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Peptide-Chelating Agent Conjugate for Selective Targeting of Somatostatin Receptor Type 1: Synthesis and Characterization*

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Abstract: *Previously reported results suggest that the analogue of the somatostatin des-AA^{1,2,5}[D-Trp^{8},IAmp^{9}]-somatostatin (CH-275) peptide bearing chelating agents able to coordinate radioactive metals could be used for scintigraphic imaging of tumor lesions overexpressing sstr1. An efficient synthetic procedure for the preparation of the somatostatin analogue CH-275 and its conjugate DTPAGlu–Gly–CH-275, bearing the chelating agent DTPAGlu (DTPAGlu = N,N-bis[2-[bis(carboxy-ethyl)amino]ethyl]-L-glutamic acid) on the N-terminus, by solid-phase peptide synthesis and 9-flourenymethyoxycarbonyl (Fmoc) chemistry, is here reported. Rapid and efficient labeling of DTPAGlu–Gly–CH-275 was achieved by addition of ^{111}In(III) to the compound. Typical yields*

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*This manuscript is dedicated to the memory of Murray Goodman. His scientific insights and his friendly and warm personality contributed to expand the research and studies in peptides among generations of scientists from the 1960s onward.

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were greater than 97% as determined by reversed phase high performance liquid chromatography (HPLC) at specific activities in the range 4–9 GBq/μmol (100–250 Ci/mmol). A preliminary biological assay of the binding ability of ^{111}In–DTPAGlu–Gly–CH-275 indicates, however, that the labeled compound does not display any specific interaction with somatostatin sstr1 receptors in the tested cell lines. To confirm this unexpected negative result, competition binding experiments were carried out, in which fixed tracer amounts of the ^{125}I-labeled somatostatin-14 were incubated with the receptor-expressing cells in the presence of DTPAGlu–Gly–CH-275 or CH-275 at concentrations ranging from 10^{-10} to 10^{-3} M. While CH-275 shows a IC_{50} of 80 nM similar to that already found in displacement experiments on CHO–K1 sstr1-transfected cells, DTPAGlu–Gly–CH-275 displays instead very low or negligible affinity towards this receptor. The NMR solution characterization indicates that the presence of DTPAGlu does not influence the conformational and chemical features of the peptide moiety, thus suggesting that the loss in binding activity should be due to steric hindrance of either the chelating agent DTPAGlu or its indium complex. © 2004 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 76: 527–534, 2004

Keywords: *somatostatin analogue; indium complexes; nuclear medicine techniques; NMR*

INTRODUCTION

Somatostatin, a cyclic tetradecapeptide hormone secreted by the hypothalamus, plays important physiological roles: it is a potent inhibitor of the release of several hormones (i.e., growth hormone, insulin, glucagons, gastrin) and it regulates many others biological activities.[1–3] It is also highly active in acromegalic patients, lowering the plasma level of growth hormone, and therefore it is of potential therapeutic value in clinical treatment of agromegaly and gastroenteropancreatic tumors.[4]

Somatostatin mediates its biological activities through G-protein-coupled receptors, which possess seven transmembrane-spanning helical domains. To date, five human somatostatin receptor subtypes (hsst1–5) have been cloned and characterized, but the physiological role of each receptor subtype has not been totally elucidated, although all of them are overexpressed by many cancer cells.[5] The wide range of physiological roles of somatostatin and its very short half-life in plasma have led to substantial efforts to synthesize analogues that exhibit potent and selective biological activity with longer duration of action. The octapeptide Octreotide[6] is the most widely known somatostatin analogue, being particularly efficient in the treatment of neuroendocrine tumors.[7] Moreover, Octreotide derivatives bearing a chelating agent on the N-terminal residue and able to coordinate with high stability radioactive metals [such as ^{111}In(III)] have been developed for applications in cancer diagnosis and therapy by nuclear medicine techniques.[8] In fact, scintigraphy using somatostatin analogues is a unique tool for diagnosis of neuroendocrine tumors, and radionuclide therapy offers promising possibilities to limit tumor growth.[9] Compared to natural somatostatin, Octreotide contains a D-tryptophan residue that enhances its potency and stability. However, Octreotide does not show a high affinity for all five human sstr subtypes, since it prefers sstr2 and sstr5, and to a lesser extent, sstr3, while it displays no affinity for sstr1 and sstr4.[10] Therefore, Octreotide is ineffective for the treatment of tumors expressing only sstr1 or sstr4, and the radiometal-labeled Octreotide does not help in the diagnosis and therapy by nuclear medicine of sstr1- or sstr4-positive tumors.

Recently, a new family of 11-residue cyclic peptides[11] has been developed for selective binding of sstr1. These peptides are characterized by the presence of the noncoded amino acid N-*iso*-propylaminomethylphenylalanine (IAmp) in the β-turn region that is considered responsible for receptor binding.[12] The lead compound in this family is des-AA^{1,2,5}[D-Trp^8,IAmp^9]-somatostatin, known as CH-275[13]; it displays very high affinity and selectivity for sstr1. From these observations, it appear reasonable to believe that CH-275 peptide derivatives, bearing chelating agents able to coordinate radioactive metals, could be useful tools for scintigraphic imaging of tumor lesions overexpressing sstr1.

