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DOTA derivatives for site-specific biomolecule-modification via click chemistry: Synthesis and comparison of reaction characteristics

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ABSTRACT

Due to the high stability of its complexes with many M^{2+} and M^{3+}-ions, DOTA (1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid) is the most commonly used chelator for the derivatization and radiolabeling of bioactive molecules. Most of the currently used DOTA derivatives comprise amine-reactive functionalities, limiting their application to the derivatization of fully protected molecules or otherwise resulting in randomly distributed conjugation sites of undefined number.

Click chemistry reactions are a valuable alternative to this unspecific conjugation as they proceed efficiently and chemoselectively under mild conditions allowing a site-specific derivatization of unprotected biomolecules.

In this work, we describe straightforward syntheses of DOTA derivatives containing thiol, maleimide, aminooxy, aldehyde, alkyne, and azide functionalities, amenable to the currently most often used click chemistry reactions. Furthermore, the efficiency of the respective click reactions introducing DOTA into bioactive molecules was investigated.

For each of the synthesized DOTA synthons, the site-specific and efficient conjugation to Tyr^3-octreotate could be shown. Among these, the addition and oxime formation reactions proceeded fast and without side reactions, giving the products in high yields of 64–83% after purification. The copper-catalyzed triazole formation reactions produced some side-products, giving the desired products in lower, but still reasonable overall yields of 19–25%.

All synthesized peptide–DOTA-conjugates were labeled with ^{68}Ga in high radiochemical yields of 96–99% and high specific activities providing compounds of high purity, demonstrating the applicability of all synthons for biomolecule modification and subsequent radiolabeling.

1. Introduction

DOTA is one of the most important chelators used for radiolabeling purposes as it forms very stable complexes with various M^{2+} and M^{3+} ions,[1,2] including most of the radiometals used in nuclear medicine such as ^{68}Ga, ^{90}Y, ^{111}In, and ^{177}Lu. Thus, DOTA is the commonly used chelator for the derivatization and radiolabeling of bioactive molecules intended for non-invasive in vivo imaging and therapeutic applications.

Among these biomolecules, peptides—especially several radiolabeled somatostatin-analogs for imaging and therapy of somatostatin positive tumors—are of particular interest. They can easily be derivatized with a chelator and radiolabeled and have thus gained much importance in nuclear medicine.[3–6]

The introduction of a DOTA chelator into a peptide is mostly performed during solid phase peptide synthesis using tris-*t*Bu-DOTA[7] that has become the standard synthon for this application as it is commercially available and can easily be conjugated using standard coupling procedures during Fmoc-solid phase peptide synthesis. Apart from this synthon, several other protected and unprotected DOTA derivatives have been described[8–13] for the conjugation to peptides and even proteins. However, most of these are based on de facto or in situ generated active esters or isothiocyanates that can only be reacted with amino functionalities of fully protected compounds to introduce a predefined number of introduced chelators and a site-specific derivatization. Otherwise, the coupling results in randomly distributed conjugation sites of undefined number potentially diminishing the bioactivity of the derivatized compound.

A promising approach to overcome these limitations is the utilization of click chemistry reactions proceeding efficiently and chemoselectively, generating products that are stable under physiological conditions.[14] In addition, these reactions are orthogonal, allowing a successive site-specific introduction of several different moieties during the synthesis of complex molecules. As these

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reactions are performed under mild conditions within short reaction times, they are particularly suitable for the derivatization of even sensitive bioactive molecules.

Therefore, DOTA derivatives comprising different functionalities suitable for application in the most often used click-chemistry reactions such as (1) addition of thiols to maleimides, (2) oxime formation between aldehydes (or ketones) and hydroxylamines (3) and the copper-catalyzed 1,3-dipolar cycloaddition reaction are desirable synthons allowing the synthesis of well-defined biomolecules with undiminished activity.[15]

So far, several DOTA derivatives comprising functionalities for site-specific conjugation are known.[16–26] However, their syntheses often require complex multi-step procedures and others have not been shown to be introducible into complex molecules such as peptides but into small molecules only. Furthermore, some of these synthons show a low reactivity, resulting in inefficient and sometimes cumbersome coupling reactions.

