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Direct Synthesis of [DOTA-DPhe^1]-Octreotide and [DOTA-DPhe^1,Tyr^3]-Octreotide (SMT487): Two Conjugates for Systemic Delivery of Radiotherapeutical Nuclides to Somatostatin Receptor Positive Tumors in Man

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Abstract: Direct attachment of unprotected DOTA (1,4,7,10-tetraazacyclododecane-N',N'',N''',N''''-tetraacetic acid) to partially suitably protected octreotide or [Tyr^3]-octreotide leads after deprotection to [DOTA-DPhe^1]-octreotide (III) and [DOTA-DPhe^1,Tyr^3]-octreotide (IV). These DOTA-containing somatostatin analogs, when labeled with a radiotherapeutic nuclide, are useful as antitumor agents. The partially protected peptides are accessible via solid phase peptide synthesis (SPPS) followed by selective cleavage under mild acidic conditions from the resin.

A large number of hormone secreting tumors, such as gastro-entero-pancreatic (GEP) tumors, small cell lung cancers and medullar thyroid carcinoma, express high densities of somatostatin receptors[1]. OctreoScan®-111 ([^{111}In-DTPA-DPhe^1]-octreotide)[2], the γ-emitting ^{111}In labeled radiopharmaceutical, consisting of DTPA (**D**iethylene**T**riamine**P**entaacetic **A**cid) conjugated to the metabolically stabilized somatostatin-14[3] analog octreotide[4], has proven to be an excellent diagnostic tool for gamma camera imaging of somatostatin receptor positive tumors in man[5]. For therapeutic purposes however, an octreotide analog capable of stably chelating rare earth ß-emitters like ^{90}Y or ^{161}Tb is desirable. Such a compound can be expected to selectively eradicate somatostatin receptor positive tumors by delivering a highly localized lethal radiation dose. Among the various bifunctional DTPA[6] chelators evaluated in our laboratory, the macrocycle DOTA was selected for conjugation with octreotide analogs because of its capability to stably chelate a number of rare earth metals. In order to circumvent the complicated and time consuming synthesis of partially protected DOTA[7], required for conjugation of the peptides, we worked out reaction conditions for the direct conjugation of commercially available unprotected DOTA.2H$_2$O[8] to the selectively protected [Lys^5(Boc)]-octreotide[2] (I) and [Tyr^3,Lys^5(Boc)]-octreotide (II). These conditions require only a simple conjugation step and a final deprotection step to give access to the target molecules (III) and (IV). The synthesis of the partially protected peptidic starting materials (I) and (II) was performed according to an in-house developed solid phase peptide synthesis protocol of octreotide[9] with a slight modification of the cleavage procedure at the end of the

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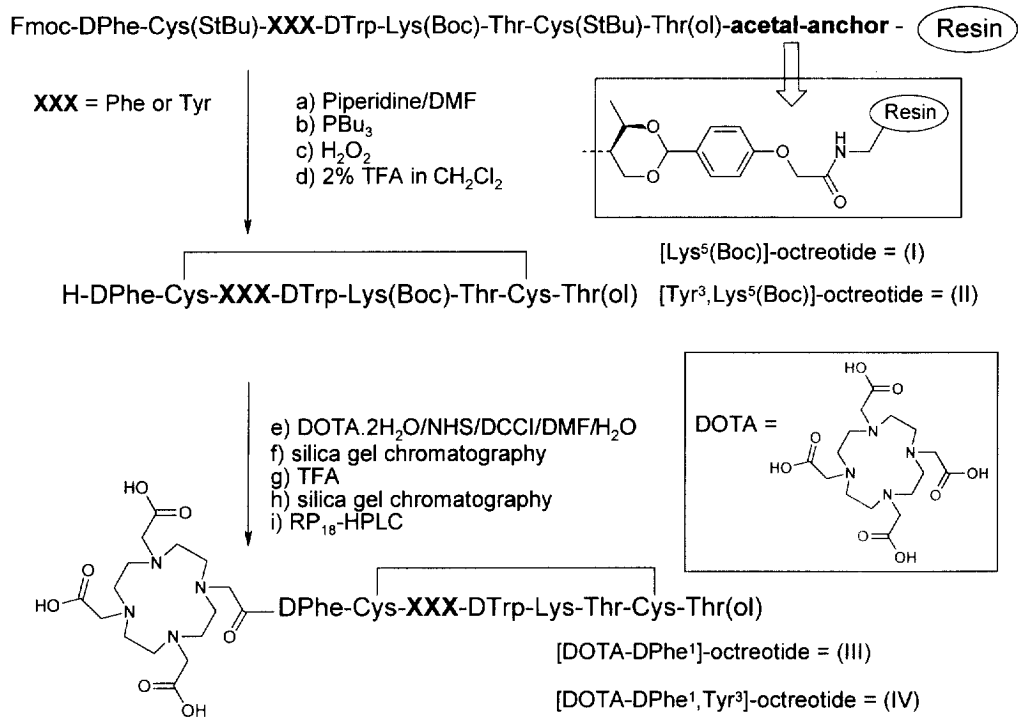
synthesis. The cyclic acetal between Fmoc-threoninol and (4-formylphenoxy)acetic acid linked to the resin was used in the very first step and Fmoc-amino acids in the following steps. For the peptide chain elongation hydroxybenzotriazole and N,N-dicyclohexylcarbodiimide were used as activating agents, while Piperidine/DMF (1/4) was employed to split-off the Fmoc-protecting group. Finally, using 2% trifluoroacetic acid (TFA) in CH_2Cl_2 instead of concentrated TFA as originally described for the cleavage step from the solid support gave access to Lys^5-Boc-protected peptides in high yields (~80%, Reaction scheme).

