Radiometal-Labelled Macrocyclic Chelator-Derivatised Somatostatin Analogue with Superb Tumour-Targeting Properties and Potential for Receptor-Mediated Internal Radiotherapy

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Abstract: A monoreactive DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) prochelator (4,7,10-tricarboxymethyl-*tert*-butyl ester 1,4,7,10-tetraazacyclododecane-1-acetate) was synthesised which is useful in solid-phase and solution-phase peptide synthesis; it was coupled to the somatostatin analogue Tyr^3-Lys^5(BOC)-octreotide. Deprotection in one step afforded DOTA^0-D-Phe^1-Tyr^3-octreotide (DOTATOC) in ≈65% yield. This peptide, modified with a chelator, was complexed with the radiometals $^{67}Ga^{3+}$, $^{111}In^{3+}$ and $^{90}Y^{3+}$ in high yields and with high specific activities. The three radiopeptides show high stability in human serum and high affinity to the somatostatin receptor: it is four to five times higher for ^{67}Ga-DOTATOC compared to ^{90}Y-DOTATOC and ^{111}In-DOTATOC. The ^{67}Ga-labelled compound also shows significantly higher tumour and lower kidney uptake than the two congeners. ^{67}Ga-DOTATOC was compared in patients with the commercially available gold standard ^{111}In-DTPA^0-D-Phe^1-octreotide. The new compound delineates SRIF-receptor positive tumours very favourably and shows distinctly lower uptake by the kidneys. Evidently, the differences in the coordination chemistry of the metals causes the differences in the biological behaviour. Indeed, a crystallographic study of the Ga^{3+} and Y^{3+} complexes of the model peptide DOTA-D-PheNH$_2$ showed differences in the geometry of the complexes. The gallium complex is hexacoordinated with pseudooctahedral *cis* geometry and a folded macrocyclic unit. The equatorial plane is formed by two transannular nitrogens of the cyclen ring and two oxygens of the corresponding carboxylate groups. The two axial positions are formed by the two remaining ring nitrogen atoms. The amide carboxy oxygen is not bound to the metal and one carboxylate group is free and most likely contributes to the favourable handling of the radiopeptide by the kidneys. In contrast, the structure of Y-DOTA-D-PheNH$_2$ has eight-fold coordination, and includes the amide carboxy oxygen. The geometry is a compact and somewhat distorted square-antiprism with two almost perfect planes (N4 and O4) with a maximum deviation of 0.025 Å. The dihedral angle between the two planes is only 0.36°.

Keywords: gallium · macrocyclic ligands · peptides · radiopharmaceuticals · yttrium

Introduction

Radiolabelled (bio)molecules are potentially useful tools for tumour targeting in order to obtain diagnostic information (spread and localisation of the disease) if they contain γ-emitting radiometals, such as ^{99m}Tc,[1] ^{111}In[2] or ^{67}Ga (^{68}Ga).[3] They also can be used for selective internal radiotherapy when complexed with particle-emitting isotopes, such as the β-emitters ^{90}Y,[4] ^{186}Re,[5] etc. Among potential biomolecules, the regulatory peptides are of special interest because of the high expression of their receptors on different malignancies.[6] In this respect, somatostatin (SRIF) analogues are very promising peptides which, because of their high clinical potential in

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the control of tumours and other diseases, were the subject of many structure–bioactivity studies and conformational analyses.[7] Moreover, as an exciting new development, the metabolically stabilised somatostatin analogue octreotide[8] was conjugated to the chelator diethylenetriaminepentaacetic acid (DTPA) and labelled with ^{111}In. This compound, known as OctreoScan (Figure 1), retains its binding affinity to SRIF receptors and is currently used very successfully in the localisation of SRIF-receptor positive tumours in man.[9, 10] It has some drawbacks; it cannot be labelled with particle-emitting metallic radionuclides, such as the hard Lewis acid β-emitters and the bone seekers $^{90}Y^{3+}$ or $^{153}Sm^{3+}$, for therapeutic applications. Their DTPA complexes are too labile, these particular radiometals are partially released in vivo, causing irreversible bone marrow damage.

Abstract in German: *Ein monoreaktiver DOTA (1,4,7,10-tetraazacyclododecane-1,4,7, 10-tetraessigsäure) Prochelator (1,4,7,10-tetraazacyclododecane-1-essigsäure-4,7,10-tricarboxymethyl-tert-butylester) wurde synthetisiert, der sowohl für die Fest- wie auch die Flüssigphasen Peptidsynthese geeignet ist. Die Kupplung an das Somatostatin Analogon Tyr^3-Lys^5(BOC)-octreotide und anschliessende Entschützung in einem Schritt ergab DOTA^0-D-Phe^1-Tyr^3-octreotide (DOTATOC) in einer Ausbeute von ≈65 %. Dieses mit einem Chelator modifizierte Peptid wurde mit den Radiometallen $^{67}Ga^{3+}$, $^{111}In^{3+}$ und $^{90}Y^{3+}$ mit hoher Ausbeute und hoher spezifischer Aktivität komplexiert. Alle drei Radiopeptide zeigten hohe Stabilität in humanem Serum und hohe Affinität zum Somatostatin Rezeptor; im Falle von ^{67}Ga-DOTATOC um den Faktor 4–5 höher als bei ^{90}Y-DOTATOC und ^{111}In-DOTATOC. Im Vergleich zu diesen beiden Radiopeptiden zeigt die ^{67}Ga-markierte Verbindung außerdem eine signifikant höhere Tumoranreicherung und geringere Nierenaufnahme. ^{67}Ga-DOTATOC wurde am Patienten mit dem kommerziellen Gold Standart ^{111}In-DTPA^0-D-Phe^1-octreotide verglichen. Die neue Verbindung stellt SRIF Rezeptor positive Tumore ausgezeichnet dar und zeigt darüber hinaus eine viel geringere Nierenaufnahme. Offensichtlich wird das unterschiedliche biologische Verhalten durch Unterschiede in der Koordinationsgeometrie bewirkt. Kristallographische Untersuchungen der Ga^{3+} und Y^{3+} Komplexe des Modellpeptids DOTA-D-PheNH$_2$ zeigen in der Tat Unterschiede in der Komplexgeometrie auf. Der Galliumkomplex ist 6-fach koordiniert mit einer cis-pseudooktaedrischen Geometrie und einer gefalteten makrocyclischen Einheit. Die Äquatorialebene wird dabei durch zwei transannulare Stickstoffatome des Cyclen Rings und zwei Sauerstoffatome der entsprechenden Carboxylatgruppen gebildet. Die zwei axialen Positionen werden von den zwei verbleibenden Ringstickstoffatomen eingenommen. Das Amido Carboxy Sauerstoffatom ist nicht metallgebunden, die verbleibende Carboxylatgruppe ist frei und trägt höchstwahrscheinlich zum ausgezeichneten Nierenverhalten des Radiopeptids bei. Im Unterschied zu dieser Struktur zeigt Y-DOTA-D-PheNH$_2$ 8-fach Koordination mit einem metallgebundenen Amido Carboxy Sauerstoff und einer kompakten, leicht verzerrten quadratisch antiprismatischen Geometrie. Der Komplex weist zwei nahezu perfekte Ebenen (N4 und O4) auf mit einer maximalen Abweichung von 0.025 Å, wobei der Diederwinkel zwischen diesen beiden Ebenen 0.36° beträgt.*