Thus, we intended to develop straightforward synthesis routes comprising as few as possible reaction steps for DOTA derivatives containing thiol, maleimide, aminooxy, aldehyde, azide, and alkyne functionalities to be applicable in the commonly used click chemistry reactions. In addition, the different reaction types should be compared with regard to coupling yields and efficiencies achievable in the site-specific introduction of the DOTA synthons into functionalized Tyr^3-octreotate (TATE) derivatives. This peptide comprises several different amino acid side-chain functionalities such as an amine, an aliphatic and aromatic alcohol and a dithiol bond that have to remain intact during the coupling reactions of the various DOTA synthons. Finally, the radiolabeling of the obtained peptide–DOTA-conjugates with a radiometal nuclide—such as ^{68}Ga—has to be shown.

2. Materials and methods

2.1. General

All commercially available chemicals were of analytical grade and used without further purification. DOTA, tris-*t*Bu-DOTA, and coupling reagents, resins as well as amino acids for peptide synthesis were purchased from Macrocyclics (Dallas, USA) and NovaBiochem (Nottingham, UK), respectively. *N*-(2-Aminoethyl)maleimide trifluoroacetate salt, mono-Fmoc-ethylene diamine hydrobromide and *p*-formylbenzoic acid *N*-hydroxysuccinimide ester (SFB) were purchased from Sigma–Aldrich (Schnelldorf, Germany). Bis-maleimidoethane (BMOE), 1,11-bis-maleimido-triethyleneglycol ($BM(PEG)_3$), and 1,4-bismaleimidyl-2,3-dihydroxybutane (BMDB) were purchased from Thermo Scientific, Ulm, Germany. Thiol-DOTA and DOTA-M-$(PEG)_3$ were synthesized according to published procedures.[26,27] Peptides were synthesized according to standard solid phase peptide synthesis protocols.[28]

The analytical and semipreparative HPLC system used was an Agilent 1200 system equipped with a Raytest Gabi Star radioactivity detector. The columns used for chromatography were a Chromolith Performance (RP-18e, 100–4.6 mm, Merck, Germany) and a Chromolith (RP-18e, 100–10 mm, Merck, Germany) column, operated with a flow of 4 and 8 mL × min^{-1}, respectively.

ESI, MALDI and NMR spectra were obtained by using a Finnigan MAT95Q, a Bruker Daltonics Microflex, and a Jeol AS500 spectrometer, respectively.

2.2. DOTA chelator derivatives

2.2.1. Maleimido-dihydroxybutyl-DOTA (MDB-DOTA) (3)

A solution of thiol-DOTA (**1**) (16.8 mg, 36.3 μmol) in phosphate buffer (0.1 M, pH 5, 200 μL) was added to a solution of 1,4-bismaleimidyl-2,3-dihydroxybutane (BMDB) (10 mg, 45.4 μmol) in a mixture of DMF (200 μL) and phosphate buffer (0.1 M, pH 5, 150 μL) and the pH of the mixture was adjusted to 7.2 by adding phosphate buffer (0.5 M, pH 7.2, ~100 μL). After 5 min the product was purified by semipreparative HPLC with 0–30% MeCN + 0.1% TFA in 8 min as the gradient (R_t = 2.6 min). The product was obtained as white, hygroscopic solid after lyophilization (12.2 mg, 17.6 μmol, 48% yield).

^1H NMR (500 MHz, water-d_2 + MeCN-d_3, 25 °C, TMS): δ = 8.17 (s, 0.2H); 6.75 (s, 2H); 3.91–3.88 (m, 1H); 3.68–3.62 (m, 7H); 3.46–2.82 (m, 24H); 2.74–2.66 (m, 2H); 2.49–2.43 (m, 1H). ^{13}C NMR (125 MHz, water-d_2 + MeCN-d_3, 25 °C, TMS): δ = 179.45; 178.25; 173.11; 173.00; 135.29; 57.27; 57.24; 55.56; 53.87; 52.03; 51.60; 49.00; 48.97; 48.81; 39.99; 38.78; 38.16; 36.81; 35.86; 31.63. ESI-MS (*m*/*z*) for $[M+H]^+$ (calculated): 744.29 (744.28); (*m*/*z*) for $[M+Na]^+$ (calculated): 766.27 (766.27); (*m*/*z*) for $[M+K]^+$ (calculated): 782.23 (782.24).

