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Structural Study by NMR of an Oxorhenium–RGD Decapeptide Complex for Application in Radiotherapy

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The decapeptide Arg-Gly-Asp-Ser-Cys-Arg-Gly-Asp-Ser-Tyr, which contains two Arg-Gly-Asp (RGD) moieties in its sequence, has been successfully labeled with radioactive rhenium (Re-188) yielding a single, stable oxorhenium complex. This complex is being evaluated for possible application in oncology as a target-specific radiotherapeutic agent, because its radioactive technetium-99m analogue has already been applied for the scintigraphic detection of malignant melanoma in humans. For structural characterization purposes, the complex of the decapeptide was synthesized at the macroscopic level using nonradioactive rhenium (Re-185/Re-187). NMR and mass spectral analysis of the nonradioactive oxorhenium complex revealed that the decapeptide coordinates to the oxorhenium core through the N_{amide} of Asp3, the N_{amide} of Ser4, and the N_{amide} and $S_{thiolate}$ atoms of Cys5 to form a complex of the $ReO[N_3S]$ type.

Introduction

The amino acid sequence Arg-Gly-Asp (RGD) is considered to be the common epitope that many members of the integrin family of cell surface receptors recognize on their ligands.[1] Integrins are the main receptors by which cells attach to extracellular matrixes, and they also mediate important cell–cell adhesion processes including platelet aggregation and thrombus formation.[2] In addition, they appear to play a key role in tumor invasion, dissemination, and cell proliferation of various neoplasias including malignant melanoma, osteosarcoma, and glioblastoma.[3] A number of synthetic peptides that contain the RGD sequence have been shown to bind to cancer cells via the corresponding cell–surface integrins and to inhibit invasion in vitro and tumor dissemination in vivo.[4]

A number of technetium-99m-labeled RGD-containing molecules have been designed, synthesized, and evaluated for the detection and imaging of thrombi.[5] Within this framework, the synthetic linear decapeptide Arg-Gly-Asp-Ser-Cys-Arg-Gly-Asp-Ser-Tyr (RGDSCRGDSY), which contains two RGD moieties in its sequence, was complexed with the oxotechnetium-99m ($^{99m}TcO(V)^{3+}$) core and applied successfully for the detection of experimentally induced

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thrombi in rabbits.[6] Furthermore, it was administered to patients with malignant melanoma,[7] a disease of high metastatic potential with cells expressing RGD-binding integrins,[3] and was found to bind specifically to adhesion molecules on tumors, permitting thus the in vivo detection of neoplastic metastases.

In the present study, we report the synthesis and structural characterization of the complex of the RGDSCRGDSY decapeptide with the oxorhenium (ReO(V)$^{3+}$) core and the transfer of the synthetic procedure at the radioactive oxorhenium-188 tracer level, with the ultimate goal of preparing a radiotherapeutic agent. In recent years, attention has been focused on the development of β-emitting radiopharmaceuticals for therapy[8] that will selectively accumulate and deliver therapeutically significant radiation doses at malignant sites, while keeping the radiation exposure of the surrounding normal tissues to a minimum. ^{188}Re possesses nuclear characteristics ($t_{1/2}$ 17 h, γ 155 keV, β^- 2.12 MeV) favorable for use in radiotherapy. Because the coordination chemistry of rhenium is analogous to that of technetium,[9] it is expected that reaction of the RGDSCRGDSY peptide with the oxorhenium core will yield a complex analogous to that of the radioactive oxotechnetium-99m core that will display affinity for metastatic melanoma in vivo and may be potentially applied in oncology as a target-specific radiotherapeutic agent.

