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Synthesis and biological activity profile of new analogues of the cyclic opioid peptide H-Tyr-c(D-Cys-Gly-Phe(pNO_2)-D-Cys)-NH_2 containing (S)-α-hydroxymethylcysteine in place of cysteine

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Abstract: To examine the effect on biological activity of replacing D-Cys in the opioid peptide H-Tyr-c[D-Cys-Gly-Phe(pNO_2)-D-Cys]-NH_2 in position 2 or/and 5 with α-hydroxymethylcysteine (α-Hmc), three analogues were synthesized. These compounds exhibit agonist activity at both μ and δ receptors. However, the most active analogue, with (*S*)-α-Hmc residue in position 5, was 3360- and 2190-fold less active than the parent peptide in the GPI and MVD assays, respectively.

Keywords: α-hydroxymethylamino acids; α-hydroxymethylcysteine; opioid activity; opioid peptides

INTRODUCTION

The search for the bioactive conformations of opioid peptides at their receptors has continued for over 20 years and is still a matter of intensive study in many laboratories.

The highly flexible nature of the linear natural peptides renders them relatively nonselective in binding to the commonly accepted μ, δ and κ receptors [1–4]. The incorporation of conformational and topographical constraints into linear peptides may force the structure into the binding conformation at one particular receptor [5–8]. DPDPE is an early example of a cyclic constrained ligand which is a potent opioid agonist, selective for the δ receptor [9]. The extremely high potency in the GPI assay of the conformationally constrained analogues of enkephalin H-Tyr-c[D-Cys-Gly-Phe-L-Cys]-NH_2 and H-Tyr-c[D-Cys-Gly-Phe-D-Cys]-NH_2 was shown to be a consequence of the conformational restrictions introduced by cyclization [10]. Furthermore, the cysteine-containing analogues were highly resistant to enzymatic degradation.

This paper describes the synthesis of three analogues of the nonselective opioid peptide H-Tyr-c[D-Cys-Gly-Phe(pNO_2)-D-Cys]-NH_2 [11], in which the D-cysteine residue in position 2 or/and 5 is replaced by a (*S*)-α-Hmc residue. These analogues permit an assessment of steric and hydrophilic influences on opioid activity and receptor selectivity.

MATERIALS AND METHODS

Chemistry

Racemic α-Hmc was synthesized by selective Cys α-hydroxymethylation, and was resolved into its enantiomers by fractional crystallization of the diastereomeric salts of their *N*-benzoyl derivatives with (–)-quinine, using the method previously described [12]. (*R*)- and (*S*)-Boc-α-Hmc(Bzl)-OH were prepared in acetonitrile using TMAH pentahydrate and Boc_2O according to a published procedure [13].

The peptide analogues were synthesized by the manual solid-phase method using a 4-methylbenzhydrylamine resin × HCl (100–200 mesh, 0.59 mmol/g, Novabiochem). Boc-protected amino acids were obtained from commercial sources. Starting with 0.24g (0.4 mmol) of resin the following protected amino acids were added in a stepwise fashion to the growing peptide chain: Boc-(*S*)-α-Hmc(Bzl)-OH or Boc-D-Cys(SMeBzl)-OH, Boc-Phe(p-NO_2)-OH, Boc-Gly-OH, Boc-(*S*)-Hmc(Bzl)-OH or Boc-D-Cys(MeBzl)-OH and Boc-Tyr-OH. Amino acids were coupled in 3-fold excess using TBTU (3 equiv.) in the presence of DIEA (6 equiv.). When the α-hydroxymethylamino acid was acylated or used as an acylating component, a prolonged reaction time (24 h for repeated coupling) was necessary. After Boc-(*S*)-α-Hmc(Bzl)-OH had been coupled to the growing peptide chain, the unreacted amino groups as well as the hydroxyl groups were acetylated with an excess of acetic

Abbreviations: DIEA, diisopropylethylamine; DPDPE, H-Tyr-c[D-Pen-Gly-Phe-D-Pen]-OH; GPI, guinea-pig ileum; Hmc, hydroxymethylcysteine; HPLC, high performance liquid chromatography; MeBzl, *p*-methylbenzyl; MVD, mouse vas deferens; Pen, penicillamine; TBTU, O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate; TMAH, tetramethylammonium hydroxide.

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anhydride in the presence of a catalytic amount of DMAP. Removal of the Boc protecting group was performed with 50% (v/v) TFA in CH_2Cl_2 for 5 min as a prewash, followed by the 20 min treatment as a normal deprotecting reaction. The resin was washed with CH_2Cl_2 (3×1 min), neutralized with freshly prepared 10% DIEA in CH_2Cl_2 for 5 min as a prewash, followed by the usual 10 min treatment, and finally washed with CH_2Cl_2 (3×1 min). Completion of the coupling reactions was monitored by the ninhydrin test [14]. The peptides were cleaved from the resin with anhydrous HF (5 ml/g resin) with anisole added as a scavenger (1 ml/g resin) for 1 h at 0 °C and 1 h at 20 °C to remove the Bzl or MeBzl group from (*S*)-α-Hmc or D-Cys, respectively. After evaporation of HF, the resin was extracted three times with anhydrous diethyl ether and, subsequently, with a 50% aqueous solution of acetic acid. The crude linear peptides were obtained in the solid form by lyophilization of the acetic acid extracts and were purified by preparative reversed-phase HPLC on a Vydac C_{18} column (25×2.2 cm) with a linear gradient 25%–50% of B [A: 0.05% TFA in water, B: 0.038 TFA in acetonitrile/water (90 : 10, v/v)] at a flow rate of 12 ml/min. Disulfide bond formation was performed in methanol using a 5% solution of iodine in acetic acid [15]. Each peptide was >98% pure as determined by analytical reversed-phase HPLC on a Vydac C_{18} column (25×0.46 cm) using a linear gradient of 20%–50% B [A: 0.05% TFA in water, B: 0.038% TFA in acetonitrile/water (90 : 10, v/v)] at a flow rate 1 ml/min, with UV detection at 220 nm. Molecular weights were confirmed by FAB-MS. Analytical data of all synthesized opioid analogues are presented in Table 1.