2.2.2. Maleimido-ethyl-DOTA (ME-DOTA) (4)

A solution of thiol-DOTA (**1**) (26.4 mg, 57.1 μmol) in phosphate buffer (0.1 M, pH 5, 200 μL) was added to a solution of bis-maleimidoethane (BMOE) (20 mg, 71.4 μmol) in DMF (200 μL) and the pH of the mixture was adjusted to 7.2 by adding phosphate buffer (0.5 M, pH 7.2, ~150 μL). After 5 min the product was purified by semipreparative HPLC with 0–30% MeCN + 0.1% TFA in 8 min as the gradient (R_t = 2.6 min). The product was obtained as white, hygroscopic solid after lyophilization (17.1 mg, 22.9 μmol, 40% yield).

^1H NMR (500 MHz, water-d_2, 25 °C, TMS): δ = 8.10 (s, 0.2H); 6.68 (s, 2H); 3.94–3.90 (m, 1H); 3.68–3.51 (m, 9H); 3.44–2.83 (m, 28H); 2.79–2.63 (m, 2H); 2.59–2.53 (m, 1H). ^{13}C NMR (125 MHz, water-d_2, 25 °C, TMS): δ = 179.67; 178.45; 173.10; 170.17; 167.44; 134.55; 68.11; 67.97; 56.45; 56.41; 55.46; 53.44; 51.47; 50.84; 50.82; 48.55; 48.22; 41.84; 40.47; 39.92; 38.40; 36.23; 30.47. ESI-MS (*m*/*z*) for $[M+H]^+$ (calculated): 684.27 (684.26); (*m*/*z*) for $[M+Na]^+$ (calculated): 706.25 (706.25); (*m*/*z*) for $[M+K]^+$ (calculated): 722.21 (722.22).

2.2.3. 2,2′,2″-(10-(2-(4-(Aminomethyl)benzylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclo-dodecane-1,4,7-triyl)triacetic acid (amino-DOTA) (5)

DOTA (1.0 g, 1.93 mmol), 1,4-bis(aminomethyl)benzene (263 mg, 1.93 mmol) and diisopropyl-carbodiimide (DIC, 244 mg, 1.93 mmol) were dissolved in a mixture of water/MeCN/pyridine (1:1:0.2, 50 mL) and reacted overnight. The solvents were removed and the crude product was purified by column chromatography on silica gel using H_2O/MeCN 1:1 + 0.1% TFA as the mobile phase. The product was isolated as lightly yellow solid after lyophilization (760 mg, 1.46 mmol, 75% yield). R_f = 0.45 (H_2O/MeCN 1:1 + 0.1% TFA)

^1H NMR (500 MHz, water-d_2, 25 °C, TMS): δ = 7.45 (d, 2H, ^3J(H,H) = 8.3 Hz); 7.38 (d, 2H, ^3J(H,H) = 8.3 Hz); 4.35 (br s, 2H); 4.14 (br s, 2H); 3.86–2.89 (br m, 24H). ^{13}C NMR (125 MHz, water-d_2, 25 °C, TMS): δ = 163.30; 162.94; 139.16; 131.72; 129.27; 128.10; 55.54; 55.45; 55.39; 50.85; 50.48; 50.42; 48.65; 48.55; 48.54; 48.50; 48.46; 48.30; 42.89. ESI-MS (*m*/*z*) for $[M+H]^+$ (calculated): 523.27 (523.28), (*m*/*z*) for $[M+K]^+$ (calculated): 561.23 (561.24).

2.2.4. [*N*-(4-(Aminomethyl)benzyl)-4-formylbenzamido]-DOTA (DOTA-aldehyde) (6)

Amino-DOTA (**5**, 100 mg, 191 μmol) and SFB (52 mg, 211 μmol) were dissolved in a mixture of phosphate buffer (0.1 M, pH 6, 350 μL) and MeCN (350 μL) and the pH was adjusted to 7.2 by adding phosphate buffer (0.5 M, pH 7.2, ~200 μL). After 60 min, the reaction mixture was acidified by using 1 M HCl and the crude product was purified by semi-preparative HPLC with

0–40% MeCN + 0.1% TFA in 8 min as the gradient (R_t = 3.2 min). The product was obtained as white, highly hygroscopic solid after lyophilization (46 mg, 70 μmol, 37% yield).