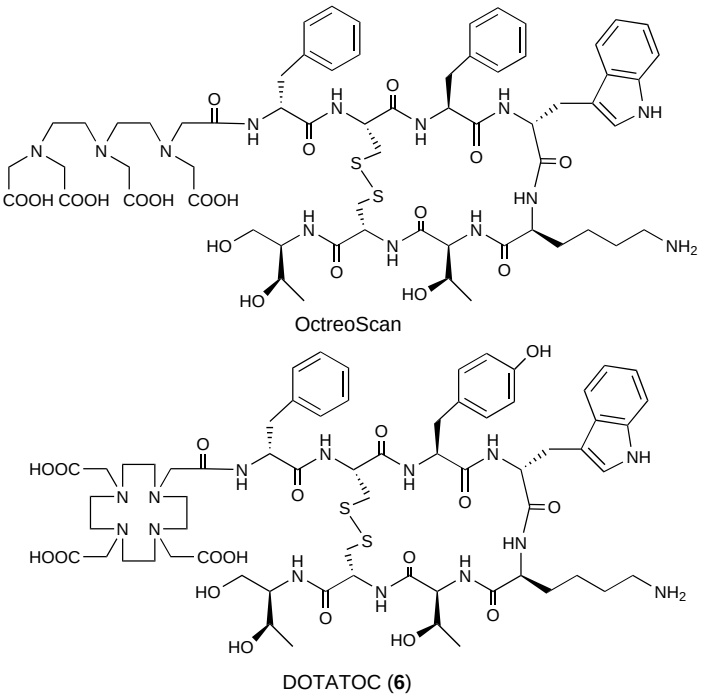
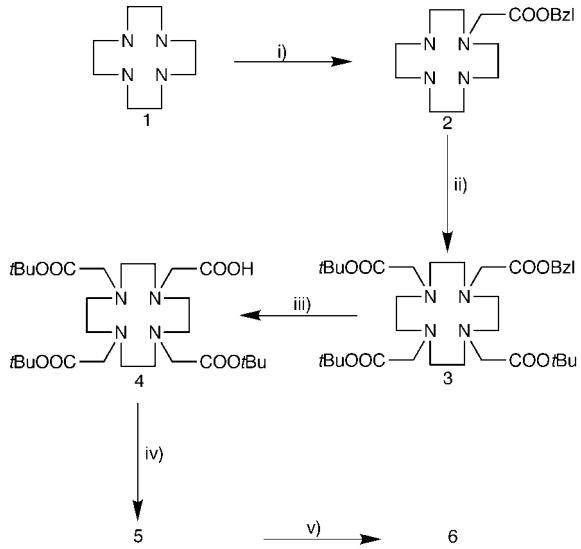


Figure 1. Structural formulae of DOTA^0-D-Phe^1-Tyr^3-octreotide (**6**, DOTATOC) and DTPA^0-D-Phe^1-octreotide (OctreoScan).

We have now designed (Scheme 1) a DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) functionalised somatostatin analogue, DOTATOC **6** (Figure 1), that can be labelled with a variety of radiometals which are useful in different areas of diagnostic and therapeutic nuclear oncology.[11] DOTA is known to form thermodynamically and kinetically stable metal complexes with a multitude of



Scheme 1. Synthesis of DOTA^0-D-Phe^1-Tyr^3-octreotide (**6**, DOTATOC). Reagents and conditions: i) $BrCH_2COOBz$, $CHCl_3$, room temperature (RT), 2h ; ii) $BrCH_2COOtBu$, AcCN, K_2CO_3, RT, 2.5 h; iii) H_2, Pd/C (10 %), THF/MeOH 1:1, RT, 5 h; iv) (ε-(*tert*-butoxycarbonyl))Lys^5-Tyr^3-octreotide, HATU, DIPEA, DMF, RT, 4 h; v) TFA/thioanisole/H_2O 92:6:2, RT, 4 h.

2+ and 3+ charged metals and forms the basis of very useful diagnostic magnetic resonance imaging (MRI) agents when coordinated with Gd^{3+}ions.[12] Moreover, when conjugated as a bifunctional version to monoclonal antibodies and labelled with ^{90}Y, it is used in monoclonal antibody-mediated internal radiotherapy.[4] We have modified the octapeptide octreotide somewhat by replacing Phe^3 by Tyr for two reasons: i) to increase the hydrophilicity and ii) to allow dual labelling; iodination of Tyr^3 with ^{125}I may be of additional benefit in therapeutic applications because of its Auger electrons. In a first step, we have performed an in vitro and in vivo evaluation of this new peptide–DOTA conjugate with three radiometals which can be used for single photon emission tomography (SPET, $^{111}In^{3+}$, $^{67}Ga^{3+}$), positron emission tomography (PET, $^{68}Ga^{3+}$, $^{86}Y^{3+}$) and receptor-mediated radionuclide therapy ($^{90}Y^{3+}$). Very important prerequisites must be fulfilled with regard to the in vivo application of a radiometal-labelled bioactive peptide: i) the chelator–peptide conjugate must be complexed with the radiometal in high yields (>99.5%) at high specific activities (Bq Mol^{-1} peptide), ii) the radiometal–peptide bond should be very stable under physiological conditions in order to minimise the premature release of the radiometal to serum proteins followed by the unspecific accumulation in non-target organs; iii) the modification by the chelate should not compromise the receptor-binding affinity of the peptide and iv) the radiopeptide should be hydrophilic and excreted by the urinary tract. We studied the performance of ^{67}Ga-, ^{111}In- and ^{90}Y-DOTATOC in vitro and in vivo with regard to these conditions.