In this article we describe the synthesis, the ^{111}In(III) radiolabeling, and the biological behavior of a CH-275 conjugate. This compound (Figure 1) con-

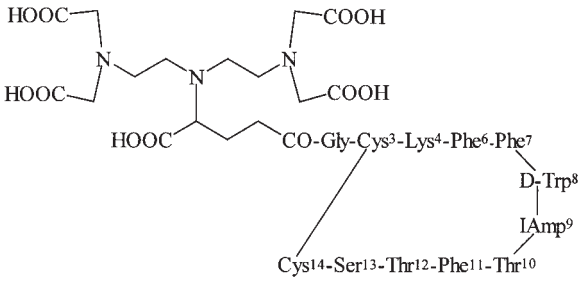

FIGURE 1 Schematic representation and amino acid numbering scheme for DTPAGlu–Gly–CH-275. CH-275, a somatostatin analogue, is des-AA^{1,2,5}[D-Trp^8,IAmp^9]-somatostatin.

tains the recently described DTPA derivative, the chelating agent DTPAGlu[14,15] bound to the N-terminus of the peptide during solid-phase peptide synthesis, and a glycine residue introduced as spacer between the chelating agent and the biologically active CH-275 peptide. The NMR characterization of DTPAGlu–Gly–CH-275 is also reported to determine structure–activity relationships on the binding mode to the sstr1 receptor subtypes of this class of somatostatin analogues.

EXPERIMENTAL

Materials and Methods

Benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium (PyBop), HOBt, all N-Fmoc-amino acid derivatives, and Rink amide 4-methylbenzhydrylamine (MBHA) resin (HOBt: 1-hydroyxbenzotriazole; Fmoc: 9-flourenylmethyoxycarbonyl) were purchased from Calbiochem-Novabiochem. Boc–Amp–OH was obtained from ChemImpex. The DTPAGlu pentaester, the chelating agent fully protected by the *tert*-butyl (tBu) groups on its carboxyl functions with the exception of the carboxyl function on the side chain of glutamic acid, *N,N*-bis[2-[bis[2-(1,1-dimethyletoxy)-2-oxoethyl]-amino]ethyl]-L-glutamic acid 1-(1,1-dimethylethyl)ester, was provided by Bracco Imaging SpA and used without further purification. Its synthesis is given in the literature.[14] Indium-111 was purchased from Nycomed Amersham Sorin Srl in the chemical form of [^{111}In]Cl_3 (740 MBq/mL). ^{125}I-Somatostatin-14 was obtained by Amersham Biosciences. All other chemicals were commercially available by Sigma-Aldrich and were used as received unless otherwise stated. Analytical reversed phase high performance liquid chromatography (RP-HPLC) runs of Fmoc–IAmp(Z)–OH were carried out on a HPLC Shimadzu, model 10A-LC, using a Phenomenex C18 column, 4.6 * 250 mm, eluted with a H_2O/0.1% triflouroacetic acid (TFA) (A) and CH_3CN/0.1%TFA (B) linear gradient from 20 to 80% over 40 min at a 1 mL/min flow rate. Preparative RP-HPLC runs were carried out on a Shimadzu apparatus using a Phenomenex 22 × 250 mm, eluted with a H_2O/0.1% TFA (A) and CH_3CN/0.1% TFA (B) linear gradient from 20 to 80% over 40 min at a 20 mL/ min flow rate.

Solid-phase peptide synthesis was performed on a fully automated peptide synthesizer Shimadzu SPPS-8. Analytical RP-HPLC runs of DTPAGlu–Gly–CH-275 and of CH-275 peptides were carried out eluting with H_2O/0.1% TFA (A) and CH_3CN/0.1% TFA (B) linear gradients from 5 to 70% over 30 min at a 1 mL/min flow rate. Preparative RP-HPLC runs were carried out eluting with H_2O/0.1% TFA (A) and CH_3CN/0.1% TFA (B) linear gradients from 5 to 70% over 30 min at a 20 mL/ min flow rate. Mass spectra were recorded on a matrix-assisted laser desorption ionization–time of flight (MALDI–TOF) Vojager-DE (Perseptive Biosystem). NMR experiments were carried out on a Varian Unity 400-MHz spectrometer of the Centro Interuniversitario di Ricerca sui Peptidi Bioattivi (CIRPeB) at the University of Naples "Federico II."