^{1}H NMR (500 MHz, DMSO-d_6, 25 °C, TMS): δ = 10.09 (s, 1H); 9.35 (t, 1H, ^3J(H,H) = 6.0 Hz); 8.97 (br s, 1H); 8.09–8.07 (m, 2H); 8.02–8.00 (m, 2H); 7.32–7.31 (m, 2H); 7.28–7.26 (m, 2H); 4.48 (br d, 2H, ^3J(H,H) = 5.9 Hz); 4.32 (br d, 2H, ^3J(H,H) = 5.5 Hz); 4.01 (br s, 2H); 3.94 (br s, 2H); 3.63 (br s, 4H); 3.33 (br s, 8H); 3.12 (br s, 8H). ^{13}C NMR (125 MHz, DMSO-d_6, 25 °C, TMS): δ = 192.84; 165.29; 158.13; 157.87; 139.24; 138.20; 137.73; 136.91; 129.36; 127.89; 127.49; 127.31; 54.60; 54.58; 53.89; 53.86; 52.79; 52.77; 52.76; 50.50; 50.48; 50.46; 50.33; 50.31; 48.53; 48.51; 48.49; 48.42; 48.39; 42.46; 42.05. ESI-MS (m/z) for $[M+H]^+$ (calculated): 655.31 (655.30).

2.2.5. [*N*-(4-(Aminomethyl)benzyl)-2-(aminooxy)acetamido]-DOTA (aminooxy-DOTA) (7)

A solution of HBTU (87.2 mg, 230 μmol) in DMF (500 μL) was added to Boc-aminooxy-acetic acid (45.8 mg, 239 μmol) followed by DIPEA (41 μL, 239 μmol) and reacted for 2 min. Subsequently, the mixture was added to a solution of amino-DOTA (**5**, 100 mg, 192 μmol) in DMF (250 μL) and reacted for 30 min. After acidification with 1 M HCl (~70 μL), the product was purified by semi-preparative HPLC with 0–20% MeCN + 0.1% TFA in 8 min as the gradient (R_t = 2.5 min). After solvent removal, the obtained colorless solid was dissolved in a mixture of neat TFA (3 mL) and TIS (100 μL) and reacted for 15 min. The product was purified by semi-preparative HPLC with 0–20% MeCN + 0.1% TFA in 8 min as the gradient (R_t = 2.0 min). The product was obtained as white, highly hygroscopic solid after lyophilization (32 mg, 53.8 μmol, 28% overall yield).

^{1}H NMR (500 MHz, water-d_2, 25 °C, TMS): δ = 7.23 (s, 4H); 4.58 (s, 2H); 4.35 (s, 2H); 4.30 (s, 2H); 3.66 (br s, 8H); 3.21 (br s, 16H). ^{13}C NMR (125 MHz, water-d_2, 25 °C, TMS): δ = 168.81; 163.26; 162.90; 136.73; 136.70; 127.91; 127.87; 71.97; 55.16; 54.07; 53.85; 53.24; 52.72; 49.90; 49.81; 49.50; 49.26; 49.23; 49.11; 48.64; 45.25; 45.15; 42.96; 42.57. ESI-MS (m/z) for $[M+K+H]^+$ (calculated): 635.34 (635.26).

2.2.6. Tris-*t*Bu-(phenylene-1,4-dimethylamino-)DOTA (P-amino-DOTA) (8)

A solution of HBTU (314.5 mg, 830 μmol) in DMF (1.5 mL) was added to tris-*t*Bu-DOTA (500 mg, 874 μmol) followed by DIPEA (149 μL, 874 μmol) and reacted for 2 min. Subsequently, the mixture was added to a solution of 1,4-bis(aminomethyl)benzene (297 mg, 2.18 mmol) in DMF (250 μL) and reacted for 30 min. After acidification with 1 M HCl (~200 μL), the product was purified by semi-preparative HPLC with 0–50% MeCN + 0.1% TFA in 8 min as the gradient (R_t = 4.3 min). The product was obtained as slightly green, highly hygroscopic solid after lyophilization (390 mg, 565 μmol, 68% yield).

