

Backbone metal cyclization: novel ^{99m}Tc labeled GnRH analog as potential SPECT molecular imaging agent in cancer

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Abstract

Gonadotropin-releasing hormone (GnRH) is a decapeptide secreted to the pituitary where it binds to specific receptors on the gonadotropes to regulate gonadotropic hormones (luteinizing hormone (LH) and follicle-stimulating hormone (FSH)) synthesis and secretion. Specific GnRH receptors are overexpressed in breast, prostatic, ovarian, and other tumors. The aim of this study was to synthesize a cyclic GnRH analog with high affinity to GnRH receptors that can be radiolabeled with ^{99m}Tc . A precyclic GnRH analog, [Cys-Gly]¹[D-Ala]⁶[N^α(η -Cys-amino hexyl)]¹⁰GnRH (Gn-2), containing two hemi-chelator groups was synthesized. It was cyclized applying the recently reported backbone metal cyclization (BMC) approach, to obtain *cyclo*[Re(O)1-10][Cys-Gly]¹[D-Ala]⁶[N^α(η -Cys-amino hexyl)]¹⁰GnRH (*cyclo*[Re(O)-Gn-2]). For comparative evaluations, Gn-2 was oxidized on-resin to yield *cyclo*(S-S,1-10)[Cys-Gly]¹[D-Ala]⁶[N^α(η -Cys-amino hexyl)]¹⁰GnRH, (*cyclo*[S-S-Gn-2]). The binding affinity of *cyclo*[Re(O)-Gn-2] to rat pituitary membranes showed IC₅₀ of 50nM, compared to IC₅₀ = 10 nM in the native GnRH. *Cyclo*(^{99m}Tc (O)1-10)[Cys-Gly]¹[D-Ala]⁶[N^α(η -Cys-amino hexyl)]¹⁰GnRH (*cyclo*[^{99m}Tc (O)-Gn-2]) was synthesized from Gn-2 and showed similar chromatographic behavior to its rhenium surrogate. © 2004 Elsevier Inc. All rights reserved.

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1. Introduction

Prostate cancer is second only to lung cancer as a cause of cancer-related deaths among men. To date, imaging tools, including single photon emission computerized tomography (SPECT) and positron emission tomography (PET), for diagnosing local recurrence and metastatic sites of prostate cancer are sub-optimal [1]. PET, using the non-specific (metabolic related) radiopharmaceutical 2-[^{18}F]-2-deoxy-D-glucose (FDG), has been shown to be an accurate technique for tumor detection, staging and monitoring of therapy in a number of malignant tumors [2]. So far, the clinical experience with FDG-PET in prostate cancer is limited for two reasons: the uptake of FDG in prostate cancer is low and

FDG is rapidly excreted in urine, causing an accumulation of activity in the bladder [3]. ^{11}C labeled choline has recently been reported as a new PET radiopharmaceutical for prostate cancer detection [4,5]. Choline is one of the components of phosphatidylcholine, an essential element of phospholipids in the cell membrane [6]. Malignant tumors show a high proliferation and increased metabolism of cell membrane components leading to an increased uptake of choline. However, ^{11}C half-life of 20 minutes limits the regional distribution of the tracer to PET centers. The preparation and use of ^{18}F Choline and analogs was also described, however high radioactivity uptake was observed in kidney and bladder at a rather early stage [7]. Several ^{18}F labeled androgen ligands have been studied as candidates for nuclear medicine imaging of prostate tumor, however, none of them yields a clinical widely used agent [8–10].

GnRH, also known as luteinizing hormone-releasing hormone (LH-RH), is a decapeptide with the sequence

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pGlu¹-His²-Trp³-Ser⁴-Tyr⁵-Gly⁶-Leu⁷-Arg⁸-Pro⁹-Gly¹⁰-NH₂. It was first elucidated in 1971 in the laboratories of Schally and co-workers [11] and Guillemin and co-workers [12]. GnRH activity is mediated by specific transmembrane receptors that belong to the G-protein coupled receptor (GPCR) family, found on the pituitary gonadotrophs [13]. GnRH is secreted into the pituitary portal circulation and plays a major role in the biology of reproduction, inducing the biosynthesis and release of LH and FSH. GnRH secretion is crucial for the control of gonadal function and normal ovarian cycle. Continuous stimulation of the pituitary, either by natural GnRH or by GnRH agonists, cause desensitization of the pituitary gonadotropes, resulting in cessation of gonadotropin secretion [14,15]. GnRH antagonists produce a competitive blockade of pituitary GnRH receptors and cause an immediate inhibition of the release of gonadotropins and therefore also of sex steroids. GnRH receptors and GnRH mRNA are overexpressed in several tumors such as breast, prostatic, and ovarian [16,17]. These findings suggest that local GnRH may be involved in the growth of these tumors, thus providing the basis for the clinical applications of GnRH analogs in gynecology and oncology.

Clinical use of GnRH analogs in the therapy of prostate cancer consists of two general approaches: first, chronic administration of GnRH analogs (agonists such as Leuprolide, Decapeptyl or Buserelin [17], and antagonists such as cetrorelix and ganirelix [13]), resulting in medical castration. The second approach utilizes the high affinity of GnRH analogs for GnRH receptors, for targeting cytotoxic, anti-tumor agents. In this approach, cytotoxic drugs which are linked to GnRH analogs are transported directly to metastases. An example of this type of therapeutic agent is AN-152 [18], which consists of doxorubicin (DOX) linked through a glutaric acid spacer to the free amino group of [D-Lys⁶]GnRH, and is currently under clinical trials.

GnRH analogs having high affinity to GnRH receptors might thus also serve as carriers to direct diagnostic or therapeutic substances to GnRH receptor-positive tumors. The successful use of ¹¹¹In-DTPA-Octreotide (Octreoscan) [19] in diagnosis of somatostatin receptor-positive tumors has stimulated the search for new target-specific radiopharmaceuticals based on small biomolecules. These findings motivate the search for synthetic, labeled GnRH analogs, for the treatment, diagnosis and monitoring of prostatic, breast, and ovarian cancers. To date, one radiolabeled analog of GnRH, ^{99m}TcO₂-P₂S₂-D-Lys⁶-LHRH, was reported [20], but no biological data were reported for this linear derivative. P₂S₂-D-Lys⁶-LHRH is derived from [D-Lys⁶]GnRH which was labeled on the ε-position of the D-Lys⁶ with ^{99m}Tc in high yields.

The generally used method for metal labeling of a given peptide is the conjugate design approach [21] that consists of attaching a bifunctional chelating agent (BFCA) to the tumor targeting molecule via a linker. A method developed by Giblin et al. for introduction of a radioactive isotope into α-melanotropin analogs [22] and by Melendez-Alafort et al.

for somatostatin analogs [23], is based on a totally different approach: binding the metal isotope through the thiol groups of Cys residues not essential for receptor binding but present in the peptide sequence, to achieve cyclization of the peptide through metal coordination. Giblin et al. also demonstrated that the ¹⁸⁸Re and ^{99m}Tc cyclic peptides showed outstanding stability in competition with phosphate, Cysteine, diethylene triamine pentaacetic acid (DTPA) and in serum [22].

