Synthesis of DOTA-conjugated multivalent cyclic-RGD peptide dendrimers *via* 1,3-dipolar cycloaddition and their biological evaluation: implications for tumor targeting and tumor imaging purposes†‡

Ingrid Dijkgraaf,§[a,b] Anneloes Y. Rijnders,§[a] Annemieke Soede,[b] Annemarie C. Dechesne,[a] G. Wilma van Esse,[a] Arwin J. Brouwer,[a] Frans H. M. Corstens,[b] Otto C. Boerman,[b] Dirk T. S. Rijkers[a] and Rob M. J. Liskamp*[a]

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This report describes the design and synthesis of a series of $\alpha_V\beta_3$ integrin-directed monomeric, dimeric and tetrameric *cyclo*[Arg-Gly-Asp-D-Phe-Lys] dendrimers using "click chemistry". It was found that the unprotected *N*-ε-azido derivative of *cyclo*[Arg-Gly-Asp-D-Phe-Lys] underwent a highly chemoselective conjugation to amino acid-based dendrimers bearing terminal alkynes using a microwave-assisted Cu(I)-catalyzed 1,3-dipolar cycloaddition. The $\alpha_V\beta_3$ binding characteristics of the dendrimers were determined *in vitro* and their *in vivo* $\alpha_V\beta_3$ targeting properties were assessed in nude mice with subcutaneously growing human SK-RC-52 tumors. The multivalent RGD-dendrimers were found to have enhanced affinity toward the $\alpha_V\beta_3$ integrin receptor as compared to the monomeric derivative as determined in an *in vitro* binding assay. In case of the DOTA-conjugated ^{111}In-labeled RGD-dendrimers, it was found that the radiolabeled multimeric dendrimers showed specifically enhanced uptake in $\alpha_V\beta_3$ integrin expressing tumors *in vivo.* These studies showed that the tetrameric RGD-dendrimer had better tumor targeting properties than its dimeric and monomeric congeners.

Introduction

Integrins are a class of heterodimeric transmembrane proteins[1] which play an important role in cell-signaling, cell–cell adhesion, apoptosis and cell-matrix interactions.[2] Integrin $\alpha_V\beta_3$, which binds to the Arg-Gly-Asp (RGD) tripeptide motif containing ligands,[3] plays a pivotal role in tumor angiogenesis[2] and metastasis. $\alpha_V\beta_3$ Integrin expressed on endothelial cells modulate cell migration and survival during angiogenesis, while $\alpha_V\beta_3$ integrin expressed on carcinoma cells potentiate metastasis by facilitating invasion and movement across blood vessels. The $\alpha_V\beta_3$ integrin is expressed on activated endothelial cells during tumor induced angiogenesis, whereas it is absent on quiescent endothelial cells and normal tissues. In addition, $\alpha_V\beta_3$ is expressed on various tumor cell types (*e.g.* breast, ovarian, and prostate cancers). Evidence exists that inhibition of $\alpha_V\beta_3$ integrin function prevents tumor growth and induces tumor regression by antagonizing angiogenesis.[4] Several peptidic[5] and peptidomimetic[6] $\alpha_V\beta_3$ antagonists have been synthesized. Among these, the *cyclo*[Arg-Gly-Asp-D-Phe-Val] (c[RGDfV]), as developed by Kessler and coworkers, is one of the most active and selective antagonists for the $\alpha_V\beta_3$ integrin.[7] Structure–activity relationship studies on this cyclic pentapeptide showed that the exchange of the valine by a lysine residue (Lys, K) did not significantly influence activity and selectivity.[8] Because the ε-amino moiety of the lysine residue can be easily modified, numerous applications of c[RGDfK] have been studied for tumor targeting and imaging.[9]

Multivalency is a well accepted approach to increase the interaction of weakly interacting individual ligands with their respective receptors.[10] Dendrimers are macromolecules consisting of multiple perfectly branched monomers and this architecture makes them versatile constructs for the simultaneous presentation of receptor binding ligands and other biologically relevant molecules.[11] Additionally, dendrimers might serve as promising molecular scaffolds containing a number of ligands thereby inducing an apparent increase of ligand concentration and increasing the probability of statistical rebinding.[10b–e,12] Alternatively, dendrimers may align these ligands and induce multivalency when receptor clustering occurs or is initiated after initial monovalent binding.[10b–e] To improve tumor targeting efficacy and to obtain better *in vivo* imaging properties, several studies explored the multivalency effect by using dimeric and tetrameric RGD peptides with affinity toward the $\alpha_V\beta_3$ integrin.[13] These studies clearly demonstrated the multivalency effect, since the *in vivo* affinity significantly increased going from monomer *via* dimer to tetramer. Moreover, also with respect to tumor-uptake and tumor-to-organ ratios, a similar increase was observed. These are promising results in view of the development of integrin-targeted radionuclide therapy.[12]

To decorate the dendrimer end-groups with biologically relevant peptides as ligands, it is of crucial importance to have the disposal of efficient and chemoselective conjugation chemistry to ensure the complete attachment of the ligands to the dendrimer. In cases of completely amino acid- or peptide-based dendrimers,[14,15]

[a]*Department of Medicinal Chemistry and Chemical Biology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, P. O. Box 80082, 3508 TB Utrecht, The Netherlands. E-mail: R.M.J.Liskamp@pharm.uu.nl; Fax: +31 30 253 6655; Tel: +31 30 253 7396/7307*
[b]*Department of Nuclear Medicine, Radboud University Nijmegen Medical Centre, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands*

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‡ Electronic supplementary information (ESI) available: Experimental details for the synthesis of **2–5**, **7**, **8**, **10**, **17**, **18** and **20–22**, as well as analysis data for **23–25** (HPLC, MALDI-TOF). See DOI: 10.1039/b615940k

§ These authors contributed equally to this work.

this is often achieved using peptide coupling reagents, however, in most cases, the peptide ligands are attached to dendrimers by chemoselective reaction of sulfhydryl groups of cysteine residues with maleimide or iodoacetamide functionalities,[16] by thiol–disulfide exchange, by native chemical ligation[17] or *via* a chemoselective oxime[13d–l,m] respectively hydrazone[18] ligation. However, new bioconjugation reactions with mutually reactive conjugation partners with increased efficiency and chemoselectivity which are synthetically easily accessible would be very welcome.

Recently, the well-known reaction between an alkyne and an azide to yield 1,4-disubstituted 1,2,3-triazoles, was reinvestigated independently by Meldal *et al.*[19a] and Sharpless *et al.*[19b] They found that an alkyne and an azide in the presence of Cu(I) undergo a 1,3-dipolar cycloaddition to the corresponding triazole under very mild reaction conditions with very high chemoselectivity and efficiency which make this reaction particularly suitable for bioconjugations. So far, this 1,3-dipolar cycloaddition denoted as a 'click reaction',[20] has led to a plethora of applications in the literature.[21] Recently, we synthesized multivalent dendrimeric peptides[22a] (up to octa- and hexadecavalent systems) respectively triazole-linked glycodendrimers[22b] *via* a microwave-assisted 1,3-dipolar cycloaddition between azido peptides respectively glycosyl azides and dendrimeric alkynes as an alternative approach to functionalize dendrimers.[22c]