^{1}H NMR (500 MHz, acetone-d_6, 25 °C, TMS): δ = 7.47 (d, 2H, ^3J(H,H) = 8.1 Hz); 7.39 (d, 2H, ^3J(H,H) = 7.9 Hz); 5.01 (br s, 2H); 4.61–3.13 (br m, 24H); 4.49 (br s, 2H); 1.53 (s, 9H); 1.46 (s, 18H). ^{13}C NMR (125 MHz, acetone-d_6, 25 °C, TMS): δ = 160.60; 160.25; 140.10; 130.29; 129.53; 128.85; 55.70; 55.68; 55.22; 55.19; 55.16; 52.27; 52.24; 50.80; 50.66; 50.09; 49.97; 28.34; 28.32. ESI-MS (m/z) for $[M+H]^+$ (calculated): 691.48 (691.47).

2.2.7. *tert*-Butyl 2,2′,2″-(10-(2-(4-(hex-5-ynamidomethyl) benzylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (P-DOTA-alkyne) (9)

A solution of HBTU (65.9 mg, 174 μmol) in DMF (500 μL) was added to 5-hexynoic acid (20.3 mg, 181 μmol) followed by DIPEA (30.9 μL, 181 μmol) and reacted for 2 min. Subsequently, the mixture was added to a solution of P-amino-DOTA (**8**, 100 mg, 145 μmol) in DMF (200 μL) and reacted for 60 min. After acidification with 1 M HCl (60 μL), the product was purified by semi-preparative HPLC with 10–65% MeCN + 0.1% TFA in 8 min as the gradient (R_t = 4.2 min). The product was obtained as brownish, highly hygroscopic solid after lyophilization (13 mg, 17 μmol, 11% yield).

^{1}H NMR (500 MHz, acetone-d_6, 25 °C, TMS): δ = 8.94 (br s, 1H); 7.62 (br s, 1H); 7.30–7.28 (m, 2H); 7.26–7.24 (m, 2H); 4.44 (br d, 2H, ^3J(H,H) = 5.5 Hz); 4.36 (br d, 2H, ^3J(H,H) = 5.6 Hz); 4.33–4.04 (br s, 8H); 3.77–3.62 (br s, 4H); 3.59–3.40 (br s, 4H); 3.35–3.10 (br s, 8H); 2.36 (t, 1H, ^4J(H,H) = 2.7 Hz); 2.35 (t, 2H, ^3J(H,H) = 7.4 Hz); 2.23 (dt, 2H, ^3J(H,H) = 7.1 Hz, ^4J(H,H) = 2.7 Hz); 1.81 (p, 2H, ^3J(H,H) = 7.2 Hz); 1.51 (s, 9H); 1.47 (s, 18H). ^{13}C NMR (125 MHz, acetone-d_6, 25 °C, TMS): δ = 172.33; 160.33; 160.06; 139.72; 128.55; 128.38; 84.46; 82.20; 70.29; 58.03; 57.93; 56.17; 56.07; 56.00; 55.88; 52.53; 52.45; 52.31; 52.28; 50.37; 50.08; 49.97; 49.85; 43.13; 43.00; 35.19; 28.34; 25.44; 18.40. ESI-MS (m/z) for $[M+H]^+$ (calculated): 785.52 (785.51).

2.2.8. *tert*-Butyl 2,2′,2″-(10-(2-(4-((5-azidopentanamido)methyl)-benzylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (P-DOTA-azide) (10)

A solution of HBTU (65.9 mg, 174 μmol) in DMF (500 μL) was added to 5-azidopentanoic acid (25.9 mg, 181 μmol) followed by DIPEA (30.9 μL, 181 μmol) and reacted for 2 min. Subsequently, the mixture was added to a solution of P-amino-DOTA (**8**, 100 mg, 145 μmol) in DMF (200 μL) and reacted for 60 min. After acidification with 1 M HCl (~50 μL), the product was purified by semi-preparative HPLC with 0–60% MeOH + 0.1% TFA in 8 min as the gradient (R_t = 6.6 min). The product was obtained as colorless, highly hygroscopic solid after lyophilization (46 mg, 56 μmol, 39% yield).