Protected Amino Acid Synthesis

Boc–IAmp–OH. The amount of 1.000 g Boc–Amp–OH (3.4 mmol) was dissolved in 200 mL of acetone at room temperature. The solution was acidified at pH 4 by adding dropwise HCl 0.012 *M*. The atmosphere was purged with N_2 for 10 min, then 0.030 g (0.79 mmol) of $NaBH_4$ and 0.020 g Pd/C 10% was added. The mixture was stirred overnight, filtered, and evaporated in vacuo. The amount of 0.900 g of the crude product was obtained as yellow oil (2.67 mmol, 79% yield). The purity was tested by analytical HPLC and ^{1}H-NMR. HPLC-retention time (Rt) = 12.9 min, purity >98%; ^{1}H-NMR(D_2O).δ: 7.38 (2H, d, ArH), 7.31 (2H, d, ArH), 4.67 (1H, m, αCH), 4.15 (2H, m, CH_2NH), 3.45 [1H, m, C*H*(CH_3)$_2$], 3.13 (1H, dd C*H*HAr), 2.85 (1H, dd CH*H*Ar), 1.34 [6H, d CH(C*H*$_3$)$_2$], 1.32 [9H, s,C(CH_3)$_3$].

Boc–IAmp(Z)–OH. The amount of 0.900 g of Boc–IAmp–OH (2.67 mmol) were dissolved in 120 mL of a tetrahydrofuran (THF)/H_2O 10/1 mixture. The pH was adjusted with 5.0 mL of NaOH 0.5 *M*. A solution of Z–OSu (703 mg, 1 eq.) in 30 mL of THF was added dropwise at 0°C. After stirring for 2 h at room temperature, the product was extracted with three portions of ethyl acetate. The organic phase was dried under sodium sulfate, filtered, and evaporated in vacuo. The purity of the crude product (0.800 g, 1.69 mmol, yield 63%) was tested by analytical HPLC and ^{1}H-NMR. HPLC-Rt = 26.0 min, purity ≥ 98%; ^{1}H-NMR($CDCl_3$). δ: 7.15 (4H, m, Phe–ArH), 7.36 (5H, br, Cbz–ArH), 5.32 (2H, s, CH_2–Cbz), 4.55 (1H, m, αCH), 4.39 (2H, m, CH_2NH), 4.25 [1H, m, C*H*(CH_3)$_2$], 3.1 (2H, m CH_2Ar) 1.10 [6H, d CH(C*H*$_3$)$_2$], 1.41 [9H, s,C(CH_3)$_3$].

H–IAmp(Z)–OH. A solution of Boc–IAmp(Z)–OH (1.69 mmol) dissolved in 50 mL of TFA/$H_2$0 80/20 was stirred for 6 h at room temperature. The solvent was removed in vacuo and the pale yellow crude oil was obtained in good yield (1.58 mmol, yield 93%). The analytical HPLC chromatogram of the crude product showed two peaks identified as H–IAmp(Z)–OH and H–IAmp–OH by ^{1}H-NMR spectroscopy. Purification of the crude mixture by preparative chromatography RP-HPLC afforded 0.250 g of H–IAmp(Z)–OH (yield 40%). HPLC-Rt = 19.1 min, purity < 98%; ^{1}H-NMR(CD_3OD). δ 7.20–7.40 (9H, br, ArH), 5.16 (2H, d, CH_2–Cbz), 4.45 (2H, s, CH_2NH), 4.22 (1H, m, αCH), 4.00 [1H, m, C*H*(CH_3)$_2$], 3.30 (1H, s, C*H*HAr), 3.05 (1H, m CH*H*Ar), 1.13 [6H, d CH(C*H*$_3$)$_2$].

Fmoc–IAmp(Z)–OH.

H–IAmp(Z)–OH (1.58 mmol) was dissolved in 40 mL of a 10% solution of Na_2CO_3. Thirty milliliters of dioxane were added and the solution was cooled at 0°C. Then, Fmoc–OSu

(1 eq.) dissolved in dioxane (10 mL) was added dropwise. The mixture was stirred for 4 h at 0°C and overnight at room temperature. The solution was concentrated and acidified to pH 1 with 12 *M* HCl at 0°C, to achieve the final compound as a solid. The crude product was purified by RP-HPLC. The amount of 0.573 g (yield 61%) was obtained and characterized by analytical HPLC and ^{1}H-NMR. HPLC-Rt = 28.0 min, purity > 99%; ^{1}H-NMR ($CDCl_3$). δ: 7.75 (2H, d, Fmoc), 7.53 (2H, br, Fmoc), 7.4–7.2 (4H, m, 2HFmoc, 2HAr), 7.30 (4H, m, 2HFmoc, 2HAr), 7.1 (5H, br, Cbz), 4.19–5.27 [8H, m, αCH, CH_2NH, CH_2Fmoc, CH_2–Cbz, C*H*(CH_3)$_2$], 3.15 (1H, s, C*H*HAr), 3.10 (1H, m CH*H*Ar), 1.09 [6H, d CH(C*H*$_3$)$_2$].

