Polish J. Chem., **78**, 951–959 (2004)

Synthesis of HYNIC- and DOTA-Conjugates with μ-Opioid Receptor Ligands: Morphiceptin and Endomorphin-2

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(Received April 7th, 2004; revised manuscript May 11th, 2004)

Opioid peptides recently attracted much attention as low molecular weight compounds, which can target malignant cells expressing opioid receptors on their cell surface. Therefore opioid peptides have a potential to be introduced as radiopharmaceuticals. In this paper, we describe the method of conjugation of two bifunctional chelating agents (BFCAs), hydrazinopyridine-3-carboxylic acid (HYNIC) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), to the N-terminal amino group of morphiceptin, endomorphin-2, and two of their analogs modified in position 3. GABA was used as a spacer molecule. μ-Opioid binding affinities of the peptides were compared with the binding affinities of BFCA-GABA-peptide conjugates. It was shown that the introduction of HYNIC to μ-opioid ligands causes only a slight decrease of μ-opioid receptor affinity, while DOTA-conjugates loose their affinity for μ-receptors completely.

Key words: solid phase peptide synthesis, bifunctional chelating agents, receptor binding assay, opioid receptors

Many tumors express an increased number of peptide receptors on their cell surface. Radiolabeled peptides, which bind to these receptors, attracted much attention as potential radiopharmaceuticals. The analogs of somatostatin [1, 2], chemotactic peptides [3], RGD [4], bombesin [5] and vasoactive intestinal peptide [6] have been used to target neoplastic cells.

Malignant tissues were also examined for the presence of opioid receptors. Opioid μ, δ and κ binding sites were identified on various tumors, such as primary human breast carcinomas, colon, prostate and lung cancer [7]. Therefore opioid peptides are another group of low molecular weight compounds which have a potential to be introduced as radiopharmaceuticals.

Two peptides that target μ-opioid receptors with high affinity and selectivity are structurally related endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2) [8] and morphiceptin (Tyr-Pro-Phe-Pro-NH_2) [9]. In our earlier paper [10] we have demonstrated the uptake of ^{131}I labeled morphiceptin by mouse mammary adenocarcinoma. However, for clinical applications labeling of peptides with metallic radionuclides, such as techne-

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tium-99m (^{99m}Tc), indium-111 (^{111}In) or lutetium-177 (^{177}Lu), is more important than labeling with ^{131}I.

Direct labeling methods are mostly based on the binding of a radionuclide to thiol groups in the peptide molecule [11]. This approach, limited to ^{99m}Tc and ^{188}Re, is rather unsuitable for small peptides, which either do not possess a disulfide linkage or are unable to maintain their activity after reduction.

The alternative is to bind a radionuclide to the targeting peptide indirectly, through a bifunctional chelating agent (BFCA) [12]. A BFCA, covalently attached to the peptide, functions as a coordinating agent for a radionuclide. The synthesis of conjugates of different BFCAs with somatostatin analogs, such as octreotide or lanreotide, has been described in numerous papers [13].

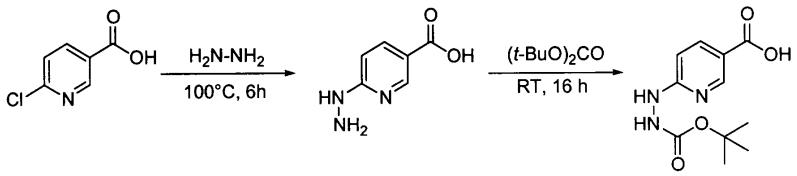
In this article we report, for the first time, simple and high-yield synthetic procedures leading to hydrazinopyridine-3-carboxylic acid (HYNIC) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) conjugates with μ-opioid receptor ligands – morphiceptin and endomorphin-2 and two of their analogs modified in position 3.

EXPERIMENTAL

Materials. All solvents were obtained from Aldrich-Sigma Chemical Co. (St. Louis, USA), except where otherwise stated, and used without further purification. Protected amino acids were purchased from Nova Biochem AG (Laufelfingen, Switzerland). 6-Chloronicotinic acid and hydrazine hydrate were obtained from Fluka (Buchs SG, Switzerland). 1,4,7,10-Tetraazacyclododecane-1,4,7-tris(acetic acid-t-butyl ester)-10-acetic acid [DOTA-(*t*Bu)$_3$] was purchased from Macrocyclics Inc (Richardson, USA).