^{99m}Tc is the most commonly used radiometal for diagnostic imaging because of its physical properties (pure γ-emitter, *t*_{1/2} = 6 h, *E*_{max} = 140 keV) and its availability [24]. When carrying out synthetic and characterization work, ⁹⁹Tc is often replaced by its third row congener, rhenium (a mixture of ¹⁸⁵Re and ¹⁸⁷Re), which is cheaper, and easier to obtain and handle.

The backbone cyclization and Cycloscan technologies [25,26] are powerful tools for generating peptide- and peptido-mimetics [27,28] with enhanced stability, selectivity and bioavailability. Ruan et al. [29] have previously described the stabilization of α-helical conformation by metal cyclization through two ω-aminodiacetic acid residues in the *i* and *i*+4 positions. Recently, we have combined the backbone cyclization, Cycloscan and metal cyclization methods to develop a novel method, called backbone metal cyclization (BMC) [30], which enables the fast discovery of backbone cyclic analogs based on the sequence of biologically active linear and/or cyclic peptides and containing a metal ion in the bridge. The application of the BMC method to somatostatin was patented [31]. We have shown the feasibility of this method in the discovery of a highly potent radiolabeled somatostatin analog based on the physiologically selective backbone cyclic somatostatin analog PTR-3173 [27].

This work describes an application of the BMC method to generate novel backbone metal cyclic GnRH analogs bearing a rhenium oxide or a technetium-99m oxide as leads for therapy, radiotherapy, imaging, and monitoring of cancer.

2. Materials and methods

Protected amino acids, 9-fluorenylmethoxycarbonyl-N-hydroxysuccinimide (Fmoc-OSu), bromo-tris-pyrrolidone-phosphonium hexafluorophosphate (PyBrop), and supports for solid phase peptide synthesis (SPPS) were purchased from Nova Biochemicals (Läufelfingen, Switzerland). Trifluoroacetic acid (TFA) and high performance liquid chromatography (HPLC) solvents were purchased from Bio-Lab (Jerusalem, Israel). Glyoxylic acid and 1,6-diamino hexane were purchased from Merck (Darmstadt, Germany), solvents for organic chemistry were purchased from Frutarom (Haifa, Israel), a ⁹⁹Mo/^{99m}Tc generator was purchased from Soreq Nuclear Research Center (Yavne, Israel), trichlorooxobis(triphenylphosphine)rhenium(V) and

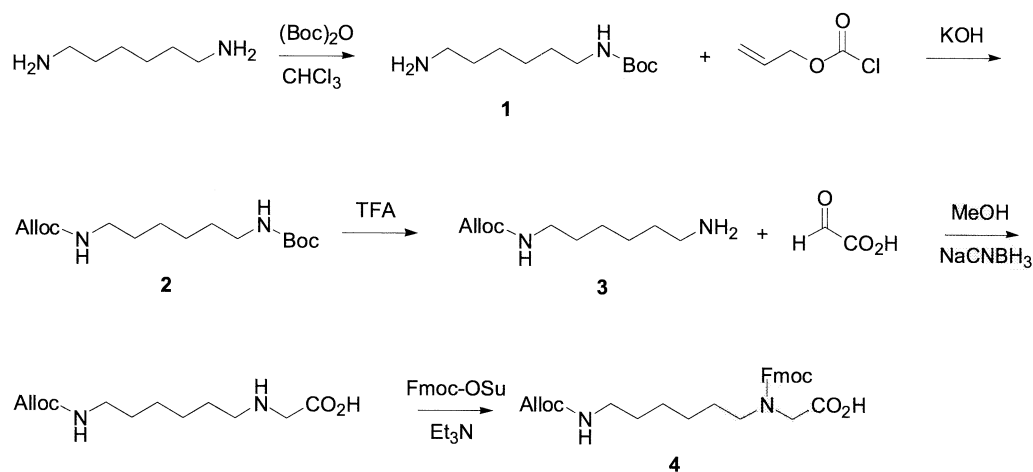


Fig. 1. Synthetic scheme of Glycine derived building unit.

all other chemicals were purchased from Aldrich (Milwaukee, WI, USA), GnRH was acquired from Sigma (St. Louis, MO, USA). Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AMX-300 MHz spectrometer. Mass spectra were performed on a Finnigan LCQ DUO ion trap mass spectrometer. Thin layer chromatography (TLC) was performed on Merck F245 60 silica gel plates (Darmstadt, Germany). Flash chromatography was performed using silica gel 60 (230–400 mesh) (Merck Darmstadt, Germany). HPLC analysis was performed using a Vydac analytical RP column (C18, 4.6×250 mm, catalog number 201TP54), and were carried out on a Merck-Hitachi L-7100 pump and a Merck-Hitachi L-7400 variable wavelength detector operating at 220 nm. The mobile phase consisted of a gradient system, with solvent A corresponding to water with 0.1% TFA and solvent B corresponding to acetonitrile (AcCN) with 0.1% TFA. The mobile phase started with 95% A from 0 to 5 min followed by linear gradient from 5% B to 95% B from 5 to 50 min. The gradient remained at 95% B for an additional 5 min, and then was dropped to 95% A and 5% B from 55 to 65 min. The gradient remained at 95% A for additional 5 min to achieve column equilibration. The flow rate of the mobile phase was 1 mL/min. Peptide purification was performed by reversed phase HPLC (RP-HPLC) (on L-6200A pump, Merck-Hitachi, Japan), using a Vydac preparative RP column (C18, 22×250 mm, catalog number 218TP1022). Preparative HPLC of radiolabeled compounds were carried out on a Varian 9012Q pump, a Varian UV-VIS 9050 variable wavelength detector operating at 215 nm, and a Bioscan Flow-Count radioactivity detector with a NaI crystal, using a Waters μ -Bondapak C18 analytical column. All preparative HPLC were carried out using a gradient system with solvent A corresponding to water with 0.1% TFA and solvent B corresponding to AcCN with 0.1% TFA. The mobile phase began with a linear gradient from 5% B to 18% B from 0 to 10 min followed by gradients from 18% B to 45% B from 10 to 45 min, and a gradient from 45% B to 100% B from 45 to 50 min. The

gradient remained at 100% B for an additional 15 min, and then was dropped to 5% B and 95% A from 65 to 80 min. The gradient remained at 95% B for additional 10 min to achieve column equilibration. The flow rate of the mobile phase was 9 ml/min.

