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Synthesis of DOTA-Conjugated Multimeric [Tyr^3]Octreotide Peptides via a Combination of Cu(I)-Catalyzed "Click" Cycloaddition and Thio Acid/Sulfonyl Azide "Sulfo-Click" Amidation and Their in Vivo Evaluation

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Herein, we describe the design, synthesis, and biological evaluation of a series of DOTA-conjugated monomeric, dimeric, and tetrameric [Tyr^3]octreotide-based analogues as a tool for tumor imaging and/or radionuclide therapy. These compounds were synthesized using a Cu(I)-catalyzed 1,3-dipolar cycloaddition ("click" reaction) between peptidic azides and dendrimer-derived alkynes and a subsequent metal-free introduction of DOTA via the thio acid/sulfonyl azide amidation ("sulfo-click" reaction). In a competitive binding assay using rat pancreatic AR42J tumor cells, the monomeric [Tyr^3]octreotide conjugate displayed the highest binding affinity (IC_{50} = 1.32 nM) followed by dimeric [Tyr^3]octreotide (2.45 nM), [DOTA^0,Tyr^3]octreotide (2.45 nM), and tetrameric [Tyr^3]octreotide (14.0 nM). Biodistribution studies with BALB/c nude mice with subcutaneous AR42J tumors showed that the ^{111}In-labeled monomeric [Tyr^3]octreotide conjugate had the highest tumor uptake (42.3 ± 2.8 %ID/g) at 2 h p.i., which was better than [^{111}In-DOTA^0,Tyr^3]octreotide (19.5 ± 4.8 %ID/g). The ^{111}In-labeled dimeric [Tyr^3]-octreotide conjugate showed a long tumor retention (25.3 ± 5.9 %ID/g at 2 h p.i. and 12.1 ± 1.3 %ID/g at 24 h p.i.). These promising results can be exploited for therapeutic applications.

Introduction

Somatostatin is a cyclic tetradecapeptide secreted in several locations in the gastrointestinal system and throughout the central nervous system. It plays an important regulatory role in neurotransmission and secretion, and the peptide hormone is known to control cell proliferation in normal tissues and tumors.[1−4] It elicits its biological effects via high-affinity interactions with a family of five different somatostatin receptors (SSTR1−5).[5−7] Pathologically up-regulated receptors, such as overexpression of especially SSTR2[a] in neuroendocrine tumors, can be exploited in functional imaging techniques, allowing radionuclide imaging of tumor lesions. The short plasma half-life and consequent limited therapeutic potential of somatostatin have been overcome with the availability of [Tyr^3]octreotide, a long-acting, synthetic octapeptide analogue of the naturally occurring hormone. This chemically engineered [Tyr^3]octreotide has an enhanced stability toward proteolytic degradation, leading to a prolonged inhibitory effect in somatostatin-responsive tumors.[8] Several radiopharmaceuticals based on [Tyr^3]octreotide, functionalized with a diethylenetriamine pentaacetic acid (DTPA) or 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), are applied for imaging and treatment of neuroendocrine tumors in cancer patients.[9−13]

There is a growing interest in the development of multivalent receptor targeting ligands because they have higher binding affinities than monovalent analogues.[14−19] A promising approach toward polyvalent systems is the use of dendrimers as a versatile scaffold. Unique features, such as the stepwise synthetic control of branching, the well-defined monodisperse structure, the possibility of specifically tailored peripheral groups, and the efficient and reproducible synthesis, make dendrimers ideal molecular scaffolds for the simultaneous presentation of biologically relevant ligands.[20−26] We have described a versatile procedure using amino acid based dendrimers, with a considerable degree of molecular diversity in the dendrimer synthetic strategy, to design and synthesize multivalent carbohydrates[27−30] and peptides.[31−34]

An important issue is the complete and efficient attachment of ligands to dendrimers. In addition to other chemoselective conjugation reactions,[35−40] the Cu(I)-catalyzed variant[41−44] of the Huisgen 1,3-dipolar cycloaddition[45] has led to a plethora of biomedical applications in drug discovery, drug delivery, and nanomedicine research involving peptidomimetics, biomaterials,

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[a]Abbreviations: SSTR2, somatostatin subtype 2 receptor; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; Fmoc, 9*H*-fluoren-9-ylmethoxycarbonyl; TMOB, 2,4,6-trimethoxybenzyl; EDCI, ethyl-3-(3-dimethylaminopropyl)carbodiimide; ^{t}Bu, *tert*-butyl; Cbz, benzyloxycarbonyl; TFA, trifluoroacetic acid; BOP, benzotriazolyloxy-tris(dimethylamino)phosphonium hexafluorophosphate; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; PBS, phosphate buffered saline; IC_{50}, half maximal inhibitory concentration; CI, confidence interval; DMF, dimethylformamide; DCM, dichloromethane; DMAP, 4-dimethylaminopyridine.

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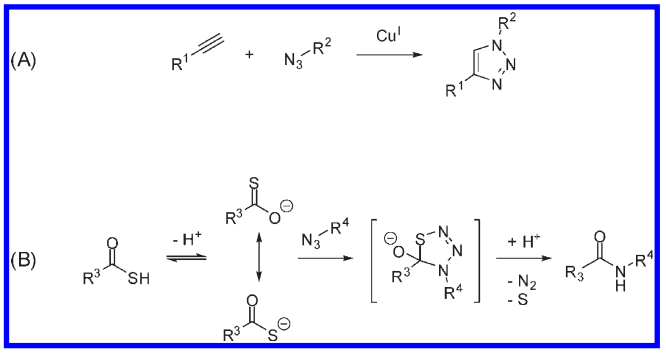
Scheme 1. Click Chemistry Reactions[a]



[a] (A) Cu(I)-catalyzed 1,3-dipolar cycloaddition of azides and terminal alkynes, yielding 1,4-disubstituted 1,2,3-triazoles ("click chemistry"). (B) Thio acid/sulfonyl azide amidation reaction via a thiatriazoline intermediate, leading to amide, nitrogen, and sulfur ("sulfo-click chemistry").

oligonucleotides, and carbohydrates.[46−55] This reaction, which regiospecifically gives 1,4-disubstituted 1,2,3-triazoles (Scheme 1A), is particularly suitable for chemoselective bioconjugation reactions with *unprotected* peptide ligands to dendrimers.[31−34]

Recently, we reported the successful synthesis of multimeric [Tyr^3]octreotide conjugates using the Cu(I)-catalyzed click reaction between dendrimeric alkynes and azido peptides.[34] However, a disadvantage of this approach is that during the click reaction, copper could be chelated by DOTA, as was indeed observed previously.[34] This will hamper efficient labeling of the DOTA moiety with radioactive metal ions and hampers (radiolabeling and) in vivo applications. To avoid copper contamination, alternative copper-free "click" approaches are particularly relevant.[56−58] In the recent literature interesting methodologies toward metal-free triazole formation are described that involve strained cyclooctyne derivatives and electron-deficient alkyne or oxanorbornadiene moieties.[59−66] However, these conjugation procedures lack regioselectivity, which prevents precise control over the product that is formed, and often suffer from poor conversion or relatively lengthy reaction times and/or formation of (noxious) undesired side products.