Peptide Synthesis

CH-275. Synthesis of des-AA^{1,2,5}-[D-Trp^8, IAmp^9]-somatostatin, CH-275, was carried out in the solid phase under standard conditions using an Fmoc strategy. Cys(Trt)–Wang preloaded resin (0.57 mmol/g, 0.057 mmol scale, 0.200 g) was used. Double couplings were performed with addition of 4 equivalents of protected amino acids activated by PyBop and HOBt and 6 equivalents of DIEA in DMF. Stirring time was 60 min. When the sequence Fmoc–Cys(trt)–Lys(Boc)–Phe–Phe–D-Trp(Boc)–IAmp(Z)–Thr(tBu)–Phe–Thr(tBu)–Ser(tBu)–Cys(trt)–resin was completed the Fmoc N-terminal protecting group was removed by piperidine/dimethylformamide (DMF) 20/80 and the peptide–resin was divided in two portions.

The first portion was cleaved with TFA containing tri-isopropylsylane (2.0%), ethandithiole (2.5%), and water (1.5%) over a period of 24 h, and the peptide products were precipitated at 0°C by adding dropwise ethyl ether. In these conditions, the Z-protecting group was partially lost. Purification of the crude mixture was carried out by RP-HPLC, affording 60 mg of linear CH-275 (yield 70%). CH-275 (linear form) HPLC-Rt = 25.6 min. MALDI–TOF MW = 1488. The disulfide bridge between Cys^3 and Cys^{14} was obtained at room temperature by 20 h spontaneous oxidation in water solution containing 0.1 *M* NH_4HCO_3 (pH = 8.0) (1 mg of peptide per 1 mL of solution). The reaction was monitored by Ellman's test and the final product analyzed by analytical HPLC and MALDI-TOF mass spectroscopy. CH-275 (cyclic form) HPLC-Rt = 25.1 min. MALDI–TOF MW = 1486.

DTPAGlu–Gly–CH-275.

The second portion of the resin containing CH-275 was transferred in a vessel for the manual coupling of Fmoc–Gly–OH and DTPAGlu pentaester. Fmoc–Gly–OH was coupled to the N-terminal moiety of CH-275 in the same way as the previous amino acid residues.

DTPAGlu pentaester was coupled to the Gly α-amino group using a 2.0 equivalent amount of DTPAGlu pentaester, 2.0 equivalents of O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium (HATU), and 4.0 equivalents of *N,N*-diisopropylethylamine (DIPEA) in a single coupling. The reaction was carried out by stirring the mixtures in DMF for 3 h. For deprotection and cleavage, the fully protected peptide resins were treated, over a period of 24 h, with TFA containing tri-isopropylsylane (2.0%), ethandithiole (2.5%), and water (1.5%). The peptide products were precipitated at 0°C by adding ethyl ether dropwise. In these conditions, the Z-protecting group was partially lost. Purification of the crude mixture was carried out by RP-HPLC affording 50 mg of linear DTPAGlu–Gly–CH-275 (yield 44%). DTPAGlu–Gly–CH-275 (linear form) HPLC-Rt = 25.4 min. MALDI–TOF MW = 1993. The disulfide bridge between Cys^3 and Cys^{14} was obtained at room temperature by 20-h spontaneous oxidation in water solution containing 0.1 *M* NH_4HCO_3 (pH = 8.0) (1 mg of peptide per 1 mL of solution). The reaction was monitored by Ellman's test and the final product analyzed by analytical HPLC and MALDI–TOF mass spectroscopy. DTPAGlu–Gly–CH-275 (cyclic form) HPLC-Rt = 24.9 min. MALDI–TOF MW = 1991.

Radiolabeling

The DTPAGlu–Gly–CH-275 peptide was dissolved in CH_3CN/ 20 % H_2O at concentrations ranging between 0.1 and 1 m*M*. Prior to labeling, [^{111}In]Cl_3 was buffered by adding 10 volumes of 0.3 *M* sodium citrate. One volume of the peptide solution was then mixed with 10 volumes of [^{111}In]citrate and incubated for at least 1 h at room temperature. Labeling efficiency was determined by Silica Gel-TLC using 0.3 *M* Na-citrate as eluent, or RP-HPLC, which was performed on a Gilson 305 system with an analytical Vydac C_{18} column connected to a Beckman 170 radiodetector, using a H_2O/0.1% TFA and CH_3CN/0.1% TFA gradient slightly different from the one described in the previous paragraph (5– 70 % CH_3CN/0.1% TFA over 31 min). The retention time was 22.0 min.

In Vitro Cellular Assays: Saturation Binding Experiments

A plasmid containing the cDNA for the human sstr type 1 was kindly provided by Dr. Graeme Bell, University of Chicago. A431 cells were stably transfected to overexpress this receptor subtype. All in vitro cellular assays were performed on cells that had been plated at a density of 1–200,000 cells/well in 12-well multiwell plates two or three days prior to the experiments. These conditions allowed for the cells to be almost confluent at the time of the assay. Experiments aimed at assessing specificity of interaction were performed by comparing cell-associated activity after incubation of the radiolabeled peptide with A431-sstr1 and A431-control cells and with A431-sstr1 cells in the presence of excess unlabeled peptide. Triplicate wells were incubated with known amounts of radiolabeled peptide for different times at 37°C. After incubation, cells were washed with phosphate-buffered saline (PBS), pH 7.4, and cells recovered by trypsinization. Cell-associated and total radioactivity was determined with a Wallac Wizard gamma

counter. The amount of bound compound was normalized for protein content that was determined by utilizing a commercially available kit (Bio-Rad Laboratories) after solubilizing cells in 1 N NaOH.