Results and Discussion

Synthesis and stability: An important step towards the synthesis of DOTA-derivatised bioactive peptides is the development of the prochelator **4**. This monoreactive DOTA prochelator (Scheme 1) was synthesised in three steps with an overall yield of 63±5% to afford a synthon which can be used in solution- and in solid-phase peptide synthesis. Prochelator **4** was then coupled to Tyr^3-Lys^5(BOC)-octreotide in DMF followed by deprotection in one step. The use of HATU (*O*-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) as a coupling reagent guaranteed the efficient and fast formation of **5**. After deprotection and purification by HPLC, DOTA^0-D-Phe^1-Tyr^3-octreotide (DOTATOC) was obtained in 65±3% yield and >97% purity, even if 10 mg amounts of Tyr^3-Lys^5(BOC)-octreotide were used. Recently, an alternative synthetic procedure was published which used a large excess of unprotected DOTA·2H_2O for the coupling reaction in an acidic water/DMF solution. This procedure afforded 40% yield when a gram scale of DOTA·2H_2O and peptide was used.[13] The stoichiometry and structure of DOTATOC were verified by MS-ESI, by the retention of a high-binding affinity to the somatostatin receptor as well as by amino acid and elemental analysis. The model DOTA peptide DOTA-D-PheNH_2 was synthesised accordingly in 55% yield. DOTATOC was complexed with the radiometals of interest (^{67}Ga, ^{111}In, ^{90}Y) in acetate buffer (pH 5, 0.1M) by heating (95°C, 25–30 min); the labelling yields were >99.5% at specific activities of >40 GBq μmol^{-1}. [Ga(DOTA-D-PheNH_2)] and [Y(DOTA-D-PheNH_2)] were synthesised by the addition of excess metal nitrate and heating (95°C, pH 5, 25–30 min) followed by precipitation of the uncomplexed metal as the trihydroxide under weakly basic conditions (pH≈8). Slow evaporation in aqueous solution (pH 7) afforded colourless single crystals of X-ray quality. The kinetic stability under physiological conditions was studied by measurements of the rate of radiometal exchange in blood serum.[14] The approximate transfer half-lives were 1250 h, 1850 h and 2100 h for ^{67}Ga-DOTATOC, ^{111}In-DOTATOC and ^{90}Y-DOTATOC, respectively; that is the reverse order of the kinetic stability of the three metal ions.[15] These values indicate the potential for in vivo use.

Receptor binding: The retention of the binding affinity was shown by competition binding measurements between ^{125}I-Tyr^3-octreotide and the metal-coordinated ^{89}Y-, ^{115}In- or $^{69/71}Ga$-DOTATOC or by Scatchard analysis, as previously reported.[16] Data obtained from a competition binding assay are shown in Figure 2. Interestingly, the IC_{50} values differed by

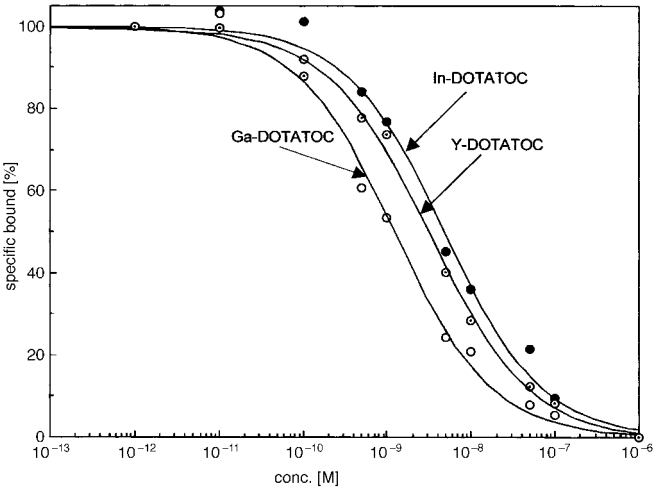


Figure 2. In vitro displacement of ^{125}I-Tyr^3-octreotide by Ga-, In-, Y-DOTATOC in rat brain cortex membranes. The maximal specific binding in absence of a competitor was taken as 100% (control). Values are the mean of three determinations.

a factor of 4–5 and K_D values obtained from a saturation experiment were 0.46±0.1 nM for ^{67}Ga-DOTATOC and 2.57±0.2 nM for ^{111}In-DOTATOC (data not shown). An evaluation of the saturation curve of ^{90}Y-DOTATOC was not possible; this is probably the consequence of radiolytic events caused by the high energy β-emitter ^{90}Y. An approximate K_D value for ^{90}Y-DOTATOC of 2.2±0.3 nM was obtained indirectly by the use of the competition data of ^{115}In-DOTATOC and ^{89}Y-DOTATOC and the K_D value determined for ^{111}In-DOTATOC.

Tumour and kidney uptake in an animal model: A biodistribution study was performed in a somatostatin-receptor positive tumour model: nude mice bearing the rat pancreatic AR4-2J tumour. Tumour and kidney uptake for ^{67}Ga-, ^{111}In- and ^{90}Y-DOTATOC and the gold standard ^{111}In-DTPA-octreotide are shown in Figure 3. The three new radiotracers

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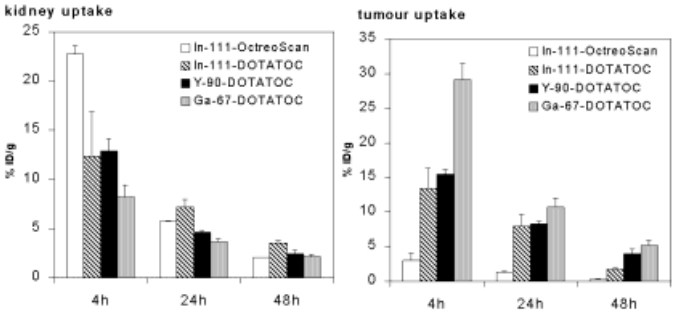

Figure 3. Biodistribution study of DOTATOC labelled with ^{67}Ga, ^{111}In and ^{90}Y, in comparison to ^{111}In-DTPA^{0}-D-Phe^{1}-octreotide, on nude mice bearing the AR4-2J tumour (% ID g^{-1}: % injected dose per gram).

show a clear advantage over the established clinically used compound ^{111}In-DTPA-octreotide, with regard to higher tumour uptake and lower kidney retention. ^{67}Ga-DOTATOC reveals a significantly higher tumour uptake followed by ^{90}Y-DOTATOC and ^{111}In-DOTATOC, which is in the same order as their receptor-binding affinities. These variations are remarkable considering that they are evidently caused by differences between the three metal cations which have all hard Lewis acid character with spherical symmetry[17] and predominantly electrostatic interaction with the macrocyclic chelator. Besides the higher tumour uptake, the favourable lower kidney uptake is of importance. The kidneys appear to be the most affected and dose-limiting organs in human studies with regard to toxicity; after glomerular filtration peptides are reabsorbed by the kidney tubuli and partially retained, which may cause long-term damage to this organ if a particle ray-emitting isotope is used for the therapy.[18]

Patient studies: In a series of patients with SRIF-receptor positive tumours, ^{111}In-DTPA-octreotide was compared with ^{67}Ga-DOTATOC. A typical example is shown in Figure 4. A