Experimental Section

Synthesis of the ReO[RGDSCRGDSY] Complex 1. The synthesis of the RGDSCRGDSY peptide has been described before.[6] For the synthesis of complex **1**, a methanolic solution of the decapeptide (3 mg, 2.7 nmol) with [Bu$_4$N]$^+$[ReOCl$_4$]$^-$ in molar ratio 1:1 was allowed to react at room temperature for 3 days. After evaporation of the solvent, the residue was purified by HPLC using a μ-Bondapack C$_{18}$ reversed-phase column from Waters Assoc. The eluents used were (A) 0.05% TFA in water and (B) 60% acetonitrile/40% water with 0.05% TFA. The linear gradient system applied ranged from 100% A at zero time to 100% B at 20 min, at a flow rate of 1.3 mL/min. The HPLC fraction corresponding to the ReO[RGDSCRGDSY] (R_T = 11.0−12.0 min) derivative was isolated and freeze-dried to leave 1.2 mg of an oily, light purple product.

NMR Spectroscopy. The NMR study was conducted in 90% H$_2$O/10% D$_2$O at 21 °C on a 400-MHz Bruker DRX-Avance spectrometer using ∼1 mg of complex **1** in 0.5 mL of solvent. Total correlation spectroscopy (TOCSY) spectra were obtained with a spectral width of 4006 Hz in both dimensions, a mixing time of 80 ms, 512 t_1 increments, 128 scans, and 2K t_2 data points. Rotating frame Overhauser spectroscopy (ROESY) spectra were obtained

with the same spectral parameters and a spin-lock of 250 ms. Suppression of the water peak was achieved by employing the Bruker Watergate pulse sequence. Chemical shifts are relative to DSS.

Mass Spectrometry. ESI spectrometry was performed on a Micromass Platform II spectrometer equipped with electrospray probe. Complex **1** was dissolved in H$_2$O/CH$_3$CN 1:1 and the solution was acidified with 0.1% trifluoroacetic acid for analysis in the positive ion mode.

^{188}**Re Labeling of RGDSCRGDSY.** *Caution!! Re-188 is a γ and β^- emitter ($t_{1/2}$ 17 h, γ 155 keV, β^- 2.12 MeV). All manipulations of solutions and solids were carried out in a laboratory approved for the handling of radioisotopes and safety procedures were followed at all times to prevent contamination.*

A 2-mL sample of perrhenate-188 (^{188}ReO$_4^-$) eluate obtained by a ^{188}W/^{188}Re generator (MAP Medical Technologies Oy, Finland) was added to a mixture of reducing agents (SnCl$_2$ 1.6 mg; ascorbic acid 2.5 mg; citric acid 16 mg), and the resulting mixture was left for 2 h at room temperature. The 2.0-mL mixture containing the reduced rhenium-188 was added to 3 mg of the "gluconate kit" mixture prepared by adding 1 g of sodium gluconate, 2 g of sodium bicarbonate, and 15 mg of SnCl$_2$ to an empty vial and shaking well. The formation of the intermediate ^{188}Re−gluconate complex was monitored by ITLC in methyl ethyl ketone and by HPLC using the same conditions given above for the ReO[RGDSCRGDSY] complex. The separation was tested by both UV and radioactivity detectors, coupled in line.

A quantity of ∼2.0 mL of the above ^{188}Re−gluconate solution, containing 2.0−10.0 mCi of ^{188}Re, was added to 0.5 mL of an aqueous RGDSCRGDSY solution, containing 0.5 mg of the decapeptide, and the mixture was left to react for a period of 60 min at 80 °C. The reaction was monitored by HPLC, with the solvent system given above, and showed the formation of a single derivative with R_t = 11.0−12.0 min corresponding to that of the nonradioactive $^{185/187}$Re derivative and also to the R_t of the ^{99m}Tc-labeled RGDSCRGDSY under the same HPLC conditions.[6]

Results and Discussion

Synthesis and NMR Characterization of the ReO-[RGDSCRGDSY] Complex 1. Reaction of the RGDSCRGDSY decapeptide with the ReO(V)$^{3+}$ core led to formation of only one product as shown by HPLC and NMR. Complexes of peptides do not usually provide crystals suitable for X-ray crystallography, so high-resolution structural analysis of the complex was based mainly on NMR spectroscopy.