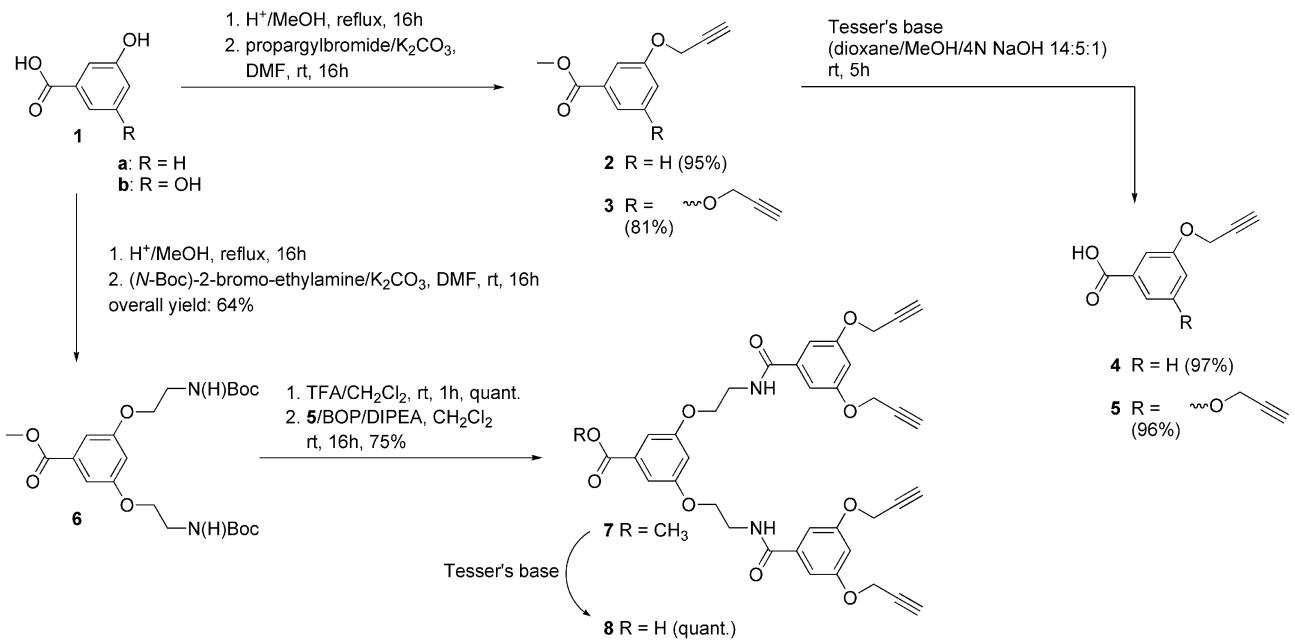
Here we describe the synthesis of monomeric, dimeric and tetrameric c[RGDfK] dendrimers *via* a microwave-assisted 1,3-dipolar cycloaddition of dendrimeric alkynes with the *N*-ε-azido derivative of *cyclo*[Arg-Gly-Asp-D-Phe-Lys] and their subsequent evaluation as $\alpha_V\beta_3$ integrin antagonists. Additionally, the RGD dendrimers were conjugated with a 1,4,7,10-tetraazadodecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (DOTA) moiety. These analogs were radiolabeled with ^{111}In to evaluate the *in vitro* receptor binding characteristics and *in vivo* tumor targeting properties.

Results and discussion

Synthesis

Schemes 1 and 2 illustrate our approach for the convergent synthesis of amino acid based dendrimers[23] and their corresponding DOTA-conjugated derivatives. Monovalent compound **2** and divalent **3** respectively, were synthesized starting from 3-hydroxy methyl benzoate or 3,5-dihydroxy methyl benzoate and propargylbromide in the presence of K_2CO_3 as a base and were obtained in 95 and 81% yield. Since these two compounds were also used as synthons in further syntheses, the resulting methyl esters **2** and **3** were treated with Tesser's base[24] to yield acids **4** and **5** in nearly quantitative yield. After treatment of the previously described **6**[23c] with TFA to remove both Boc-functionalities, the resulting bisamine TFA salt was coupled to acid **5** in the presence of BOP–DIPEA to give the tetravalent dendrimer **7** with 75% yield. To conjugate the tetravalent dendrimer with a DOTA-moiety at a later stage of the synthesis, its methyl ester was saponified with Tesser's base and acid **8** was obtained quantitatively.

The DOTA-moiety was connected to the dendrimer core *via* a short ethylene spacer. For this purpose, 1,2-diaminoethane was converted into the mono-protected Boc derivative **10** which was obtained in 56% yield. Unfortunately, although a large excess of the amine was used, the bis-protected side product was obtained in a considerable amount. Compound **10** was coupled in the presence of BOP–DIPEA to either the monovalent, divalent or tetravalent dendrimer acids **4**, **5** or **8** to obtain the corresponding amides **11**, **12** or **15**, respectively, generally in yields higher than 90%. The Boc-protected dendrimers were treated with TFA to obtain the corresponding amines and they were treated with BOP–DIPEA in the presence of 2-(4,7,10-tris(2-*tert*-butoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl) acetic acid (DOTA(O^{t}Bu)$_3$) to give the DOTA-conjugated mono-, di- and tetravalent dendrimers **13**, **14**



Scheme 1 Synthesis of the mono-, di- and tetravalent dendrimeric alkynes **2**, **3** and **7**.

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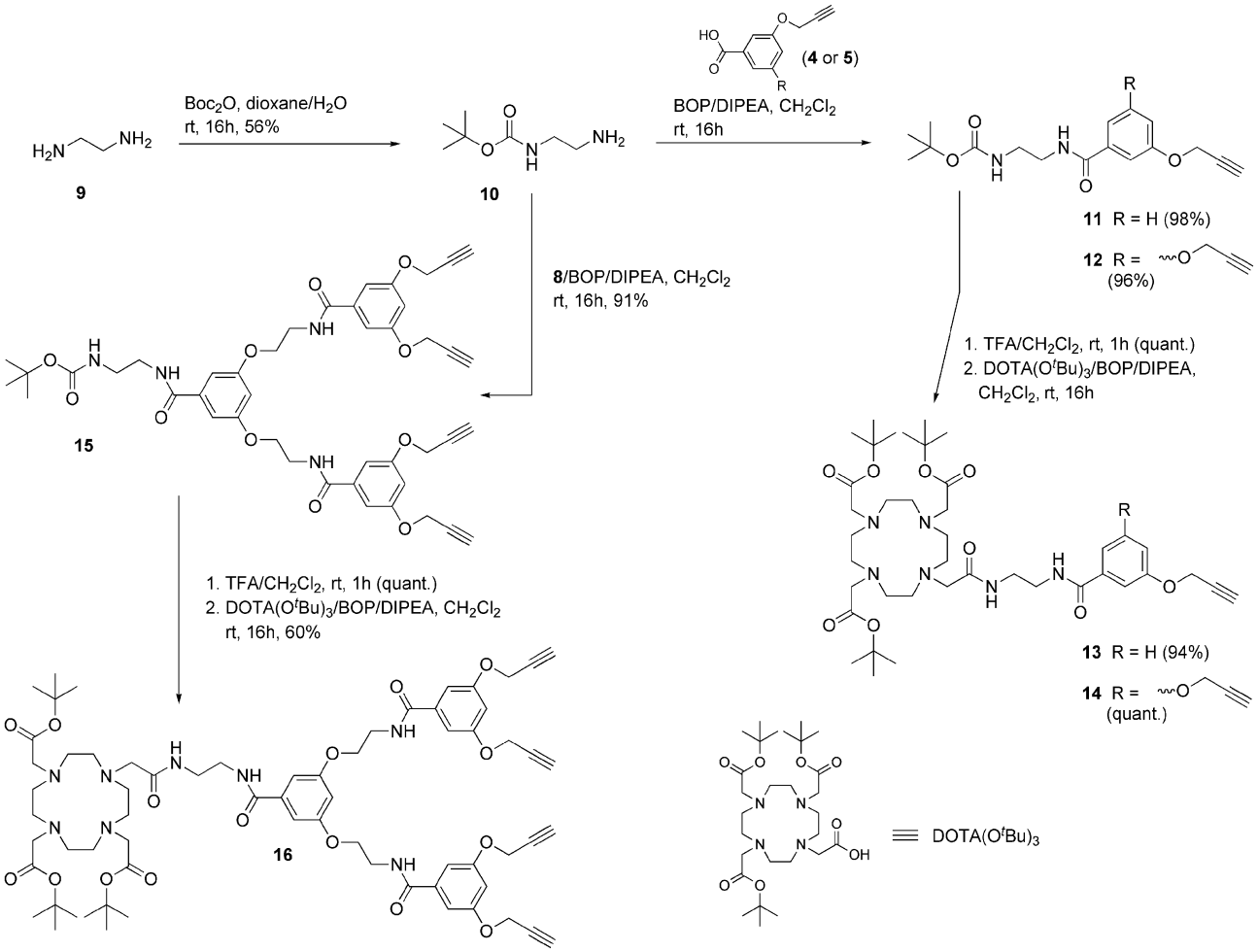