Preparation of Boc-HYNIC. 6-*tert*-Butoxycarbonyl-hydrazinopyridine-3-carboxylic acid (Boc-HYNIC) was synthesized according to the modified method of Abrams *et al*. [14]. The reaction sequence for the synthesis of Boc-HYNIC is shown in Scheme 1. 6-Chloronicotinic acid (4.0 g, 25.4 mmol) was added to hydrazine hydrate (80% in aqua, 17.5 ml, 450.1 mmol) and placed in a 100°C bath for 6 h. The reaction mixture was cooled to room temperature and concentrated to dryness. The resulting white solid was dissolved in water and acidified with concentrated hydrochloric acid to pH 5.5 to form a precipitate. The precipitate was filtered off, washed with ethanol and ethyl ether and dried in vacuo to give 6-hydrazinopyridine-3-carboxylic acid (3.55 g, 90%).

To a solution of 6-hydrazinopyridine-3-carboxylic acid (1.6 g, 11.2 mmol) in triethyl amine (1.4 ml), di-tert-butyl dicarbonate (2.6 g, 11.2 mmol) in N,N-dimethylformamide (DMF) (17 ml) was added drop-wise within 1 h. The reaction mixture was stirred for 16 h. To form a precipitate, 20 ml of ethanol/ammonia 7:3 was added, and the mixture was kept at 4°C overnight. The resulting precipitate was filtered off and dried in vacuo to give Boc-HYNIC (1.2 g, 75%).



Scheme 1. Boc-HYNIC synthesis.

Peptide synthesis. All peptides were synthesized by a standard solid-phase method on a MBHA Rink Amide resin, 100–200 mesh, 0.66 mmole/g, Nova Biochem, La Jolla, USA, using 9-fluorenylmethoxycarbonyl (Fmoc) for the protection of the α-amino group. 20% piperidine in DMF was used for Fmoc deprotection and TBTU [2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate] was employed to facilitate coupling.

Synthesis of BFCA-GABA- peptide conjugates. Fmoc-γ-aminobutyric acid (Fmoc-GABA) was coupled in N-methyl-2-pyrrolidone (NMP), to the α-amino group of the peptide linked to the resin using 1-hydroxybenzotriazole (HOBt) and N,N-diisopropylcarbodiimide (DIC) as coupling reagents. Three equivalents of Fmoc-GABA, 10.5-fold molar excess of HOBt and 10.5-fold molar excess of DIC were stirred in NMP at room temperature for 0.5 h. The mixture was added to the resin and shaken for 4 h at room temperature.

After deprotection of the amino group of GABA, DOTA-(tBu)$_3$ or Boc-HYNIC were coupled to GABA-peptide-resin. A 2.5-fold molar excess of DOTA-(tBu)$_3$ and 2.5-fold molar excess of O-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HATU) were stirred in NMP at room temperature for 15 min. The pH was adjusted to 8–9 by adding N,N-diisopropylethylamine (DIPEA). The mixture was added to the resin and shaken overnight at room temperature. The coupling reaction for Boc-HYNIC conjugation was prolonged to 48 h.

Cleavage of peptide conjugates from the resin. Trifluoroacetic acid (TFA, 4.75 ml), triisopropylsilane (TIS, 0.125 ml), and water (0.125 ml) were added to fully protected dried peptide resin. The mixture was shaken for 2 h for DOTA- and 1 h for HYNIC-conjugates. Dimethyl ether (40 ml) was then added twice to precipitate crude product. The precipitate was dissolved in water and the solution was evaporated to dryness.

