Solid-Phase Synthesis of a Cyclic NGR-Functionalized Gd^{III}DTPA Complex

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A convenient methodology has been developed for the solid-phase synthesis of a cyclic NGR-functionalized Gd^{III}DTPA complex employing an isocyanate-functionalized DTPA pentaester. This methodology is an alternative strategy for the tagging of oligopeptides with Gd^{III}DTPA as an MRI label on the solid phase.

Introduction

Magnetic Resonance Imaging (MRI) contrast agents based on the gadolinium complex of diethylenetriaminepentaacetic acid (Gd^{III}DTPA) are generally employed as diagnostic agents in medical imaging.[1,2] The low toxicity coupled with an excellent solubility in water are major advantages of Gd^{III}DTPA complexes.[1,2] One of the drawbacks, however, is their nonspecifity, which necessitates the use of relatively high concentrations of MRI contrast agent. An improvement of the effectiveness of Gd^{III}DTPA-based contrast agents can be accomplished by incorporating target-specific oligopeptides to induce accumulation of MRI probes at regions of interest.[3] Recently, a cyclic peptide containing the Cys–Asn–Gly–Arg–Cys (CNGRC) sequence (cNGR) was identified as a targeting unit for the aminopeptidase CD13 that is overexpressed on endothelial cells during angiogenesis.[4,5] We designed a cNGR–Gd^{III}DTPA construct (**7**) composed of the cNGR targeting domain, with an intramolecular disulfide bridge between the cysteine residues, and a Gd^{III}DTPA complex for imaging of angiogenesis (Scheme 1). The gadolinium chelate is introduced at the ε-amine of the lysine side chain of the peptide. The targeting peptide motif and the Gd^{III}DTPA complex are connected with a glycinylglycine spacer to enhance peptide flexibility and to minimize potential steric interactions that may hamper binding.[6]

In recent years, various types of chelates, including DTPA and DOTA derivatives (DOTA = 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*′′,*N*′′′-tetraacetic acid), have been attached to peptides.[7–15] Traditionally, peptides have been functionalized with DTPA moieties using the cyclic dianhydride of DTPA that reacts with amine functionalities of a peptide either in solution or on solid-phase.[7–9] However, this is a nonselective reaction resulting in a mixture of monoamide-DTPA and bisamide-DTPA conjugates, which is an inherent disadvantage of this methodology.[16] Moreover, Sherry et al. reported that a significant decrease in the thermodynamic stability of the Gd^{III}DTPA complex occurs upon conversion of even one of the five carboxylic acids of DTPA into an amide.[17] When designing synthetic approaches to stable Gd^{III}DTPA complexes, it is important that all five carboxylic acids remain available for Gd^{III} complexation in order to reduce toxity both in vitro and in vivo.[18–20] Alternative DTPA pentaester building blocks based on either glutamic acid (carboxylate terminated) or lysine (amine terminated) were reported by Williams et al.[21] and Anelli et al.[18] Recently, De Luca et al. reported a solid-phase route to a DTPA-functionalized cholecystokinin octapeptide (CCK8) using the glutamic acid-based DTPA pentaester.[10] So far, the lysine-based DTPA analogue was not utilized for the solid-phase peptide synthesis. The first construct of cNGR with Gd^{III}DTPA was synthesized using native chemical ligation.[22] In this paper, we present an alternative approach for the labeling of peptides with DTPA, in our case cNGR, on solid phase employing an isocyanate-functionalized lysine-based DTPA pentaester **3**, which can be readily prepared from lysine-based DTPA.