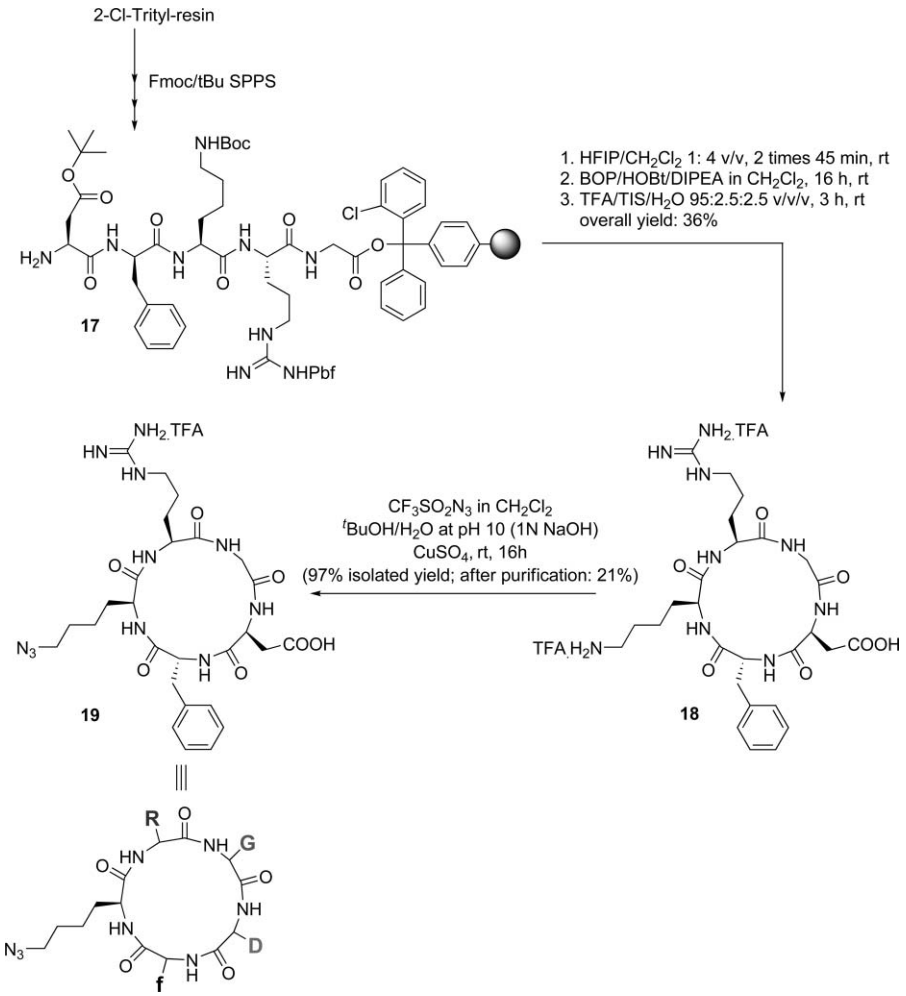
Scheme 2 Synthesis of the DOTA-conjugated dendrimeric alkynes **13**, **14** and **16**.

and **16** respectively. It is important to note that the solubility of the DOTA-conjugated dendrimer is an important factor that determines the yield of the coupling reaction. Compounds **13** and **14** were isolated in very high yields (>94%) but compound **16** was isolated with a modest yield of 60% due to its low solubility in solvents like EtOAc and CH_2Cl_2.

The next step in the synthesis was the preparation of the *N*-ε-azido *cyclo*(Arg-Gly-Asp-D-Phe-Lys) peptide **19** (Scheme 3). To obtain this compound, peptide resin **17** was synthesized using Fmoc–^{t}Bu SPPS (solid phase peptide synthesis) based on the protocol of Liu *et al.*[25] It was decided to cleave the protected peptide acid from the resin by HFIP–CH_2Cl_2[26] instead of AcOH–TFE to avoid premature acetylation during the BOP–DIPEA-mediated macrolactamization step. Cyclic peptide **18** was obtained in 36% overall yield based on the initial resin loading of 0.64 mmol g^{-1}. Subsequently, the ε-amine of the lysine residue was selectively converted into the azide moiety by a diazotransfer.[27] At pH 10, the ε-amine can be deprotonated in the presence of a guanidino functionality, since the latter is a much stronger base and will not act as a nucleophile in the diazotransfer reaction. Finally, the peptide *N*-ε-azido *cyclo*(Arg-Gly-Asp-D-Phe-Lys) **19** was obtained in 21% yield after purification by HPLC and was characterized by ^{1}H-NMR (500 MHz) and mass spectrometry (LC-MS). Incorporation of Fmoc-Lys(N_3)-OH, to avoid the diazo transfer as the final reaction step, did not substantially improve the isolated yield.

At this stage of the synthesis, the challenge was the chemoselective coupling of the different dendrimeric alkynes (**2**, **3**, **7**, **13**, **14**, or **16**) to the cyclic RGD azido peptide (**19**) to furnish the DOTA-conjugated dendrimeric *cyclo*-RGD peptides as $\alpha_V\beta_3$ integrin antagonists as shown in Scheme 4. Our first experiments were based on the literature procedure[19b] in which acetylene **3** was coupled to azido glycine ethyl ester (ethyl 2-azidoacetate) in the presence of $CuSO_4$–Na-ascorbate–Cu-wire in *tert*-BuOH–H_2O for 16 h at room temperature. Monitoring the reaction by TLC showed that formation of the monovalent cycloadduct proceeded rapidly, but the conversion into the divalent product was sluggish. However, a tremendous improvement was achieved by running this reaction under microwave irradiation. After 10 min at 100 °C using DMF–H_2O as solvent in the presence of $CuSO_4$–Na-ascorbate, the divalent cycloaddition product was obtained in 96% yield. This microwave-assisted cycloaddition of dendrimeric alkynes and azido peptides was recently reported as a versatile approach to obtain multivalent dendrimeric peptides.[22a,c] The optimized reaction conditions were used to couple the cyclic RGD azido peptide (**19**) to the different dendrimeric alkynes (**2**, **3**, **7**, **13**, **14**, or **16**).

In case of alkynes **2**, **3** and **7** the formation of the cycloadducts **20**, **21** and **22** could be followed by TLC and LC-MS. It turned



Scheme 3 Synthesis of the *N*-ε-azido *cyclo*(Arg-Gly-Asp-D-Phe-Lys) peptide **19**.

out that the formation of **20** and **21** was complete after 10 to 20 min microwave irradiation at 100 °C, whereas the formation of **22** was complete after 30 min. Although HPLC analysis of the crude cycloaddition products evidenced a complete conversion as judged by the absence of the alkyne starting material, the RGD-dendrimers **20–22** were obtained in yields varying between 14 to 57%. Then, the DOTA-conjugated alkyne dendrimers **13**, **14** and **16** were subjected to the cycloaddition reaction conditions in the presence of azido peptide **19**. It should be emphasized that the carboxyl functionalities of the DOTA-moiety needed to be protected by *tert*-butyl groups to avoid premature and irreversible sequestering of the Cu^{2+} ions. Chelated copper(II) will result in a lower efficiency of the Cu(II)–Cu(I) redox couple to generate the active Cu(I)-catalyst. More importantly, it will hamper the radiolabeling of the DOTA-moiety of compounds **23–25** with trivalent radiometals such as ^{111}In, ^{90}Y or ^{177}Lu. As a result, after the click reaction an additional reaction step was needed in which the partially protected cycloadducts were treated with TFA, in the presence of suitable scavengers, to give the unprotected DOTA-conjugated RGD-dendrimers **23–25**.

The cycloaddition reaction of the DOTA-conjugated dendrimeric alkynes **13**, **14** and **16** was difficult to monitor by mass spectrometry. As was described above, reaction times of 10 to 30 min were used and the cycloaddition reaction was directly followed by a TFA-treatment without isolation of the cycloaddition intermediates. The isolated yield (13%) of monovalent **23** was rather disappointing. Recently, optimized conditions with respect to the generation of the catalytic active Cu(I) species were published[28] and these conditions were applied in the cycloaddition of **14** and **19**. Unfortunately, an increase of the isolated yield was not observed using these modified reaction conditions. As was mentioned earlier, the cycloaddition reaction was complete according to HPLC analysis, and the low isolated yield was mainly due to the difficult purification. The DOTA-conjugated RGD-dendrimers were obtained in yields varying between 11 and 36%.

Radiolabeling of the RGD dendrimers

Dendrimers **23**, **24** and **25** were radiolabeled by dissolving these compounds in an NH_4OAc buffer of pH 6.0 and 22.2–37 MBq $^{111}InCl_3$ was added to each of the reaction mixtures. The reaction mixtures were degassed and subsequently heated at 100 °C for 15 min. Reversed phase-HPLC analysis showed a single peak for each of the three ^{111}In-labeled compounds with an elution time of 25.9 min, 29.5 min and 29.4 min for the ^{111}In-labeled monovalent **23**, divalent **24**, and tetravalent **25**, RGD peptide dendrimers respectively.