Reaction scheme



In order to avoid the multiple acylation of the unprotected hydroxy groups of the peptide by DOTA.$2H_2O$ (free acid), especially in the case of [Tyr^3,Lys^5(Boc)]-octreotide (II), N-hydroxysuccinimide (NHS) was selected as activating reagent in combination with N,N-dicyclohexylcarbodiimide (reaction scheme). In practice, to an aqueous solution of DOTA.$2H_2O$ (free acid) a solution of the peptide in combination with both activating reagents in DMF was mixed. After 72 hours of stirring at ambient temperature, the solvent was removed under reduced pressure and the crude conjugates were subjected to flash chromatography on silica gel[10]. The purified and protected conjugates were then treated with trifluoroacetic acid to give, after silica gel chromatography and RP_{18}-HPLC, pure and homogenous target molecules [DOTA-$DPhe^1$]-octreotide (III) or [DOTA-$DPhe^1$,Tyr^3]-octreotide (IV, SMT487)[10]. The correct structures were confirmed by means of ESI-MS[11] as well as amino acid analysis[12] and their purity was >97% as assessed by TLC[13] and RP_{18}-HPLC[14].

Receptor binding studies: In order to demonstrate high affinity binding of [DOTA-DPhe^1]-octreotide (III), [DOTA-DPhe^1,Tyr^3]-octreotide (IV, SMT487) and ^{90}Y-labelled SMT487 to somatostatin receptors the compounds were tested in a receptor binding assay using rat cortex membranes[16] (Table 1).

Table 1: Binding affinity of octreotide, [DOTA-DPhe^1]-octreotide (III) and [DOTA-DPhe^1,Tyr^3]-octreotide (IV, SMT487) to SRIF receptors of rat cortex membranes using ^{125}I-[Tyr^3]-octreotide or ^{90}Y-labelled SMT487 as specific ligand. N = 3, mean ± SEM

Compound	Octreotide	[DOTA-DPhe^1]-octreotide (III)	[DOTA-DPhe^1,Tyr^3]-octreotide (IV, SMT487)
^{125}I-[Tyr^3]-octreotide (pK_i)	9.3 ± 0.1	8.5 ± 0.1	8.9 ± 0.1
^{90}Y-labelled SMT487 (pIC_{50})	9.0 ± 0.3		

In conclusion, a convenient approach for the conjugation of either octreotide or [Tyr^3]-octreotide with unprotected DOTA.2H$_2$O was developed. By choice of commercially available reagents both target molecules (III) and (IV = SMT487) have been synthesized in satisfactory yield (~45% and ~40% respectively) together with doubly substituted DOTA analogs as main side product. Due to its high binding affinity to somatostatin receptors and due to the higher accumulation of ^{90}Y-labelled SMT487 (IV, [DOTA-DPhe^1,Tyr^3]-octreotide) at the tumor site (unpublished data) as compared to ^{90}Y-labelled (III, [DOTA-DPhe^1]-octreotide), SMT487 (IV) has been chosen for further clinical development[17].