Results and Discussion

Solid-phase peptide synthesis (SPPS) using the in situ neutralization/2-(1*H*-benzotriazol-1-yl)-1,1,3,3-(tetraethyl)uronium hexafluorophosphate (HBTU) activation

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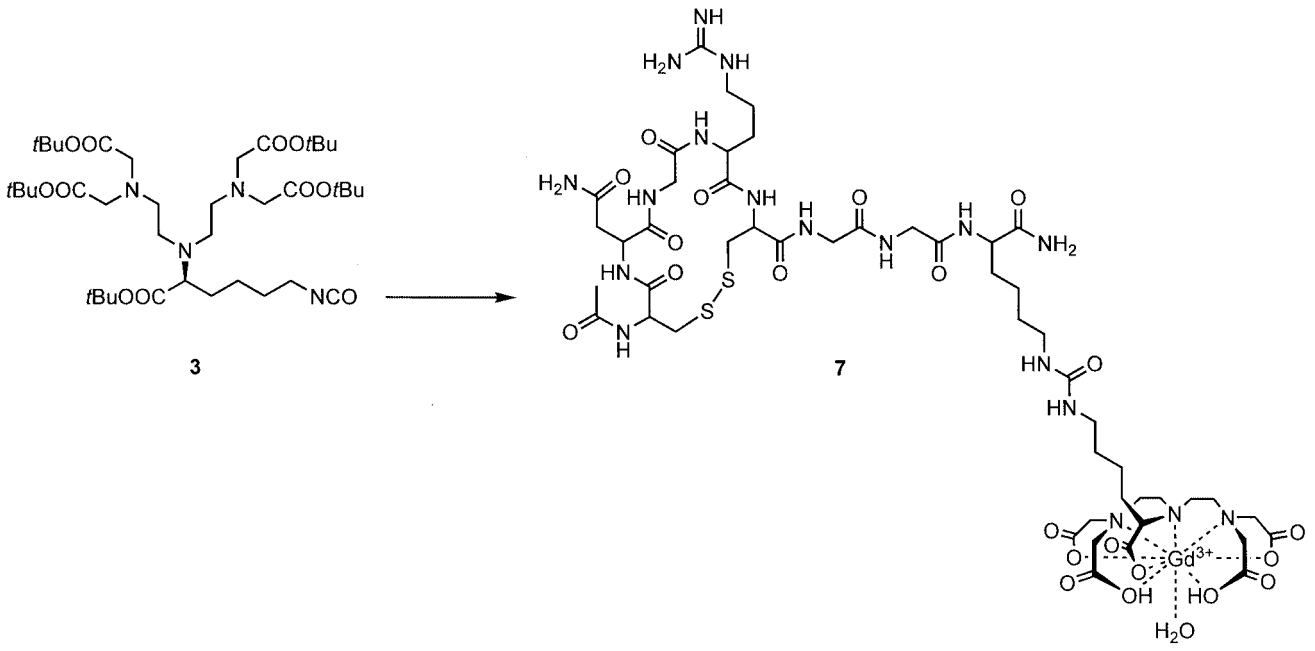