In Vitro Cellular Assays: Competition Binding Experiments

Fixed tracer amounts of the ^{125}I-labeled somatostatin-14 were incubated with the receptor-expressing cells in the presence of DTPAGlu–Gly–CH-275 or CH-275 at concentrations ranging from 10^{-10} to 10^{-3} *M*. The amount of bound radioactivity was determined after 1 h at 4°C. Nonlinear regression analysis using a model for homologous competition binding was performed using GraphPad Prism (version 3.0a for Macintosh, GraphPad Software, San Diego California USA, www.graphpad.com.) to derive the 50% inhibitory concentration (IC_{50}) for DTPAGlu–Gly–CH-275 and CH-275.

^{1}H-NMR Spectroscopy

The solutions for NMR measurements were prepared by dissolving 2.0 mg of DTPAGlu–Gly–CH-275 in 0.70 mL in CD_3CN/H_2O in the ratio of 1:1. The spin system identification and assignment of individual resonances of DTPAGlu–Gly–CH-275 solutions were carried out by using a combination of total correlated spectroscopy (TOCSY)[16] and double quantum filtered–correlated spectroscopy (DQF–COSY) spectra.[17] Sequence-specific assignment was obtained by nuclear Overhauser effect spectroscopy (NOESY)[18] and rotating frame NOESY (ROESY) experiments,[19,20] according to the standard procedures.[21]

RESULTS AND DISCUSSION

Peptide Synthesis

The synthesis of CH-275 has been already reported by Rivier and coworkers[13] using solid-phase peptide synthesis and *t*-butoxycarbonyl (Boc) chemistry. In order to synthesize the CH-275 derivative containing the chelating agent, we have changed the synthetic strategy, setting up the best condition for solid-phase peptide synthesis with Fmoc chemistry. For this purpose, the non-natural amino acid derivative Fmoc–IAmp(Z)–OH has been obtained according to scheme reported in Figure 2 and it was fully characterized. The secondary amine group on the IAmp side chain is protected with the benzyl group (Z). This group is partially lost when TFA treatment is performed to remove the Boc-protecting group from the primary α-amine function. Therefore, the product mixture has been separated by preparative HPLC in order to isolate the H–IAmp(Z)–OH-protected derivative. This derivative is successively protected on the primary α-amino group with Fmoc, and then used in solid-phase peptide synthesis. Solid-phase peptide synthesis was performed on a fully automated peptide synthesizer. When the peptide synthesis was complete, half the amount of the resin containing the peptide was treated for deprotection and cleavage to obtain CH-275; the remaining part was used for peptide derivatization with the glycine spacer and the DTPAGlu chelating agent. The coupling step of the DTPAGlu pentaester was very efficient, while the subsequent deprotection reaction required prolonged time to ensure complete removal of all the *tert*-butyl-protecting groups. The prolonged treatment in TFA during the deprotection and cleavage steps allows the almost complete removal of the protecting group (Z) from the secondary amine of the IAmp residue. At the end of the synthesis, CH-275 and DTPAGlu–Gly–CH-275 were obtained as linear compounds in high yields, and after purification, spontaneous Cys–Cys oxidation in basic buffer gave the desired cyclic compounds at high purity.

The DTPAGlu chelating agent, chosen to derivatize the CH-275 peptide, presents some advantages among the chelating agents in use for radiometal coordination in nuclear medicine applications. First, the use of the chelating agent DTPAGlu allows us to obtain a peptide conjugate having five carboxyl functions and three amino groups available for radiometal coordination. The total of eight chelating functions constitute a coordinative set able to give stable metal complexes, in particular with metal ions in the +3 oxidation state. There is one additional chelating function compared to the coordinative set displayed by DTPA directly bound to a peptide. The eight functions displayed in the coordination set by our peptide conjugate are in equal number to those utilized by DTPA alone, which yields very stable complexes. The additional coordinating moiety may provide better stability for chelating other radiometals of interest, such as, for example, ^{90}Y, which is used in therapeutic applications and is known to form rather unstable complexes with conventional DTPA-coupled peptides. Second, the entire peptide conjugate is synthesized in the solid phase, which allows us to obtain high yields of the compound and high purity of the raw product. The final HPLC purification step yields a well-characterized compound that corresponds to the desired molecule.