For the NMR studies, the decapeptide complex was dissolved in 0.5 mL of 90% H$_2$O/10% D$_2$O resulting in a pH 4.3 solution. Assignment of the individual amino acid residues (Table 1) relied upon the combined use of TOCSY and ROESY spectra. Inspection of the amide proton region of the spectra revealed that the amide protons of Asp3, Ser4, and Cys5 were missing (Figure 1). It is known that cysteine acts as a bidentate chelator and coordinates to oxotechnetium and oxorhenium cores both through the sulfur and the amide nitrogen.[10−12] Thus, it was reasonable to propose that the decapeptide coordinates to the oxorhenium core through the

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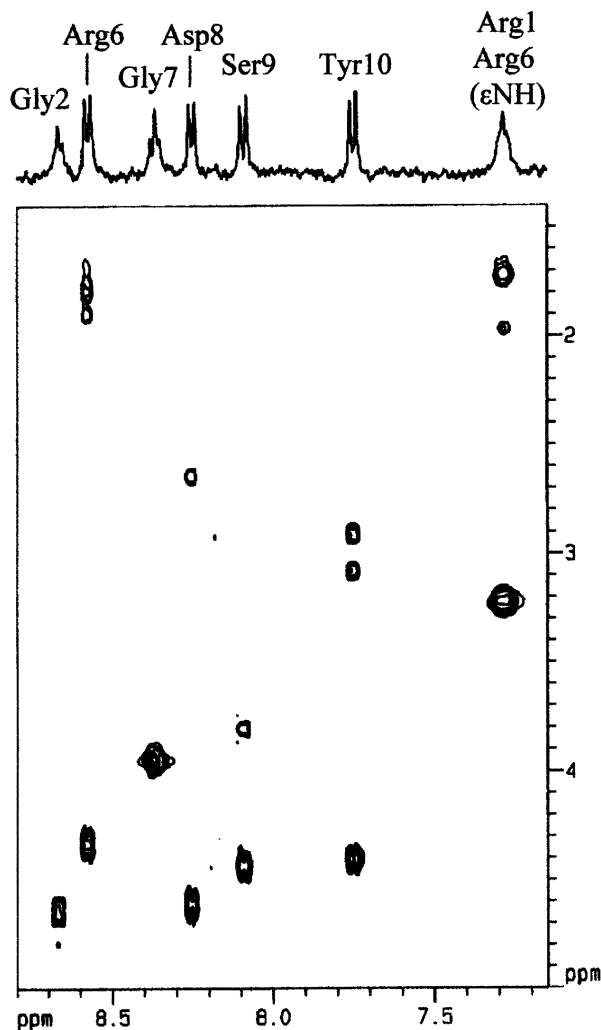
Figure 1. Amide to aliphatic proton region (F2, 8.80−7.15 ppm; F1, 5.00−1.40 ppm) of the 80-ms TOCSY spectrum of the ReO[RGDSCRGDSY] complex **1** in 90% H_2O/10% D_2O at 21 °C.

Figure 2. Proposed structure for the ReO[RGDSCRGDSY] complex **1** based on NMR and mass spectral data.

structure, the resonances of the α-CH protons of Asp3, Ser4, and Cys5 are shifted downfield, compared to the free decapeptide,[6] by 0.24, 0.37, and 0.26 ppm, respectively, while the β-CH protons of Cys5 are shifted downfield by 0.58 ppm.