As an alternative metal-free "click" reaction, the chemoselective amidation reaction between thio acids and sulfonyl azides is receiving increased attention as an important coupling method. The reaction between thio acids and (sulfonyl) azides was first mentioned in the literature in 1980,[67] and it was assumed that the reaction took place via reduction of the azide to the corresponding amine prior to acylation by the thio acid, a very rapid but otherwise conventional, nucleophilic substitution.[68,69] Thorough mechanistic studies by Williams et al.[70,71] revealed that this amidation reaction proceeded via a thiatriazoline intermediate rather than via acetylation of the free amine (Scheme 1B). The potentially chemical orthogonality of this reaction has led to a renewed interest in the thio acid/sulfonyl azide amidation reaction as a valuable synthetic tool for the modification/coupling of unprotected peptides in solution or on solid support[72−75] and the chemoselective modulation of bioactive proteins for biomedical research.[76,77] Encouraged by these promising results, we extended the application of the thio acid/sulfonyl azide amidation, which we denote as "sulfo-click",[78] to the metal-free introduction of a DOTA-sulfonyl azide in multimeric [Tyr^3]octreotide thio acids. This resulted in a highly efficient synthesis and radiometal-labeling of

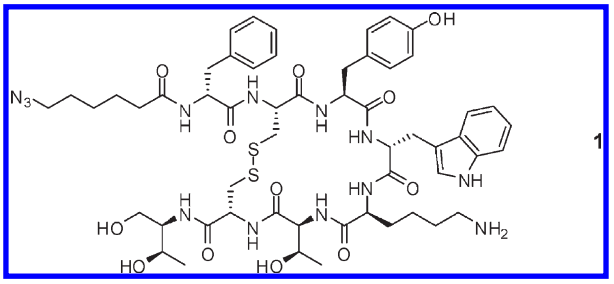


Figure 1. Chemical structure of N_3-Ahx-D-Phe-cyclo(Cys-Tyr-D-Trp-Lys-Thr-Cys)-Threol **1**.

DOTA-conjugated mono-, di-, and tetrameric [Tyr^3]octreotide peptides.

Thus, we describe a two-stage ligation procedure that combines the established Cu(I)-catalyzed "click" reaction between dendrimeric alkynes and peptidic azides, and the subsequent copper-free thio acid/azide "sulfo-click" amidation reaction between the obtained dendrimeric peptide thio acids and a DOTA-derived sulfonyl azide. The receptor binding characteristics of the resulting mono-, di-, and tetrameric [Tyr^3]-octreotide conjugates were determined in a competitive binding assay using AR42J tumor cells and compared to native [Tyr^3]octreotide. Biodistribution of ^{111}In-labeled compounds was evaluated in an in vivo study with BALB/c nude mice with subcutaneous AR42J tumors. The uptake of the ^{111}In-labeled DOTA-functionalized dimeric [Tyr^3]octreotide conjugate was higher at 6 and 24 h after administration when compared to the tumor uptake of [^{111}In-DOTA^0,Tyr^3]octreotide. These findings are promising results for novel therapeutic applications of [Tyr^3]octreotide-based conjugates.

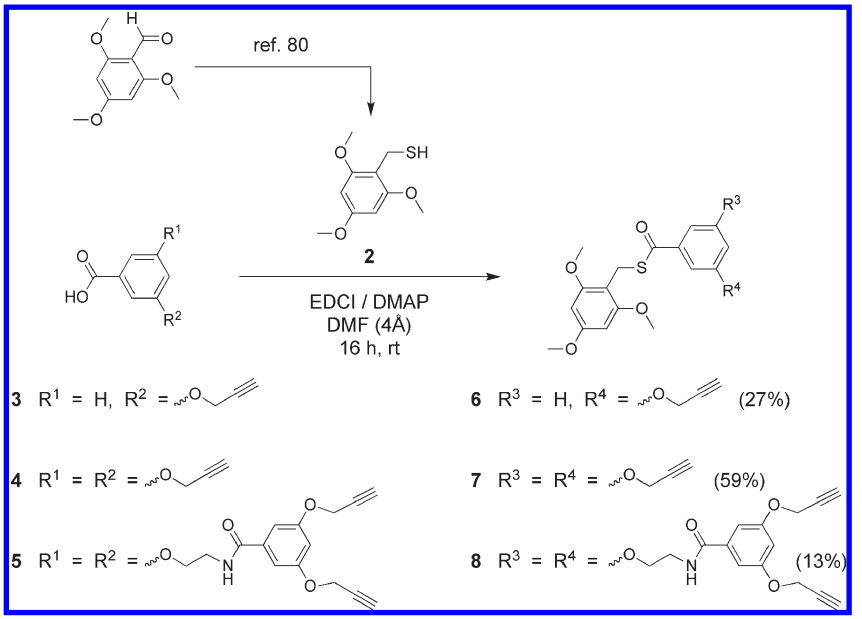
Results and Discussion

Chemistry. Syntheses of Cyclic [Tyr^3]Octreotide Azides and Dendrimeric Alkyne-Based Thio Esters. Cyclic [Tyr^3]-octreotide **1** with an N-terminal azide functionality (Figure 1) was prepared on the 2-chlorotrityl chloride resin via Fmoc/^{t}Bu solid phase chemistry, as was described previously.[34]

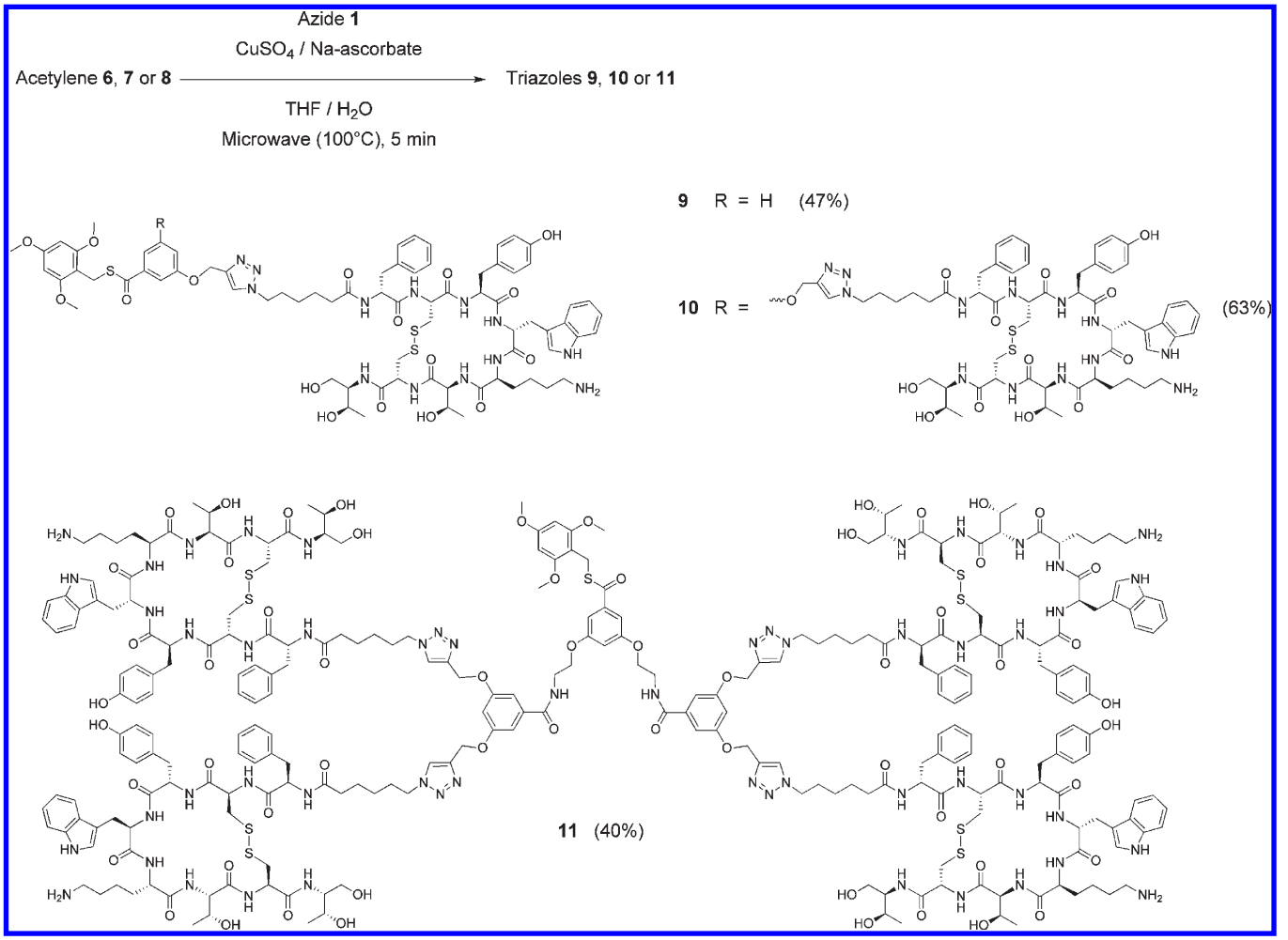
The combination of two orthogonal conjugation methods, as outlined in the Introduction, requires a bifunctional scaffold with a peripheral acetylene moiety to facilitate the Cu(I)-catalyzed click reaction and a thio acid functionality to initiate the thio acid/sulfonyl azide amidation reaction. We have developed a modular approach using easily accessible building blocks, with a considerable degree of molecular diversity in monomer structure, for the synthesis of bifunctional amino acid based dendrimers. This flexible synthetic strategy, based on the dihydroxybenzoic acid moiety, can be easily modified with a variety of branching units, rigidity, cavity size, and tunable surface functionalities.[79] Alkyne-derived benzoic acids **3**−**5** (Scheme 2) were prepared following a procedure that was previously described.[31,32,34] The thio acid moiety can be readily prepared from a 2,4,6-trimethoxybenzyl (TMOB) thio ester precursor, according to Vetter.[80] Hence, mono-, di-, and tetrameric dendrimeric alkynes **6**−**8** with TMOB-protected thio acids were synthesized from the corresponding carboxylic acids **3**−**5** and TMOB-thiol **2** by EDCI-mediated esterification, as is depicted in Scheme 2.