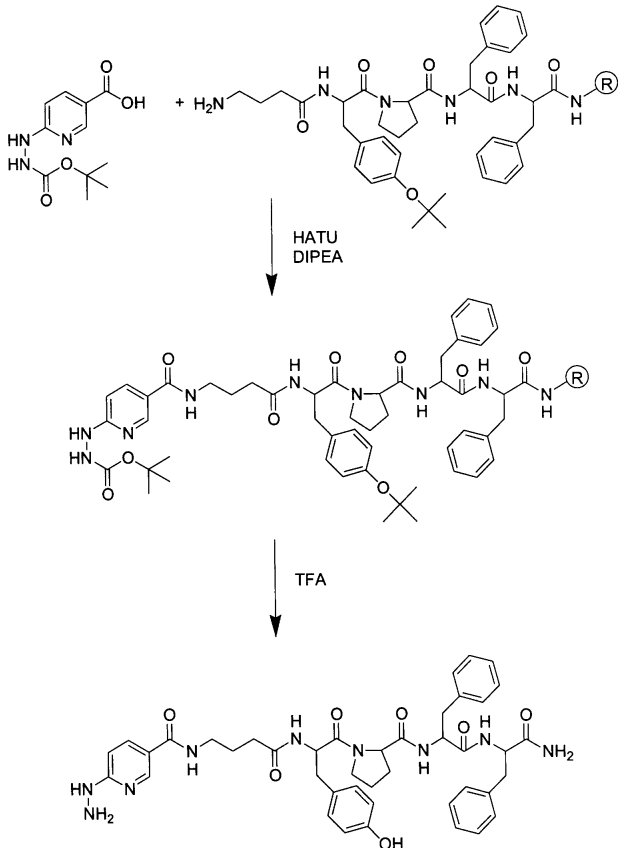
Crude peptide conjugates were purified by RP HPLC (HP Series 1050 chromatography system, Hewlett Packard, Palo Alto, USA) on a Macherey-Nagel C_{18} column (VP 250/21 Nucleosil 100-5) using the solvent system of 0.1% TFA in water (A)/ 80% acetonitrile in water containing 0.1% TFA (B) with a linear gradient of 20–60% B over 25 min, λ = 214 nm. Calculated values for protonated molecular ions were in agreement with those obtained using FAB mass spectrometry (Fisons VG-BIO-Q, Manchester, UK).

Receptor binding assay. Receptor binding assays have been performed as described previously [15] after obtaining permission from the Local Ethical Committee for Animal Research. Adult male Wistar rats (200–250 g) were killed and their brain immediately removed and placed on ice. Whole brains were homogenized in 20 volumes of 50 nM Tris/HCl stock buffer (pH 7.4). The homogenate was centrifuged (48 000 x g for 15 min), resuspended and preincubated (25°C for 30 min) to remove endogenous opioids. The homogenate was centrifuged and resuspended (0.5% concentration). Binding affinities of the compounds for the μ-receptor were measured against [^{3}H]naloxone, synthesized by Dr. G. Toth, Institute of Biochemistry, Hungarian Academy of Sciences, Szeged, Hungary. A 100 μl aliquot of rat brain homogenate was incubated at 25°C for 120 min with 0.5 nM [^{3}H]naloxone in a total volume of 1 ml of 50 mM Tris/HCl (pH 7.4) containing bovine serum albumin (BSA) (1 mg/ml), bacitracin (50 μg/ml), bestatin (30 μM) and captopril (10 μM). All reactions were carried out in duplicate, at 10 μM concentration. Naltrexone hydrochloride was used to determine nonspecific tissue binding. The binding reaction was terminated by rapid filtration through GF/B Whatman glass fiber strips with a Brandel Cell Harvester (Brandel 24 Sample Semi-Auto Harvester, Gaithersburgh, USA), followed immediately by three rapid washes with 4 ml aliquots of ice-cold saline solution. The filter was removed and soaked in 10 ml of scintillation fluid and radioactivity was measured. The data were analyzed by a nonlinear least square regression analysis computer program GraphPad Prism (GraphPad Software, Inc, San Diego, USA).

RESULTS AND DISCUSSION

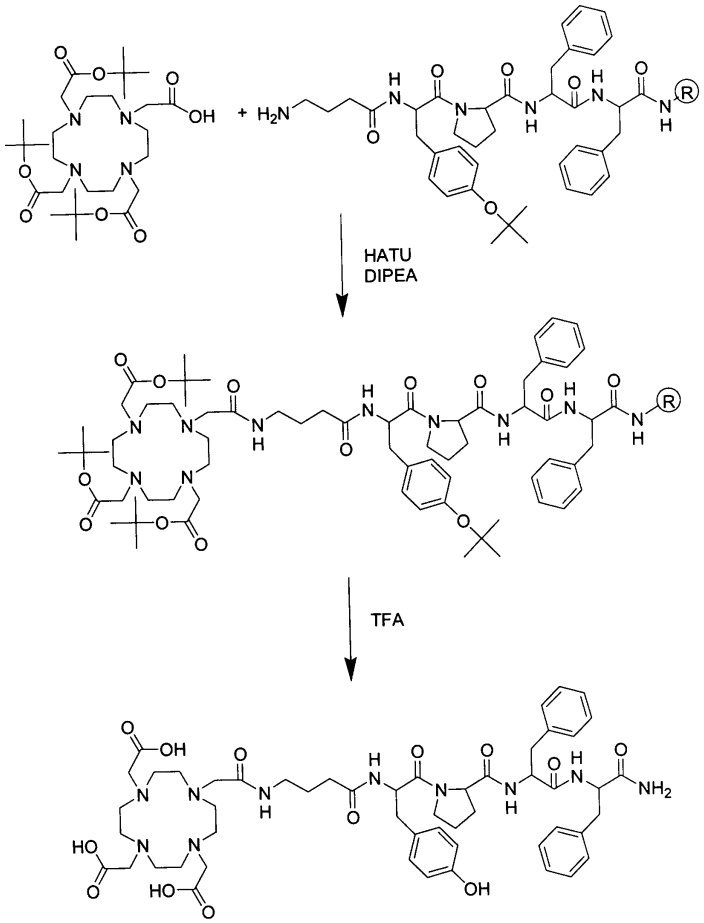
Synthesis. Endomorphin-2 **1**, morphiceptin **2**, and two potent morphiceptin analogs modified at the position 3, [D-Phe^3]morphiceptin **3** and [D-1-Nal^3]morphiceptin **4** (1-Nal = 3-(1-naphthyl)alanine), were chosen for the coupling reaction with two BFCAs, HYNIC and DOTA. GABA was used as a spacer molecule. Peptides were