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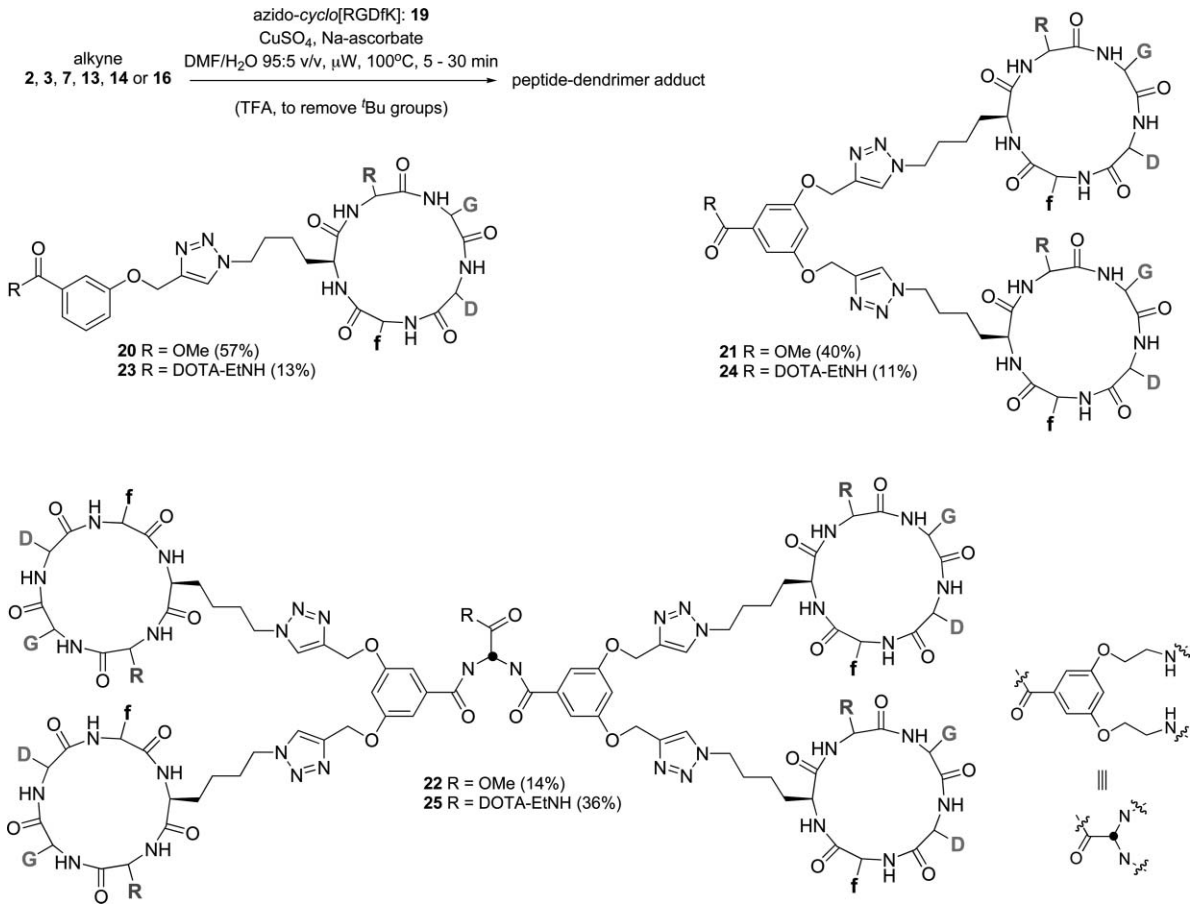

Scheme 4 Synthesis of the mono-, di- and tetravalent *cyclo*[RGDfK] peptide dendrimers **20**, **21** and **22** and their respective DOTA-conjugated counterparts **23**, **24** and **25**.

Solid phase $\alpha_v\beta_3$ binding assay

The affinity of the DOTA-conjugated RGD dendrimers **23**, **24**, and **25** for the $\alpha_v\beta_3$ integrin was determined in a competitive binding assay. The results of these analyses are shown in Fig. 1. Binding of the ^{111}In-labeled dimeric peptide, ^{111}In-DOTA-Glu-(c[RGDfK])$_2$,[29] to $\alpha_v\beta_3$ was competed by unlabeled **23**, **24**, and **25** in a concentration dependent manner. The IC_{50} values were 212 nM for monovalent **23**, 356 nM for divalent **24**, and 50 nM for tetravalent **25**. The dendrimer containing four c[RGDfK] units (**25**) showed an increased affinity for $\alpha_v\beta_3$ compared to the dendrimers containing one (**23**) or two (**24**) c[RGDfK] units. Multimerization of c[RGDfK] resulted in enhanced affinity for $\alpha_v\beta_3$ as was evidenced by a decrease of the IC_{50} concentration.

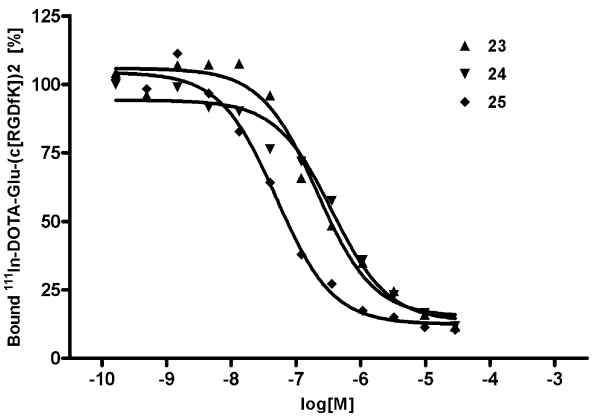

Fig. 1 Competition of specific binding of ^{111}In-DOTA-Glu-(c[RGDfK])$_2$ with RGD dendrimers **23**, **24**, and **25**.

Biodistribution studies

In athymic mice with subcutaneously (s.c.) growing SK-RC-52 renal cell carcinoma, the tumor uptake of the ^{111}In-labeled tetrameric RGD dendrimer **25** at 2 h post-injection (p.i.; 7.27 ± 2.06%ID/g) was significantly higher ($P < 0.05$) compared to that of the ^{111}In-labeled monomeric RGD dendrimer **23** (1.69 ± 0.41%ID/g) as shown in Fig. 2A. At 2 h p.i., the tumor uptake of tetrameric RGD dendrimer **25** was also significantly higher ($P < 0.05$) than the dimeric analog **24** (3.15 ± 0.51%ID/g). The tumor-to-blood ratios of the tetramer **25** (5.66 ± 1.74%ID/g, 34.73 ± 5.95%ID/g) were significantly higher ($P < 0.05$)—both at 2 h p.i. and at 24 h p.i.—than those of the monomer **23** (3.12 ± 1.92%ID/g, 19.65 ± 12.42%ID/g) and dimer **24** (1.70 ± 0.50%ID/g, 14.66 ± 0.25%ID/g). At 24 h post injection, the tumor uptake of the tetrameric RGD dendrimer **25** (5.83 ± 1.18%ID/g) was significantly higher compared to the dimeric RGD dendrimer **24** (2.82 ± 0.59%ID/g, $P < 0.05$) and the monomeric RGD dendrimer **23** (1.19 ± 0.31%ID/g, $P < 0.01$) which is shown in Fig. 2B. Co-injection of an excess of non-radiolabeled RGD

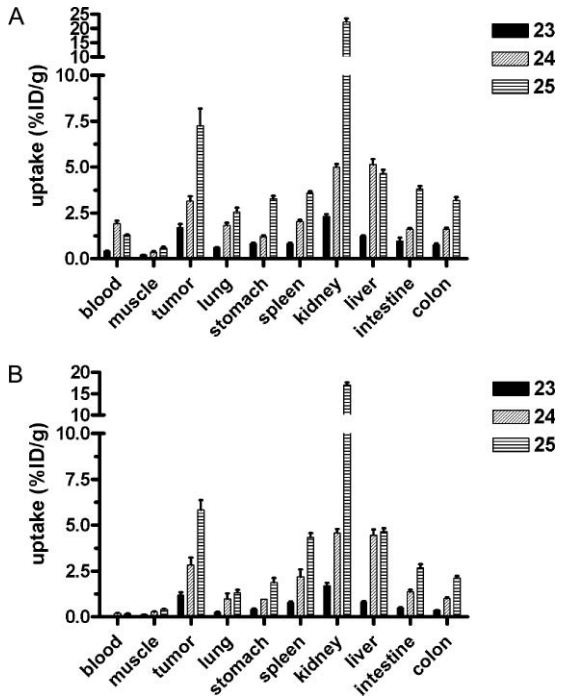

Fig. 2 **A** Biodistribution of ^{111}In-labeled monomer **23**, dimer **24**, and tetramer **25** at 2 h p.i. in athymic mice with s.c. SK-RC-52 tumors. **B** Biodistribution of ^{111}In-labeled monomer **23**, dimer **24**, and tetramer **25** at 24 h p.i. in athymic mice with s.c. SK-RC-52 tumors.