Conjugation of [Tyr^3]Octreotide to Dendrimers by the Copper-Catalyzed Click Cycloaddition Reaction Procedure. The Cu(I)-catalyzed coupling of azide **1** to TMOB-alkynes **6**, **7**, and **8** was performed as previously reported.[31,32,34]

Scheme 2. Synthesis of TMOB-Protected Mono-, Di-, and Tetrameric Alkyne-Derived Thio Esters **6**, **7**, and **8**, Respectively



Scheme 3. Synthesis of Mono-, Di-, and Tetrameric [Tyr^3]Octreotide Triazoles **9**, **10**, and **11**, Respectively

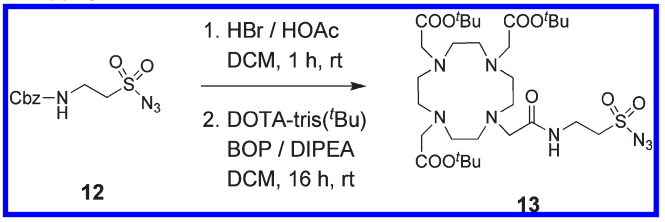


The synthetic route toward the corresponding mono-, di-, and tetrameric [Tyr^3]octreotide conjugates **9**, **10**, and **11**, respectively, is outlined in Scheme 3. Following our procedure for microwave-assisted cycloaddition chemistry, azide **1** was reacted with acetylene **6**, **7**, or **8** in the presence of $CuSO_4$/sodium ascorbate in THF/H_2O (1:1 v/v) under microwave irradiation at 100 °C. HPLC analysis showed complete conversion within 5 min. Optimization studies showed that using

50 mol % $CuSO_4$, accompanied by 2.5 equiv of sodium ascorbate, gave the best results for the examined click reactions. The identity of the cycloadducts **9**−**11** was confirmed by mass spectrometry.

Conjugation of DOTA to Mono-, Di-, and Tetrameric [Tyr^3]Octreotide Triazoles by the Thio Acid/Sulfonyl Azide Amidation Ligation Method. For use in the thio acid/sulfonyl azide amidation reaction, DOTA was derivatized with a sulfonyl azide linker. For this purpose the Cbz-protected taurylsulfonyl azide **12** was used.[81] The N-terminal Cbz functionality of **12** was removed by treatment with HBr/glacial acetic acid, and the hygroscopic hydrobromide was transformed into the hydrochloride using an ion-exchange resin. Subsequently, DOTA-Tris(^{t}Bu) was coupled using BOP to give ^{t}Bu-protected DOTA-derived sulfonyl azide **13** in 20% yield (Scheme 4), after column chromatography. Several attempts using different coupling reagents, concentrations, reaction times, and workup procedures were studied to improve yields for the synthesis of compound **13**; however, the yield obtained with BOP reagent was the highest in this optimization series.

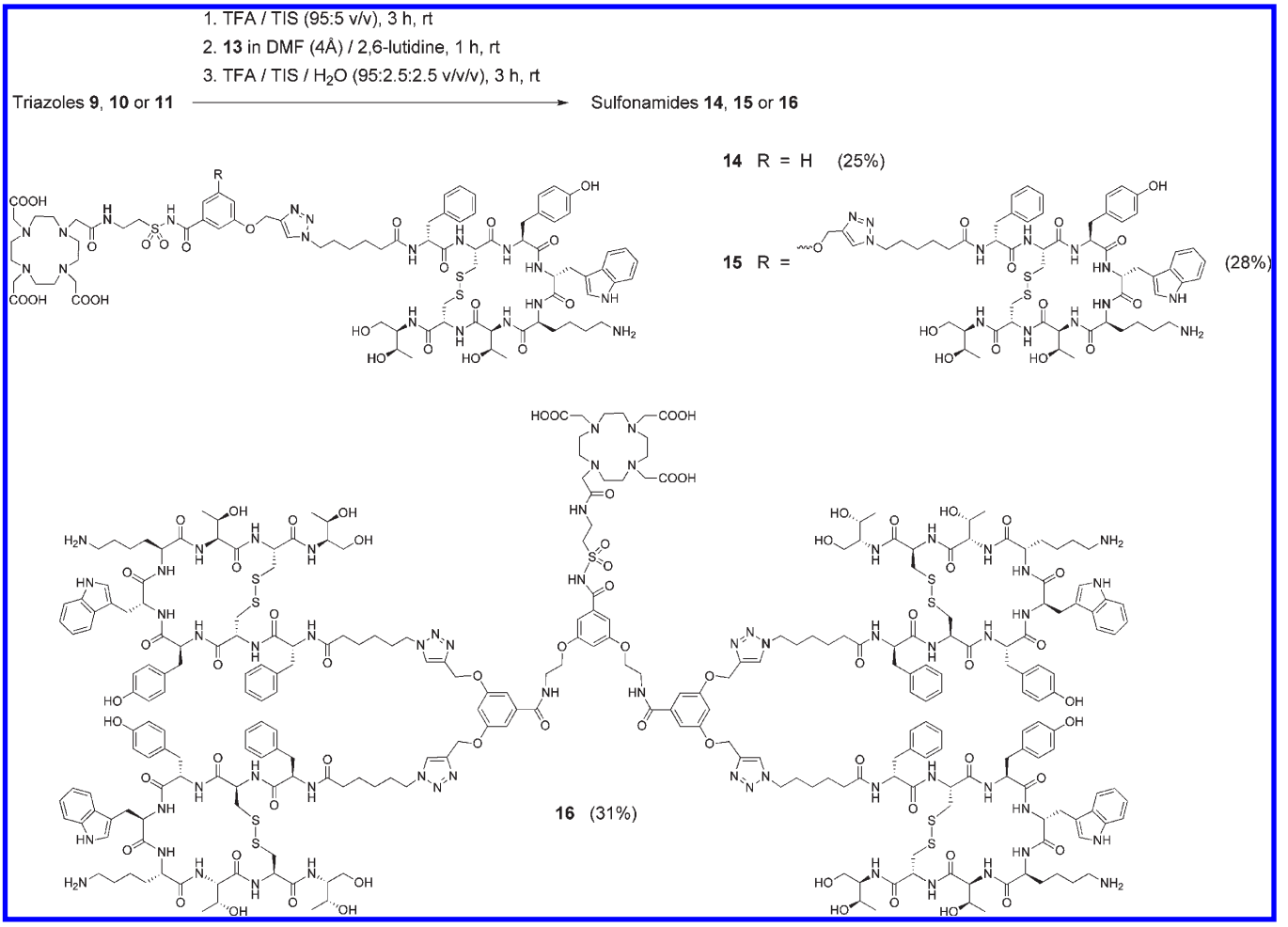
Scheme 4. Synthesis of ^{t}Bu-Protected DOTA-Derived Sulfonyl Azide **13**



