, 592 mg of For-peptide was obtained. The formyl group was removed by treatment with 1M NaOH in the presence of hydrazine [8], and the solution was submitted to gel filtration on a Sephadex G-10 column (2.5×90 cm, elution with 0.5 N acetic acid). Yield 126 mg after purification 22 mg of homogeneous product; r.t. 10.7 min (20–80% B in 13 min); ESI-MS: $[M + 4H]^{4+} = 1018.5$ (calcd 1017.9); $[M + 5H]^{5+} = 815.0$ (calcd 814.5); $[M + 6]^{6+} = 679.4$ (calcd 678.9); $[M + 7]^{7+} = 582.2$ (calcd 582.0).

[Har^6]β-neoendorphin (**8**) was synthesized using Merrifield resin (0.2 mmol), and Lys was protected with the Fmoc group. One half of the peptide-resin was saved for the synthesis of [Har^{6,7}]β-neoendorphin (see the next paragraph) and the other half was treated with 50% piperidine in DMF and then with TFA in DCM, and HF to obtain the product. Yield of crude product 46 mg. 17.8 mg of homogeneous product was obtained after preparative HPLC. ESI-MS: $[M + 1H]^{1+} = 1114.8$ (calcd 1115.0); $[M + 2H]^{2+} = 557.9$ (calcd 558.0).

[Har^6]α-neoendorphin (**7**) was synthesized using Boc-Lys[(Z(2Cl)]-PAM- resin (0.35 meq/g, 0.1 mmol, 0.286 g) ESI-MS: $[M + 1H]^{1+} = 1243.2$ (calcd 1243.4); $[M + 2H]^{2+} = 622.2$ (calcd 622.0).

[Har^{6,7},Leu^{5,12}]BI amide (10) was synthesized using BMH-resin (0.52 meq/g).ESI-MS: $[M + 1H]^{1+} = 1432.5$ (calcd 1433.6); $[M + 2H]^{2+} = 717.4$ (calcd 717.3).

Synthesis of (Har^{6,7})β-neoendorphin (9). The portion of peptide-resin prepared in the synthesis of [Har^6]β-neoendorphin was treated with 50% piperidine in DMF and then was guanidinylated using *N*,*N*′-[Z(2Cl)]$_2$-*S*-methylisothiourea (0.5 mmol). The Boc group was removed and the peptide-resin was treated with HF. The crude peptide (48 mg) was purified using preparative HPLC. Yield 14 mg. ESI-MS: $[M + 1H]^{1+} = 1156.9$ (calcd 1157.0); $[M + 2H]^{2+} = 578.9$ (calcd 579.0). The ESI-MS spectrum indicated that about 2% of the peptide was not guanidinylated.

Synthesis of α-neoendorphin. α-Neoendorphin (**6**) was synthesized using Boc-Lys[(Z(2Cl)]-PAM- resin (0.35 meq/g, 0.1 mmol, 0.286 g). Boc-Arg-{ω, ω'[Z(2Cl)]$_2$}-OH [9] was used for the incorporation of Arg, and (2Cl)Z and (2Br)Z for Lys and Tyr, respectively. The resulting peptide-resin (436 mg) was treated with HF to obtain the crude product (170 mg). Homogeneous peptide was obtained (32 mg) after purification by preparative HPLC. Analytical HPLC (10–80% B in 13 min): r.t. = 11.95 min. ES-MS: $[M + 1H]^{1+} = 1229.2$ (calcd 1228.4); $[M + 1Na]^{1+} = 1251.2$ (calcd 1251.4); $[M + 2H]^{2+} = 615.1$ (calcd 615.2).

Bioassay

The GPI [10] and MVD [11] bioassays were carried out as reported in detail elsewhere [12,13]. A log-response curve was determined with [Leu^5]-enkephalin as standard for each

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ileum and vas preparation, and the IC_{50} values of the compounds being tested were normalized according to a published procedure [14]. The results are presented in Table 2.

RESULTS AND DISCUSSION

In order to analyze the effect of replacement of dibasic coded amino acids in opioid peptides with homoarginine (Har) on activity and selectivity, we synthesized several Har-containing analogues (Table 1). The peptides were obtained by using two different protocols. In the first one (compounds **2** and **4**), Boc-Lys(Fmoc)-OH was used for peptide chain elongation, and after removal of Fmoc protection the resulting peptide-resin was guanidinylated using the previously described reagents N,N'-[Z(2Cl)]$_2$-*S*-methylisothiourea or N,N'-[Z(2Br)]$_2$-*S*-methylisothiourea [7].We found that the yield of guanidinylation greatly depended on the concentration of the reagent and on the solvent used for this reaction. In the synthesis of **2**, in which a relatively diluted solution in DCM was used, about 10% (5% per residue) of nonguanidinylated peptide was present in the crude product, as judged from the mass spectrum. In this case, the impurity was successfully removed by preparative HPLC. When a concentrated solution of the reagent in DMF: DCM (1 : 1) was used, the reaction was complete (peptide **4**) or less than 2.5% of nonguanidinylated peptide was detected (peptide **9**).

In the second protocol, we used the new protected Har derivative, Boc-Har{ω, ω'-[Z(2Cl)]$_2$}-OH, which was obtained by guanidinylation of Boc-Lys-OH. This protection of the guanidino group was recently used by us for synthesis of Arg-containing peptides [15]. It was demonstrated that this protection was superior to Tos protection. Using this protocol, we successfully obtained **5**, **6**, **7**, **8**, **9** and **10**.