2.1. Organic synthesis—synthesis of glycine-derived building unit was performed according to Fig. 1

2.1.1. Preparation of *t*-butoxycarbonyl-NH(CH₂)₆NH₂ (Boc-NH(CH₂)₆NH₂) (1)

1,6-Diamino hexane (86.49 g, 0.744 mol) was dissolved in Chloroform (1 L) and cooled in an ice bath. To the cooled solution, a mixture of (Boc)₂O (21.86 g, 0.1 mol) in Chloroform (500 mL) were added at 0°C dropwise over 5 h and then stirred overnight at room temperature. The reaction mixture was washed with water (500 mL \times 4), dried over sodium sulfate and evaporated *in vacuo*. The crude product was further purified by flash chromatography on silica gel. The crude product was adsorbed on silica gel (50 g). The silica gel adsorbed product was added onto a column with silica gel (450 g). The column was eluted with a mixture of dichloromethane (DCM):methyl alcohol (MeOH) 4.3:1, and was monitored by TLC (DCM:MeOH 4.3:1). Yield after evaporation: 19.54 g, 90.3% (0.0903 mol). ¹H NMR (300 MHz, CDCl₃): δ 1.31 (s, 2H), 1.34 (m, 4H), 1.44–1.50 (m, 13H), 2.68 (t, *J* = 6.8, 2H), 3.10 (q, *J* = 6.4, 2H).

2.1.2. Synthesis of Boc-NH(CH₂)₆NH-Alloc (allyloxycarbonyl) (2)

The mono-Boc protected diamine (1) (19.54 g, 0.0903 mol) was dissolved in 4 M KOH (60 mL), and cooled in an ice bath. Allyl chloroformate (12.5 mL, 0.118 mol, 1.3 eq) was added dropwise to the solution and a precipitate was formed immediately. 4 M KOH (100 mL) were added and the solution was allowed to stir overnight. Petroleum ether (PE) (60 mL) and water (60 mL) were added to the white suspension. The solid was filtered, washed with PE (60 mL

× 2) and dissolved in DCM (200 mL). The organic solution was washed with 4M KOH (60 mL × 3), and with water (25 mL), dried over Na₂SO₄ and evaporated, yielding 23.57 g (86.7%) of colorless oil. ¹H NMR (300 MHz, CDCl₃): δ 1.31–1.35 (m, 4H), 1.44–1.52 (m, 13H), 3.07–3.20 (m, 4H), 4.55 (d, *J* = 5.3, 2H), 5.23 (dd, 2H), 5.93 (m, 1H).

2.1.3. Synthesis of H₂N(CH₂)₆NH-Alloc (3)

TFA (60 mL) was added to compound (2) (23.57 g, 0.0785 mol) and the mixture was stirred at room temperature for 1.5 h. Three portions of ether (200 mL) were added and evaporated to get rid of excess TFA. The acidic residue was dissolved in DCM (300 mL) and the solution was brought to pH=9 with 4M NaOH (75 mL). The organic layer was separated, dried over Na₂SO₄ and evaporated *in vacuo* yielding 9.1 g (0.0454 mol, 57.9%) of colorless oil. ¹H NMR (300 MHz, CDCl₃): δ 1.33 (m, 4H), 1.50 (m, 4H), 2.77 (t, *J* = 7.0, 2H), 3.16 (q, *J* = 6.5, 2H), 4.55 (d, *J* = 5.0, 2H), 5.2 (d, 1H), 5.28 (d, 1H), 5.92 (m, 1H).

2.1.4. Synthesis of Fmoc-N^α-[ω-NH(Alloc)-hexyl] glycine-OH (Fmoc-GlyN6(Alloc)-OH) (4)

NaCNBH₃ (3.25 g, 0.052 mol) was dissolved in MeOH (40 mL). Compound (3) (9.1 g, 0.0454 mol) was dissolved in MeOH (50 mL) and added to the NaCNBH₃ solution. Glyoxilic acid (3.43g, 0.0463 mol) was added and the reaction was stirred for 18 h. The MeOH was evaporated under reduced pressure, and the residue was dissolved in water (110 mL), and triethyl amine (11 mL, 0.079 mol) was added. Fmoc-OSu (9.82 g, 0.0291 mol) in AcCN (170 mL) was added, and the reaction was stirred for 4 h whereas the pH was kept alkaline with triethyl amine. The reaction mixture was washed with petroleum ether PE (180 mL × 3) and ether:PE 7:3 (180 mL × 3). The aqueous layer was acidified under cooling to pH=3–4 with 2M HCl (~10 mL), and extracted with ethyl acetate (EA) (150 mL × 4). The organic layer was washed with 1M HCl (100 mL × 2) and sat. KHSO₄ (100 mL × 2), dried over Na₂SO₄ and evaporated *in vacuo* to yield: 5.50 g, 0.0115 mol (39.5%) of colorless oil that was later solidified. The product was used for SPPS without further purification. ¹H NMR analysis of the product indicated that at room temperature, the product is a mixture of two isomers (E and Z at the Fmoc-urate bond). ¹H NMR (300 MHz, CDCl₃): δ 1.16–1.52 (m, 8H), 3.18 (m, 3H), 3.36 (t, *J* = 6.9, 1H), 3.94 (s, 1H), 4.02 (s, 1H), 4.27 (m, 1H), 4.47 (d, *J* = 6.0, 1H), 4.57 (m, 3H), 5.28 (dd, 2H), 5.95 (m, 1H), 7.37 (m, 4H), 7.60 (t, *J* = 8.7, 2H), 7.78 (t, *J* = 6.1, 2H).

2.2. Peptide synthesis

2.2.1. Solid phase peptide synthesis of the pre-cyclic GnRH analogs (Fig. 2)

The synthesis was performed in a reaction vessel equipped with a sintered glass bottom, following general Fmoc chemistry protocols: Rink amide methylbenzhydryl-

amine (MBHA) resin (0.4 g, 0.66 mmol/g) was pre-swollen in N-methylpyrrolidone (NMP) for 2 h. Fmoc deprotection step was carried out with 20% piperidine in NMP (2 × 30 min), followed by washing with NMP (5 × 2 min) and DCM (2 × 2 min). Couplings of the building unit to the resin and of Fmoc-amino-acid-OH (Fmoc-AA-OH) to the building unit were carried out according to the procedure published by Falb *et al.* [32]. Fmoc-GlyN6(Alloc)-OH (5 eq., 1.32 mmol) and bis-(trichloromethyl) carbonate (BTC, triphosgene) (1.613 eq., 0.44 mmol) were suspended in DCM. 2,4,6-collidine (8 eq., 2.112 mmol) was added to the pre cooled suspension in an ice bath. After all the solids were dissolved (about 1 min), the solution was poured onto the resin and shaken for 1 h at room temperature. This coupling cycle was repeated once more. At the end of the second coupling cycle, the peptidyl-resin was washed with DCM (5 × 2 min). Capping was carried out after the first amino acid and was repeated twice by reaction of the peptidyl-resin with a mixture of acetic anhydride (1.1 mL, 0.5 M), diisopropyl amine (DIEA) (0.5 mL, 0.125 M) and N-hydroxybenzotriazole (HOBT) (0.05 g, 0.015M) in dimethyl formamide (DMF) (25 mL). Capping was followed with resin wash with DMF (5 × 2 min), DCM (2 × 2 min), and NMP (2 × 2 min). Two cycles of the difficult coupling of Fmoc-Pro-OH to the building unit were carried out at 50°C as follows: Fmoc-Pro-OH (5 eq., 1.32 mmol) and BTC (1.613 eq., 0.44 mmol) were suspended in dibromomethane (DBM) and cooled in an ice bath for 5 min. 2,4,6-collidine (8 eq., 2.112 mmol) was added, and after all the solids were dissolved (~ 1 min), the solution was added to the peptidyl-resin and the vessel was shaken at 50°C for 3 h. Washing steps were carried out with DCM (5 × 2 min). From this point all coupling steps were carried out using PyBrop as the coupling agent. A sample of a coupling cycle: Fmoc-AA-OH (3 eq.) and PyBrop (3 eq.) were dissolved in NMP and DIEA (7 eq.) was added. The reaction mixture was pre-activated for 10 min prior to being added to the reaction vessel, and shaken for 2 h. Coupling was followed by washing the peptidyl-resin with NMP (3 × 2 min) and DCM (2 × 2 min). After coupling of Fmoc-Gly-OH, the resin was dried overnight in a desiccator and removal of the Alloc protecting group from the building unit was performed with tetrakis(triphenylphosphine)Pd(0) (0.75 g per g resin) in DCM containing acetic acid (5%) and N-methyl morpholin (2.5%) under Argon. This step was carried out for 4 h with vigorous shaking in the dark. After Alloc deprotection, Boc-Cys(Trt)-OH was coupled to the ω-nitrogen of the building unit using the PyBrop coupling protocol described above. After Fmoc-deprotection at the N-terminus, Boc-Cys(Trt)-OH was coupled using PyBrop as coupling agent. All Fmoc removal and couplings were monitored by the chloranil test [33,34], and coupling was repeated if necessary. Chloranil test, after the coupling of Fmoc-His(Trt)-OH, was negative, but became blue (positive) after 1 h. A small amount of resin was cleaved (small cleavage procedure). MS analysis indicated that a partial