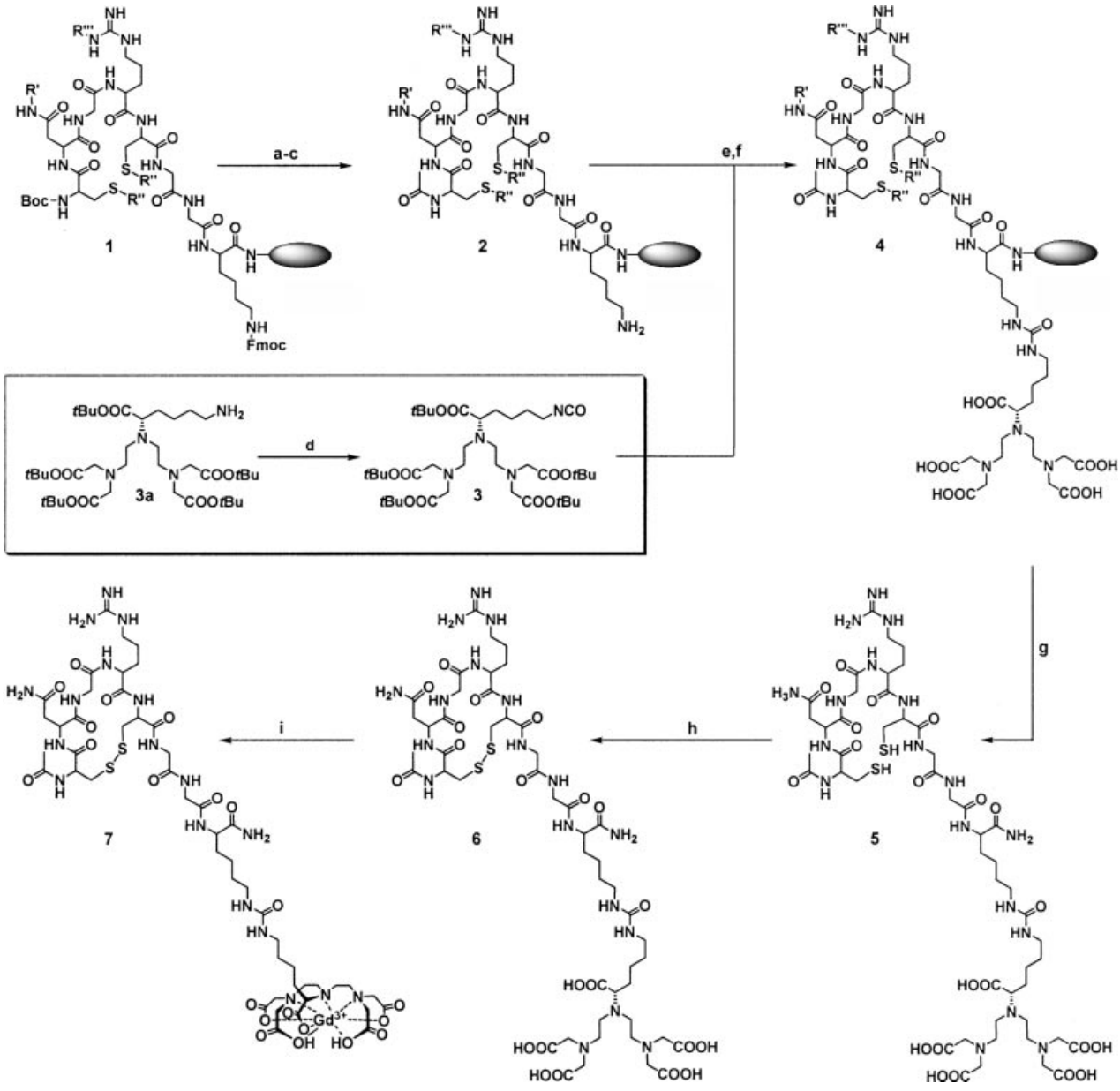
Scheme 1. Preparation of cyclic NGR-functionalized Gd^{III}DTPA (cNGR–Gd^{III}DTPA) **7** from the isocyanate-functionalized DTPA pentaester **3**. The gadolinium chelate is introduced at the ε-amine of the lysine side chain of the peptide.

procedure for Boc chemistry on a MBHA resin[23] was applied to synthesize side chain-protected BocCNGRCGGK(Fmoc)-MBHA (**1**) containing the target-specific NGR sequence (Scheme 2). The N^{α}-Boc group of the oligopeptide was removed with trifluoroacetic acid (TFA) and the corresponding primary amine was acetylated with acetic anhydride and pyridine. Subsequently, the Fmoc protective group of the lysine side chain was removed with 20% piperidine in DMF to give partially side chain-protected NAcCNGRCGGK(NH_2)–MBHA (**2**).