^{1}H NMR (500 MHz, acetone-d_6, 25 °C, TMS): δ = 8.58 (br s, 1H); 7.72 (br s, 1H); 7.30–7.24 (m, 4H); 4.47 (br d, 2H, ^3J(H,H) = 5.2 Hz); 4.37 (br d, 2H, ^3J(H,H) = 5.8 Hz); 4.30 (br s, 2H); 3.93–3.16 (br m, 22H); 3.35 (t, 2H, ^3J(H,H) = 6.6 Hz); 2.28 (t, 2H, ^3J(H,H) = 7.1 Hz); 1.74–1.58 (m, 4H); 1.54 (s, 9H); 1.47 (s, 18H). ^{13}C NMR (125 MHz, acetone-d_6, 25 °C, TMS): δ = 172.76; 160.33; 159.96; 139.84; 137.99; 128.61; 128.42; 82.93; 56.00; 55.96; 55.48; 55.42; 54.96; 54.93; 54.92; 52.21; 52.19; 52.14; 52.09; 51.77; 49.97; 49.95; 49.91; 49.86; 49.77; 43.50; 42.99; 35.91; 29.11; 28.35; 23.63. ESI-MS (m/z) for $[M+H]^+$ (calculated): 816.53 (816.53).

2.2.9. 2,2′,2″-(10-(2-(4-(Hex-5-ynamidomethyl)benzylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (DOTA-alkyne) (11)

A solution of HBTU (43.6 mg, 115 μmol) in DMF (500 μL) was added to 5-hexynoic acid (13.4 mg, 120 μmol) followed by DIPEA (22 μL, 120 μmol) and reacted for 2 min. Subsequently, the mixture was added to a solution of amino-DOTA (**5**, 50 mg, 96 μmol) in DMF (250 μL) and reacted for 30 min. At this time, water (250 μL) was added and the reaction was continued for further 15 min. After acidification with HCl (1 M, 35 μL), the product was purified by semi-preparative HPLC with 0–20% MeCN + 0.1% TFA in 8 min as the gradient (R_t = 3.5 min). The product was obtained as colorless, highly hygroscopic solid after lyophilization (17.2 mg, 28 μmol, 29% yield).

^{1}H NMR (500 MHz, water-d_2, 25 °C, TMS): δ = 7.27–7.22 (m, 4H); 4.79–4.78 (m, 4H); 4.29 (br s, 2H); 4.01–3.53 (br m, 6H); 3.50–2.94 (br s, 16H); 2.34 (t, 2H, ^3J(H,H) = 7.4 Hz); 2.26 (t, 1H, ^4J(H,H) = 2.6); 2.15 (dt, 2H, ^3J(H,H) = 6.9, ^4J(H,H) = 2.6); 1.75 (p, 2H, ^3J(H,H) = 7.1). ^{13}C NMR (125 MHz, water-d_2, 25 °C, TMS): δ = 176.19; 172.32; 137.37; 127.89; 127.70; 84.74; 70.04; 57.33; 57.25; 55.12; 54.04; 51.82; 49.56; 42.97; 42.80; 34.72; 24.24; 17.19. ESI-MS (m/z) for $[M+H]^+$ (calculated): 617.33 (617.32); (m/z) for $[M+Na+K+H]^+$ (calculated): 679.24 (679.27).

2.2.10. 2,2′,2″-(10-(2-(4-((5-Azidopentanamido)methyl)-benzylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (DOTA-azide) (12)

A solution of HBTU (43.6 mg, 115 μmol) in DMF (500 μL) was added to 5-azidopentanoic acid (17.1 mg, 120 μmol) followed by DIPEA (22 μL, 120 μmol) and reacted for 2 min. Subsequently, the mixture was added to a solution of amino-DOTA (**5**, 50 mg, 96 μmol) in DMF (250 μL) and reacted for 30 min. At this time, water (250 μL) was added and the reaction was continued for further 15 min. After acidification with HCl (1 M, 35 μL), the product was purified by semi-preparative HPLC with 0–30% MeCN + 0.1% TFA in 8 min as the gradient (R_t = 3.4 min). The product was obtained as colorless, highly hygroscopic solid after lyophilization (19.9 mg, 31 μmol, 32% yield).