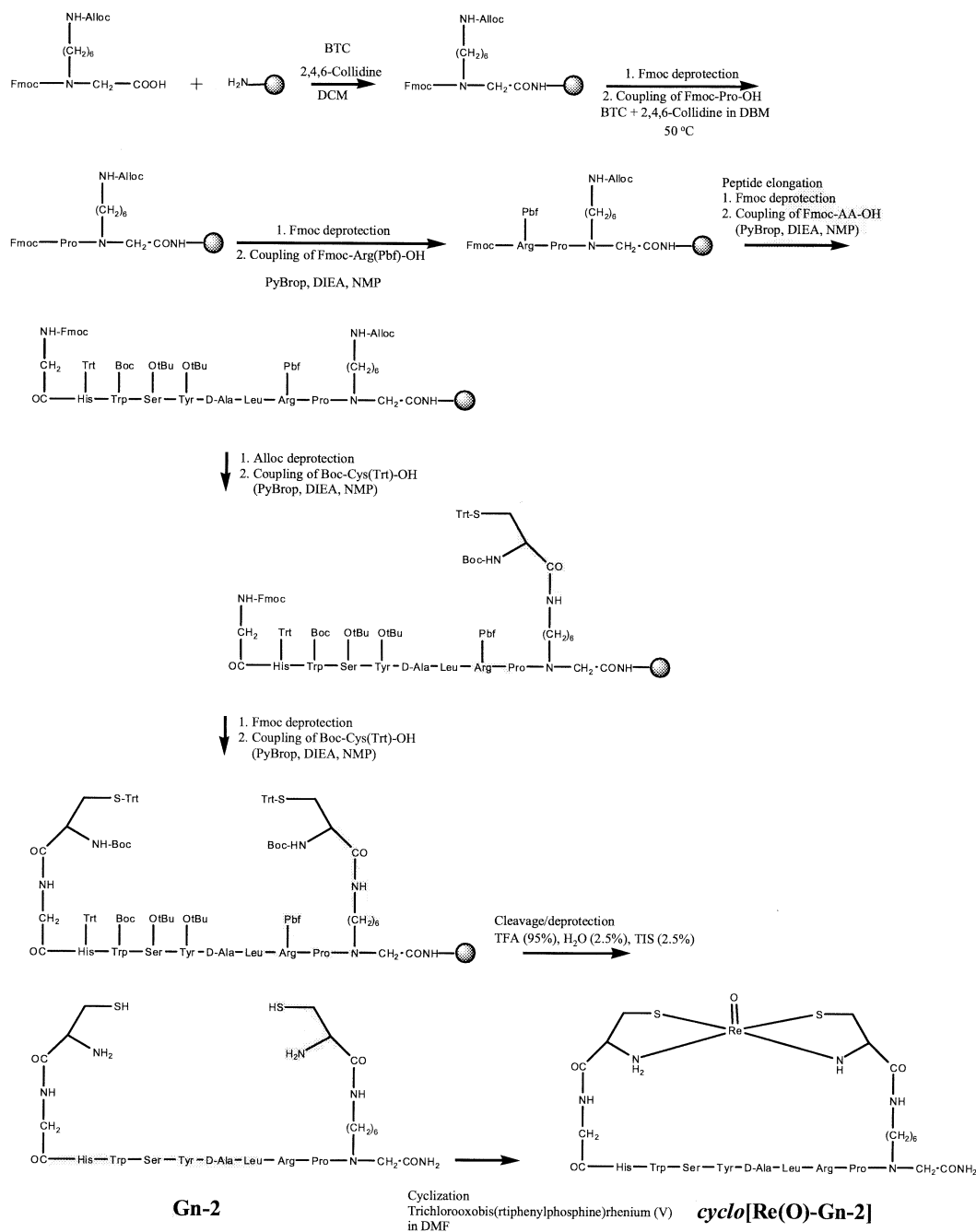


Fig. 2. Synthetic scheme of Gn-2 using SPPS and its cyclization in solution with Re(O).

coupling of Fmoc-His(Trt)-OH was achieved. After coupling of the second Boc-Cys(Trt)-OH, the resin was split to four portions (each of ~0.11 g peptidyl-resin). The first portion (portion A = linear peptide) was cleaved from the resin applying TFA, as will be described later, and biological activity was tested without cyclization (Table 1). The second portion (portion B=S-S bridged cyclic peptide) was washed with DMF (2 × 2 min), and on-resin oxidation was performed with I₂ (10 eq.) [35], in DMF:triple-distilled water (TDW) 4:1 for 70 min. The resin was washed with DMF (2 × 2 min), 2% ascorbic acid in DMF (2 × 2 min),

NMP (5 × 2 min) and DCM (2 × 2 min) and then cleaved from the resin. The third portion (portion C = Re(O) cyclic peptide) and the fourth portion (portion D = ^{99m}Tc(O) cyclic peptide) were cleaved from the resin as the pre cyclic peptide and cyclization was performed in solution as will be described later. Cleavage from the resin and removal of side chain protecting groups were carried out simultaneously using a pre cooled mixture of 95% TFA, 2.5% TDW and 2.5% triisopropylsilane (TIS). After the resin was added, the mixture was cooled for 30 min in an ice bath, and then was shaken for 2.5 h at room temperature. The resin was then

Table 1
Binding affinity values of the library synthesized compared to the affinity of native GnRH