peptide (DOTA-Glu-(c[RGDfK])$_2$) to saturate all $\alpha_v\beta_3$ receptors *in vivo*, resulted in a significantly reduced tumor uptake of each of the three compounds: **23**: 0.46 ± 0.04%ID/g (2 h p.i.), 0.36 ± 0.31%ID/g (24 h p.i.), **24**: 0.76 ± 0.09%ID/g (2 h p.i.), not determined (24 h p.i.) and **24**: 1.56 ± 0.02%ID/g (2 h p.i.), 1.19 ± 0.03%ID/g (24 h p.i.), indicating that each of the three RGD dendrimers of this study showed receptor mediated uptake in the tumor. These *in vivo* results were in line with the *in vitro* binding assay. The tetrameric RGD dendrimer showed enhanced affinity for $\alpha_v\beta_3$, as compared to the monomeric and dimeric RGD dendrimer, respectively. The results of this study correlated nicely with the results observed in a previous study in which we evaluated multimeric RGD peptides in the same animal model.[13o]

The affinity of the dendrimers as determined in an *in vitro* binding assay are in agreement with the results obtained from the *in vivo* experiment: the IC_{50} concentration of the tetrameric RGD dendrimer **25** was lower compared to those of the monomeric **23** and dimeric **24** analogs, resulting in a significantly higher uptake of the former in $\alpha_v\beta_3$-expressing tumors and better tumor-to-blood ratios compared to the monomeric and dimeric RGD dendrimers.

In conclusion, a series of $\alpha_V\beta_3$ integrin-directed monomeric, dimeric and tetrameric *cyclo*[Arg-Gly-Asp-D-Phe-Lys] dendrimers using "click chemistry" was successfully synthesized, since the unprotected *N*-ε-azido derivative of *cyclo*[Arg-Gly-Asp-D-Phe-Lys] underwent a highly chemoselective conjugation to amino acid-based dendrimers bearing terminal alkynes using a microwave-assisted Cu(I)-catalyzed 1,3-dipolar cycloaddition. The $\alpha_V\beta_3$ binding characteristics and $\alpha_V\beta_3$ targeting properties of the dendrimers were determined both *in vitro* and *in vivo*. In the case of the DOTA-conjugated ^{111}In-labeled RGD-dendrimers, it was found that the radiolabeled multimeric dendrimers showed specifically enhanced uptake in $\alpha_V\beta_3$ integrin expressing tumors *in vivo*. These studies showed that the tetrameric RGD-dendrimer had better tumor targeting properties than its dimeric and monomeric congeners.

Experimental

Instruments and methods

Peptides were synthesized on an ABI 433A automatic Peptide Synthesizer using the FastMoc solid phase peptide synthesis protocols. Microwave-assisted reactions were carried out in a Biotage microwave reactor. Analytical HPLC runs were carried out on a Shimadzu HPLC system and preparative HPLC runs were performed on a Gilson HPLC workstation. Analytical HPLC runs were performed on Alltech Prosphere C4 or C8 and Adsorbosphere XL C18 columns (250 × 4.6 mm, pore size 300 Å, particle size: 5 μm) or on a Merck LiChroCART CN column (250 × 4.6 mm, pore size 100 Å, particle size: 5 μm) at a flow rate of 1.0 mL min^{-1} using a linear gradient of buffer B (0–100% in 25 min) in buffer A (buffer A: 0.1% TFA in H_2O, buffer B: 0.1% TFA in CH_3CN–H_2O 95 : 5 v/v). Preparative HPLC runs were performed on an Alltech Prosphere C4 or C8 column (250 × 22 mm, pore size 300 Å, particle size: 10 μm), and semi-prep HPLC runs were performed on an Alltech Adsorbosphere XL C18 column (250 × 10 mm, pore size 300 Å, particle size: 10 μm) or on a Merck LiChroCART CN column (250 × 10 mm, pore size 100 Å, particle size: 10 μm) at a flow rate of 10.0 mL min^{-1} (semi-prep HPLC: 4.0 mL min^{-1}) using a linear gradient of buffer B (0–100% in 50 min) in buffer A (buffer A: 0.1% TFA in H_2O, buffer B: 0.1% TFA in CH_3CN–H_2O 95 : 5 v/v). Liquid chromatography electrospray ionization mass spectrometry was measured on a Shimadzu LCMS-QP8000 single quadrupole bench-top mass spectrometer operating in a positive ionization mode. LC/MS(MS) runs were performed on a Finnigan LCQ Deca XP MAX LC/MS equipped with a Shimadzu 10A VP analytical HPLC system. The samples were dissolved in 10% formic acid in CH_3CN–H_2O 1 : 1 v/v and analyzed using a Phenomenex Gemini C18 column (150 × 4.6 mm, particle size: 3 μm, pore size: 110 Å) at a flow rate of 1.0 mL min^{-1} using a linear gradient of 100% buffer A (0.1% TFA in H_2O–CH_3CN 95 : 5 v/v) to 100% buffer B (0.1% TFA in CH_3CN–H_2O 95 : 5 v/v) in 50 min. MALDI-TOF analysis was performed on a Kratos Axima CFR apparatus with bradykinin(1–7) (monoisotopic [M + H]$^+$ 757.399), human ACTH(18–39) (monoisotopic [M + H]$^+$ 2465.198) and bovine insulin oxidized B chain (monoisotopic [M + H]$^+$ 3494.651) as external references and α-cyano-4-hydroxycinnamic acid or sinapinic acid as matrices. ^{1}H NMR spectra were recorded on a Varian G-300 (300 MHz) spectrometer and chemical shifts are given in ppm (δ) relative to TMS. ^{13}C NMR spectra were recorded on a Varian G-300 (75.5 MHz) spectrometer and chemical shifts are given in ppm relative to $CDCl_3$ (77.0 ppm). The ^{13}C NMR spectra were recorded using the attached proton test (APT) sequence. ^{1}H NMR spectra in H_2O–D_2O 9 : 1 v/v were recorded on a Varian Inova-500 (500 MHz) spectrometer and chemical shifts are given in ppm (δ) relative to 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (0.00 ppm). Peak assignments are based on DQF-COSY, TOCSY (mixing times: 20 or 60 ms) and ROESY (mixing times: 150 or 250 ms) spectra. HSQC and HMBC spectra were measured on a Varian Inova-500 spectrometer and

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chemical shifts are given in ppm (δ) relative to 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (0.00 ppm). Fourier transform infrared spectra (FTIR) were measured on a Bio-Rad FTS-25 spectrophotometer. Melting points were measured on a Büchi Schmelzpunktbestimmungsapparat and are uncorrected. Elemental analyses were done by Kolbe Mikroanalytisches Labor (Mülheim/Ruhr, Germany). R_f values were determined by thin layer chromatography (TLC) on Merck precoated silica gel 60F254 plates. Spots were visualized by UV-quenching, ninhydrin or Cl_2–TDM.[30] The 2-chlorotrityl chloride resin (Hecheng Science & Technology Company) was used in all solid phase syntheses. The coupling reagents 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and benzotriazol-1-yloxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) were obtained from Biosolve. *N*-Hydroxybenzotriazole (HOBt) was from Advanced ChemTech and N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc) amino acids were obtained from MultiSynTech. The side-chain protecting groups were chosen as *tert*-butyl for aspartic acid, *tert*-butyloxycarbonyl (Boc) for lysine and 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf) for arginine. Peptide-grade *tert*-butanol (^{t}BuOH), dichloromethane, *N*,*N*-dimethylformamide (DMF), 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), *tert*-butyl methylether (MTBE), *N*-methylpyrrolidone (NMP), and trifluoroacetic acid (TFA) and HPLC-grade acetonitrile were purchased from Biosolve. 2-(4,7,10-Tris(2-*tert*-butoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl) acetic acid (DOTA(O^{t}Bu)$_3$) was purchased from Macrocyclics. Piperidine, *N*,*N*-diisopropylethylamine (DIPEA), $CuSO_4$ and sodium ascorbate were obtained from Acros Organics. Triisopropylsilane (TIS) and HPLC-grade TFA were obtained from Merck. Triflic anhydride and propargylbromide were purchased from Aldrich.