synthesized by solid-phase method using Fmoc strategy. After deprotection of the peptide N-terminal amino group Fmoc-GABA was coupled, followed by Boc-HYNIC (Scheme 2) or DOTA-(*t*-Bu)$_3$ (Scheme 3). HATU was used as a coupling reagent. In the case of DOTA 24 h reaction time was sufficient. For HYNIC conjugates a negative ninhydrin test was obtained after 48 h.



Scheme 2. Conjugation of Boc-HYNIC and GABA-endomorphin-2.

The chromatograms in Fig. 1 show the conjugation reaction of DOTA-(*t*-Bu)$_3$ with GABA-endomorphin-2 after 24 h. After cleavage from the resin and deprotection three peaks were obtained. The main peak with the retention time t_R = 10.8 min had the molecular weight 1042, corresponding to DOTA-GABA-endomorphin-2 **9**. Similar elution profiles were obtained for DOTA-GABA-morphiceptin **10** and for HYNIC-GABA-peptide conjugates. Retention times for purified BFCA-GABA-peptide conjugates as well as their calculated and found molecular weights are summarized in Table 1.



Scheme 3. Conjugation of [DOTA-(tBu)$_3$] and GABA-endomorphin-2.

Receptor binding. The binding affinities of morphiceptin, endomorphin-2 and two analogs at the μ-opioid receptor were compared with the affinities of HYNIC-GABA- and DOTA-GABA-conjugates of these peptides The results are summarized in Table 2. The lowest IC_{50} value was found for [D-1-Nal^3]morphiceptin **4**, which was about 2-fold lower than for endomorphin-2 **1** (1.9±0.11 and 3.9±0.13 nM, respectively). IC_{50} values for morphiceptin **2** and [D-Phe^3]morphiceptin **3** were an order of magnitude higher (79.4±6.04 and 50.1±3.27 nM, respectively). Coupling of HYNIC-GABA to the N-terminal amino group of these peptides caused a 3 to 5-fold decrease in μ-receptor affinity, however the values were still in the nanomolar range. For DOTA-conjugates, however, a complete loss of μ-opioid receptor affinity was observed.