Peptide no.	Peptide name	Structure*	AA	Calc. mass	Obs. Mass	IC 50 (μ M)
1	Gn-2	A	His	1447.73	1448.32	>1000
2	cyclo[S-S-Gn-2]	B	His	1445.72	1446.37	1.00
3	cyclo[Re(O)-Gn-2]	C	His	1648.93	1648.15	0.05
4	des-His-Gn-2	A	none	1310.59	1311.58	1.00
5	cyclo[S-S-des-His-Gn-2]	B	none	1308.58	1309.43	>1000
6	cyclo[Re(O)-des-His-Gn-2]	C	none	1511.79	1511.22	1.00
	GnRH			1182		0.01

* See structures in Fig. 3

removed by filtration washed with a small amount of neat TFA, and the combined TFA filtrates were evaporated under a stream of nitrogen. The oily product was triturated with cold ether and the ether was decanted. The residue was dissolved in AcCN:TDW 1:1 and lyophilized. Analysis of the crude peptides by analytical HPLC and MS showed that in each portion, two peptides were the major products: 1. the desired peptide, and 2. a peptide with deletion of His² (named des-His). The peptides were purified by preparative HPLC, and analyzed by analytical HPLC and by mass spectra (MS).

2.2.2. Small cleavage procedure

A small amount of the peptidyl-resin was treated with a pre cooled mixture of TFA (2 mL); H₂O (1 drop) and TIS (1 drop) for 30 min. The resin was removed by filtration. TFA was evaporated with a stream of nitrogen. The residue was dissolved in an AcCN:TDW 1:1 (v/v) and analyzed by MS.

2.3. A typical procedure for peptide cyclization through Re coordination

Lyophilized peptide (14.3 mg) was dissolved in TDW (2 mL), and a solution of trichlorooxobis(triphenylphosphine)rhenium(V) (1 eq.) in DMF (~2 mL) was added. DMF was further added to the mixture to form a peptide concentration of 1 mg/mL, and shaking was carried out for 2 h. Cyclization was monitored by analytical HPLC. The solvents were evaporated by centrifugal vacuum apparatus (Savant Speed-Vac, Holbrook, NY), and the dried residue was analyzed by analytical HPLC and MS, and purified by preparative HPLC.

2.4. Binding of peptides to the GnRH receptor on pituitary membranes

The procedures for preparation of radioligand and the binding assay have been published [36]. In brief, GnRH was iodinated by the chloramine T method, and the iodinated analog was purified (1,700 μ Ci nmol⁻¹) by analytical HPLC system [37]. Pituitary membranes (0.1 pituitary equivalent/tube, prepared from Wister-derived proestrous

rats) were incubated for 90 min at 4°C with 50,000–80,000 cpm ¹²⁵I-GnRH alone or in the presence of various concentrations of the unlabeled peptides in a total volume of 0.5 mL of assay buffer (10 mM Tris-HCl containing 0.1% BSA). The reaction was terminated by rapid filtration through Whatman GF/C filters. The filters were washed three times with cold assay buffer and counted in a Packard Auto-Gamma Spectrometer. The experiments were performed in triplicates. Non-specific binding was calculated by subtracting the non-specific binding from the maximal binding, determined in the absence of any competing peptide.

2.5. Peptide cyclization and ^{99m}Tc radiolabeling

To a 15 mL corning polypropylene tube equipped with a magnetic stirrer, the following aqueous solution were added: sodium glucoheptanoate (93.9 μ mol, 0.5 mL of a 188 mM solution), ethylenediaminetetraacetic acid (EDTA) (1.35 μ mol, 394 μ L of a 3.43 mM solution), Gn-2 (0.3445 μ mol, 50 μ L of a 6.9 mM solution), saline eluent from a ⁹⁹Mo/^{99m}Tc generator (927 μ Ci sodium [^{99m}Tc] pertechnetate, 0.5 mL) and stannous chloride (1.11 μ mol, 210 μ L of freshly prepared 5.27 mM solution). The tube was capped, mixed and then heated to 100°C in an oil bath with stirring for 10 min. After cooling to room temperature, the reaction mixture was purified by preparative HPLC to obtain the two radiolabeled isomers with 30% radiochemical yield. Chemical and radiochemical purities (97% and 100% respectively) were determined using analytical HPLC C18 column. The retention time of the labeled product was identical to the Re cyclic peptides.

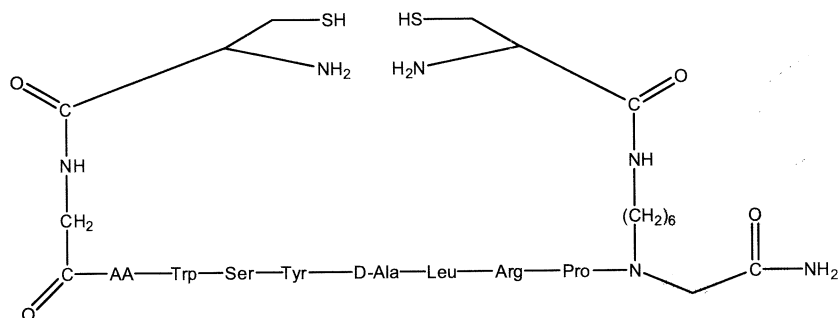
3. Results

3.1. Design

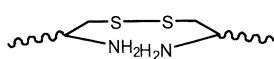
Using the backbone cyclization method [38], it was possible to design a cyclic peptide based on the native GnRH sequence, without modification of any side chain moieties essential for bioactivity. Taking into account structure-activity relationship studies [39] and bioactive analogs of



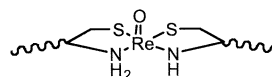
GnRH



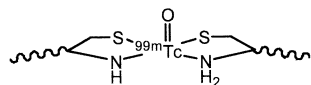
A1 = Gn-2 (AA=His)
A2 = des-His Gn-2 (AA=none)



B1 = *cyclo*[S-S-Gn-2]
B2 = *cyclo*[S-S-des-His-Gn-2]



C1 = *cyclo*[Re(O)-Gn-2]
C2 = *cyclo*[Re(O)-des-His-Gn-2]



D1 = *cyclo*[^{99m}Tc(O)-Gn-2]

Fig. 3. A general structure of the Gn-2 (A1), des-His-Gn-2 (A2) and their cyclic analogs: *cyclo*[S-S-Gn-2] (B1), *cyclo*[Re(O)-Gn-2] (C1), *cyclo*[^{99m}Tc(O)-Gn-2] (D1), *cyclo*[S-S-des-His-Gn-2] (B2) and *cyclo*[Re(O)-des-His-Gn-2] (C2), respectively. Wavy line represents attachment to the Cys C α , of the bridge and AA represents His or none.

GnRH [40,41], the following modifications were introduced into the GnRH sequence, with the purpose of conserving and stabilizing the consensus β -turn at positions 5–8 of the peptide.