Next, *N*-acylsulfonamides **14**−**16** were prepared from thio esters **9**−**11** in three steps according to previously established protocols.[70−72] Treatment with TFA of mono-, di-, or tetrameric [Tyr^3]octreotide thio ester **9**, **10**, or **11** released the corresponding thio acid intermediate which was immediately reacted with DOTA(^{t}Bu)sulfonyl azide **13** in the presence of 2,6-lutidine as a base (Scheme 5). This amidation process was monitored by HPLC and was found to be complete within 1 h. The obtained *N*-acylsulfonamides with a ^{t}Bu-protected DOTA moiety were treated with TFA to give, after purification by HPLC, mono-, di-, and tetrameric DOTA-conjugated [Tyr^3]octreotide sulfonamides **14**, **15**, and **16** in a decent yield of 67% per step. Figure 2 shows the ESI-MS spectrum of dimeric [Tyr^3]octreotide sulfonamide **15** with no indication of additional Cu-DOTA complexes. A clear advantage of both the applied cycloaddition chemistry and the thio acid/sulfonyl azide amidation reaction is the orthogonality with functional groups in this peptide. This study has therefore clearly demonstrated the compatibility of these chemoselective coupling methods using *unprotected* peptide derivatives leading to no metal contamination of the DOTA moiety.

Radiolabeling. ^{111}In-Labeling of the DOTA-conjugated mono-, di-, and tetrameric [Tyr^3]octreotide peptides **14**−**16** was performed in NH_4OAc buffer (pH 5.5) with $^{111}InCl_3$ by

Scheme 5. Synthesis of Mono-, Di-, and Tetrameric DOTA-Conjugated [Tyr^3]Octreotide Sulfonamides **14**, **15**, and **16**, Respectively



heating the reaction mixture for 10−15 min at 95 °C, and the labeling process was monitored by radio-HPLC. The radiochemical yield was more than 98% with a specific activity of 7.4 MBq/μg for both ^{111}In-labeled **14** (corresponding to 13.6 GBq/μmol) and **15** (22.7 GBq/μmol) and was 80% for ^{111}In-labeled **16** (43.3 GBq/μmol). These efficient labeling yields are evidently the result of the current approach, which comprises a significant synthetic improvement toward DOTA-conjugated mono- and multimeric [Tyr^3]octreotide biomolecules without contamination by Cu-DOTA adducts resulting from the remaining copper ions in a Cu(I)-catalyzed "click" cycloaddition.[34]

Lipophilicity Studies. The log(D) values of ^{111}In-labeled DOTA-conjugated mono-, di-, and tetrameric [Tyr^3]octreotide sulfonamides **14**, **15**, and **16** were −2.20 ± 0.17, −1.79 ± 0.11, and −0.71 ± 0.01, respectively, and obtained from *n*-octanol/PBS partition coefficient measurements. Compared to [^{111}In-DOTA^0,Tyr^3]octreotide (log(D) = −3.59 ± 0.14), ^{111}In-labeled compound **14** was more lipophilic reflected by the log D value which was 25-fold higher. This could be attributed to the aromatic character of the dendrimer-derived linker moiety.

Receptor Binding Studies. Binding affinities for the somatostatin receptor of the DOTA-conjugated mono-, di-, and tetrameric [Tyr^3]octreotide peptides **14**, **15**, and **16**, respectively, were determined using a competitive binding assay with AR42J tumor cells, using [^{111}In-DOTA^0,Tyr^3]octreotide as a radiotracer. The IC_{50} values were calculated from fitted sigmoidal displacement curves as depicted in Figure 3A and are summarized in Table 1. As a reference, commercially available [DOTA^0,Tyr^3]octreotide was included as one of the competing ligands in the same in vitro assay. The obtained IC_{50} values for the AR42J cell line were in the low nanomolar range, indicating the high binding affinity of the synthesized compounds.

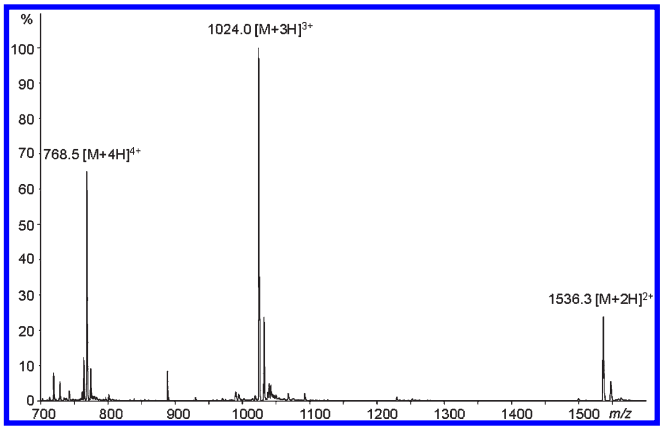


Figure 2. ESI-MS spectrum of dimeric [Tyr^3]octreotide sulfonamide **15**: m/z found, 3070.0; calcd, 3069.3 (isotopic)/3071.6 (average).

Both the monomeric [Tyr^3]octreotide conjugate **14** and the dimeric [Tyr^3]octreotide conjugate **15** had IC_{50} values of 1.32 and 2.45 nM, respectively, comparable to that of the reference compound [DOTA^0,Tyr^3]octreotide (IC_{50} = 2.45 nM). When the valency was increased from two to four [Tyr^3]-octreotide moieties (compound **16**), a 5.7-fold decrease in affinity (IC_{50} = 14.0 nM) was observed. The displacement curve of conjugate **16** (Figure 3A) did not show a sigmoidal shape, suggesting that the compound also bound aspecifically. The hypothesized increase in receptor affinity due to multimerization was not observed, especially in the case of [Tyr^3]octreotide tetramer **16**, which may be attributed to the limited water solubility of these [Tyr^3]octreotide conjugates.

To assess the binding properties of In-containing DOTA-complexes, an additional binding experiment was performed with ^{115}In-labeled DOTA-conjugated compounds **14**−**16** using similar protocols. Overall, the IC_{50} values for all ^{115}In-labeled compounds improved by a factor of 1.4−2.9 (Figure 3B and Table 1). This improvement in binding strength was of importance and justified the use of these compounds in in vivo experiments.