The amine-functionalized DTPA analogue **3a**, in which the five carboxylic acid residues are protected with *tert*-butyl esters, was prepared according to a literature procedure.[18,21] Our strategy deals with the conversion of the amine of lysine-based DTPA analogue **3a** into the corresponding isocyanate **3** using di-*tert*-butyl tricarbonate, which is a versatile reagent for the quantitative conversion of primary amines into isocyanates under mild reaction conditions.[24,25] This reaction was performed in DCM since this procedure cannot be performed in DMF (the preferred solvent for solid-phase peptide synthesis) due to decomposition of di-*tert*-butyl tricarbonate. A droplet of pyridine was added to quench the excess di-*tert*-butyl tricarbonate and dichloromethane (DCM) was removed under an argon flow. The crude mixture was redissolved in DMF and directly used for the reaction with the peptide on the solid phase. The ε-amine group of the lysine side-chain was reacted in DMF with a sevenfold excess of isocyanate-functionalized DTPA analogue **3**. The excess of **3** was easily removed by washing with DMF. Subsequently, the *tert*-butyl esters were removed with trifluoroacetic acid (TFA), to yield side chain-protected NAcCNGRCGGK(DTPA)–MBHA (**4**). The coupling efficiency between the isocyanate-functionalized DTPA pentaester **3** and the primary ε-amine of the lysine side chain of **2** was ca. 67% based on the mass increase of MBHA resin. The DTPA-functionalized oligopeptide was cleaved from the resin with anhydrous HF and lyophilized to give NAcCNGRCGGK–DTPA (**5**).

The coupling efficiency of ca. 67% between **3** and the primary ε-amine of the lysine side chain was confirmed by analytical reversed phase HPLC (RP HPLC) on the crude mixture. The reaction mixture containing **5** was used for the next reaction without further purification, avoiding additional product loss due to HPLC purification procedures. The oxidation of the sulfhydryl functionalities of the cysteine residues was performed in 1 M of guanidine in 0.1 M Tris buffer at pH 8 and was monitored with analytical RP HPLC and ESI-mass spectrometry. Quantitative formation of the disulfide bridge was accomplished after 3 h [Figure 1, note the mass difference between **5** and cNGR–DTPA (**6**) in the ESI-MS spectra below is only 1 amu, due to the doubly charged nature of **5** and **6**]. Semi-preparative RP HPLC purification and subsequent lyophilization yielded **6** in an overall isolated yield of 19%. The gadolinium complex **7** was prepared by adding 0.9 equivalents of gadolinium chloride to a solution of **6** in water. The pH was mainained at 6.5–7 by adding ammonium hydroxide. A slight excess of **6** was used to ensure the absence of free gadolinium, which is highly toxic. The gadolinium complex **7** was characterized with IR spectroscopy and ESI-MS (see supporting information; for supporting information see also the footnote on the first page of this article), while the gadolinium content was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES). The value for the observed gadolinium content was found to be 94% of the theoretical value.

Gd^{III}DTPA-based MRI contrast agents are used to improve the contrast in MR images by lowering the longitudi-



Scheme 2. Synthesis of cNGR-Gd^{III}DTPA **7**. R′–R′′′ represent the protective groups of the amino acids, with R′ = xanthyl, R′′ = 4-methylbenzyl and R′′′ = *p*-tolylsulfonyl. (a) TFA, (b) acetic anhydride (0.25 M), pyridine (0.25 M) in DMF, (c) 20% piperidine in DMF, (d) di-*tert*-butyl tricarbonate in DCM, 2 h, argon, (e) **3** (7 equiv.) in DMF, (f) TFA, (g) cleavage from MBHA resin with anhydrous HF, (h) 1 M guanidine in 0.1 M Tris buffer, (i) $GdCl_3$ (0.9 equiv.), NH_4OH, H_2O, pH 6.5–7.

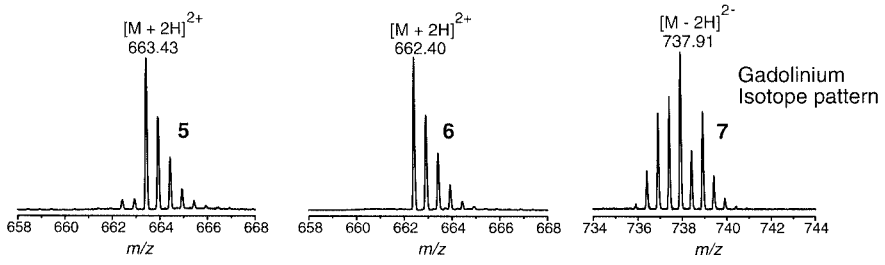


Figure 1. ESI-MS spectra of **5**, cNGR-DTPA (**6**) and cNGR-Gd^{III}DTPA (**7**). The *m*/*z* region of interest shows the doubly charged state of the constructs.