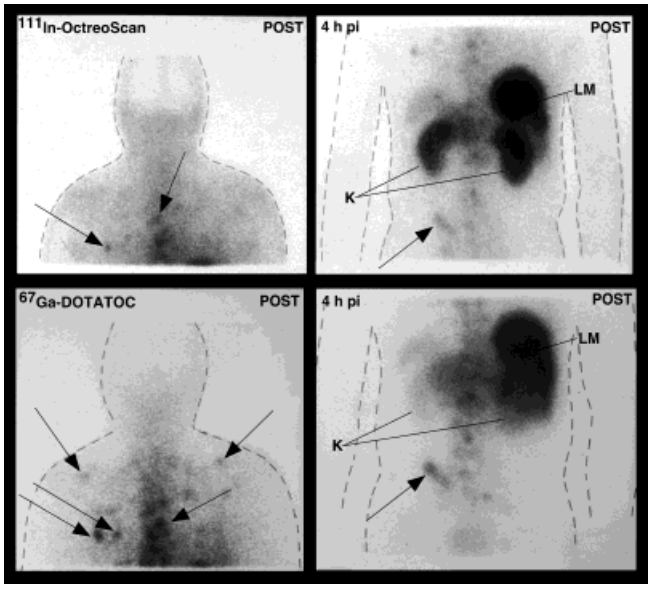

Figure 4. Scintiscans of a patient 4 hours after intravenous injection of: ^{111}In-DTPA^{0}-D-Phe^{1}-octreotide (OctreoScan) (top row) and ^{67}Ga-DOTATOC (bottom row) within two weeks. There is a large liver metastasis (LM) of a carcinoid tumour and multiple metastases throughout (arrows); note the lower uptake in kidneys (K) and the higher tumour-to-background ratio of ^{67}Ga-DOTATOC in comparison to ^{111}In-DTPA-octreotide.

patient with a carcinoid tumour was studied with both radiotracers within two weeks. This patient has a large liver metastasis (LM, Figure 4) and smaller metastases (arrows) throughout the body. The new compound shows tumour and metastases more clearly, and most remarkably, the kidney uptake is again much lower. The first human data with ^{90}Y-DOTATOC as a new therapeutic modality show very promising results.[19]

Coordination chemical aspects: Besides the important clinical potential[20] of these new radiopeptides, it is of major interest to understand why the radiometal ion influences the receptor-binding affinity and uptake in tumours in such a way. One approach to analyse and possibly understand this mechanism is to study the structural differences of the respective metal complexes. As a model peptide-chelate we synthesised a DOTA-derivatised D-PheNH$_2$ conjugate and crystallised the respective Ga^{3+} and Y^{3+} complexes as neutral complexes, and ORTEP representations of these are shown in Figures 5 and 6,

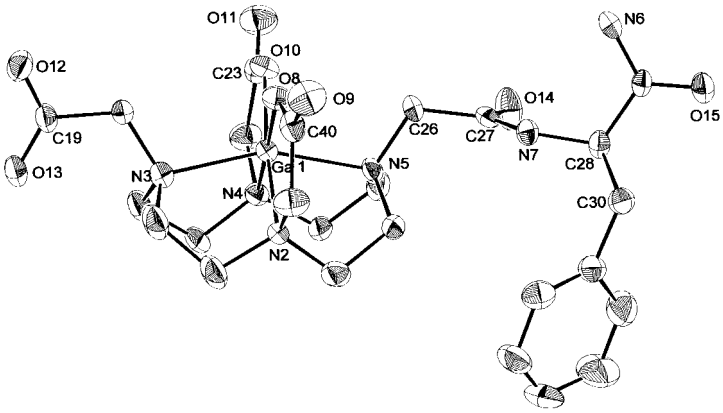

Figure 5. ORTEP plot of the crystal structure of [Ga-DOTA-D-PheNH$_2$] (thermal ellipsoids at 50% probability level). Selected bond lengths [Å] and angles [°]: Ga1–N4 2.120(5), Ga1–N5 2.163(5), Ga1–N2 2.135(5), Ga1–N3 2.145(5), Ga1–O8 1.904(5), Ga1–O10 1.932(4), O9–C40 1.201(8), O11–C23 1.224(8), O12–C19 1.254(8), O14–C27 1.221(8), N7–C27 1.343(8); N3-Ga1-N5 156.3(2), N2-Ga1-O10 169.7(2), N4-Ga1-O8 170.0(2); C26-C27-N7-C28 177.9, C27-N7-C28-C30 –141.7.

respectively. The two geometries show remarkable differences: in Ga-DOTA-D-PheNH$_2$, the chelator adopts a *cis*-pseudo-octahedral geometry with a folded macrocyclic unit (2424 conformation). The equatorial plane is formed by two transannular nitrogens of the cyclen ring and two oxygens of the corresponding carboxlate groups. These four atoms form an almost perfect plane with a maximum deviation of 0.028 Å. The Ga^{3+} ion is positioned 0.043 Å from this plane. The axial positions are occupied by the two remaining ring nitrogen atoms. The equatorial Ga–N bonds are slightly shorter (2.120(5) and 2.135(5) Å) than the axial ones (2.145(5) and 2.163(5) Å) whereas the Ga–O bonds are 1.904(5) Å and 1.932(4) Å. The main deviation from octahedral geometry is exhibited by the N3-Ga-N5 angle of 156.3°, which deviates from the linearity expected for an octahedral arrangement. Most importantly, one carboxylate group is free and is deprotonated at the physiological pH; this structural feature is known to contribute to a quick and efficient excretion of

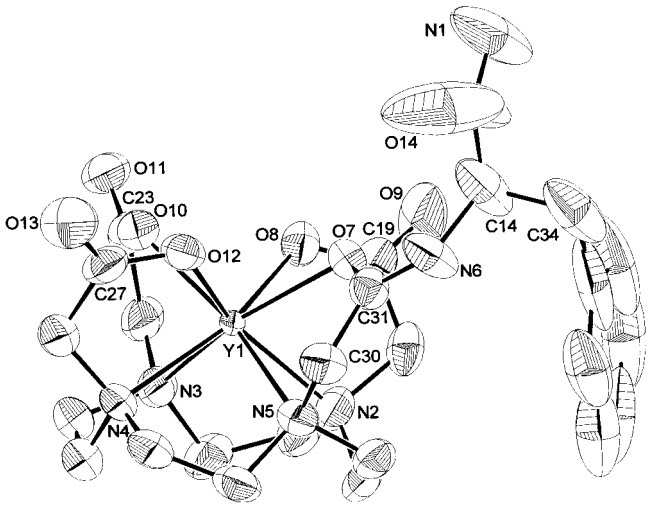

Figure 6. ORTEP representation of the crystal structure of [Y-DOTA-D-PheNH$_2$] (thermal ellipsoids at 50% probability level). Selected bond lengths [Å] and angles [°]: Y1–O10 2.241(6), Y1–O12 2.254(6), Y1–O8 2.282(6), Y1–O7 2.318(5), Y1–N3 2.388(7), Y1–N4 2.414(6), Y1–N2 2.434(7), Y1–N5 2.437(7), O7–C31 1.260(9), O9–C19 1.221(12), O11–C23 1.249(11), O13–C27 1.237(11), N6–C31 1.308(11); N4-Y1-O8 157.7(2), N2-Y1-O12 154.3(2), N5-Y1-O10 158.1(2), N2-Y1-O7 155.4; C30-C31-N6-C14 174.1, C31-N6-C14-C34 – 124.9.