Biodistribution Study. The in vivo characteristics and tumor targeting of ^{111}In-labeled **14**−**16** were investigated in BALB/c nude mice with subcutaneous SSTR2 expressing AR42J tumors. ^{111}In-labeled [DOTA^0,Tyr^3]octreotide was taken as the reference compound (see Supporting Information Figure SI 11A and Table SI 11B), with tumor uptake of 19.5 ± 4.84 %ID/g at 2 h p.i. and 7.47 ± 0.78 %ID/g at 24 h p.i. The organ distribution of ^{111}In-labeled **14** and **15** after 2, 6, and 24 h p.i. is summarized in Figure 4 and in Table 2. ^{111}In-Labeled compound **14** (Figure 4A) had a rapid and high tumor uptake of 42.33 ± 2.80 %ID/g at 2 h p.i., and this could be blocked by co-injection of an excess of octreotide, an indication that tumor accumulation was a receptor-mediated

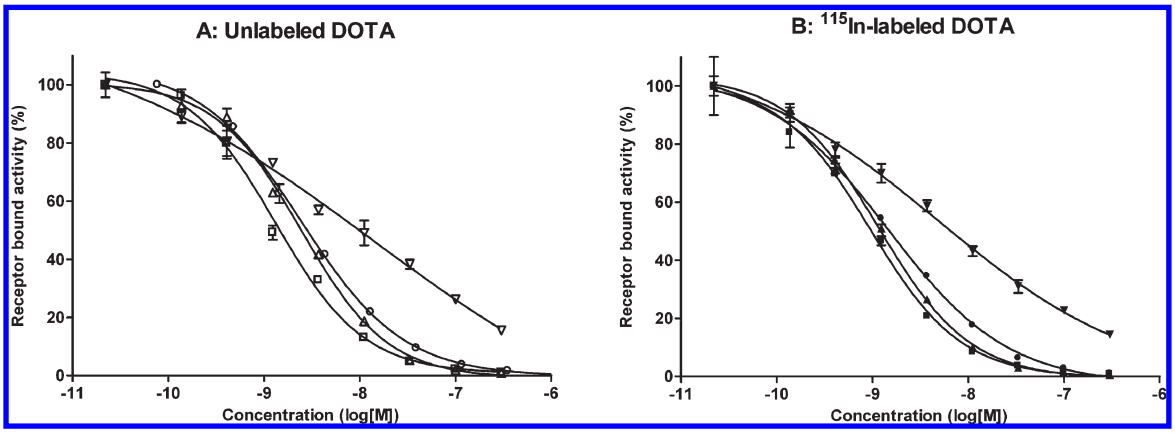


Figure 3. (A) Competitive binding assay of mono-, di-, and tetrameric DOTA-conjugated systems of [Tyr^3]octreotide **14** (□), **15** (△), and **16** (▽), respectively, displacing [^{111}In-DOTA^0,Tyr^3]octreotide on AR42J tumor cells (n = 2 for each data point). [DOTA^0,Tyr^3]octreotide (○) was used as reference compound. (B) Competitive binding assay of mono-, di-, and tetrameric ^{115}In-DOTA-conjugated systems of [Tyr^3]octreotide **14** (■), **15** (▲), and **16** (▼), respectively, displacing [^{111}In-DOTA^0,Tyr^3]octreotide on AR42J tumor cells. [^{115}In-DOTA^0,Tyr^3]octreotide (●) was used as reference compound. The error bars indicate the range of the 95% confidence interval.

Table 1. IC_{50} Values for Competitive Binding Assay of Mono-, Di-, and Tetrameric [0]DOTA- and [^{115}In]DOTA-Conjugated Systems of [Tyr^3]-Octreotide Displacing [^{111}In-DOTA^0,Tyr^3]Octreotide with AR42J Tumor Cells[a]

competing ligand	IC_{50} (nM)	95% CI (nM)
DOTA		
monomer **14**	1.32	1.10 − 1.57
dimer **15**	2.45	2.14 − 2.81
tetramer **16**	14.0	4.30 − 45.5
[DOTA^0,Tyr^3]octreotide	2.45	2.26 − 2.65
[^{115}In]DOTA		
monomer **14**	0.950	0.845 − 1.07
dimer **15**	1.16	1.05 − 1.28
tetramer **16**	4.75	3.49 − 6.45
[^{115}In-DOTA^0,Tyr^3]octreotide	1.45	1.26 − 1.68

[a] $n = 2$ for each data point.

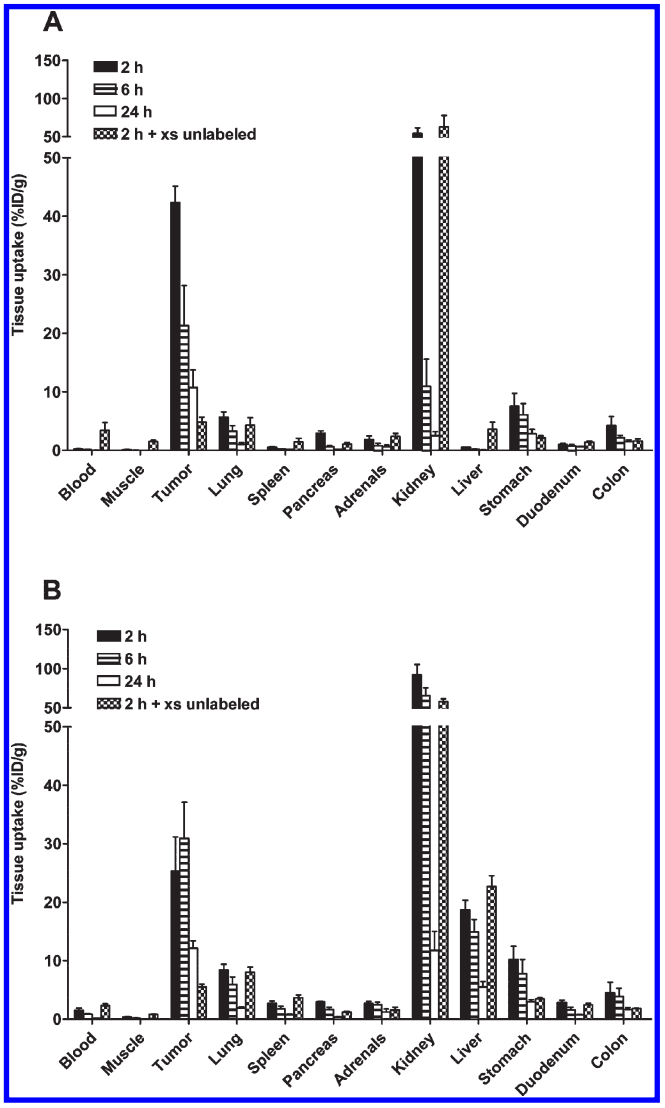


Figure 4. Biodistribution of [^{111}In]DOTA-conjugated [Tyr^3]octreotide analogue **14** (A) and [^{111}In]DOTA-conjugated dimeric octreotide **15** (B) in BALB/c nude mice bearing subcutaneous AR42J expressing tumors in the right flank. Values are given as percentage of the injected dose per gram of tissue ($n = 5$ mice/group). Blocking was performed by co-injection of, respectively, 900 and 1500 molar excess of octreotide. Mice were dissected at 2, 6, and 24 h p.i.

process. Lower, but specific, uptake was observed in the pancreas, stomach, and colon. Apart from the kidney, the tumor was the tissue with the highest radioactivity concentration. Its