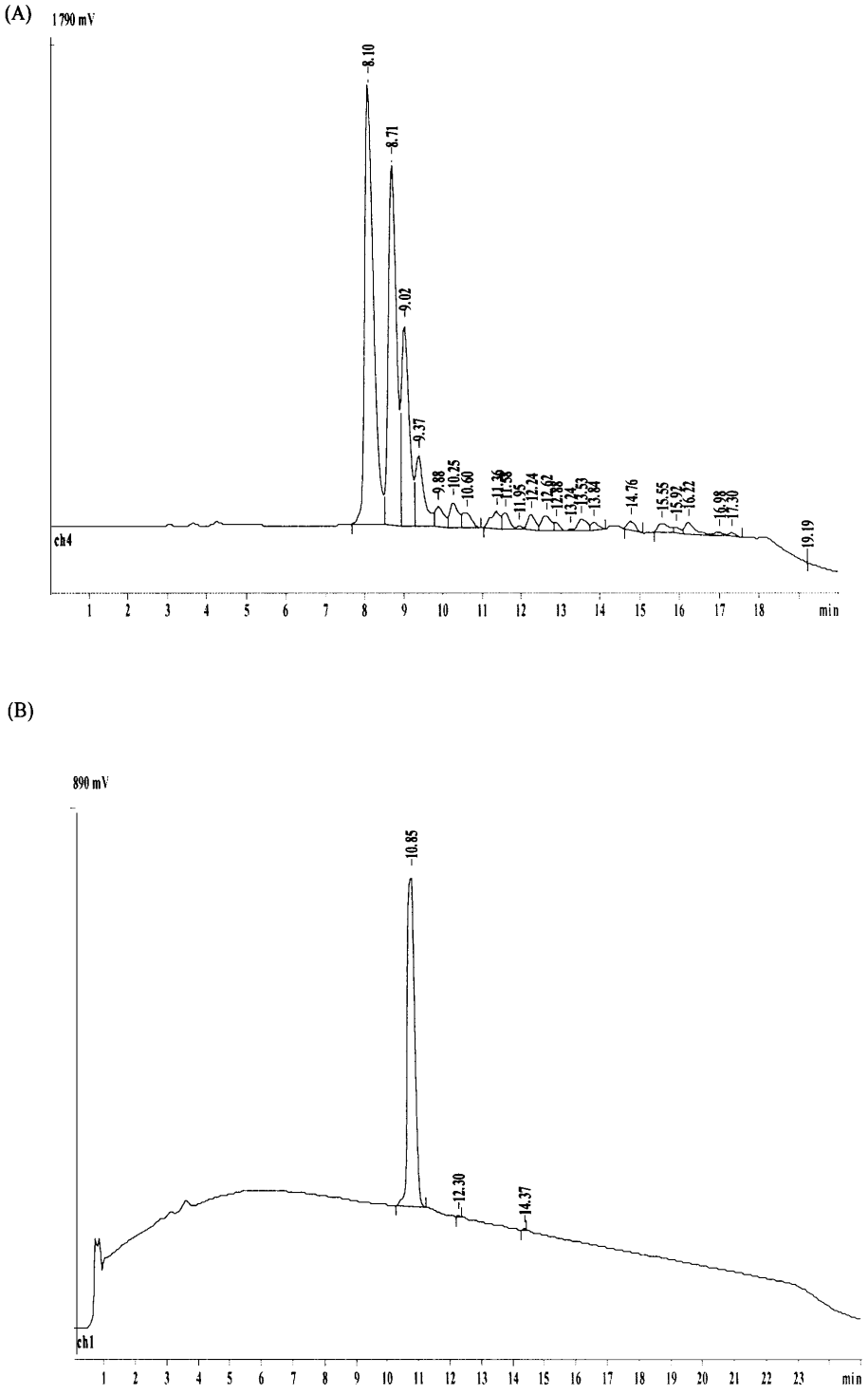


Figure 1. Chromatograms of DOTA-GABA-endomorphin-2 **9**. (A) crude product; (B) purified conjugate.

The most widely used method for labeling small highly specific peptides is by conjugation of BFCAs to the peptide. HYNIC is one of a number of BFCAs suitable for labeling with ^{99m}Tc, the radionuclide of choice for labeling peptides for imaging applications [16,17].

Peptides labeled with ^{111}In, ^{67}Ga, ^{90}Y and ^{177}Lu, group IIIb metallic radionuclides, are suitable for both scintigraphy and radionuclide therapy. The chelating agent DOTA has been shown to have widespread application for labeling peptides with these radiometals. The widest use of this compound has been in combination with octreotide analogs for imaging and treating tumors expressing high levels of somatostatin receptors [18], but it has been used to conjugate a much wider range of peptides [19–21].

Table 1. Physicochemical data of BFCA-GABA-peptide conjugates.

No	Peptide	FAB-MS			HPLC[a] t_r
		Formula	MW	$[M + H]^+$	
5	HYNIC-GABA-endomorphin-2	$C_{42}H_{49}N_9O_7$	791	792	8.1
6	HYNIC-GABA-morphiceptin	$C_{38}H_{47}N_9O_7$	741	742	10.1
7	HYNIC-GABA-[D-Phe^3]morphiceptin	$C_{38}H_{47}N_9O_7$	741	742	10.2
8	HYNIC-GABA-[D-1-Nal^3]morphiceptin	$C_{42}H_{49}N_9O_7$	791	792	9.7
9	DOTA-GABA-endomorphin-2	$C_{52}H_{70}N_{10}O_{13}$	1042	1043	10.8
10	DOTA-GABA-morphiceptin	$C_{48}H_{68}N_{10}O_{13}$	992	993	11.5

[a]HPLC elution on a Macherey-Nagel C_{18} column (VP 250/21 Nucleosil 100-5) using the solvent system of 0.1% TFA in water (A)/80% acetonitrile in water containing 0.1% TFA (B) with a linear gradient elution of 20–60% B over 25 min.