Radiolabeling of the RGD dendrimers

Dendrimers **23** (25 µg, 20 nmol), **24** (25 µg, 13 nmol), and **25** (120 µg, 33 nmol) were radiolabeled by dissolving these compounds in 500 µL 0.5 M NH_4OAc buffer, pH 6.0, containing 0.6 mg mL^{-1} gentisic acid. Then 22.2–37 MBq $^{111}InCl_3$ was added to each of the reaction mixtures. The reaction mixtures were degassed and subsequently heated at 100 °C for 15 min. The ^{111}In-labeled dendrimers were further purified on a Waters C-18 SepPak cartridge (Milford, MA). After applying the sample on the methanol-activated cartridge, the cartridge was washed with 5 mL 25 mM NH_4OAc and eluted with 25% CH_3CN in 25 mM NH_4OAc. The radiochemical purity was determined by reversed-phase HPLC (HP 1100 series, Hewlett Packard, Palo Alto, CA, USA) using a Zorbax RX-C18 column (250 × 4.6 mm) eluted with a linear gradient of buffer B (8–20% in 25 min or 8–100% in 30 min in buffer A (buffer A: 25 mM NH_4OAc, buffer B: CH_3CN) at a flow rate of 1 mL min^{-1}. The radioactivity of the eluate was monitored using an in-line radiodetector (Flo-One Beta series, Radiomatic, Meriden, CT, USA).

Solid phase $\alpha_v\beta_3$ binding assay

The affinity of the DOTA-conjugated monovalent **23**, divalent **24** and tetravalent **25** RGD dendrimers for the $\alpha_v\beta_3$ integrin was determined using a solid-phase competitive binding assay. ^{111}In-labeled DOTA-Glu-(c[RGDfK])$_2$ (3 MBq µg^{-1}) was prepared as described above and was used as the tracer in the assay. Microtiter 96-well vinyl assay plates (Corning B.V., Schiphol-Rijk, The Netherlands) were coated with 100 µL/well of a solution of purified human integrin $\alpha_v\beta_3$ (150 ng mL^{-1}) in Triton X-100 Formulation (Chemicon International, Temecula, CA, USA) in coating buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 1 mM $MnCl_2$) for 17 h at 4 °C. The plates were washed twice with binding buffer (0.1% bovine serum albumin (BSA) in coating buffer). The wells were blocked for 2 h with 200 µL blocking buffer (1% BSA in coating buffer). The plates were washed twice with binding buffer. Then 100 µL binding buffer containing 11.1 kBq of ^{111}In-DOTA-Glu-(c[RGDfK])$_2$ and appropriate dilutions of non-labeled monovalent **23**, divalent **24** and tetravalent **25** RGD dendrimers in binding buffer were incubated in the wells at 37 °C for 1 h. After incubation, the plates were washed three times with binding buffer. The retained radioactivity in each well was determined in a γ-counter (1480 Wizard, Wallac, Turku, Finland). The IC_{50} values of the RGD dendrimers were calculated by nonlinear regression using GraphPad Prism (GraphPad Prism 4.0 Software, San Diego, CA, USA). Each data point represents the average of three individual determinations.

Biodistribution studies

In the right flank of 6–8 weeks old female nude BALB/c mice, 0.2 mL of a cell suspension of 8.5 × 10^6 cells/mL SK-RC-52 cells was injected subcutaneously (s.c.). Two weeks after inoculation of the tumor cells, mice were randomly divided into three groups. The mice were injected with 0.25–0.29 MBq of the ^{111}In-labeled dendrimers **23**, **24**, or **25** *via* a tail vein. The mice were euthanized by CO_2 asphyxiation, 2 and 24 h postinjection (p.i.) (2–5 mice/group). Blood, tumor, and the major organs and tissues were collected, weighed, and counted in a γ-counter. The percentage injected dose per gram (%ID/g) was determined for each sample. To investigate whether the uptake of each of the three RGD dendrimers is $\alpha_v\beta_3$-mediated, a separate group of mice was co-injected with an excess (50 µg) of non-radiolabeled DOTA-Glu-(c[RGDfK])$_2$ to saturate all the $\alpha_v\beta_3$ integrin receptors.

Statistical analysis

All mean values are given ± standard deviation (S.D.). Statistical analysis was performed using the One-way Analysis of Variance. Tukey corrections for multiple comparisons were applied. The level of significance was set at $P < 0.05$.

Syntheses

Details of the synthetic procedures for compounds **2–5**, **7**, **8**, **10**, **17**, **18** and **20–22** are given in the ESI‡.

***tert*-Butyl-2-(3-(prop-2-ynyloxy)benzamido)ethylcarbamate (11).** Acid **4** (774 mg, 4.40 mmol) and amine **10** (704 mg, 4.40 mmol) were dissolved in CH_2Cl_2 (25 mL) and BOP (1.95 g, 4.41 mmol) followed by DIPEA (1.77 mL, 10 mmol, 2.27 equiv) were added and the obtained reaction mixture was stirred for 16 h. Then, the solvent was removed by evaporation and the residue was redissolved in EtOAc (50 mL) and subsequently washed with H_2O

(3 × 20 mL), 1 N $KHSO_4$ (3 × 20 mL), H_2O (3 × 20 mL), 5% $NaHCO_3$ (3 × 20 mL) and brine (3 × 20 mL), dried (Na_2SO_4) and evaporated to dryness. The residue was purified by column chromatography (eluents: EtOAc–hexane 1 : 1 v/v) and was obtained as a white solid with 98% yield (1.38 g). Mp: 118–121 °C; R_f (EtOAc–hexane 1 : 1 v/v): 0.20; ^{1}H NMR ($CDCl_3$) δ: 1.42 (s, 9H, $(CH_3)_3$ Boc), 2.54 (s, 1H, CH), 3.38 (m, 2H, ~NH–CH_2–CH_2~), 3.54 (m, 2H, ~CH_2–CH_2–NH~), 4.70 (s, 2H, ~O–CH_2), 5.35 (m, 1H, NH urethane), 7.10–7.47 (broad m, 5H, arom H/NH amide); ^{13}C NMR ($CDCl_3$) δ: 28.3, 39.9, 41.7, 55.8, 75.7, 78.1, 79.7, 113.4, 118.3, 119.8, 129.4, 135.6, 157.3, 157.6, 167.5; MS analysis: calcd for $C_{17}H_{22}N_2O_4$ 318.16, found ES-MS 319.27 [M + H]$^+$, 341.33 [M + Na]$^+$; Elemental analysis: calcd for $C_{17}H_{22}N_2O_4$ C 64.13, H 6.97, N 8.80 found C 63.81, H 6.81, N 8.63%.

***tert*-Butyl-2-(3,5-bis(prop-2-ynyloxy)benzamido)ethylcarbamate (12).** This compound was synthesized using acid **5** (506 mg, 2.20 mmol) and amine **10** (352 mg, 2.20 mmol) as described for **11**. Compound **12** was obtained in 96% yield (760 mg) after column chromatography with EtOAc–hexane 8 : 2 v/v as eluents. Mp: 128–134 °C; R_f(EtOAc–hexane 7 : 3 v/v): 0.31; ^{1}H NMR ($CDCl_3$) δ: 1.42 (s, 9H, $(CH_3)_3$ Boc), 2.55 (s, 2H, CH), 3.37 (m, 2H, ~NH–CH_2–CH_2~), 3.52 (m, 2H, ~CH_2–CH_2–NH~), 4.68 (s, 4H, ~O–CH_2), 5.30 (m, 1H, NH urethane), 6.72 (s, 1H, arom H4), 7.06 (s, 2H, arom H2/H6), 7.44 (m, 1H, NH amide); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 28.3, 40.0, 41.7, 56.0, 75.9, 78.0, 79.8, 105.5, 106.6, 136.4, 157.3, 158.6, 167.2; MS analysis: calcd for $C_{20}H_{24}N_2O_5$ 372.17, found ES-MS 373.24 [M + H]$^+$, 395.27 [M + Na]$^+$; Elemental analysis: calcd for $C_{20}H_{24}N_2O_5$ C 64.50, H 6.50, N 7.52 found C 63.61, H 6.21, N 7.05%.