Table 2. Biodistribution of [^{111}In]DOTA-Conjugated [Tyr^3]Octreotide Analogue **14** and [^{111}In]DOTA-Conjugated Dimeric [Tyr^3]Octreotide **15** in AR42J Tumor-Bearing BALB/c Nude Mice after 2, 6, and 24 h p.i.[a]

	time after injection			
	2 h	6 h	24 h	2 h + cold
	[Tyr^3]Octreotide Monomer **14**			
blood	0.27 ± 0.03	0.13 ± 0.06	0.04 ± 0.01	3.46 ± 1.28
tumor	42.33 ± 2.80	21.32 ± 6.85	10.75 ± 2.98	4.82 ± 0.87
pancreas	2.92 ± 0.43	0.63 ± 0.21	0.17 ± 0.03	1.07 ± 0.27
adrenals	1.86 ± 0.58	0.78 ± 0.41	0.63 ± 0.27	2.38 ± 0.53
kidney	54.19 ± 7.44	10.96 ± 4.62	2.53 ± 0.67	62.74 ± 15.28
liver	0.52 ± 0.03	0.22 ± 0.06	0.09 ± 0.01	3.61 ± 1.21
stomach	7.54 ± 2.22	6.11 ± 1.89	2.83 ± 0.76	2.16 ± 0.34
	[Tyr^3]Octreotide Dimer **15**			
blood	1.52 ± 0.36	0.86 ± 0.11	0.17 ± 0.01	2.31 ± 0.30
tumor	25.31 ± 5.86	30.91 ± 6.16	12.14 ± 1.28	5.55 ± 0.43
pancreas	2.96 ± 0.09	1.69 ± 0.35	0.36 ± 0.02	1.18 ± 0.18
adrenals	2.69 ± 0.37	2.49 ± 0.43	1.27 ± 0.50	1.56 ± 0.50
kidney	92.20 ± 13.08	65.61 ± 9.71	11.74 ± 3.31	57.78 ± 3.79
liver	18.67 ± 1.66	14.92 ± 2.13	5.54 ± 0.90	22.67 ± 1.86
stomach	10.21 ± 2.26	7.78 ± 2.44	2.97 ± 0.35	3.46 ± 0.24

[a] Each value represents the mean % injected dose per gram of tissue ± SD of five animals (2 h + cold: three animals). Mean tumor weight was 0.24 g.

blood clearance was very fast (0.27 ± 0.03 %ID/g at 2 h p.i. and 0.04 ± 0.01 %ID/g at 24 h p.i.) with the tumor/blood ratios increasing from 156 ± 18.9 at 2 h p.i. to 268 ± 104 at 24 h p.i. The higher tumor uptake of ^{111}In-labeled compound **14** compared to [^{111}In-DOTA^0,Tyr^3]octreotide emphasizes the importance of the implemented sulfonamide linker and dendrimer-derived core for tumor targeting.

^{111}In-Labeled dimer [Tyr^3]octreotide **15** showed a high and specific tumor uptake of 25.31 ± 5.86 %ID/g at 2 h p.i. (Figure 4B, Table 2). Interestingly, the activity in the tumor remained high at 6 h p.i. (30.91 ± 6.16 %ID/g), which was an indication for a prolonged retention of this compound in the tumor. The tumor uptake of ^{111}In-labeled **15** at 6 h was similar to the tumor uptake of the ^{111}In-labeled [Tyr^3]octreotide monomer **14** (21.32 ± 6.85 %ID/g at 6 h p.i.) ($P = 0.063$).

Apart from the undesirable kidney accumulation, radiolabeled **15** had a high liver uptake (18.67 ± 1.66 %ID/g at 2 h p.i. to 5.54 ± 0.90 %ID/g at 24 h p.i.), which was completely absent for radiolabeled **14** (0.52 ± 0.03 %ID/g at 2 h p.i. to 0.09 ± 0.01 %ID/g at 24 h p.i.). However, from the results of the octanol/PBS partition coefficient studies it was expected that the increased lipophilic character of the dimer **15** could have implications for liver uptake and kidney accumulation.

Also, the in vivo tumor uptake of ^{111}In-labeled dimeric [Tyr^3]octreotide conjugate **15** was significantly higher than [^{111}In-DOTA^0,Tyr^3]octreotide at 6 and 24 h after administration ($P < 0.05$). From this, the dimeric [Tyr^3]octreotide conjugate **15** showed interesting properties for radionuclide therapy studies because of its long tumor retention. The receptor binding affinity may be improved, and the undesirable kidney and liver accumulation may be limited, by further optimizing the spacer length and hydrophilic character.[28,30] These considerations are currently addressed in a study in which the effects of different spacers are investigated.

The biodistribution of ^{111}In-labeled [Tyr^3]octreotide tetramer **16** indicated that this compound behaved quite differently compared to its monomeric and dimeric counterparts, **14** and **15**, respectively. Besides the high liver uptake, biodistribution

data of ^{111}In-labeled **16** showed a high spleen uptake (Supporting Information Figure SI 12A and Table SI 12B), which could be attributed to the rather lipophilic character and thus reduced water solubility of ^{111}In-labeled **16**. Unfortunately, ^{111}In-labeled [Tyr^3]octreotide tetramer **16** showed negligible tumor accumulation. On the basis of the octanol/PBS partition coefficient, receptor binding properties, and tissue distribution data, it became clear that tetramerization of [Tyr^3]octreotide had profound implications on its lipophilic character and consequently on receptor affinity and tumor uptake.

Conclusions

In the present study, we have demonstrated that the combined approach using the Cu(I)-catalyzed 1,3-dipolar cycloaddition between dendrimeric alkynes and peptidic azides and subsequent copper-free thio acid/sulfonyl azide amidation reaction with DOTA-derived sulfonyl azides is a powerful method for the synthesis of DOTA-conjugated mono- and multimeric [Tyr^3]octreotide biomolecules. Since the DOTA moiety was not contaminated with residual Cu^+/Cu^{2+}, radiolabeling with $^{111}In^{3+}$ of the final compounds was highly efficient, resulting in a specific activity of up to 43 GBq/μmol.

In a competitive binding assay using AR42J tumor cells it was shown that the IC_{50} values of mono- and multimeric [Tyr^3]octreotide-based conjugates were all in the low nanomolar range. The monomeric [Tyr^3]octreotide conjugate **14** displayed the highest binding affinity (IC_{50} = 1.32 nM) followed by dimeric [Tyr^3]octreotide **15** (2.45 nM), and tetrameric [Tyr^3]octreotide (14.0 nM). The effect of [Tyr^3]octreotide multimerization on its receptor-binding affinity was less than expected, which may be attributed to the decreased water solubility, especially in the case of tetramer **16**. Thus, the high lipophilic character of tetrameric [Tyr^3]octreotide conjugate **16** could have compromised the receptor binding affinity of this compound.

^{111}In-Labeled [Tyr^3]octreotide conjugate **14** showed the highest tumor uptake (42.3 ± 2.8 %ID/g at 2 h p.i.) in BALB/c nude mice inoculated with sc AR42J tumors, which was considerably higher than that for [^{111}In-DOTA^0, Tyr^3]octreotide (19.5 ± 4.8 %ID/g at 2 h p.i.). Radiolabeled [Tyr^3]octreotide dimer **15** exhibited a long tumor retention, probably due to the bivalency effect, which implied that dimeric [Tyr^3]octreotide conjugate **15** could have interesting properties for therapeutic and clinical applications.