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10. Experimental procedure for SMT487: DOTA.2H$_2$O (6.0 g, 14.8 mmol, free acid) was dissolved in 50

mL of water (pH-value ~ 3.7). After addition of 60 mL of DMF, N-hydroxysuccinimide (0.86 g, 7.4 mmol), N,N-dicyclohexylcarbodiimide (3.7 g, 29.6 mmol) and [Tyr^3,Lys^5(Boc)]-octreotide (3.1 g, 2.7 mmol) was added. The reaction was kept at room temperature for 72 hours under continuous stirring. Isolation of Lys^5 protected SMT487 was performed by purification on a silica gel column (Silica gel 60 (230-400 mesh), Merck 1.0985). Mobile phase: CH_2Cl_2/MeOH/AcOH/H_2O (90/10/0.6/0.6 → 70/40/10/10). Subsequent cleavage of the Lys^5 protecting group with trifluoroacetic acid (TFA) and purification on silica gel (mobile phase: CH_2Cl_2/MeOH/AcOH/H_2O (70/30/5/5 → 70/50/20/20)) followed RP_{18}-HPLC[15] column, using H_2O/CH_3CN/TFA as mobile phase, gave a pure and homogenous title compound in an overall yield of ~40%.

11. ESI-MS: Compound III: $(M-H)^-$ = 1403.1 (calc.: 1405.67 for $C_{65}H_{92}N_{14}O_{17}S_2$)
Compound IV (SMT487): $(M-H)^-$ = 1419.9 (calc.: 1421.67 for $C_{65}H_{92}N_{14}O_{18}S_2$)

12. Amino acid analysis for compound III: 84% peptide content ($DPhe^1$/Phe^3 & Lys^5 as standard) amino acid ratios: Phe (2.05), Thr (0.83), Lys (0.95), Trp (0.20), Cys/2 (0.39), Thr(ol) (0.67). Amino acid analysis for compound III (SMT487): 83% peptide content ($DPhe^1$ & Tyr^3 as standard); amino acid ratios: Phe (1.00), Thr (0.90), Tyr (1.00), Lys (0.83), Trp (0.56), Cys/2 (0.38), Thr(ol) (1.09). Conditions: hydrolysis with 6M HCl in sealed tubes, 24 hours at 115° C; tryptophan and cystein are partially destroyed under these conditions

13. TLC: Silica gel 60 HPTLC plates (Merck 1.05628)
Mobile phase: CH_2Cl_2/MeOH/AcOH/H_2O: 70/30/5/5 (for compound (I) and (II))
Mobile phase: CH_2Cl_2/MeOH/AcOH/H_2O: 70/40/10/10 (for compound (III) and (IV))

14. Analytical RP_{18}-HPLC conditions: Column: Spherisorb ODS 4.5 x 250 mm (2.5μ);
UV detection λ = 220 nm; Flow: 1.25 ml/min; mobile phase A: 1% TFA in H_2O; mobile phase B: 1% TFA in H_2O/CH_3CN (20/80); linear gradient: 0 % to 100 % B over 20 min.
R_t: 15.19 min. (III) and 13.66 min (IV)

15. Preparative HPLC conditions: Column: Spherisorb RP 18 (3μ) 20 x 250 mm;
UV detection λ = 240 nm; Flow: 3 ml/min; Mobile phase A: H_2O/CH_3CN (+0.1% AcOH) 100/0; mobile phase B: 20/80; Linear gradient: 0 % to 100 % B over 60 min.

16. In brief, for the SSTR2-binding assays, rat cortex membranes were diluted with incubation buffer and homogenized. The final protein concentration per assay tube was 20-50μg. The assay (300 μL) consisted of cortex membranes (200 μL), approximately 20 pM ^{125}I-[Tyr^3]-octreotide (70 μL) respectively 1 nM of ^{90}Y-labelled SMT487 and various concentrations of competitor compound (30 μL). After the incubation for 60 min. at room temperature, the samples were filtered through glass-fibre filters which were presoaked with buffer containing 1% wt/vol BSA. The filters were then rinsed with 10 mL of ice-cold buffer; bound radioactivity was measured in a γ-counter. Specific binding was defined as the difference between total and non-specific binding in the presence of an excess (1 μM) of octreotide.

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