***tert*-Butyl-2,2′,2″-(10-(2-oxo-2-(2-(3-(prop-2-ynyloxy)benzamido)-ethylamino)ethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (13).** To a solution of compound **11** (100 mg, 0.31 mmol) in CH_2Cl_2 (5 mL), TFA (5 mL) was added to remove the Boc protecting group. After 1 h of stirring at room temperature, the volatiles were removed by evaporation and the residue was coevaporated with CH_2Cl_2 to remove any residual TFA. The obtained solid was used without further purification. Then, the TFA-salt was dissolved in CH_2Cl_2 (10 mL) and 2-(4,7,10-tris(2-*tert*-butoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)acetic acid (DOTA(O^{t}Bu)$_3$; 177 mg, 0.31 mmol), BOP (137 mg, 0.31 mmol) followed by DIPEA (220 µL, 1.24 mmol, 4 equiv) were added and the obtained reaction mixture was stirred for 16 h at room temperature. Subsequently, the solvent was removed by evaporation and the residue was redissolved in EtOAc (50 mL) and this solution was washed with H_2O (3 × 20 mL), 1 N $KHSO_4$ (3 × 20 mL), H_2O (3 × 20 mL), 5% $NaHCO_3$ (3 × 20 mL), brine (3 × 20 mL) and dried (Na_2SO_4). Finally, the solvent was evaporated *in vacuo* after which **13** was obtained as a pale yellow oil with 94% yield (227 mg). R_f (CH_2Cl_2–MeOH 9 : 1 v/v): 0.49; R_t: 18.10 min (C8); ^{1}H NMR ($CDCl_3$) δ: 1.42 (s, 27H, $(CH_3)_3$ ^{t}Bu), 2.20–3.70 (broad s, 28H, CH_2 DOTA (24H)/~NH–CH_2–CH_2–NH~(4H)), 2.52 (s, 1H, CH), 4.75 (s, 2H, ~O–CH_2), 6.85 (m, 1H, NH), 7.08 (m, 1H, arom H), 7.25–7.32 (m, 2H, arom H), 7.51 (m, 2H, arom H/NH); ^{13}C NMR ($CDCl_3$) δ: 27.9, 39.6, 39.7, 55.6, 55.8, 56.0, 75.5, 78.4, 81.8, 112.9, 118.9, 120.3, 129.6, 135.5, 157.6, 167.4, 172.0, 172.4; MS analysis: calcd for $C_{40}H_{64}N_6O_9$, 772.47, found ES-MS 773.90 [M + H]$^+$.

***tert*-Butyl-2,2′,2″-(10-(2-(2-(3,5-bis(prop-2-ynyloxy)benzamido)-ethylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (14).** This compound was synthesized as described for **13** starting from **12** (107 mg, 0.30 mmol). Compound **14** was obtained as a yellowish solid with nearly quantitative yield (250 mg). Mp: 74–84 °C; R_f ($CHCl_3$–MeOH–AcOH 95 : 20 : 3 v/v/v): 0.45; R_t: 18.70 min (C8); ^{1}H NMR ($CDCl_3$) δ: 1.43 (s, 27H, $(CH_3)_3$ ^{t}Bu), 2.04–3.70 (broad s, 28H, CH_2 DOTA (24H)/~NH–CH_2–CH_2–NH~(4H)), 2.52 (s, 2H, CH), 4.73 (s, 4H, ~O–CH_2), 6.73 (m, 2H, arom H4/NH), 7.10 (s, 2H, arom H2/H6), 7.11 (m, 1H, NH); ^{13}C NMR ($CDCl_3$) δ: 27.9, 39.6, 50.0*, 55.6, 55.8, 56.2, 75.6, 78.4, 81.9, 106.4, 106.5, 136.4, 158.7, 167.2, 172.0, 172.4 (*broad signal: CH_2 DOTA); MS analysis: calcd for $C_{43}H_{66}N_6O_{10}$, 826.48, found ES-MS 827.65 [M + H]$^+$; Elemental analysis: calcd for $C_{43}H_{66}N_6O_{10}\cdot K_2SO_4$ C 51.58, H 6.64, N 8.39 found C 52.05, H 6.54, N 8.04%.

***tert*-Butyl-2-(3,5-bis(2-(3,5-bis(prop-2-ynyloxy)benzamido)-ethoxy)benzamido)ethylcarbamate (15).** This compound was synthesized as described for **11** using amine **10** (292 mg, 2.0 mmol) and acid **8** (460 mg, 2.0 mmol). Compound **15** was obtained as a pale yellow solid with 91% yield (1.46 g). Mp: 110 °C; R_f (EtOAc–hexane 4 : 1 v/v): 0.53; R_t: 18.38 min (C8); ^{1}H NMR (DMSO-d$_6$) δ: 1.37 (s, 9H, $(CH_3)_3$ Boc), 3.10 (m, 2H, ~NH–CH_2–CH_2–NH~), 3.28 (m, 2H, ~NH–CH_2–CH_2–NH~), 3.58 (s, 4H, CH), 3.65 (m, 4H, ~O–CH_2–CH_2–NH~), 4.17 (m, 4H, ~O–CH_2–CH_2–NH~), 4.85 (s, 8H, ~O–CH_2), 6.72 (m, 1H, arom H4), 6.80 (m, 2H, arom H2/H6), 6.90 (m, 1H, NH urethane), 7.05 (m, 2H, arom H4′), 7.15 (m, 4H, arom H2′/H6′), 8.44 (m, 1H, NH amide), 8.68 (m, 2H, NH amide); ^{13}C NMR (DMSO-d$_6$) δ: 28.0, 39.2, 55.9, 75.8, 75.9, 77.8, 79.6, 104.7, 105.4, 105.9, 106.7, 135.9, 136.1, 157.2, 158.5, 159.5, 167.7, 167.8, 168.0, 168.1; MS analysis: calcd for $C_{44}H_{46}N_4O_{11}$, 806.32, found ES-MS 807.65 [M + H]$^+$, 707.55 [(M–$C_5H_8O_2$) + H]$^+$; Elemental analysis: calcd for $C_{44}H_{46}N_4O_{11}$ C 65.50, H 5.75, N 6.94 found C 65.28, H 5.71, N 6.80%.

***tert*-Butyl-2,2′,2″-(10-(2-(2-(3,5-bis(2-(3,5-bis(prop-2-ynyloxy)-benzamido)ethoxy)benzamido)ethylamino)-2-oxyethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (16).** Compound **16** was synthesized as described for **13** starting from **15** (242 mg, 0.30 mmol). After workup, the crude product was purified by column chromatography (eluents DCM–MeOH 98 : 2 v/v → DCM–MeOH 9 : 1 v/v) to yield a white solid (227 mg, 60%). Mp: 108–114 °C; R_f (CH_2Cl_2–MeOH 9 : 1 v/v): 0.30; R_t: 19.70 min (C8); ^{1}H NMR ($CDCl_3$) δ: 1.45 (s, 27H, $(CH_3)_3$ ^{t}Bu), 2.04–4.50 (broad s, 36H, CH_2 DOTA (24H)/~NH–CH_2–CH_2–NH~(4H)/~O–CH_2–CH_2–NH~(8H)), 2.55 (s, 4H, CH), 4.72 (m, 8H, ~O–CH_2), 6.60 (m, 1H, arom H4), 6.70 (m, 2H, arom H2/H6), 7.18–7.33 (m, 6H, arom H2′/H4′/H6′), 7.80 (m, 2H, NH), 8.75 (m, 2H, NH); ^{13}C NMR ($CDCl_3$) δ: 27.9, 28.0, 38.8, 39.3, 39.7, 55.7, 55.9, 56.2, 66.5, 75.8, 76.0, 78.3, 82.0, 106.0, 106.3, 106.5, 106.7, 136.3, 136.8, 158.6, 159.4, 166.7, 166.9, 171.5, 172.3; MS analysis: calcd for $C_{67}H_{88}N_8O_{16}$, 1260.63, found ES-MS 1261.75 [M + H]$^+$; Elemental analysis: calcd for $C_{67}H_{88}N_8O_{16}\cdot H_2SO_4$ C 59.19, H 6.67, N 8.24 found C 59.60, H 6.82, N 7.71%.