Experimental Section

General Procedures. Materials and Analysis Methods. Unless stated otherwise, chemicals were obtained from commercial sources and used without purification. Peptide-grade solvents were purchased from Biosolve and used directly except for dimethylformamide (DMF), *N*-methylpyrrolidone (NMP), and dichloromethane (DCM), which were dried on 4 Å molecular sieves prior to (solid phase) synthesis. 2-Chlorotrityl chloride resin was purchased from Tianjin Nankai Hecheng Science & Technology Company Ltd., and N^{α}-fluorenylmethyloxycarbonyl (Fmoc) amino acids were purchased from Advanced ChemTech, Alexis, or Novabiochem. Commercially available (Novartis) octreotide (Sandostatin) was used as a receptor-selective ligand. Solid phase synthesis was carried out in plastic syringes with a polyethylene frit (20 μm) obtained from Applied Separations Inc. The resin loading was determined by measuring the UV absorbance of the piperidine−dibenzofulvene adduct (λ_{max} = 300 nm).[82] Thin layer chromatography (TLC) and R_f values were determined on Merck precoated silica gel 60 F-254 (0.25 mm) plates. Spots were visualized with UV light, ninhydrin, or Cl_2/*N*,*N*,*N*′,*N*′-tetramethyl-4,4′-diaminodiphenylmethane (TDM).[83] Column chromatography was performed with Silicycle silica gel 60 Å (particle size of 41−63 μm). Melting points were measured on a Büchi Schmelzpunktbestimmungsapparat (according to Dr. Tottoli) and are uncorrected. Microwave assisted reactions were carried out in a Biotage initiator. ^{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 (0.00 ppm). ^{13}C NMR spectra were recorded on a Varian G-300 (75.5 MHz) spectrometer, and chemical shifts are given in ppm (δ) relative to $CDCl_3$ (77.0 ppm). The ^{13}C NMR spectra were recorded using the attached proton test (APT) sequence. (Semi)Preparative HPLC runs were performed on a Kratos HPLC workstation, and analytical HPLC runs were carried out on a Shimadzu HPLC system. Two buffer systems were used: (I) buffer A consisting of 0.1% trifluoroacetic acid (TFA) in CH_3CN/H_2O, 5:95 v/v, and buffer B consisting of 0.1% TFA in CH_3CN/H_2O, 95:5 v/v, and (II) buffer C consisting of 0.1% TFA in CH_3OH/H_2O, 20:80 v/v, and buffer D consisting of 0.1% TFA in CH_3OH/H_2O, 95:5 v/v. Preparative HPLC runs were performed on an Alltech Adsorbosphere XL C_8 column (250 mm × 22 mm, pore size of 90 Å, particle size of 10 μm) using a linear gradient of buffer B (0−100% in 120 min) in buffer A at a flow rate of 12.0 mL/min, and semipreparative HPLC runs were performed on an Alltech Alltima C_8 column (250 mm × 10 mm, pore size of 90 Å, particle size of 10 μm) using a linear gradient of buffer B (0−100% in 120 min) in buffer A at a flow rate of 4.8 mL/min. Analytical runs were performed on an Alltech Alltima C_8 column (250 mm × 4.6 mm, pore size of 90 Å, particle size of 5 μm) using a linear gradient of buffer B (0−100% in 20 min) in buffer A at a flow rate of 1.0 mL/min or a linear gradient of buffer D (0−100% in 40 min) in buffer C at a flow rate of 0.5 mL/min. The purity of all newly synthesized compounds was judged by analytical HPLC and was found to be at least 98%. Characterization of the compounds was performed with electrospray ionization (ESI) mass spectrometry on a Micromass LCT mass spectrometer calibrated with CsI using nano-ESI at 1200 V capillary voltage and 50 V at the sample cone.

Chemistry. The syntheses of N_3-Ahx-D-Phe-cyclo(Cys-Tyr-D-Trp-Lys-Thr-Cys)-Threol (**1**), 3-(prop-2-ynyloxy)benzoic acid (**3**), 3,5-bis(prop-2-ynyloxy)benzoic acid (**4**), and 3,5-bis(2-(3,5-bis(prop-2-ynyloxy)benzamido)ethoxy)benzoic acid (**5**) have been described previously.[34] The synthesis of 2,4,6-trimethoxybenzyl thiol (**2**) was performed as described by Vetter.[80] Details of the synthesis and characterization of compounds **7**, **8**, **10**, **11**, and **16** are described in the Supporting Information.

***S*-2,4,6-Trimethoxybenzyl 3-(Prop-2-ynyloxy)benzothioate (6).** To a solution of **3** (100 mg, 568 μmol, 1.1 equiv) in dry DCM (10 mL), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) (149 mg, 775 μmol, 1.5 equiv) and 4-dimethylaminopyridine (DMAP) (4.0 mg, 33 μmol, 0.05 equiv) followed by **2** (112 mg, 516 μmol, 1.0 equiv) were added. After being stirred for 72 h at room temperature under an N_2 atmosphere, the reaction mixture was concentrated in vacuo. The yellow oily residue was dissolved in DCM (50 mL) and washed with 1 N $KHSO_4$, brine, dried (Na_2SO_4), and concentrated in vacuo. The crude product was purified by column chromatography and isolated as a white solid in 39% yield (210 μmol, 75 mg). R_f(EtOAc/hexane, 2:8 v/v): 0.32. Mp: 84 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.61 (m, 1H), 7.55 (m, 1H), 7.33 (m, 1H), 7.14 (m, 1H), 6.13 (s, 2H), 4.72 (m, 2H), 4.40 (s, 2H), 3.81 (m, 9H), 2.53 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 192.8, 161.2, 159.6, 157.8, 139.0, 129.8, 120.9, 120.4, 112.9, 104.8, 90.8, 78.3, 76.1, 56.2, 56.1, 55.6, 22.8.

Monomeric [Tyr^3]Octreotide Peptide Thio Ester (9). Alkyne **6** (3.0 mg, 7.9 μmol) and azido peptide **1** (9.3 mg, 7.9 μmol, 1.0 equiv) were dissolved in THF/H_2O (1:1, v/v, 0.4 mL). To this solution, aqueous $CuSO_4$ (16 μL, 0.1 M, 1.6 μmol, 0.2 equiv) and sodium ascorbate (16 μL, 0.5 M, 7.9 μmol, 1.0 equiv) were added. The obtained reaction mixture was stirred for 5 min

under microwave irradiation (100 °C). Then the volatiles were removed under reduced pressure and the residue was taken up in CH_3CN/H_2O (1:1, v/v, 2.0 mL) and lyophilized. After purification by semipreparative HPLC (C_8) compound **9** was obtained in 47% yield (3.7 μmol, 5.8 mg). t_R(C_8, buffer system I): 22.6 min. ESI-MS: m/z = 1546.63 $[M + H]^+$, 1569.61 $[M + Na]^+$, 773.83 $[M + 2H]^{2+}$ ($C_{75}H_{95}N_{13}O_{17}S_3$, M = 1545.61).

^{t}Bu-Protected DOTA-Derived Sulfonyl Azide (13). HCl·H-Tau-N_3 (prepared from Cbz-Tau-N_3 as described by Brouwer et al.[81]) (205 mg, 1.11 mmol, 1.8 equiv) was dissolved in DCM (5 mL). To this solution, DOTA(Tris-^{t}Bu) (354 mg, 618 μmol, 1.0 equiv) and benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) (331 mg, 749 μmol, 1.2 equiv) followed by *N*,*N*-diisopropylethylamine (DIPEA) (305 μL, 1.75 mmol, 2.8 equiv) were added. The reaction mixture was stirred at room temperature overnight and subsequently diluted with DCM (50 mL). This solution was washed with 5% $NaHCO_3$ (3 × 50 mL) and brine (50 mL), dried (Na_2SO_4), and concentrated in vacuo. Purification of compound **13** by column chromatography required two runs (DCM/MeOH, 95:5 v/v). Compound **13** was isolated in 20% yield (124 μmol, 87 mg). R_f(DCM/MeOH, 95:5 v/v): 0.29. Mp: 86 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.73 (m, 1H), 3.75 (m, 2H), 3.58 (m, 2H), 3.55−1.90 (broad m, 24H), 1.47 (s, 27H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.8, 82.3, 82.2, 55.9, 54.2, 34.1, 28.2.