Radiolabeling and Biological Assays

Rapid and efficient labeling of DTPAGlu–Gly–CH-275 was obtained as early as 30 min after addition of

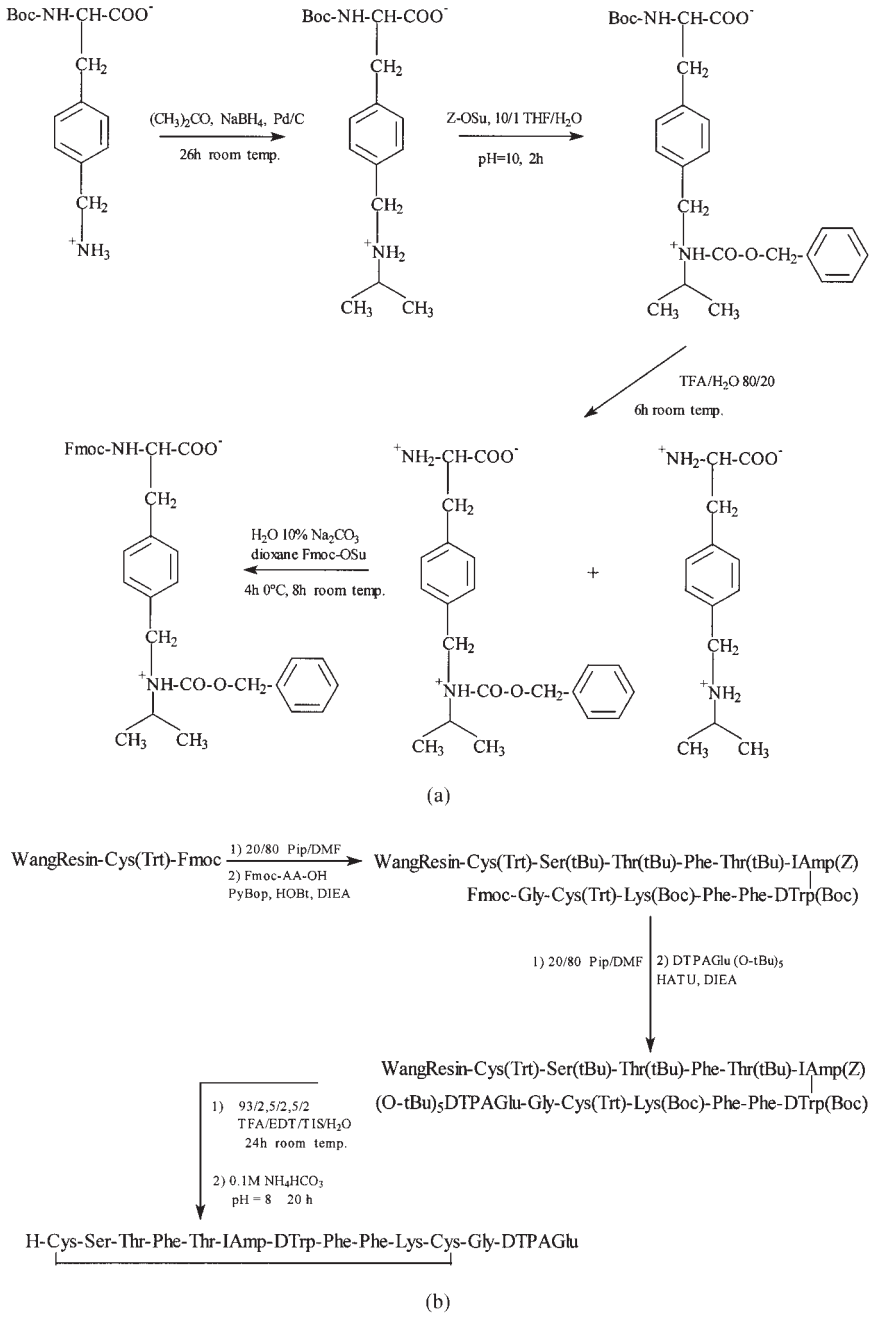

FIGURE 2 Synthetic schemes for the preparation of the amino acid derivative Fmoc–IAmp(Z)–OH (a) and for the DTPAGlu–Gly–CH-275 peptide derivative (b).

^{111}In(III) to the compound. Typical yields were greater than 97% as determined by RP- HPLC at specific activities ranging from 4 to 9 GBq/μmol (100–250 Ci/mmol). The good radiolabeling property of the compound was consistent with the predicted high efficiency of the DTPAGlu moiety to coordinate In(III). A preliminary biological assay was performed in order to verify the binding ability of ^{111}In–DTPA-Glu–Gly–CH-275 towards the sstr1 receptors expressed in cultured cells. Unexpectedly, the labeled compound did not display any specific interaction with sstr1 subtype receptors in the tested cell lines. In fact, after cell incubation with the ^{111}In-labeled compound, cell-associated radioactivity was very low, and the amount of bound compound, normalized for protein content, was negligible. To confirm this surprising negative result, we have also performed competition binding experiments in which fixed tracer