Gly⁶ was replaced by a D-amino acid, and cyclization was carried out from the N α of Gly¹⁰ to the amino end of the peptide. It is well established that substitutions in positions 6 and/or 10 can lead to super-active peptides. For example, [D-Trp⁶]GnRH (triptorelin), [D-Leu⁶,Pro⁹-NHET]GnRH (Leuprolide), and [D-Ser(Bu¹)⁶,Pro⁹-NHET]GnRH (Busereilin) are 50–100 times more potent than GnRH [17,42,43]. Substitution of Gly⁶ by D-amino acids provides analogs more resistance to degradation [44] in addition to enhancing the β -II type conformation. Although pGlu¹ has an essential role in GnRH activity [45], and replacement of pGlu¹ with Gly¹ produced an inactive analog [46], we assumed that replacing of pGlu¹ with the dipeptide Cys-Gly¹ will afford an amide bond in the same position of the pGlu amide and

thus will retain activity [46]. Backbone metal cyclization was carried out from Cys attached to η -amino group of the Gly building unit (Fig. 1) in position 10 to a Cys amino acid attached to the amino end of the peptide. This agrees with the observed activity of analogs possessing N-alkylation at Gly¹⁰ [47,48]. There is a supreme importance to the flexible character of the peptide when interacting with its receptor. Since the parent peptide is linear, it was important to preserve some of its flexible nature to promote induced fit [49]. Hence, we assumed that a glycine building unit containing a spacer of six methylenes should maintain this flexibility. In this work, Gly⁶ was replaced by D-Ala to enhance biological activity [50]. In order to achieve backbone metal cyclization, using Re(O) and ^{99m}Tc(O) as the metals for complexation, the metal binding site composed of two Cys amino acids linked through their carboxy groups to the ω -amino groups of the C-terminal Gly building unit and to the N-terminus of the peptide (Fig. 3A). In this manner,

each side of the peptide possessed a hemi-chelating arm composed of a free thiol and a free amine available for coordination with the metal atom. N_2S_2 diaminedithiols (DADT) bind the $(Tc(O))^{3+}$ core strongly to form a stable complex. The ^{99m}Tc -DADT-IgG antibody conjugate was stable, and the presence of 1000-fold excess of DTPA did not cause transchelation of ^{99m}Tc [51]. As demonstrated by Fridkin et al. [30], a stable N_2S_2 complex was predicted here as well.

3.2. Organic synthesis

In order to perform backbone cyclization, a novel procedure for the preparation of Fmoc protected N^α -[ω -N(Alloc)-alkyl] Glycine building unit with an alkyl chain, bearing 6 methylenes, was developed (Fig. 1). Utilizing the procedure described by Gazal et al. [52] for N^α -[ω -S(protected)-alkyl] Glycine building units, a glycine building unit with an Alloc protected amine at the ω position of the alkyl chain was synthesized. This glycine building unit is orthogonally protected and is suitable for solid phase peptide synthesis (SPPS). This route for N^α glycine building units is an improvement of the procedure described by Gellerman et al. [53]. Utilizing this novel procedure with some modifications, we have obtained building units with a variety of lengths of the alkyl chains [54].

3.3. Solid phase peptide synthesis

Gn-2 was synthesized following Fmoc protocols on a solid support. The pre cyclic peptidyl-resin was divided into four portions. Each portion was treated as described earlier, affording four types of analogs: Gn-2, cyclic disulfide bridged *cyclo*[S-S-Gn-2], *cyclo*[Re(O)-Gn-2] and *cyclo*[$^{99m}Tc(O)$ -Gn-2]. The cyclization through a disulfide bond was performed on-resin by oxidizing the two free thiols of the Cys amino acids using I_2 [55]. Cyclization through site-specific rhenium metal coordination was carried out as described by Fridkin et al., [30]. Cyclization through site-specific technetium coordination was achieved by ligand exchange with ^{99m}Tc -glucoheptonate [30].

During the synthesis of Gn-2, we found that partial coupling of His² to Trp³ took place, and a mixture of two peptidyl-resins was obtained in the reaction vessel. Hence, in addition to the expected, Gn-2 and its two cyclic analogs *cyclo*[S-S-Gn-2] and *cyclo*[Re(O)-Gn-2], three more analogs, missing His² were obtained: [Cys-Gly]¹ [des-His]² [D-Ala]⁶ [N^α (η -Cys-amino hexyl)]¹⁰ GnRH (des-His-Gn-2), *cyclo*(1-10) [Cys-Gly]¹ [des-His]² [D-Ala]⁶ [N^α (η -Cys-amino hexyl)]¹⁰ GnRH (*cyclo*[S-S-des-His-Gn-2]) and *cyclo*-(Re(O)1-10) [Cys-Gly]¹ [des-His]² [D-Ala]⁶ [N^α (η -Cys-amino hexyl)]¹⁰ GnRH (*cyclo*[Re(O)-des-His-Gn-2]) (Fig. 3). Modifications at position 2 were found to provide antagonistic activity [42]. [Des-His]² GnRH was the first reported competitive antagonist [56]. These facts motivated us to

evaluate the GnRH receptor binding affinity of the [des-His]² derivatives, as well.

HPLC and MS analysis revealed that the post-cleavage crude peptides obtained; A (linear), B (disulfide), C (Re(O) precyclic), and D ($^{99m}Tc(O)$ precyclic), were actually a mixture of products. Preparative HPLC of portion A generated two pure peptides: 1. Gn-2, the desired analog, and 2. des-His-Gn-2. Preparative HPLC of portion B produced two pure peptides: 1. the desired *cyclo*[S-S-Gn-2], and *cyclo*[S-S-des-His-Gn-2]. The MS results are listed in Table 1. Portion C was cyclized to give the Re(O)-cyclic analogs in two ways: route 1. Each of the purified peptides, Gn-2 and des-His-Gn-2 were cyclized with trichlorooxobis(triphenylphosphine)rhenium(V) and the products were purified by preparative HPLC (Figs. 4B and 4C, respectively). For *cyclo*[Re(O)-Gn-2], two peaks with the same molecular mass were obtained in prep HPLC. This phenomenon, known as anti/syn isomerism around the metal core, was reported previously [57]. Preparative HPLC was not effective for the separation between the two isomers of each of the peptides. Route 2, the crude product, containing a mixture of the two linear peptides (Gn-2 and des-His-Gn-2) was cyclized as described above and the products were purified by preparative HPLC (Fig. 4A) affording *cyclo*[Re(O)-Gn-2], and *cyclo*[Re(O)-des-His-Gn-2]. The separation between the two stereoisomers was not effective, and the mixture was referred to as one molecule.

3.4. Binding assay

A library of 6 analogs based on the Gn-2 prototype was obtained (A1, A2, B1, B2, C1, and C2 in Fig. 3). The library contained two pre-cyclic and 4 cyclic peptides. Of the cyclic peptides, 2 were cyclized through a disulfide bond, and 2 peptides were cyclized through site-specific rhenium metal coordination. The results of GnRH receptor binding affinity assay for the 6 peptides and GnRH are listed in Table 1. Gn-2 and *cyclo*[S-S-des-His-Gn-2] gave no activity whatsoever. IC₅₀ levels of most of the analogs were at the micromolar range. The analog *cyclo*[Re(O)-Gn-2] was the most active analog, with IC₅₀ = 50 nM, almost as active as the native GnRH (IC₅₀ = 10 nM) (Extraction glassware-Soxhlet for ROT-X-TRACT-S, Organomation Associates Inc., MA, USA).