nal relaxation times (T_1) of the protons of water molecules. The ability of Gd^{III}DTPA-based complexes to lower the T_1 values is expressed in terms of longitudinal relaxivity $\{r_1 = [(1/T_1)_{observed} - (1/T_1)_{diamagnetic}]/[Gd^{III}]\}$.[2] The longitudinal relaxivity (r_1) of **7** (normalized to the ICP-AES data) was determined by measuring the concentration dependency of its relaxation time at 1.5 T and 20 °C (Figure 2). The data gave a good linear fit ($R^2 > 0.999$) to the equation $(1/T_1)_{observed} = (1/T_1)_{diamagnetic} + r_1[Gd]$ and an r_1 of 9.8 $mM^{-1}\,s^{-1}$ was calculated. The r_1 of **7** is at least twice the r_1 value of the parent Gd^{III}DTPA complex (r_1 = 4.2 $mM^{-1}\,s^{-1}$ at 1.5 T and 20 °C), which is presumably due to the slower molecular tumbling of the gadolinium complex as a result of the higher molecular weight (1.5 $kgmol^{-1}$ for **7** vs. 0.6 $kgmol^{-1}$ for Gd^{III}DTPA).[1] The r_1 of **7** is in line with previously reported values on oligopeptide-functionalized Gd^{III}DTPA complexes.[26]

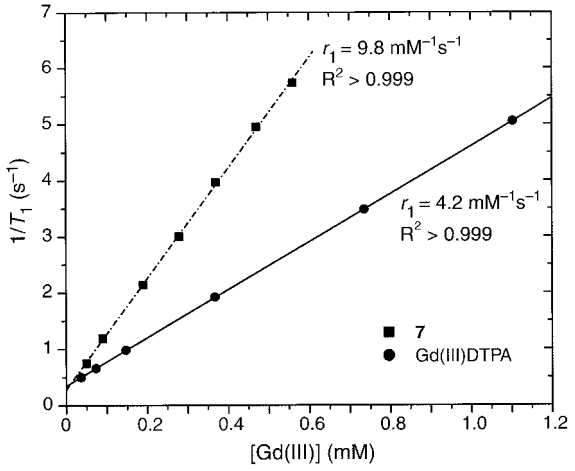


Figure 2. Longitudinal relaxation rate ($1/T_1$) vs. the concentration of gadolinium in water at pH 7 at 1.5 T and 20 °C.

Conclusions

A convenient methodology has been developed for the solid-phase synthesis of a cNGR-functionalized Gd^{III}-DTPA complex **7** using an isocyanate-functionalized DTPA pentaester. This methodology is an alternative strategy for the tagging of oligopeptides with Gd^{III}DTPA as an MRI label on the solid-phase. *In vivo* studies using mouse models will be performed to demonstrate the efficacy of **7** as an MRI contrast agent for target-specific imaging of angiogenesis.