Biology

For the determination of their *in vitro* opioid activities, the synthesized opioid peptides were tested in bioassays based on inhibition of electrically evoked contractions of the GPI and the MVD. The GPI assay is usually considered as being representative for μ opioid receptor interactions, even though the ileum does also contain κ opioid receptors. In the MVD assay opioid effects are primarily mediated by δ opioid receptors, but μ and κ receptors also exist in this tissue. The GPI and MVD bioassays were carried out as reported in detail elsewhere [16–18].

RESULTS AND DISCUSSION

All three synthesized cyclic analogues were examined in the GPI and MVD bioassays. The results are presented in Table 2. The analogues are agonists at both the μ and δ receptors. The most active analogue, containing

Table 1 Analytical Parameters of Linear and Cyclic Opioid Peptide Analogues

Compound	Chemical formula	Calc. mol. weight	FAB-MS $[M + H]^+$	HPLC t_R[a]	TLC[b]
H-Tyr-D-Cys-Gly-Phe(*p*NO_2)-(**S**)-α-**Hmc**-NH_2	$C_{27}H_{35}N_7O_9S_2$	665.73	666.32	12.41	0.53
H-Tyr-(**S**)-α-**Hmc**-Gly-Phe(*p*NO_2)-D-Cys-NH_2	$C_{27}H_{35}N_7O_9S_2$	665.73	666.56	11.59	0.52
H-Tyr-(**S**)-α-**Hmc**-Gly-Phe(*p*NO_2)-(**S**)-α-**Hmc**-NH_2	$C_{28}H_{37}N_7O_{10}S_2$	701.75	702.61	12.75	0.52
H-Tyr-c[D-Cys-Gly-Phe(*p*NO_2)-(**S**)-α-**Hmc**]-NH_2	$C_{27}H_{33}N_7O_9S_2$	663.73	664.78	16.64	0.61
H-Tyr-c[(**S**)-α-**Hmc**-Gly-Phe(*p*NO_2)-D-Cys]-NH_2	$C_{27}H_{33}N_7O_9S_2$	663.73	664.70	15.97	0.61
H-Tyr-c[(**S**)**Hmc**-Gly-Phe(*p*NO_2)-(**S**)-α-**Hmc**]-NH_2	$C_{28}H_{35}N_7O_{10}S_2$	699.75	700.63	15.54	0.62

[a] See Materials and Methods.
[b] 1-Butanol/acetic acid/ethyl acetate/water 1 : 1 : 1 : 1, by volume.

Table 2 GPI and MDV Activities[a] of Cyclic Analogues of the Opioid Peptide Modified by (*S*)-α-Hydroxymethylcysteine

Compound	GPI		MVD		GPI/MVD
	IC_{50} (nM)	Relative potency	IC_{50} (nM)	Relative potency	IC_{50} ratio
H-Tyr-c[D-Cys-Gly-Phe(*p*NO_2)-(**S**)-α-**Hmc**]NH_2	118 ± 22	2.08 ± 0.39	41.0 ± 3.4	0.278 ± 0.023	2.88
H-Tyr-c[(**S**)-α-**Hmc**-Gly-Phe(*p*NO_2)-D-Cys]NH_2	3050 ± 340	0.0807 ± 0.0090	3490 ± 260	0.00327 ± 0.00024	0.874
H-Tyr-c[(**S**)-α-**Hmc**-Gly-Phe(*p*NO_2)-(**S**)-α-**Hmc**]NH_2	3740 ± 850	0.0658 ± 0.0149	1540 ± 30	0.00740 ± 0.00014	2.43
H-Tyr-c-[D-Cys-Gly-Phe(*p*NO_2)-D-Cys]NH_2	0.9351 ± 0.0100	7010 ± 1990	0.0187 ± 0.0023	610 ± 75	1.88
[Leu[5]]-enkephalin[b]	246	1	11.4 ± 1.1	1	21.6

[a] Mean of three determinations (±SEM).
[b] [Leu[5]]-enkephalin is H-Tyr-Gly-Gly-Phe-Leu-OH.

(S)-α-Hmc in position 5, is 3360- and 2190-fold less active than the parent peptide in the GPI and the MVD assay, respectively. Interestingly, the incorporation of (S)-α-Hmc in position 5 changed the selectivity in favour of the μ receptor. On the basis of the obtained results it may be concluded that the hydroxymethyl group which was introduced on the C^{α}-atom of the Cys residue is too large and causes steric interference during receptor binding. The IC_{50}(GPI)/IC_{50}(MVD) ratios are indicative of the δ vs μ receptor selectivity of the compounds (Table 2). The ratios of the three α-Hmc-analogues are similar to that of the H-Tyr-c[D-Cys-Gly-Phe(*p*NO_2)-D-Cys]NH_2 parent peptide, indicating that introduction of the hydrophilic hydroxymethyl group at the two peptide backbone positions had no significant effect on the δ vs μ selectivity profile. On the other hand, introduction of a hydroxyl group in the side chains of linear enkephalin analogues in the 2- and 6-position of the peptide sequence (Ser or Thr substitution) has been shown to result in increased δ receptor selectivity [19].

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