3.5. Synthesis of radioactive analog

Based on the high binding affinity of *cyclo*[Re(O)-Gn-2] we synthesized the ^{99m}Tc equivalent of *cyclo*[Re(O)-Gn-2], as described above *Cyclo*[$^{99m}Tc(O)$ -Gn-2] was analyzed by HPLC and showed two radioactive signals at 27.22 and 27.86 min, their retention times were similar to those of *cyclo*[Re(O)-Gn-2] (Fig. 5). Yield of purification by preparative HPLC was 30% (9.4 μ Ci).

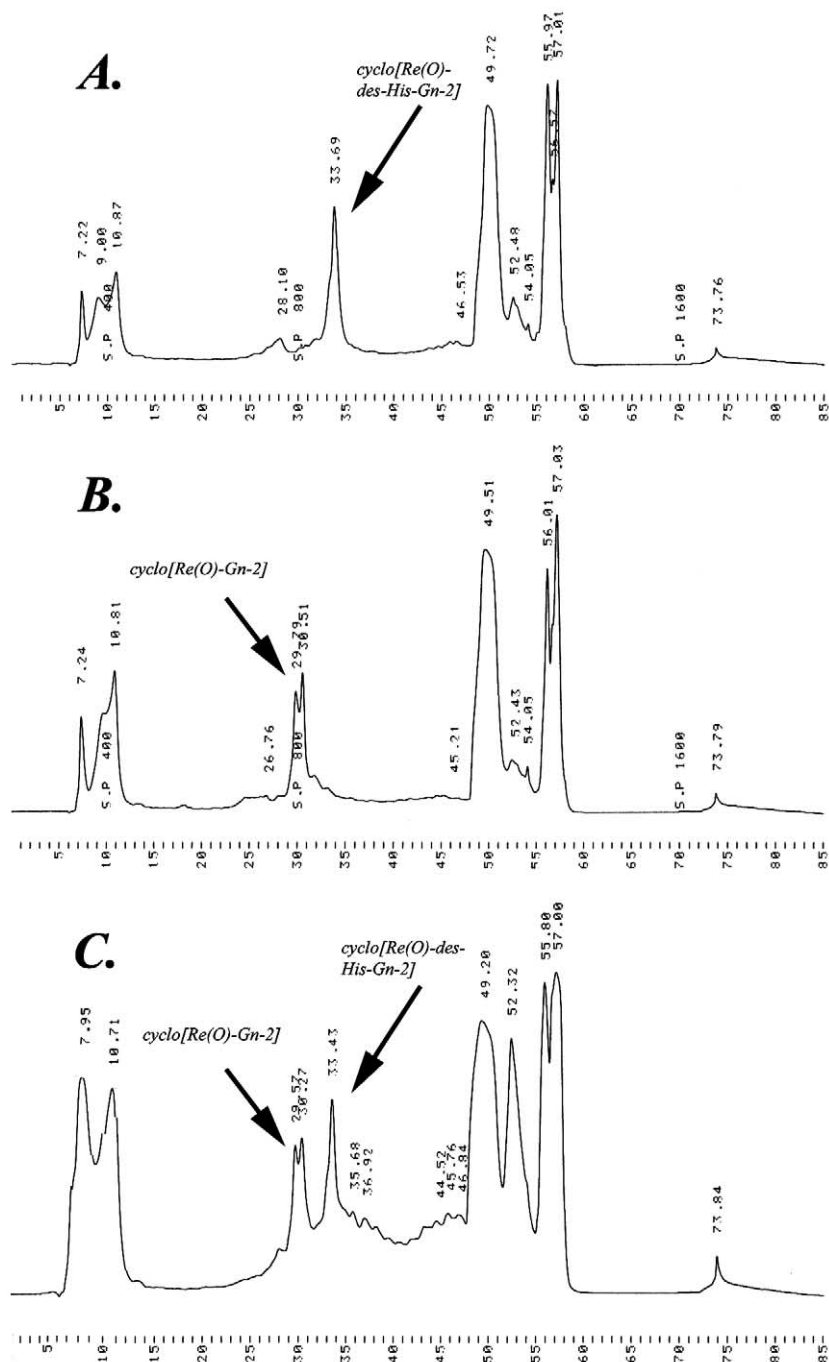


Fig. 4. Preparative HPLC chromatograms of Re cyclization of the crude mixture of peptides (A), compared to the chromatograms of Re cyclization of the purified peptides: Gn-2 (B) and des-His-Gn-2 (C).

4. Discussion

In their review, Liu and Edwards [21] describe two strategies for the design of ^{99m}Tc radiopharmaceuticals: the integrated approach and the conjugate approach. In the integrated approach, applicable for antibodies and proteins, a part of a known high-affinity receptor ligand is replaced by a technetium chelate with minimal changes in size, conformation and receptor binding affinity. The conjugate

design approach is the preferred strategy in the design of receptor-based radiopharmaceuticals. It consists of hybridization of the high binding receptor ligand as the targeting molecule, a BFCA for the chelation of the radionuclide, and a linker for connecting the BFCA to the targeting fragment.

We present an additional, novel approach for Re and Tc metal coordination. This method combines the recently introduced method for metal coordination, by Giblin *et al.* [22], with backbone cyclization, creating a novel method