Experimental Section

General: 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and Boc-amino acids were obtained from NovaBiochem (USA). Boc-Arg (p-toluenesulfonyl)-OH and Boc-Asn(xanthyl)-OH were purchased from Midwest Biotech (Fischers, USA). 4-Methylbenzhydrylamine (MBHA) resin was obtained from AnaSpec (USA). Trifluoroacetic acid (TFA) was obtained from Halocarbon (USA). *N*,*N*-Diisopropylethylamine (DIEA) was obtained from Applied Biosystems (USA). *N*,*N*-Dimethylformamide (DMF) and HPLC grade acetonitrile (CH_3CN) were purchased from Biosolve. HF was purchased from Matheson Gas (USA). DCM was obtained by distillation from P_2O_5. Analytical reversed phase high pressure liquid chromatography (RP HPLC) of the DTPA-functionalized peptide was performed on a Varian Prostar HPLC system with a Vydac™ protein peptide C18 column (0.5 × 15 cm, flow 1 mL/min), eluted with a linear gradient of 0–60% CH_3CN in 0.1% aqueous TFA in 30 minutes. The HPLC system was coupled to a UV/Vis detector probing at 214 nm. Semi-preparative HPLC was performed with a Vydac C18 column (2.5 × 20 cm, 10 mL/min), eluted with a linear gradient of 0–20% CH_3CN in 0.1% aqueous TFA in 90 minutes. Electrospray ionization mass spectrometry (ESI-MS) was performed on a PE API-150 SCIEX Turbo Ionspray. Relaxivity (r_1) measurements were performed using a 1.5 Tesla whole-body MR system (Philips Medical Systems, Best, Netherlands). Aqueous solutions of cNGR-Gd^{III}-DTPA (2 mL) were prepared with a gadolinium concentration ranging from 0.05–0.60 mM. The longitudinal relaxation times (T_1) were determined with MR experiments using a 2D-mixed dual-echo sequence (voxel size 10 × 10 × 8 mm).[27] Gd^{III} concentrations in the aqueous solutions used for the T_1-relaxivity measurements were determined by means of inductively coupled plasma atomic emission spectroscopy (ICP-AES) on a Leeman Labs Echelle spectrometer at 342.247 nm. Infrared spectra were recorded at 298 K with a Perkin–Elmer 1605 FT-IR spectrometer.

Solid-Phase Peptide Synthesis (NAcCNGRCGGK(NH_2)–MBHA) (2): Manual solid-phase peptide synthesis (SPPS) was employed on a 0.4 mmol scale using the in situ neutralization/ HBTU activation procedure for Boc chemistry as described previously by Schnolzer et al.[23] Each synthetic cycle consisted of N^{α}-Boc removal by a 1–2 minute treatment with neat TFA, a 1 min DMF-flow wash, a 10 to 20 min coupling time with 2.0 mmol activated Boc-N^{α}-protected amino acid in the presence of an excess of DIEA, followed by a second DMF-flow wash. N^{α}-Boc amino acids (2.2 mmol) were pre-activated for 3 min with 2.0 mmol HBTU (0.5 M in DMF) in the presence of excess DIEA (3 mmol). Side chain-protected amino acids were: Boc-Arg(p-toluenesulfonyl)-OH, Boc-Asn(xanthyl)-OH, Boc-Cys(4-methylbenzyl)-OH, and Boc-Lys(Fmoc)-OH. After each coupling step, the yields (> 99%) were determined by measuring residual free amine with the quantitative ninhydrin assay.[28] The peptide on MBHA resin was washed with DMF, DCM, and DCM/MeOH (v/v, 1:1), to give side chain-protected BocCNGRCGGK(Fmoc)–MBHA **1** (1.46 g, 99%). The removal of the Boc group at the N-terminus of **1** (0.363 g, 0.10 mmol) was achieved by a 1–2 min treatment with neat TFA, a 2 min DMF-flow wash, followed by neutralization with 10% DIEA in DMF. The acetylation reaction was carried out with a solution of acetic anhydride (0.25 M) and pyridine (0.25 M) in DMF for 2 min (2 times), followed by a DMF-flow wash. The Fmoc group was removed with 20% piperidine in DMF for 4 min (3 times), followed by a DMF flow wash, giving partially side chain-protected NAcCNGRCGGK(NH_2)–MBHA **2** (0.35 g, 96%).

Isocyanate-Functionalized Lysine-Based DTPA Building Block (3): The amine-functionalized DTPA pentaester **3a** was prepared according a four-step literature procedure of Williams et al.[21] and Anelli et al.[18] To a stirred solution of di-*tert*-butyl tricarbonate[24] (0.21 g, 0.80 mmol) in DCM (5 mL) was injected a solution of **3a** (0.516 g, 0.69 mmol) in DCM (5 mL) under argon. The colourless solution was vigorously stirred for 1 h at room temperature. IR spectroscopy revealed the presence of the characteristic isocyanate absorption at 2272 cm^{-1}. The excess of di-*tert*-butyl tricarbonate was quenched by the addition of three droplets of dry pyridine.