chemicals through the kidney.[21] We assume that this feature is also responsible for the efficient kidney clearance of ^{67}Ga-DOTATOC. Unlike the Ga^{III} structure in Y-DOTA-D-PheNH$_2$, the coordination number of Y^{III} is eight and includes the amide carboxy oxygen of the amide functional group used for coupling. The functionalised DOTA acts as an octadentate chelator to form a very compact although somewhat distorted square-antiprismatic geometry. The twelve-membered ring adopts the usual square [3333] conformation, as found in many cyclen-derivatised pendant-arm complexes[22] and in Na[Y(DOTA)(H$_2$O)],[23] where the average Y–N bond length is 2.645(18) Å and the Y–O bond length is 2.326(5) Å. In the present complex, the Y^{III} ion is buried more deeply inside the N_4O_4 cage at a distance of 1.289 Å above the N_4 plane, as compared to 1.616 Å in Na[Y(DOTA)(H$_2$O)]. Somewhat surprisingly, the bond between the amide carboxy oxygen atom and the metal is only slightly longer (2.318(5) Å) than the bond between the carboxylic acid oxygen atom and Y (variation between 2.241(6) and 2.282(6) Å). Significant differences are seen in the C–O and C–N bond lengths of the two metal complexes, depending on the coordination number and involvement of the amide oxygen in the metal coordination. Metallation of the amide oxygen should increase the ionic character of the C–O bond and also its bond length; the C–N double bond character should increase as well while the bond length would decrease. This is indeed the case: whereas in Ga-DOTA-D-PheNH$_2$ the C–N bond length (1.343(8) Å) and the C–O bond length (1.221 (8) Å) are within values for typical amide bonds,[24] the C–O bond in Y-DOTA-D-PheNH$_2$ increases to 1.260(9) Å while the C–N bond decreases to 1.308(11) Å. According to the literature,[25] several isomers are possible for the yttrium complex: two of them, which are related to the conformation of the macrocyclic unit, are of special interest. Both isomers have the same square [3333] conformation. The difference is caused by changes in the orientation of the side arms, which leads to a pair of diastereoisomers M (major isomer) and m (minor isomer) with a square-antiprismatic or a twisted-antiprismatic geometry for M and m, respectively. In the solid state, Y-DOTA-D-PheNH$_2$ is present only in the m form whereas the M isomer was found in Na[Y(DOTA)(H$_2$O)]. In both structures the stereochemistry on the secondary amide bond is *trans*.

At this stage of the investigation we must assume that the remarkable differences in receptor affinity and tumour and kidney uptake in animals as well as in patients is caused by the differences in the complex geometry of the coordination compound of the radiometal. This appears to be the case, despite the fact that the chelate is coupled to the exocyclic D-Phe and is remote from the four amino acids (Tyr, D-Trp, Lys, Thr), which are responsible for the bioactivity and the binding to the SRIF receptor. The more open and flexible structure of the Ga complex may allow the octapeptide to adjust more easily to the ideal conformation for receptor binding whereas the coordinative use of the amide oxygen forces the chelate closer to the peptide. This would introduce steric strain with a probability of disturbing the proper spatial arrangement of the key side chain groups. So far, we have not been able to crystallise either of the two peptides. The improved kidney clearance of Ga-DOTATOC is most likely caused by the free carboxylate group, which is a known structural feature that enables a rapid handling of carboxylic acid derivatives in the kidney. Interestingly, the two radiometal-labelled peptides are also handled differently by endopeptidases in patients. Whereas ^{90}Y-DOTATOC is excreted essentially unchanged into the urine, ^{67}Ga-DOTATOC is less stable in vivo: two metabolites were found in the urine after only 4–6 hours; the main metabolite is ^{67}Ga-DOTA-D-Phe along with very small amounts of ^{67}Ga-DOTA (data not shown).

Conclusions

A new monoreactive DOTA prochelator was synthesised which is amenable to solid- and solution-phase peptide synthesis. It allowed the synthesis of a DOTA-substituted octapeptide, DOTA^0-D-Phe^1-Tyr^3-octreotide (DOTATOC), which can be complexed with different radiometals (^{67}Ga^{3+}, ^{111}In^{3+}, ^{90}Y^{3+}). It has been used successfully in different fields of nuclear oncology in several university hospitals in Europe. A highly stable octacoordinated Y-DOTA-D-Phe-amide unit provided the first example of the use of the powerful β-emitter, ^{90}Y, coupled to a bioactive peptide, in the internal radiotherapy of some forms of cancer. In contrast, the same peptide, labelled with the γ-emitter ^{67}Ga, showed improved biodistribution in tumour-bearing animals and humans with regard to tumour uptake and kidney clearance. This behaviour is most likely the result of the hexacoordination of the Ga^{III} complex with a pendant, ionised, acetic acid group.

Experimental Section

Spectroscopy, mass spectrometry, and analyses: All chemicals were obtained from commercial sources and used without further purification. Octreotide and Tyr^3-Lys^5(BOC)-octreotide were commercially available, DTPA-octreotide was synthesised as described in the literature.[26] ^{111}In-

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DTPA-octreotide, $^{111}InCl_3$ and $^{67}GaCl_3$ were purchased from Mallinckrodt Med. and $^{90}YCl_3$ from Batelle (Pacific North West Lab). Reactions were carried out at ambient temperature, unless otherwise noted, and were monitored by thin-layer chromatography (TLC) on Merck plates precoated with silica gel 60 F-254 (0.25 mm). Spots were visualised either by UV light, I_2 or ninhydrin. Flash column chromatography was performed on silica gel 60 (Fluka); Sephadex G-50 (Pharmacia) was used for gel chromatography, Dowex 1x8 (Fluka) anion exchange resin was used for ion exchange chromatography. Electrospray ionisation (ESI) was carried out with a Finnigan SSQ 7000 spectrometer, fast atom bombardment (FAB) with a VG 70 SE spectrometer. Analytical and semipreparative HPLC was performed on a Hewlett Packard 1050 HPLC system with a multiwavelength detector and a flow-through Berthold LB 506 C1 γ-detector. Quantitative γ-counting was performed on a COBRA 5003 γ-system well counter from Packard Instrument Company. ^1H and ^{13}C NMR was performed with either a Bruker spectrometer at 360/99 MHz or a Varian VXR 400 at 400/101 MHz. Chemical shifts reported are relative to TMS.