In the proposed structure, the RGDSCRGDSY ligand complexes with $ReO(V)^{3+}$ via three deprotonated amide nitrogen atoms and one deprotonated thiol sulfur atom and the $ReO(V)^{3+}$ binding region is expected to carry a negative charge. The proposed structure of complex **1** is similar to the well-known structure of ^{99m}TcO−mercaptoacetyltri-glycine (^{99m}TcO−MAG_3), the standard radiopharmaceutical in commercial use for renal imaging,[13] in which the oxo-technetium core binds to the three deprotonated glycine amide nitrogen atoms and to the thiol sulfur atom to form a stable, negatively charged complex of the $TcO[N_3S]$ type and of its oxorhenium analogue.[14] In support of the proposed structure for complex **1** is also the currently reported NMR and mass spectral characterization of the $^{99}TcO(V)^{3+}$ complex of the synthetic tridecapeptide, apcitide,[11] in which oxotech-netium binds to three deprotonated amide nitrogens and one deprotonated thiol to form a complex of the $TcO[N_3S]$ type with the oxotechnetium core bearing a negative charge.

The overall charge of complex **1** will naturally depend on the pH of the aqueous solution and the state of the ionizable amino acids. At pH 4.3 of the NMR studies, complex **1** is expected to have an overall negative charge, because the side chains of the two basic amino acids (Arg1, Arg6) are expected to be fully protonated while the side chains of the two acidic amino acids (Asp3, Asp8) will be mostly deprotonated. Positive counterions that may accompany complex **1** are the Bu_4N^+ or the Na^+, which are present in the synthesis reaction mixture. The NMR data, however, exclude the presence of the Bu_4N^+ cation because no corresponding peaks are present in the ${}^1H$ spectrum of complex **1**.

Only one isomeric form of complex **1** was obtained even though the chelating part of the decapeptide is composed of chiral amino acids. Comparison of the NMR chemical shifts and coupling constants of the Cys5 residue to existing data

Table 1. ${}^1H$ Chemical Shifts (ppm) of the ReO[RGDSCRGDSY] Complex in 90% H_2O/10% D_2O at 21 °C

residue	NH	α-CH	β-CH	others
Arg1		4.10	1.96	γ-CH_2 1.71
				δ-CH_2 3.22
				ε-NH 7.27
Gly2	8.67	4.64		
Asp3		5.04	2.85	
Ser4		4.89	4.12, 4.23	
Cys5		4.98	3.58, 3.97	
Arg6	8.57	4.32	1.89	γ-CH_2 1.74
				δ-CH_2 3.18
				ε-NH 7.28
Gly7	8.36	3.94		
Asp8	8.24	4.62	2.64	
Ser9	8.08	4.42	3.80	
Tyr10	7.74	4.40	2.89, 3.07	arom o-CH 7.07
				arom m-CH 6.78

N_{amide} of Asp3, the N_{amide} of Ser4, and the N_{amide} and $S_{thiolate}$ atoms of Cys5 to form a complex of the ReO[N_3S] type (complex **1**, Figure 2). In accordance with the proposed

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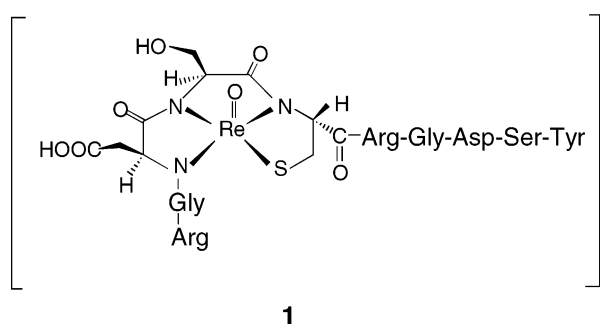
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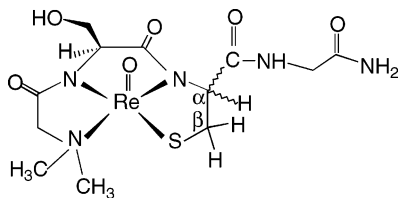
Figure 3. Structure of the ReO(V)$^{3+}$ complex of the peptide dimethyl-glycyl-L-seryl-L-cysteinylglycinamide as determined by X-ray crystal-lography.[15] The configuration of the α-CH proton of the cysteinyl residue can be syn or anti with respect to the oxygen of the ReO(V)$^{3+}$ core.