DCM was removed under an argon flow and the crude mixture was redissolved in DMF (10 mL) and directly used for the reaction with the peptide on the solid phase.

DTPA-Functionalization of the Peptide on Solid Phase: A solution of **3** (0.69 mmol) in DMF (10 mL) was added to **2** (0.347 g peptide on resin, 0.10 mmol peptide). The reaction mixture was incubated overnight at 200 rpm under argon, followed by a DMF flow wash, a 10 min treatment with neat TFA (2 times), a second DMF flow wash, a DCM flow wash, an DCM/MeOH (v/v, 1:1) flow wash, and then dried overnight, yielding crude peptide-resin **4** (0.380 g, 0.10 mmol). The gain in weight of 33 mg corresponds to a coupling efficiency of ca. 67%.

Cleavage of the Peptide from the Resin: The crude peptide on resin **4** (0.25 g, 0.066 mmol peptide) was cleaved from the MBHA resin by treatment with anhydrous HF for 1 h at 0 °C with 4% *p*-cresol as a scavenger. Subsequently, the deprotected free peptide was precipitated in cold Et_2O, collected on a filter and subsequently dissolved in 0.1% aqueous TFA and lyophilized, yielding crude peptide **5** (80 mg, nearly quantitative yield of HF cleavage) as a white powder. Analytical RP HPLC: product **5** eluting at 10.9 min. ESI-MS calcd. for $C_{49}H_{85}N_{18}O_{21}S_2$ ($[M + H]^+$): 1325.56, found 1325.86 and calcd. for $C_{49}H_{86}N_{18}O_{21}S_2$ ($[M + 2H]^{2+}$): 663.28, found 663.43.

Formation of the Disulfide Bridge: The intramolecular disulfide bond formation between the two cysteine residues was performed by dissolving the crude peptide **5** (80 mg) in 0.1 M Tris, pH 8.0 (200 mL), containing 1 M guanidine. The reaction was monitored with analytical RP HPLC. Stirring for 3 h while exposed to air at 4 °C yielded quantitative formation of the disulfide bridge. Semi-preparative RP HPLC purification and freeze drying yielded **6** (17 mg, 0.013 mmol, isolated yield of ca. 29%) as a white powder. The peptide was stored at –20 °C until further use. Analytical RP-HPLC: Product **6** eluting at 8.8 min. ESI-MS calcd. for $C_{49}H_{83}N_{18}O_{21}S_2$ ($[M + H]^+$): 1323.54, found 1323.80 and calcd. for $C_{49}H_{84}N_{18}O_{21}S_2$ ($[M + 2H]^{2+}$): 662.27, found 662.40. FT-IR (ATR): $\tilde{\nu}$ (cm^{-1}) = 3281, 1628, 1541, 1406, 1376, 1200, 1178, and 1132.

Gadolinium Complex 7: Compound **6** (8.8 mg, 6.7 μmol) was dissolved in demineralised water (9 mL). The pH of the aqueous solution was adjusted to 6.5–7.0 by adding small aliquots of 0.25% NH_4OH (aq). Subsequently, a solution of gadolinium chloride hexahydrate (2.2 mg, 5.9 μmol, 0.9 equiv.) in water (0.4 mL) was added while maintaining the pH at 7 with a 0.25% NH_4OH (aq) solution. The solution was vigorously stirred for 2 h at room temperature. The formation of the complex was confirmed with ESI-MS. After freeze drying the desired gadolinium complex **7** was obtained as a white hygroscopic powder (9.7 mg). ESI-MS calcd. for $C_{49}H_{79}GdN_{18}O_{21}S_2$ ($[M - H]^-$): 1476.43, found 1476.83 and calcd. for $C_{49}H_{78}GdN_{18}O_{21}S_2$ ($[M - 2H]^{2-}$): 737.71, found 737.91. ICP-AES (Gd^{III}): Aqueous solution of **7** Calcd. 12.5 μM, Obsd. 11.7 μM. FT-IR (ATR): $\tilde{\nu}$ (cm^{-1}) = 3252, 1634, 1577, 1447, 1413, 1202, and 1141.

Acknowledgments

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