1,4,7,10-Tetraazacyclododecane-1-carboxymethyl-benzylester (2): To a stirred solution of 1,4,7,10-tetraazacyclododecane (cyclen **1**) (2.9 g, 16.8 mmol) in $CHCl_3$ (20 mL) was added benzyl bromoacetate (1340 µL, 8.4 mmol) in $CHCl_3$ (4 mL) within 1 h. Stirring was continued for an additional hour. The solution became cloudy (cyclen · HBr). TLC control showed complete disappearance of the benzyl ester. The precipitate was filtered and the filtrate concentrated in vacuo. The resulting oil was purified by column chromatography (30×2 cm, SiO_2 (35 g), $CHCl_3$/EtOH/NH_3 8:9:4 v/v). Evaporation afforded **2** as an oil (2.3 g, 7.2 mmol) in 85% yield. MS-ESI: *m*/*z* (%): 321.0 $[M+H]^+$ (86), 244.9 $[M+H-Phe]^+$ (100), 213.9 $[M+H-OCH_2Phe]^+$ (72); ^1H NMR (360 MHz, 27 °C, [D$_6$]DMSO): $\delta = 2.4-2.7$ (m, 18H, CH_2N), 5.2 (s, 2H, CH_2Phe), 7.3–7.4 (m, 5H, Phe); ^{13}C NMR (90.56 MHz, 25 °C, [D$_6$]DMSO): $\delta = 44.9$, 45.8, 46.8, 51.1 (CH_2N), 55.3 ($NCH_2C(O)$), 65.3 (CH_2Phe), 127.8, 128.3 (CH, Phe), 136.0 (aromatic quaternary), 170.9 ($C(O)OCH_2Phe$).

1,4,7,10-Tetraazacyclododecane-4,7,10-tricarboxymethyl-*tert*-butylester-1-carboxymethyl-benzylester (3): To a stirred solution of **2** (1.41 g, 4.4 mmol) in acetonitrile (25 mL) was added K_2CO_3 (2.5 g, 18.1 mmol) followed by the dropwise addition of *tert*-butyl bromoacetate (2.65 mL, 18 mmol) in acetonitrile (5 mL) within 30 min. Stirring was continued for 2 h to complete the reaction. K_2CO_3 was filtered off and the solvent removed on a rotary evaporator. The resulting yellowish oil was immediately purified by column chromatography (30×3.5 cm; SiO_2 (35 g), cooling 8–10 °C; gradient of $CHCl_3$ to $CHCl_3$/EtOH, 9:1, v/v). Evaporation of the solvent afforded an oil (2.9 g, 4.4 mmol) in 100% yield. MS-ESI: *m*/*z* (%): 685.3 $[M+Na]^+$ (100), 663.4 $[M+H]^+$ (45); ^1H NMR (360 MHz, 27 °C, [D$_6$]DMSO): $\delta = 1.42$ (s, 27H, *t*Bu), 1.95–3.90 (m, 24H, CH_2N), 5.10 (s, 2H, CH_2Phe), 7.33–7.40 (m, 5H, Phe); ^{13}C NMR (90.56 MHz, 25 °C, [D$_6$]DMSO): $\delta = 27.47$, 27.65 (*t*Bu), 55.8–60.8 (CH_2N), 65,8 (CH_2Phe), 79.05, 81,2 (*t*Bu), 127.7–134.05 (CH, Phe), 172.05, 173.0 (C=O).

4,7,10-Tricarboxymethyl-*tert*-butyl ester 1,4,7,10-tetraazacyclododecane-1-acetate (DOTA(*t*Bu)$_3$, 4): Pd/C (10% Pd, 400 mg) was added to a solution of **3** (2.92 g, 4.4 mmol) in THF/MeOH (1:1, v/v, 500 mL), and H_2 was bubbled through the solution at normal pressure. Hydrogenation was conducted for 5–12 h at normal pressure. The catalyst was removed by filtration through Celite. The solvent was evaporated to afford a pale yellow oil (2.5 g, 4.3 mmol) which was crystallised from acetone/diisopropyl ether by adding small amounts of HBr to afford the product (2.9 g, 3.3 mmol) in 74% yield. MS(70 eV, FAB): *m*/*z* (%): 595 $[M+Na]^+$ (18), 573 $[M+H]^+$ (100); ^1H NMR (360 MHz, 27 °C, [D$_6$]DMSO): $\delta = 1.4$ (s, 18H, *t*Bu), 1.5 (s, 9H *t*Bu), 2.6–3.45 (m, 16H, CH_2N), 3.5–4.2 (m, 8H, $CH_2NC(O)$); ^{13}C NMR (90.56 MHz, 25 °C, [D$_6$]DMSO): $\delta = 27.7$ (*t*Bu), 48.9, 50.1, 50.7, 50.8, 53.8, 54.2, 54.4 (CH_2N), 81.3 (*t*Bu), 169.50 (C=O); $C_{28}H_{51}N_4O_8K \cdot 2.8\,HBr \cdot 2.36\,H_2O$ (879.9): calcd C 38.22; H 6.70; N 6.37; Br 25.82; found C 38.40; H 6.78; N 6.49; Br 25.82.

1,4,7,10-Tetraazacyclododecane-4,7,10-tricarboxymethyl-*tert*-butylester-1-yl-acetyl-D-Phe-Cys-Tyr-D-Trp-Lys(BOC)-Thr-Cys-L-threoninol (disulfide bond) (DOTA^0(*t*Bu)$_3$-Phe^1-Tyr^3-Lys^5(BOC)-octreotide, 5): Compound **4** (63.0 mg, 90 µmol), HATU (*O*-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (34.2 µL, 90 µmol), and DIPEA ((*N*,*N*′-diisopropylethylamine) (15.3 µL, 90 µmol) were preincubated in DMF (1.5 mL). After 10 min, Tyr^3-Lys^5(BOC)-octreotide (87.9 mg, 75 µmol) and DIPEA (15.1 µL, 90 µmol) dissolved in DMF (1 mL) were added. Stirring was continued for 4 h to complete the reaction, then EtOAc (5 mL) and an aqueous solution of $NaHCO_3$ (5%, 2 mL) were added. The organic layer was washed with $NaHCO_3$ solution (5%, 3×2 mL) and the water layer with EtOAc (4×2 mL). The combined organic layers were washed with H_2O (4×2 mL). Evaporation afforded a crude product (151.8 mg) as a white solid which was not purified further.