The peptides were tested *in vitro* in the guinea-pig ileum (GPI) and mouse vas deferens (MVD) assays. The results presented in Table 2 indicate that the activities of these peptides were not much different from those of the native peptides. Interestingly, in three cases the activities in the MVD assay within the pairs of peptides were very similar (**1** and **2**; **6** and **7**; **8** and **9**), but in the GPI assay the activities of the Har-containing peptides were about 2 times lower than those of their native counterparts in the case of peptides **1** and **2**, and **6** and **7**, and in one case the peptide containing 2 Har residues (**9**) was twice as active as the one containing only one (**8**). It should also be noted that the analogue of dynorphin-32 containing 6 Har residues is quite active in the GPI assay.

CONCLUSIONS

Analogues of opioid peptides containing Har residues were successfully obtained by two approaches using either new tris-alkoxycarbonyl homoarginine derivatives or guanidinylation of the side-chain amino group in the peptide chain by previously described guanidinylation reagents. Analysis of the crude products obtained with the use of Boc-Har{ω, ω'-[Z(2Cl)]$_2$}-OH indicated that this method of synthesis is superior to the one using N,N'-[Z(2Cl)]$_2$-*S*-methylisothiourea for the guanidinylation of a Lys residue already incorporated in the peptide chain. In the case of the guanidinylation of a peptide-resin, attention should be paid to the reaction conditions in order to avoid loss of material due to incomplete guanidinylation.

Table 1 Opioid peptides

Peptide	Sequence	Method of synthesis
1 Dynorphin A (1–8)	H-TyrGlyGlyPheLeuArgArgIle-OH	Ref. 15
2 [Har^{6,7}]dynorphin A(1–8)	H-TyrGlyGlyPheLeuHarHarIle-OH	Method 1
3 Dynorphin A(1–13)	H-TyrGlyGlyPheLeuArgArgIleArgProLysLeuLys-OH	Ref. 15
4 [Har^{6,7,9}]dynorphin A(1–13)	H-TyrGlyGlyPheLeuHarHarIleHarProLysLeuLys-OH	Method 1
5 [Har^{6,7,9,19,25,26}]dynorphin-32	H-TyrGlyGlyPheLeuHarHarIleHarProLysLeuLys-TrpAspAsnGlnLysHarTyrGlyGlyPheLeuHarHar-GlnPheLysValValThr-OH	Method 2
6 α-Neoendorphin	H-TyrGlyGlyPheLeuArgLysTyrProLys-OH	Method 2
7 [Har^{6}]α-neoendorphin	H-TyrGlyGlyPheLeuHarLysTyrProLys-OH	Method 2
8 [Har^{6}]β-neoendorphin	H-TyrGlyGlyPheLeuHarLysTyrPro-OH	Method 2
9 [Har^{6,7}]β-neoendorphin	H-TyrGlyGlyPheLeuHarHarTyrPro-OH	Method 2 + 1
10 [Leu^{5,12}, Arg^{6}]BI amide	H-TyrGlyGlyPheLeuArgArgTyrGlyGlyPheLeu-NH_2	Ref. 16
11 [Har^{6,7}, Leu^{5,12}]BI amide	H-TyrGlyGlyPheLeuHarHarTyrGlyGlyPheLeu-NH_2	Method 2

Method 1. Guanidinylation on the resin.
Method 2. The use of tris-alkoxycarbonyl homoarginine derivatives.

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Table 2 GPI and MVD Assays of Opioid Peptides

Compound	GPI		MVD		MVD/GPI
	IC_{50} (nM)[a]	Rel. potency	IC_{50} (nM)[a]	Rel. potency	IC_{50} ratio
1 Dynorphin A(1–8)	678 ± 50	0.363 ± 0.027	208 ± 22	0.0548 ± 0.058	0.30
2 [$Har^{8,7}$]dynorphin A(1–8)	1070 ± 60	0.230 ± 0.013	209 ± 26	0.0545 ± 0.0068	0.19
3 Dynorphin A(1–13)	0.535 ± 0.062	460 ± 53	7.45 ± 0.39	1.53 ± 0.008	13.9
4 [$Har^{6,7,9}$]dynorphin A(1–13)	1.17 ± 0.23	210 ± 41	16.4 ± 1.1	0.695 ± 0.047	14.0
5 [$Har^{6,7,9,19,25,26}$]dynorphin-32	6.36 ± 0.53	38.7 ± 3.2	146 ± 26	0.078 ± 0.014	23.0
6 α-Neoendorphin	11.6 ± 0.8	21.2 ± 1.5	31.7 ± 1.6	0.360 ± 0.018	0.366
7 [Har^{6}]α-neoendorphin	20.3 ± 1.2	12.1 ± 0.7	35.4 ± 1.3	0.322 ± 0.012	0.573
8 [Har^{6}]β-neoendorphin	244 ± 22	1.01 ± 0.09	79.2 ± 11.0	0.144 ± 0.020	0.32
9 [$Har^{6,7}$]β-neoendorphin	145 ± 6	1.70 ± 0.07	75.5 ± 9.5	0.151 ± 0019	0.52
10 [$Leu^{5,12}$, Arg^{6}]BI amide[b]	24.9 ± 1.5	9.88 ± 058	32.1 ± 5.5	0.355 ± 0.061	1.29
11 [$Leu^{5,12}$, $Har^{6,7}$]BI amide	27.0 ± 3.3	9.11 ± 1.11	213 ± 6	0.0535 ± 0.0016	7.9
[Leu^{5}]enkephalin	246 ± 39	1	11.4 ± 1.1	1	0.0463

[a] Mean of 3–6 determinations ± SEM.
[b] Ref. 16.

The modified peptides were fairly potent when examined *in vitro* using the GPI and MVD assays. In some cases the activity was very similar to that of the corresponding native peptide, but also some changes in activity and selectivity were observed. These results indicate that using this approach it is possible to obtain active analogues, which, due to the presence of Har, would be resistant to trypsin-like enzymes.

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