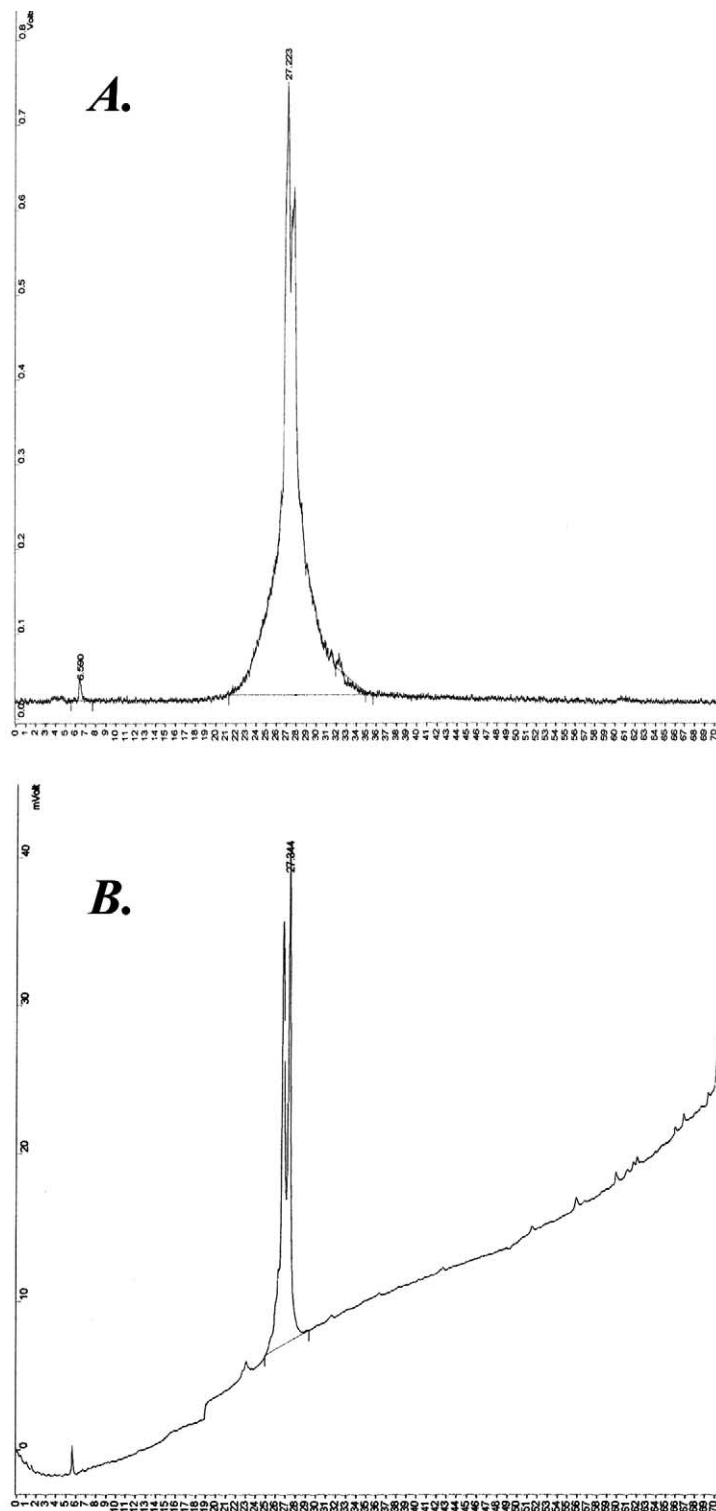


Fig. 5. A typical preparative HPLC chromatogram of *cyclo*[Re(O)-Gn-2] (A), and a radiochromatogram of the *cyclo*[^{99m}Tc(O)-Gn-2] (B).

called BMC. In this method, the metal ion is an integral part of the peptide, and the complexation is crucial for gaining its three-dimensional structure. In the traditional approach, the analog is composed of three parts: the bioactive component, a linker, and a chelating group. According to the BMC method, the peptide backbone is equipped with two

chelating arms complexing the metal ion core, to form the bioactive compound. The linker and the chelating group used in the conjugated approach are discarded in the BMC method. Thus, avoiding their steric, electronic and conformational effect, that may obstruct the bioactivity. This offers several advantages: first, in the complexation step, in

addition to the act of labeling the peptide, a conformational constraint is achieved through cyclization. Backbone cyclization is known for its quality in improving peptide selectivity, stability, and bioavailability [27,58–61] and thus, the BMC method is also expected to improve the biological performance of the backbone cyclic labeled peptide, compared to its linear parent analogs. Second, by applying Cycloscan [25], each of the chelating arms can vary in its length, chemistry, stereochemistry, and position on the peptide backbone. This method is called metal cycloscan [30], providing a library with a large number of elements of diversity, that allows efficient screening of a large number of constraint conformers, based on the same amino acid sequence. Third, since the screening is performed on the Re(O) complex, once a bioactive analog is detected, no further structural modifications are needed for radiolabeling. Thus, different therapeutic and diagnostic applications can be achieved by merely altering the metal ion core as desired.

A novel route for the synthesis of Gly building units is described herein. This route is general and allows the synthesis of Fmoc-N^α-[ω-NH(Alloc)-alkyl]glycine-OH with various alkyl chain lengths. The method relies on *in situ* carboxy methylation of mono Alloc alkyl diamine followed by Fmocilation previously applied for N^α-[ω-S(protected)-alkyl] Glycine building units [52]. Building units having ethyl and propyl chains were previously prepared by a different method by Gellerman *et al.* [53].

The labeling step, either with rhenium or with technetium-99m, is straightforward, and preparative HPLC purification provides the pure backbone metal cyclic peptides. The precyclic peptides are very stable, and can be prepared and kept in a dry atmosphere for long period of time before performing the cyclization, thus, allowing the peptide labeling before patient injection. The oxorhenium cyclic peptides are also stable, and can be kept in a dry atmosphere for long periods of time.

The pre-cyclic peptide, Gn-2, was found to have a low binding affinity, whereas both of its cyclic analogs, *cyclo*[S-S-Gn-2] and *cyclo*[Re(O)-Gn-2] were found to be active, where the later was capable of binding more avidly to rat pituitary receptors (Table 1). The peptides, however, did not enhance the release of LH from rat pituitary cells. The possibility that they are antagonists will be evaluated *in vitro* and *in vivo* assays. Gn-2 showed no binding activity, probably because the replacement of pGlu¹ with Cys-Gly did not produce the cis amide bond, typical for the pGlu ring, which was essential for the activity [45]. *Cyclo*[S-S-Gn-2] restored some of the binding affinity. This can be explained either by the possible formation of a cis amide bond of Cys-Gly via cyclization, or by the stabilization of the bioactive conformation. Cyclization through the Re(O) core to produce *cyclo*[Re(O)-Gn-2] largely increased and almost regained the binding affinity of GnRH itself, indicating that the steric arrangement and the chemical properties of the metal ion bridge plays a dramatic role in adopting

the exact conformation needed for efficient binding to the GnRH receptor.

5. Conclusion

An application of the recently reported technology of backbone metal cyclization to generate GnRH radiolabeled analogs is presented herein. This method achieves three goals: 1. Increase of the diversity of cyclic peptide libraries through the ring chemistry and thus increasing the probability of finding candidates as drugs. 2. Simple synthesis of a metal cyclized GnRH analog possessing high binding affinity to the GnRH receptor that might be suitable for cancer therapy. 3. Simple synthesis of radiolabeled GnRH analogs, suitable for the diagnosis of tumors and for monitoring the course of recovery of cancer patients. Using the method of backbone metal cyclization we have designed and synthesized a novel backbone cyclic analog of GnRH with high binding affinity, containing rhenium oxide in the bridge. The precyclic peptide was labeled with 99m-technetium to obtain a radio labeled analog. We have further demonstrated the feasibility of the backbone metal cyclization method by its application to GnRH. Backbone metal cyclization affords a simple route for gaining radio labeled peptide analogs without use of a bulky chelating connected via a linker. The pharmacokinetic and pharmacodynamic properties of this novel backbone metal cyclic GnRH analog is currently under further examination. New analogs based on Gn-2 have been synthesized and their binding affinity is being investigated. The successful application of BMC method to somatostatin and GnRH demonstrated the feasibility of this method in generating an *in-vitro* active radiolabeled cyclic peptide based on a sequence of a cyclic or a linear parent peptide. This method is being currently applied to other peptides with diagnostic value and the peptide described herein is currently undergoing pharmacological evaluation in cells and animals.

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