1,4,7,10-Tetraazacyclododecane-4,7,10-tricarboxymethyl-1-yl-acetyl-D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Cys-L-threoninol (disulfide bond) (DOTA^0-Phe^1-Tyr^3-octreotide, 6): Compound **5** (151.8 mg raw product) was dissolved in a deprotection mixture (TFA/thioanisole/H_2O, 92:6:2, v/v, 2 mL). After stirring for 4 h the solvent was removed by evaporation and the residue redissolved in H_2O (2 mL) and EtOAc (1 mL). The organic layer was washed with H_2O (3×0.5 mL) and the water layer with EtOAc (3×0.5 mL). The combined water layers were purified by RP-HPLC (Vydac 218TP510, 5 µm, C_{18}, 1×25 cm, eluent: A: NH_4OAc (20 mM, pH 5); B: AcCN; gradient: from 0–50% in 30 min at 1 mL min^{-1}). Lyophilisation afforded the pure compound (83.1 mg, 58.5 µmol) in 65% yield. MS-ESI: *m*/*z* (%): 1421.7 $[M+H]^+$ (16), 711.7 $[M+2H]^{2+}$ (97), 474.7 $[M+3H]^{3+}$ (100); $C_{65}H_{92}N_{14}O_{18}S_2 \cdot 7\,H_2O \cdot 0.4\,AcOH \cdot 0.05\,TFA$ (1577.37): calcd C 50.18; H 6.81; N 12.43; found C 50.38; H 6.74; N 12.40; amino acid analysis: Thr 0.86 (1), Cys 1.20 (2), Tyr 1.00 (1), Phe 0.99 (1), Lys 1.07 (1), Trp det. (1); purity (HPLC): >97%.

1,4,7,10-Tetraazacyclododecane-4,7,10-tricarboxymethyl-*tert*-butylester-1-yl-acetyl-D-PheNH$_2$ (DOTA(*t*Bu)$_3$-D-PheNH$_2$, 7): Compound **4** (350.0 mg, 500 µmol), HOBT (1-hydroxy-benzotriazol) (80.0 mg, 500 µmol) and H-D-PheNH$_2$ (82.0 mg, 500 µmol) were preincubated in DMF (7 mL). After 10 min, DIPEA (85.6 µL, 500 µmol) was added and the mixture cooled to 4 °C. DCC (*N*,*N*′-dicyclohexyl-carbodiimide) (270.0 mg, 1.310 mmol) in DMF (1 mL) was added and the pH adjusted to 7–8. Stirring was continued for 20 h to complete the reaction. The reaction mixture was filtered and EtOAc (25 mL) and H_2O (5 mL) were added to the filtrate. The organic layer was washed with a $NaHCO_3$ solution (5%, 3×3 mL) and the water layer with EtOAc (4×3 mL). The combined organic layers were washed with a saturated NaCl solution (4×2 mL), dried over Na_2SO_4, filtered and evaporated to dryness to afford the product (217 mg, 302 µmol) in 60% yield.

1,4,7,10-Tetraazacyclododecane-4,7,10-tricarboxymethyl-1-yl-acetyl-D-Phe-NH$_2$ (DOTA-D-PheNH$_2$, 8): Compound **7** (217.0 mg, 302 µmol) was dissolved in a deprotection mixture (TFA/H_2O, 95:5, 3 mL), the mixture was stirred for 5 h and then isopropyl ether/petrol ether (1:1, 15 mL) was added. The oily product was decanted from the solvent and washed with isopropyl ether/petrol ether (1:1, v/v, 3×4 mL) and evaporated to dryness. The crude product was dissolved in water (2 mL) and purified by ion exchange chromatography (DOWEX 1x8, Cl^-, 50–100 mesh, 1.33 meq mL^{-1}). Lyophilisation afforded the compound (152.8 mg, 278 µmol) in 92% yield. MS-ESI: *m*/*z* (%): 573.3 $[M+Na]^+$ (4), 551.3 $[M+H]^+$ (37), 276.3 $[M+2H]^{2+}$ (100); purity (HPLC): >99%; ^1H NMR (400 MHz, 27 °C, D_2O): $\delta = 2.45-4.10$ (m, 26H, CH_2N, CH_2Phe), 4.64 (br s, 1H, CH), 7.22–7.40 (m, 5H Phe); IR (KBr): $\tilde{\nu} = 3410$, 3084, 1733, 1675, 1555, 1490, 1456, 1389, 1353, 1218, 1163, 1087, 1022, 992, 764, 705 cm^{-1}.

Ga-DOTA-D-PheNH$_2$ (9): The Ga complex was obtained from the reaction of **8** (10 mg, 18.1 µmol) with $Ga(NO_3)_3 \cdot 9\,H_2O$ (9.1 mg, 21.7 µmol) in an aqueous solution (1 mL, pH 4, 80 °C, 30 min). After cooling to room temperature, the reaction mixture was adjusted to pH 8 by the addition of NaOH in order to remove the excess uncomplexed gallium by precipitation of $Ga(OH)_3$, which was then removed by filtration. The pH of the filtrate was adjusted to pH 7 by the addition of HCl. Slow evaporation from DMSO/H_2O afforded colourless single crystals which were suitable for X-ray analysis. MS-ESI: *m*/*z* (%): 617.2 $[M+H]^+$ (100), 639.1 $[M+Na]^+$ (70); ^1H NMR (400 MHz, 27 °C, D_2O): $\delta = 2.2-3.95$ (m, 26H, CH_2N, CH_2Phe), 4.64 (br s, 1H, CH), 7.22–7.35 (m, 5H Phe); IR (KBr): $\tilde{\nu} = 3417$, 3263, 1705, 1667, 1610, 1560, 1495, 1446, 1385, 1361, 1308, 1081, 921, 985, 771, 745, 705 cm^{-1}.

Y-DOTA-D-PheNH$_2$ (10): The Y complex was obtained from the reaction of **8** (9.7 mg, 16.9 µmol) with $Y(NO_3)_3 \cdot 5\,H_2O$ (7.4 mg, 20.3 µmol) in an aqueous solution (1 mL, pH 4, 80 °C, 30 min). After cooling to room temperature, the reaction mixture was adjusted to pH 9 by the addition of NaOH in order to remove the excess uncomplexed yttrium by precipitation of $Y(OH)_3$, which was then removed by filtration. The pH of the filtrate was adjusted to pH 7 with HCl. Slow evaporation from DMSO/H_2O afforded colourless single crystals which were suitable for X-ray analysis.

MS-ESI: m/z (%): 637.3 $[M+H]^+$ (100), 323.2 $[M+H+Li]^{2+}$ (88); ^1H NMR (400 MHz, 27 °C, D_2O): $\delta = 2.6-3.75$ (m, 26H, CH_2N, CH_2Phe), 4.75 (br s, 1H, CH), 7.25–7.40 (m, 5H Phe); IR (KBr): $\tilde{\nu} = 3417$, 3263, 1688, 1626, 1590, 1495, 1455, 1398, 1328, 1280, 1246, 1085, 1015, 973, 930, 833, 795, 764, 725, 705 cm^{-1}.

X-ray diffraction studies of 9 and 10: All measurements were performed on a Nicolet P3 diffractometer with graphite-monochromated $Mo_{K\alpha}$ radiation. The crystallographic data are summarised in Table 1 and the average bond lengths, angles and planes are given in Table 2. The structures were solved with Patterson methods, expanded by Fourier techniques and refined against F^2 by full-matrix least-squares methods with the ShelX 93 crystallographic software package.[27]

Table 1. Crystallographic data for the compounds **9** and **10**.