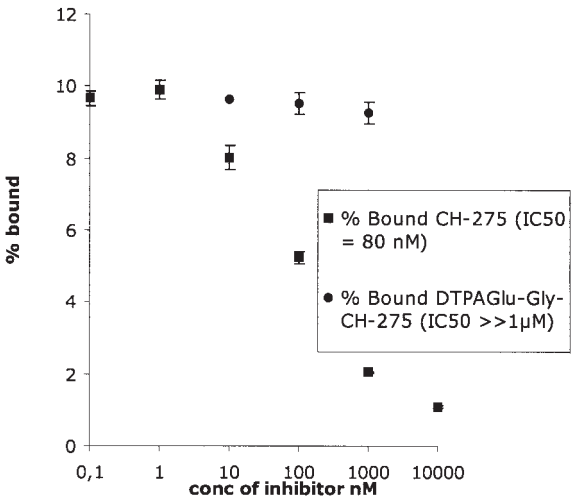

FIGURE 3 Displacement of ^{125}I somatostatin-14 with the CH-275 peptide and the DTPAGlu–Gly–CH-275 peptide conjugate, on A431 cells overexpressing the human sstr1 receptor subtype by stable transfection. IC_{50} values are similar to published values for the CH-275 peptide, whereas the DTPAGlu–Gly–CH-275 conjugate shows higher IC_{50} values (much lower affinity). Similar results were obtained in saturation experiments with ^{111}In labelled DTPAGlu–Gly–CH-275, where low affinity for the sstr1 overexpressing cells was observed (data not shown).

amounts of the ^{125}I-labeled somatostatin-14 were incubated with the receptor expressing cells in the presence of DTPAGlu–Gly–CH-275 or CH-275 at concentrations ranging from 10^{-10} to 10^{-3} *M*. The amount of bound radioactivity was determined after 1 h at 4°C and the results are reported in Figure 3. Nonlinear regression analysis using a model for homologous competition binding allows us to derive the 50% inhibitory concentration (IC_{50}) for DTPAGlu–Gly–CH-275 and CH-275. While CH-275 shows a IC_{50} of 80 n*M*, similar to that already observed in displacement experiments on CHO–K1 sstr1-transfected cells (IC_{50} = 30.9),[11] DTPAGlu–Gly–CH-275 again displays very low or negligible affinity towards the sstr1 subtype receptor.

NMR Studies

Identification of spin systems and assignment of individual resonances for DTPAGlu–Gly–CH-275 were obtained by a combination of TOCSY[16] and DQF-COSY spectra.[17] Sequence-specific assignment was obtained by NOESY[18] and ROESY experiments.[19,20,21] Proton chemical shifts for all the resonances at 298 K are listed in Table I. The NOE pattern of DTPAGlu–Gly–CH-275 (data not shown) does not indicate the presence of a clear preferred conformation of the peptide moiety, which is presumably highly flexible in the used experimental conditions. Furthermore, the absence of NOE contacts between DTPAGlu and CH-275 protons suggests that DTPAGlu does not appreciably influence the conformation behavior of the cyclic peptide. In particular, the four amino acids (residues 7 $\rightarrow$ 10) usually in a β-turn structure and involved in the interaction with the receptor are not influenced by the presence of DTPAGlu and should be able to adopt the right conformation receptor binding.

Previous studies[12] on the CH-275 peptide family and its analogues have led to a model in which the positively charged IAmp side chain interacts with Asp137 in the receptor sstr1 subtype, while the iso-

Table I **^{1}H-NMR Parameters for the Peptide Derivative DTPAGlu–Gly–CH-275**

	Chemical Shifts (ppm) in CD_3CN/ H_20 1/1					
Amino Acid	NH	α (α')	β (β')	γ (γ')	δ (δ')	Others
Gly	8.69	4.36				
Cys^3	8.64	5.12	3.70–3.60			
Lys^4	8.58	4.82		1.62	2.01	CHε 3.03; εNH 7.89
Phe^6	8.59	5.12	3.49–3.38			
Phe^7	8.56	4.98	3.38–3.38			
D-Trp^8	8.34	4.75	3.35–3.35			NH 10.34; H_2 7.38; H_4 7.87; H_5–H_7 7.56–7.20
IAmp^9	8.36	5.11	3.41–3.34			H_{ar} 7.71–7.51; CH_2 3.30; CH 3.72; CH_3 1.71
Thr^{10}	8.17	4.78	4.75	1.53		OH 5.04
Phe^{11}	8.61	5.11	3.48–3.29			H_{ar} 7.52–7.40
Thr^{12}	8.45	4.88	4.56	1.56		
Ser^{13}	8.44	4.93	4.32–4.32			
Cys^{14}	8.61	5.11	3.48–3.29			
DTPAGlu	3.96	2.34	3.85			CH_2CO 2.95; CH_2CH_2N 2.55–2.94

propyl group experiences hydrophobic interaction with the side chain of Leu107. The proposed interactions thus indicate that this class of peptide ligand interacts with the sstr1 receptor subtype in a binding pocket within the transmembrane 2 and 3 domains. On the basis of these observations, DTPAGlu–Gly–CH-275 derivatives bearing a chelating agent would be expected to get deeper in the pocket of receptor sstr1 subtype, as the biologically active CH-275 peptide does, since the same conformational and chemical features should have been maintained. This assumption, however, has not been confirmed by the experimental data, presumably because the presence of DTPAGlu does not allow the DTPAGlu–Gly–CH-275 derivative to entry into the binding pocket of sstr1 receptor subtype, due to the increased steric hindrance of the chelating moiety.