in the literature[15] on related ReO(V)$^{3+}$ complexes of known configuration indicates that the configuration of the α-CH of Cys6 is syn with respect to the oxygen of the oxorhenium core. Specifically, the values of the chemical shifts and coupling constants of the protons of the cysteinyl residue of the ReO[RGDSCRGDSY] complex **1** determined in our study (α-CH = 4.98 ppm, β-CH$_2$ = 3.58, 3.97 ppm, $^3J_{Hα−Hβ}$ = 8.9 Hz, $^3J_{Hα−Hβ'}$ = 0.8 Hz, and $^2J_{Hβ−Hβ'}$ = 13.1 Hz) are compared to the values reported for the protons of the cysteinyl residue of the stereoisomers of the oxorhenium complex (Figure 3) of the peptide dimethylglycyl-L-seryl-L-cysteinylglycinamide (α-CH = 5.09 ppm and β-CH$_2$ = 3.50, 3.97 ppm, $^3J_{Hα−Hβ}$ = 8.2 Hz, $^3J_{Hα−Hβ'}$ = 1.1 Hz, and $^2J_{Hβ−Hβ'}$ = 12.8 Hz for the syn configuration, and α-CH = 5.31 ppm and β-CH$_2$ = 3.51, 3.55 ppm, $^3J_{Hα−Hβ}$ = 4.6 Hz, $^3J_{Hα−Hβ'}$ = 7.7 Hz, and $^2J_{Hβ−Hβ'}$ = 12.4 Hz for the anti configuration). This comparison of the NMR data clearly indicates that in complex **1** the configuration of α-CH of Cys5 is syn with respect to the oxygen of the ReO(V)$^{3+}$ core with the rest of the Arg6-Gly7-Asp8-Ser9-Tyr10 chain occupying the anti configuration, as shown in Figure 2. Under this assumption, the configurations of the CH$_2$OH side chain of Ser4 and of the CH$_2$COOH side chain of Asp3 have to be syn as well with respect to the oxygen of the ReO core because these are the only configurations that allow the approach of the amide nitrogens of the L-amino acids to the oxorhenium core for binding. Preferred formation of one diastereomer over another is not uncommon in oxotech-netium[11] and oxorhenium[16] complexes with chiral amino acids, even though the position of the pendant side chains relative to the oxygen of the oxometal core leads in many cases to the observation of sets of diastereomers.[12,15,17]

Shown in Figure 4 is the three-dimensional structure of the ReO[RGDSCRGDSY] complex **1** as derived from the Hyperchem molecular modeling program (Hypercube, Inc.) incorporating the above structural data and utilizing crys-tallographical data from related ReO complexes in the literature.[15] Specifically, bond lengths and angles for the ReO environment were introduced as constraints and the molecule was energy minimized using the MM+ force field. In the geometrically optimized structure of Figure 4, the distance

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between the oxygen of the ReO core and the oxygen of the Ser4 hydroxyl group is ∼3.3 Å, the distance between the oxygen of the ReO core and the oxygen of the Asp3 carbonyl group is ∼3.2 Å, and the distance between the oxygen of the Ser4 hydroxyl group and the oxygen of the Asp3 carbonyl group is ∼3.0 Å. It therefore appears possible that the hydrogen of the Ser4 hydroxyl group is shared through hydrogen bonding between the three oxygen atoms of the ReO core, the Ser4 hydroxyl group, and the Asp3 carbonyl group, rendering additional stability to the complex. The $^3J_{Hα−Hβ}$ coupling constants for the Ser4 residue are both <4 Hz, being in agreement with the proposed orientation of the β-C−OH bond of Ser4, while the corresponding coupling constants for the Asp3 residue cannot be measured due to overlapping and second-order distortions.

During the course of the NMR studies, no interconversion between isomers in solution was observed.