	9	**10**
chem. formula	$C_{25}H_{35}GaN_6O_8 \cdot 3H_2O$	$C_{25}H_{35}N_6O_8Y \cdot 9H_2O$
fw	664.30	764.50
cryst. size [mm]	$0.4 \times 0.4 \times 0.2$	$0.4 \times 0.2 \times 0.2$
space group	$P2_12_12_1$	$P3_1$
cryst. syst.	orthorhombic	trigonal
a [Å]	9.563(19)	11.958(17)
b [Å]	13.616(3)	11.958(17)
c [Å]	21.878(4)	20.238(4)
V [Å^3]	2848.7(10)	2506.2(7)
Z	4	3
ρ_{calcd} [g cm^{-3}]	1.549	1.520
T [K]	293(2)	183(2)
μ [mm^{-1}]	1.037	1.825
$\lambda(Mo_{K\alpha})$ [Å]	0.71073	0.71073
$2\theta_{max}$ [°]	52	56
measured refl.	3329	9435
unique refl.	3301	6797
obs. refl., $I > 2\theta(I)$	2632	6330
no. of parameters	389	445
restraints	0	4
$R(F_o)$[a]	0.0505	0.0698
$Rw(F_o)$[b]	0.1133	0.1764
$(\Delta/\rho)_{max}$ [e Å^{-3}]	0.659	1.637
$(\Delta/\rho)_{min}$ [e Å^{-3}]	−0.531	−0.525
goodness of fit	1.030	1.046

[a] $R = \Sigma||F_o| - |F_c||/\Sigma|F_o|$ (obsd. reflections). [b] $Rw = [\Sigma w(|F_o| - |F_c|)^2/\Sigma|F_o|^2]^{1/2}$ (all reflections).

Table 2. Selected average bond lengths [Å], angles [°] and planes [°] for the compounds **9** and **10**.[a]

	9	**10**
CN	6	8
ring config.	2424	3333
M–N [Å]	eq 2.128(7)	2.418(35)
	ax 2.154(9)	
N-M-N [°]	eq 156.3(2)	73.5(6)
	ax 105.6(2)	
O-M-O [°]	eq 86.2(2)	73.25(10)
M–O [Å]	eq 1.918(5)	2.274(30)
D_N [Å]		1.289
D_O [Å]		1.221
D_{N2O2} [Å]	0.043	
R_N [Å]		0.025
R_O [Å]		0.025
R_{N2O2} [Å]	0.028	
P_N–P_O [°]		0.36

[a] CN is the coordination number of the metal; D_N, D_O, D_{N2O2} are metal-to-plane distances; R_N, R_O, R_{N2O2} are the deviations of the defining atoms from the different planes (P_N, P_O, P_{N2O2}); P_N–P_O is the angle between the planes.

Crystallographic data (excluding structure factors) for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication nos. CCDC-101 668 (gallium complex) and CCDC-101 669 (yttrium complex). Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: (+44) 1223-336-033; e-mail: deposit @ccdc.cam.ac.uk).

Radiotracers: ^{111}In-, ^{67}Ga- and ^{90}Y-DOTATOC were prepared as follows: DOTATOC (8 μg, 5.1 nmol) and gentisic acid (7 mg, 45 μmol) were dissolved in sodium acetate buffer (190 μL, 0.4 M, pH 4.8–5.5); after the addition of $^{111}InCl_3$, $^{67}GaCl_3$ or $^{90}YCl_3$ (6 mCi), the solution was heated at 95 °C for 25 min to afford the very pure radioligands with specific activities > 40 GBq μmol^{-1}. Iodination of Tyr^3-octreotide was performed by the reported chloramine T method.[28] In all cases, a quality control was performed with HPLC (column: Machery Nagel, Nucleosil 120-C_{18}; eluents: A = 0.1 % TFA in H_2O and B = AcCN: gradient: 0–5 min, 100 % A; 25 min, 75 % A; 30–35 min, 100 % A).

In vitro studies of the serum stability: Measurements of the rate of exchange of the radiometal from ^{111}In-, ^{67}Ga- or ^{90}Y-labelled DOTATOC to serum proteins was performed with a reported procedure:[29] in brief, radiolabelled DOTATOC was mixed with human blood serum (5 μCi per 3 mL serum) and the exchange kinetics measured at 37 °C (CO_2/air, 5:95 %), by taking aliquots of the serum solution at various time intervals. Subsequently, a gel-filtration (Sepahadex G-50, column 20 × 0.5 cm, eluent: 0.01 M PBS buffer, pH 7.4, flowrate: 0.5 mL min^{-1}) was performed with the lower molecular fractions (intact DOTA conjugate) which elute between 10 and 25 min, whereas serum proteins eluted between 5 and 9 min. The percentage of radiometal scavenged by the serum proteins was than calculated from the ratio of the radioactivity measured in the respective fractions.

In vitro studies of receptor binding: Studies of the SRIF-receptor binding were performed in a rat brain cortex membrane assay, as described in the literature:[30] briefly, cerebral cortex from male Sprague Dawley rats was dissected and homogenised in HEPES buffer (10 mM, pH 7.6), centrifuged and washed several times with the same buffer. For the binding assay, the membranes were diluted to 50 μg protein/assay tube in HEPES buffer containing $MgCl_2$ (10 mM) and Bacitracin (14 mM). Binding assays consisted of radioligand (70 μL), buffer (1 % bovine serum albumin in 10 mM HEPES buffer, pH 7.6, 30 μL) or increasing concentrations of the SRIF analogues and membrane suspension (200 μL). The tubes were incubated for 30 min at room temperature with ≈20 000–70 000 cpm (6—40 fmol) of radiolabelled peptide per tube (300 μL). Incubation was stopped by rapid filtration through Whatmen GF/C glass-fibre filters and subsequent washing with TRIS (10 mM)/NaCl (150 mM) buffer (pH 7.5, 4 °C, 3 × 2 mL). All experiments were carried out in triplicate. Binding data were best-fitted according to a one-site receptor model. The dissociation constants (K) were calculated by Scatchard analysis.

Biodistribution studies: Nude mice: IFFA-CREDO (France) bearing the AR4-2J tumour (rat pancreatic tumour, size: 0.05–0.1 g), implanted subcutaneously, (5 mio cells), received the radioligand (5 μCi) in NaCl buffer (pH 7.5, 0.1 % bovine serum albumine, 200 μL) intravenously in the lateral tail vein. The animals were sacrificed at various time intervals by cervical dislocation, organs of interest and blood were collected and the radioactivity in these samples measured by use of a γ-detector. For the determination of non-specific binding, cold octreotide (50 μg) was injected intravenously in the lateral tail vein together with the radiopeptide. Urine samples were analysed for possible metabolites by HPLC. All animal experiments were performed in compliance with the Swiss regulations for animal treatment (Bundesamt für Veterinärwesen, approval no. 798).

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