CONCLUSIONS

The peptide family of CH-275 and its analogues represent a very important class of somatostatin analogues for their ability in selective binding of sstr1receptor subtype. Unfortunately, as indicated by saturation and competition binding experiments on cells expressing sstr1, the CH-275 derivative bearing the DTPAGlu chelating agent on the N-terminal end loses its binding property towards the sstr1 receptor. The NMR results indicate that the presence of DTPAGlu does not influence the conformational and chemical features of peptide moiety, and particularly that of the four amino acids (residues 7 $\rightarrow$ 10) usually responsible for binding; therefore the loss of binding activity should be attributed to the steric hindrance or to the charges of both DTPAGlu (5 additional negative charges) and the complex ^{111}In–DTPAGlu (2 additional negative charges). In fact, it is known[7] that the binding sites for somatostatin in its receptors are deeply embedded in the membrane and therefore the surface charge on the membrane could repel our charged conjugates. The introduction of a long space between the bioactive peptide CH-275 and the indium complex of DTPAGlu should enable us to recover the specific binding ability toward the sstr1 receptor subtype.

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REFERENCES

1. Brazeau, P.; Vale, W. W.; Burnus, R.; Ling, N.; Butcher, M.; Rivier, J. E.; Guillemin, R. Science 1973, 179, 77–79.
2. Guillemin, R.; Gerich, J. E. Annu Rev Med 1976, 27, 379–388.
3. Reichlin, S. N Engl J Med 1983, 309, 1495–1563.
4. Heppeler, A.; Froidevaux, S.; Eberle, A. N.; Maecke, H. R. Curr Med Chem 2000, 7, 971–994.
5. Bell, G.; Reisine, T. Trends Neurosci 1993, 16, 34–38.
6. Lamberts, S. W. L. Octreotide: The Next Decade; Bioscientifica: Bristol, 1999.
7. Patel, C. Y. Frontiers Neuroendocrinol Rev 1999, 20, 157–198.
8. Lamberts, S. W. J.; Krenning, E. P.; Reubi, J. C. Endocrinol Rev 1991,12, 450–482.
9. Krenning, E. P.; Kwekkeboom, D. J.; Pauwels, S.; Kvols, L. K.; Reubi, J. C. Nuclear Med Annu 1995, 1–50.
10. Reisine, T.; Bell, G. I. Endocrinol Rev 1995, 16, 427–442.
11. Rivier, J.; Hoeger, C.; Erchegyi, J.; Gulyas, J.; DeBoard, R.; Craig, A. G.; Koerber, S. C.; Wenger, S.; Waser, B.; Shaer, J. C.; Reubi, J. C. J Med Chem 2001, 44, 2238–2246.
12. Chen, L.; Hoeger, C.; Rivier, J.; Fitzpatrick, V. D.; Vandlen, R. L.; Tashjian, A. H., Jr. Biochem Biophys Res Commun 1999, 258, 3, 689–694.
13. Liapakis, G.; Hoeger, C.; Rivier, J.; Reisine, T. J Pharm and Exp Therapeutics 1996, 276, 1089–1094.
14. Anelli, P. L.; Fedeli, F.; Gazzotti, O.; Lattuada, L.; Lux, G.; Rebasti, F. Bioconjugate 1999, 10, 137–140.
15. Aloj, L.; Caracò, C.; Panico, M.; Zannetti, A.; Del Vecchio, S.; Tesauro, D.; De Luca, S.; Arra, C.; Pedone, C.; Morelli, G.; Salvatore M. J Nuclear Med 2004, 45, 485–494.
16. Braunschwetler, L.; Ernst, R. R. J Magn Reson 1983, 53, 521–528.
17. Rance, M.; Sorensen, O. W.; Bodenhausen, G.; Wagner, G.; Ernst, R. R.; Wuthrich, K. Biochem Biophys Res Commun 1983, 117, 479–485.
18. Kumar, A.; Ernst, R. R.; Wuthrich, K. Biochem Biophys Res Commun 1980, 95, 1–6.
19. Griesinger, C.; Otting, G.; Wuthrich, K.; Ernst, E. E. J Am Chem Soc 1998, 110, 7870–7872.
20. Bothner-By, A. A.; Stephens, R. L.; Lee, J.; Warren, C. D.; Jeanlotoz, R. W. J Am Chem Soc 1994, 106, 811–813.
21. Wuthrich, K. NMR of Proteins and Nucleic Acids; Wiley: New York, 1986.