Monomeric [Tyr^3]Octreotide DOTA-Conjugated Sulfonamide (14). Triazole **9** (2.5 mg, 1.6 μmol) was treated with TFA/triisopropylsilane (TIS) (95:5 v/v, 400 μL) for 3 h at room temperature. After concentration in vacuo, the residue was dissolved in dry DMF (120 μL) and sulfonyl azide **13** (1.1 mg, 1.6 μmol, 1.0 equiv) followed by 2,6-lutidine (1.0 μL, 8.5 μmol, 5.2 equiv) were added. The reaction mixture was stirred for 1 h at room temperature and subsequently concentrated in vacuo. This residue was treated with TFA/TIS/H_2O (95:2.5:2.5, v/v/v) for 3 h at room temperature. Compound **14** was obtained in 25% yield (0.40 μmol, 0.76 mg) after purification by semipreparative HPLC (C_8). t_R(C_8, buffer system I): 18.28 min. ESI-MS: m/z = 921.87 $[M + 2H]^{2+}$, 614.90 $[M + 3H]^{3+}$ ($C_{83}H_{115}N_{19}O_{23}S_3$, M = 1841.76).

Dimeric [Tyr^3]Octreotide DOTA-Conjugated Sulfonamide (15). The synthesis of compound **15** was performed as described for compound **14** starting from triazole **10** (3.9 mg, 1.4 μmol) and sulfonyl azide **13** (1.0 mg, 1.4 μmol, 1.0 equiv). Compound **15** was obtained in 28% yield (0.40 μmol, 1.2 mg) after purification by semipreparative HPLC (C_8). t_R(C_8, buffer system I): 18.53 min. ESI-MS: m/z = 1536.31 $[M + 2H]^{2+}$, 1024.00 $[M + 3H]^{3+}$, 768.51 $[M + 4H]^{4+}$ ($C_{141}H_{192}N_{32}O_{36}S_5$, M = 3071.55).

Cell Culture. The AR42J cell line (rat tumor of the exocrine pancreas) was a generous gift from Erasmus University Rotterdam, The Netherlands. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 1% glutamine. Cells were grown in a humidified atmosphere with 5% CO_2 at 37 °C.

Radiolabeling. DOTA-conjugated compounds **14**, **15**, and **16** were labeled with $^{111}InCl_3$ (Covidien) in 0.25 M NH_4OAc solution (pH 5.5) for 15 min at 95 °C. Labeling reactions were performed in acid-washed Protein LoBind safe-lock tubes (Eppendorf GmbH). Radiochemical purity was determined by instant thin-layer chromatography (ITLC) on silica gel (Tec-Control chromatography strips, Biodex Medical Systems, Inc.). The ITLC analysis was performed using two different mobile phases: 0.1 M EDTA/0.1 M NH_4OAc (1:1, v/v; R_f(bound ^{111}In) = 0; R_f(unbound ^{111}In) = 1) and THF/0.25 M NH_4OAc (1:1, v/v; R_f(colloid) = 0; R_f(unbound ^{111}In or ^{111}In-labeled compound) > 0.5). Radiochemically pure (>99%) tracers were obtained by preparative RP-HPLC prior to use for partition coefficient measurements (log(D)) and animal experiments. Preparative isolation of ^{111}In-labeled compounds were carried out on an Agilent HPLC system with an in-line NaI radiodetector (Raytest GmbH) using a Zorbax Eclipse C_8 column (150 mm × 4.6 mm, pore size: of 300 Å, particle size of 5 μm) at a flow rate of 1.0 mL/min using a linear gradient of buffer B (5−100% in 20 min) in buffer A (buffer A consisting of 0.1% TFA in water; buffer B consisting of 0.1% TFA in ethanol). Retention times were 17.3, 18.3, and 18.4 min for **14**, **15**, and **16**, respectively.

Determination of the Octanol−PBS Partition Coefficients. A total of approximately 148 kBq (4 μCi, 3.7 MBq/μg) of the ^{111}In-labeled compounds **14**, **15**, and **16** in 500 μL of PBS (pH 7.4) was mixed with 500 μL of *n*-octanol. The resulting biphasic system was shaken vigorously for 2 min. The two layers were subsequently separated by centrifugation (5 min, 250*g*), and the counts in 100 μL aliquots (n = 3) of both the organic and the aqueous layers were measured in a γ-counter (Perkin-Elmer). The partition coefficient (log(D)) was determined from at least two independent experiments and calculated from the formula log(D) = $\log_{10}$[(counts in octanol layer)/(counts in aqueous layer)].

Receptor Binding Studies. The half maximal inhibitory concentration (IC_{50}) of compounds **14**, **15**, and **16** for the SSTR2 on AR42J tumor cells was determined in a competitive binding assay, using [^{111}In-DOTA^0,Tyr^3]octreotide as a tracer. Radiolabeling was performed as described above. AR42J cells were seeded into six-well plates at 8 × 10^5 cells/well and cultured until confluent. Cells were washed twice with binding buffer (DMEM with 0.1% w/v bovine serum albumin). Subsequently, binding buffer (1.5 mL), unlabeled peptide **14**, **15**, or **16** in a range from 0.1 to 300 nM, and ^{111}In-labeled [Tyr^3]octreotide (40 000 dpm) were added to each well. After incubation at 37 °C for 3 h, the medium was removed and cells were washed twice with binding buffer and extracted from the wells, and cell-associated radioactivity was determined in a γ-counter. For the "cold" labeling of monomeric (**14**), dimeric (**15**), and tetrameric (**16**) [Tyr^3]-octreotide conjugates with $^{115}In^{3+}$, each of the peptides was dissolved in an aqueous solution (25 μL, 10 mM NH_4OAc). Subsequently, a 3 molar excess of $InCl_3$ (Aldrich Chemical Co.) was added. The In(III) complexation was performed at 40 °C for 2 h.[84] GraphPad Prism software (version 4.00 for Windows, GraphPad Software, Inc.) was used to calculate IC_{50} values and to determine statistical significance at the 95% confidence interval with a P value of <0.05 being considered significantly different.

Biodistribution. All animal experiments were conducted in compliance with the animal welfare committee requirements of our institution and performed according to national regulations. The rat pancreatic AR42J tumor was grown in the right flank of male BALB/c nude mice weighing 20−25 g (6−8 weeks) by subcutaneous injection of 5 × 10^6 cells (0.2 mL) from cell culture. After approximately 2 weeks, when tumors had a diameter of 4−6 mm, mice were randomly divided into groups of five mice each. Each group received an intravenous injection via the tail vein with 0.37 MBq (0.1 μg) ^{111}In-DOTA-labeled [Tyr^3]octreotide conjugate **14**, **15**, or **16**. At 2, 6, or 24 h postinjection (p.i.) the animals were euthanized by CO_2/O_2 asphyxiation. Blood, tumor, and tissues of interest were dissected, weighed, and counted in a γ-counter along with three 100 μL aliquots of the diluted standard representing 1% of the injected activity. From this the percentage injected dose per gram (%ID/g) for each tissue was calculated. Additional groups of three mice were co-injected intravenously with 50 μg of octreotide (Sandostatin) and sacrificed after 2 h to determine nonspecific binding of the radiotracers. Data are expressed as the mean ± SD, calculated in Microsoft Excel. Statistical analysis of biodistribution data used the unpaired t test (SPSS Inc. software, version 16.0.01).

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Supporting Information Available: Synthesis and characterization of compounds **7**, **8**, **10**, **11**, and **16**; ^{1}H and ^{13}C NMR spectra of TMOB-thio esters **6**−**8** and DOTA-derived sulfonyl azide **13**; HPLC and high resolution ESI-MS analyses of triazoles **9**−**11** and DOTA-conjugated [Tyr^3]octreotide compounds **14**−**16**; biodistribution data of ^{111}In-labeled tetrameric [Tyr^3]octreotide **16** and [^{111}In-DOTA^0,Tyr^3]octreotide. This material is available free of charge via the Internet at http://pubs.acs.org.

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