***N*-ε-Azido *cyclo*(Arg-Gly-Asp-D-Phe-Lys) (19).** Cyclic peptide **18** (200 mg, 0.33 mmol) was dissolved in *tert*-BuOH–H_2O (5 mL; 1 : 1 v/v) and the pH was adjusted to 10 by the addition of

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1 N NaOH. To this solution were added: $CuSO_4 \cdot 5H_2O$ (8 mg, 0.03 mmol, 0.1 equiv) and a solution of triflic azide (587 mg, 3.3 mmol, 10 equiv) in CH_2Cl_2 (freshly prepared from triflic anhydride (555 µL, 3.3 mmol, 10 equiv) and NaN_3 (975 mg, 15 mmol, 4.5 equiv) in CH_2Cl_2–H_2O (13 mL; 10 : 3 v/v).[27] The obtained two-phase reaction mixture was firmly stirred for 16 h at room temperature. Then, the solvents were removed by evaporation and the residue was mixed with *tert*-BuOH–H_2O and subsequently lyophilized to yield 202 mg (97%) crude reaction product. Pure azido peptide **19** was obtained in 21% yield (44 mg) after purification by HPLC (C8). R_f ($CHCl_3$–MeOH–AcOH 90 : 20 : 3 v/v/v): 0.25; R_t: 16.81 min (C4); R_t: 17.33 min (CN); FTIR (KBr) υ: 2100 cm^{-1}; ^1H NMR (500 MHz, H_2O–D_2O 9 : 1 v/v, 293 K, 6.4 mM, pH 4): Arg, δ: 1.43 (m, 2H, γCH_2), 1.65/1.86 (double m, 2H, βCH_2), 3.18 (m, 2H, δCH_2), 4.36 (m, 1H, αCH), 7.20 (t (*J* 5.8 Hz), 1H, δNH), 8.04 (d (*J* 8.7 Hz), 1H, αNH); Gly, δ: 3.49 (dd (*J* 4.5 Hz, *J* 14.8 Hz), 1H, αCH_2), 4.21 (dd (*J* 7.7 Hz, *J* 14.8 Hz), 1H, αCH_2), 8.33/8.36 (dd, (*J* 4.7 Hz, *J* 7.4 Hz), 1H, αNH); Asp, δ: 2.63/2.66 (dd (*J* 6.7 Hz, *J* 16.4 Hz), 1H, βCH_2), 2.79/2.83 (dd (*J* 7.7 Hz, *J* 16.4 Hz), 1H, βCH_2), 4.73 (m, 1H, αCH), 8.12 (d, (*J* 8.8 Hz), 1H, αNH); D-Phe, δ: 2.93/2.98 (dd (*J* 10.3 Hz, *J* 13.2 Hz), 1H, βCH_2), 3.07/3.10 (dd (*J* 5.9 Hz, *J* 13.2 Hz), 1H, βCH_2), 4.45 (m, 1H, αCH), 7.25 (d (*J* 7.3 Hz), 2H, arom H), 7.33–7.38 (m, 3H, arom H), 8.42 (d (*J* 5.9 Hz), 1H, αNH); azido Lys, δ: 0.95 (m, 2H, γCH_2), 1.46/1.65 (double m, 2H, βCH_2), 1.49 (m, 2H, δCH_2), 3.24 (t (*J* 7.1 Hz), 2H, εCH_2), 3.85 (m, 1H, αCH), 8.44 (d (*J* 5.6 Hz), 1H, αNH); ^{13}C NMR (H_2O–D_2O 9 : 1 v/v, 293 K, 6.4 mM, pH 4): Arg, δ: 29.8 γC, 30.0 βC, 43.3 δC, 55.2 αC, 176.0 αCO, 176.3 guanidino C; Gly, δ: 46.3 αC, 172.9 αCO; Asp, δ: 38.5 βC, 52.9 αC, 175.1 αCO, 178.6 βCO; D-Phe, δ: 39.6 βC, 58.1 αC, 130.0 arom CH, 131.5 arom CH, 131.9 arom CH, 138.8 arom qC, 176.5 αCO; azido Lys, δ: 25.1 γC, 27.2 δC, 32.6 βC, 53.3 εC, 58.2 αC, 177.9 αCO; MS analysis: calcd for $C_{27}H_{39}N_{11}O_7$, 629.30, found ES-MS 630.55 $[M + H]^+$, 652.70 $[M + Na]^+$, 668.25 $[M + K]^+$.

General procedure for the microwave-assisted click reaction.[22] The alkyne (1 equiv) and the azide (1.3 equiv per arm) were dissolved in DMF–H_2O. To this solution, $CuSO_4 \cdot 5H_2O$ (0.05 equiv) and Na-ascorbate (0.50 equiv) were added. The reaction mixture was placed in a microwave reactor and irradiated during 10–30 min at 100 °C. The cycloaddition was monitored on TLC and LC-MS for completion of the reaction.

DOTA-conjugated monovalent *cyclo*[RGDfK] peptide dendrimer (23). Alkyne **13** (5.5 mg, 7.1 µmol) and azido peptide **19** (6.0 mg, 8.1 µmol, 1.1 equiv) were dissolved in DMF (500 µL) and 0.05 M Na-ascorbate (72 µL, 3.6 µmol, 0.50 equiv) followed by 6 mM $CuSO_4 \cdot 5H_2O$ (60 µL, 0.36 µmol, 0.05 equiv) were added. The reaction mixture was placed in the microwave reactor and irradiated for 3 × 5 min at 100 °C. Then, the solvents were removed under reduced pressure and the residue was dissolved in *tert*-BuOH–H_2O 1 : 1 v/v and lyophilized. The obtained fluffy solid was dissolved in TFA–H_2O (1 mL; 95 : 5 v/v) and stirred for 4 h at room temperature. Subsequently, the reaction mixture was concentrated *in vacuo* and the residue was redissolved in *tert*-BuOH–H_2O 1 : 1 v/v, lyophilized and purified by semi-prep HPLC (C18) to give compound **23** in 13% yield (1.1 mg). R_t: 10.3 min (C18); MS analysis: calcd for $C_{55}H_{79}N_{17}O_{16}$, 1234.340 (M_{ave}), found MALDI-TOF 1234.807 $[M + H]_{ave}^+$.

DOTA-conjugated divalent *cyclo*[RGDfK] peptide dendrimer (24).[28] Alkyne **14** (4.9 mg, 5.9 µmol) and azido peptide **19** (11 mg, 14.8 µmol, 1.3 equiv) were dissolved in DMF–2,6-lutidine (1 mL, 7 : 3 v/v) and to this solution the following reagents were subsequently added: CuOAc (1.8 mg, 14.7 µmol, 2.5 equiv), Na-ascorbate (5.9 mg, 29.8 µmol, 5.1 equiv) and DIPEA (9.8 µL, 7.1 µmol, 1.2 equiv). The obtained reaction mixture was heated by microwave irradiation to 100 °C for 3 × 5 min. Then, the solvents were removed under reduced pressure and the residue was dissolved in *tert*-BuOH–H_2O 1 : 1 v/v and lyophilized. The obtained fluffy solid was dissolved in TFA–H_2O (1 mL; 95 : 5 v/v) and stirred for 4 h at room temperature. Subsequently, the reaction mixture was concentrated *in vacuo* and the residue was redissolved in *tert*-BuOH–H_2O 1 : 1, lyophilized and purified by semi-prep HPLC (C18) to obtain compound **24** in 11% yield (1.3 mg). R_t: 12.1 min (C18); MS analysis: calcd for $C_{85}H_{120}N_{28}O_{24}$, 1918.067 ($M_{ave}$), found MALDI-TOF 1918.431 $[M + H]_{ave}^+$.

DOTA-conjugated tetravalent *cyclo*[RGDfK] peptide dendrimer (25). Alkyne **16** (3.8 mg, 3.0 µmol) and azido peptide **19** (11 mg, 14.8 µmol, 1.2 equiv) were dissolved in DMF (500 µL) and to this solution, 0.05 M Na-ascorbate (30 µL, 1.5 µmol, 0.50 equiv) followed by 6 mM $CuSO_4 \cdot 5H_2O$ (25 µL, 0.15 µmol, 0.05 equiv) were added. The obtained reaction mixture was heated by microwave irradiation to 100 °C for 2 × 5 min. Then, the solvents were removed under reduced pressure and the residue was dissolved in *tert*-BuOH–H_2O 1 : 1 v/v and lyophilized. The obtained fluffy solid was dissolved in TFA–H_2O (1 mL; 95 : 5 v/v) and stirred for 4 h at room temperature. Subsequently, the reaction mixture was concentrated *in vacuo* and the residue was redissolved in *tert*-BuOH–H_2O 1 : 1 v/v, lyophilized and purified by semi-prep HPLC (C18) to give compound **25** in 36% yield (3.9 mg). R_t: 11.6 min (C18); MS analysis: calcd for $C_{163}H_{220}N_{52}O_{44}$, 3611.873 ($M_{ave}$), found MALDI-TOF 3612.646 $[M + H]_{ave}^+$.

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