Mass Spectrometry. The ESI spectrum of complex **1** gave (M + 2H)$^+$ peaks at *m/z* 1313.4 and 1315.4 as expected from the existence of two isotopes of rhenium, ^{185}Re and ^{187}Re. The calculated (M + 2H)$^+$ values for C$_{42}$H$_{64}$N$_{16}$SO$_{19}$^{185}Re and C$_{42}$H$_{64}$N$_{16}$SO$_{19}$^{187}Re corresponding to the proposed structure of **1** are *m/z* 1313.38 and 1315.39, respectively. The ratio of the intensity of the *m/z* 1313.4 peak to the intensity of the *m/z* 1315.4 peak is 58.7%, which is in rea-sonable agreement with the theoretical ratio of 59.7% based on the relative ^{185}Re/^{187}Re abundance. Prior to the calculation of the ratio, the intensity of the *m/z* 1315.4 peak was corrected for the M + 2 contribution of the *m/z* 1313.4 peak.

^{188}Re Labeling of the RGDSCRGDSY Peptide/Radio-biological Evaluation. Perrhenate, ^{188}ReO$_4$$^-$, was obtained from a ^{188}W/^{188}Re generator. Labeling of the peptide was carried out through the intermediate formation of a ^{188}Re−gluconate precursor,[18] the formation of which was monitored by instant thin-layer chromatography (ITLC) in methyl ethyl ketone and by high-performance liquid chromatography (HPLC).

A quantity of the above solution of the ^{188}Re−gluconate complex was added to an aqueous solution of the RGD-SCRGDSY decapeptide, and the mixture was heated at 80 °C for 60 min. HPLC analysis of the reaction mixture showed the formation of a single product with R_t = 11.0−12.0 min (Figure 5) corresponding to that of the nonradioactive $^{185/187}$Re derivative and indicating that complex **1** is also formed under the ^{188}Re-labeling conditions. The in vitro stability of the radiolabeled species was studied at ambient temperature and at 4 °C (storage temperature) by monitor-ing the formation of free perrhenate by HPLC. The ^{188}Re[RGDSCRGDSY] complex was stable for 64 h at both temperatures.

The evaluation of the in vivo behavior of the radiolabeled peptide was performed in normal Swiss mice (Table 2) after iv administration of the radiolabeled solution through the tail vein of the rodents. At time intervals ranging from 1 to 24 h, the main organs, as well as blood, muscle, and urine

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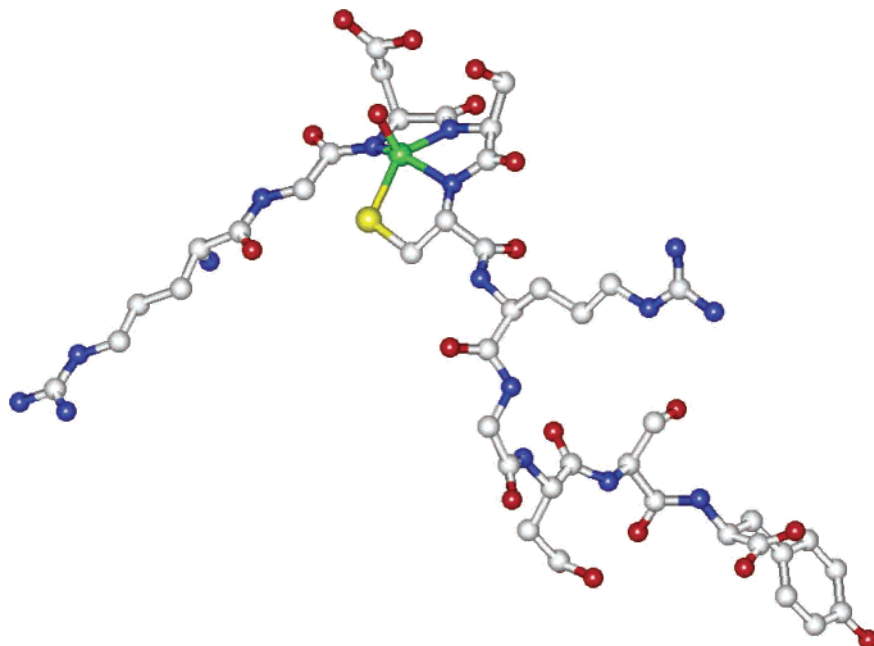
Figure 4. Three-dimensional depiction of the proposed structure for the ReO[RGDSCRGDSY] complex **1** from the Hyperchem program (Re = green, O = red, S = yellow, C = gray, and N = blue).
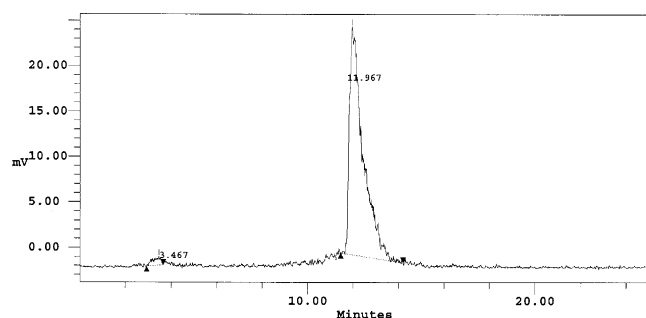