Table 2. *In vitro* binding affinities of opioid peptides and their BFCA-GABA-conjugates at μ-opioid receptor.

No	Peptide	IC_{50}[a] [nM]
1	endomorphin-2	3.9 ± 0.13
2	morphiceptin	79.4 ± 6.04
3	[D-Phe^3]morphiceptin	50.1 ± 3.27
4	[D-1-Nal^3]morphiceptin	1.9 ± 0.11
5	HYNIC-GABA-endomorphin-2	17.5 ± 1.24
6	HYNIC-GABA-morphiceptin	211.0 ± 25.6
7	HYNIC-GABA-[D-Phe^3]morphiceptin	163.0 ± 20.4
8	HYNIC-GABA-[D-1-Nal^3]morphiceptin	10.1 ± 0.92
9	DOTA-GABA-endomorphin-2	> 1000
10	DOTA-GABA-morphiceptin	> 1000

[a]Replacement of [^{3}H]naloxone. Results are the mean ± SEM of two independent experiments.

To conjugate HYNIC t-butoxycarbonyl protection on the hydrazine is most frequently used. For DOTA two methods of conjugating to peptides have been described. The first involves activation of one of the free tetra-carboxylic acid groups with carbodiimide and N-hydroxysuccinimide in aqueous solution to produce a N-hydroxysuccinimide ester *in situ*, which can react with primary amino groups at either the N-terminus or on the lysine side chains of the peptide [22]. The other method is based on the use of a monoreactive DOTA in which three of the carboxyl groups are blocked by formation of the *t*-butyl ester [2]. The remaining carboxyl group can be selectively activated to produce an active ester, which reacts with primary amino groups. Since only one carboxyl group is activated, there is a small possibility to obtain side-products.

In the present study we have conjugated endomorphin-2, morphiceptin, and two analogs with HYNIC and DOTA. Active groups of both BFCAs were protected to eliminate undesired side-reactions. The linker residue, GABA, separating the peptide from a BFCA was incorporated to increase the distance between a BFCA and the binding site of a peptide.

The reaction time for DOTA coupling was significantly shorter (24 h), although the same activating agent was used. It could be suggested that the conjugation of DOTA was less dependent on the pH of the reaction mixture. To obtain satisfactory results for HYNIC coupling, the addition of DIPEA was necessary to maintain basic conditions.

Edreira *et al*. [23] performed the synthesis of DOTA-TOC, a somatostatin analog, on a small scale (1 μmol) and reported that the best DOTA/HATU:peptide molar ratios were between 3:1 and 4:1. Heppeler *et al*. [2] used a 1:1 molar ratio for the synthesis at a larger scale of 75 μmol. In our experiments, the amount of peptide-resin used for conjugation with BFCAs was approximately 60 μmol. The best results were obtained when the 3:1 molar ratio was used. It seems that the larger excess of DOTA/HATU is advantageous for improving the yield of the conjugation reaction, but the high cost of these reagents is also an important consideration.

Amino and hydroxyl group of Tyr^1 and phenyl group of Phe^3 residue are considered as pharmacophores responsible for the binding of the peptide to the opioid receptor [24]. Coupling of a BFCA to the amino group of Tyr^1 may cause a loss of activity. The IC_{50} values at the μ-opioid receptor of BFCA-GABA-conjugates and unconjugated peptides were compared. The binding affinities of HYNIC-GABA-conjugates were found to be 3–5 fold lower than of unconjugated peptides. However, they were still in a nanomolar range. For DOTA-GABA-conjugates a complete loss of receptor affinity was observed. A much bigger size of DOTA molecule comparing to HYNIC may be the reason for such affinity differences.

In conclusion, a convenient approach for the conjugation of morphiceptin and endomorphin-2 with DOTA and HYNIC was described. The introduction of HYNIC at the N-terminal amino group of the μ-opioid receptor ligands did not influence the receptor binding significantly, while DOTA-conjugates didn't show any affinity for the μ-receptor sites.

Acknowledgments

This work was supported by the grant of the International Union Against Cancer (ICRETT 763) and by the grant of the Medical University of Lodz No. 502-12-812. The authors wish to thank Jozef Cieslak for his excellent technical assistance.

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