Figure 5. Typical HPLC profile of the [188]ReO[RGDSCRGDSY] complex under the conditions given in the Experimental Section.

Table 2. Biodistribution of the [188]ReO[RGDSCRGDSY] Complex in Swiss Mice

organ	% dose/organ[a]			
	15 min	30 min	60 min	120 min
blood	3.13	1.71	0.56	0.19
liver	0.62	0.59	0.34	0.12
lungs	0.15	0.08	0.06	0.07
stomach	0.16	1.07	0.55	0.55
kidney	2.94	1.64	0.98	0.19
intestine	0.71	1.09	0.96	1.54
muscle	5.47	2.67	2.80	0.42
urine	13.09	19.08	31.09	38.31

[a] For each time point, the average value from three animals is reported.

samples, were removed, weighed, and counted. The percentages of the injected dose per organ were calculated in comparison to a standard. The average value from three animals is reported for each time point. It was found that the [188]Re-labeled decapeptide presented fast blood clearance, since only 0.19% of the injected dose remained in circulation within 120 min pi. There was no gastrointestinal accumulation, and the radiolabeled biomolecule was practically completely eliminated via the urinary tract.

The in vivo studies were performed in compliance with the European legislation.

Concluding Remarks

The NMR data presented provide unambiguous evidence for the mode of binding of the RGDSCRGDSY decapeptide to the ReO(V)$^{3+}$ core and demonstrate, further to existing literature data,[11,12,16,17] that important structural information can be obtained from NMR studies of peptide complexes. Delineation of the structure of the ReO[RGDSCRGDSY] complex **1** is of great significance because it was shown that the same complex is formed under the labeling conditions with the radioactive rhenium-188. Furthermore, the ReO[RGDSCRGDSY] complex has the same HPLC retention time as the complex formed under the radioactive technetium-99m labeling conditions[6] suggesting that the proposed structure shown in Figure 2 applies also at technetium-99m tracer level. This structure can explain the tumor specificity of [99m]TcO[RGDSCRGDSY], which has been successfully applied for the scintigraphic detection of metastatic melanomas in humans,[7] because one of the two RGD moieties of the decapeptide sequence is left free and available to interact with surface receptors of cancer cells. Since technetium and rhenium form complexes with similar physical and chemical properties, it is expected that the [188]Re-labeled decapeptide will display tumor specificity and will prove to be a potential radiotherapeutic agent for oncology. In fact, ^{99m}Tc and [188]Re can be considered to be a matched pair for imaging and therapy, as the biodistribution studies of the [188]Re-labeled decapeptide show very fast blood clearance and complete urine elimination in accordance with the ^{99m}Tc-labeled species. Cancer cell binding studies are in progress.