^{1}H NMR (500 MHz, water-d_2, 25 °C, TMS): δ = 7.35–7.30 (m, 4H); 4.89–4.88 (m, 4H); 4.38 (br s, 2H); 4.04–3.61 (br m, 6H); 3.54–3.09 (b, 16H); 3.33 (t, 2H, ^3J(H,H) = 6.7 Hz); 2.34 (t, 2H, ^3J(H,H) = 7.1 Hz); 1.74–1.66 (m, 2H); 1.63–1.56 (m, 2H). ^{13}C NMR (125 MHz, water-d_2, 25 °C, TMS): δ = 176.57; 137.41; 137.40; 127.89; 127.68; 55.22; 55.18; 55.14; 55.11; 54.86; 54.45; 54.17; 53.47; 53.42; 53.23; 52.01; 51.95; 50.80; 42.95; 42.76; 35.33; 27.54; 22.80. ESI-MS (m/z) for $[M+H]^+$ (calculated): 648.35 (648.34).

2.3. Tyr^3-octreotate-derivatives

2.3.1. Maleimido-TATE (13), TATE-cysteine (14), aminooxy-TATE (15), TATE-5-hexynoic acid (16), and TATE-5-azidopentanoic acid (17)

Tyr^3-octreotate was synthesized on a solid support applying standard Fmoc-solid-phase-peptide synthesis methods as described by Wellings and Atherton.[28] To obtain the peptides comprising the required functional groups for the click chemistry reactions, Tyr^3-octreotate was derivatized on solid support with maleimido-hexanoic acid, Fmoc-Cys(Trt)-OH, *bis*-Boc-aminooxy-acetic acid, 5-hexynoic acid, and 5-azidopentanoic acid using standard coupling procedures. The peptides were cleaved from the resin and deprotected by incubation of the resin with a mixture of TFA/TIS/H_2O (95:2.5:2.5, 2 mL) for 60 min and suspended in diethylether. After washing the crude products three times with diethylether, they were purified by semi-preparative HPLC with 0–60% MeCN + 0.1% TFA in 8 min as the gradient and isolated as white solids after lyophilization.

2.3.1.1. Maleimido-TATE (13). 49% Yield; $R_{t(purification)}$ = 3.4 min; ESI-MS (m/z) for $[M+H]^+$ (calculated): 1242.50 (1242.49).

2.3.1.2. TATE-cysteine (14). 42% Yield; $R_{t(purification)}$ = 3.1 min; ESI-MS (m/z) for $[M+H]^+$ (calculated): 1152.43 (1152.42).

2.3.1.3. Aminooxy-TATE (15). 18% Yield; $R_{t(purification)}$ = 2.8 min; ESI-MS (m/z) for $[M+H]^+$ (calculated): 1122.44 (1122.43).

2.3.1.4. TATE-5-hexynoic acid (16). 54% Yield; $R_{t(purification)}$ = 3.6 min; ESI-MS (m/z) for $[M+H]^+$ (calculated): 1143.46 (1143.46).

2.3.1.5. TATE-5-azidopentanoic acid (17). 31% Yield; $R_{t(purification)}$ = 3.8 min; ESI-MS (m/z) for $[M+H]^+$ (calculated): 1174.47 (1174.47).

2.3.2. *N*-(2-(2,5-Dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)ethyl)-4-formylbenzamide (18)

To a solution of *N*-(2-aminoethyl)maleimide trifluoroacetate salt (15.4 mg, 60.7 μmol) in a mixture of phosphate buffer (0.1 M, pH 6, 250 μL) and MeCN (250 μL) was added *p*-formylbenzoic acid *N*-hydroxysuccinimide ester (SFB, 15.0 mg, 60.7 μmol) and the pH of the mixture was adjusted to 7.2 by adding phosphate buffer (0.5 M, pH 7.2, ~100 μL). After 60 min, the reaction mixture was acidified by using 1 M HCl and the crude product was purified by semi-preparative HPLC with 0–40% MeCN + 0.1% TFA in 8 min as the gradient (R_t = 3.2 min). The product was obtained as white solid after lyophilization (9 mg, 33 μmol, 55% yield).

^{1}H NMR (500 MHz, DMSO-d_6, 25 °C, TMS): δ = 10.07 (s, 1H); 8.81 (t, 1H, ^3J(H,H) = 6.0 Hz); 8.00–7.98 (m, 2H); 7.92–7.90 (m, 2H); 7.02 (s, 2H); 3.61–3.59 (m, 2H); 3.45–3.41 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6, 25 °C, TMS): δ = 192.80; 171.03; 165.66; 139.35; 137.62; 134.46; 129.31; 127.70; 37.66; 36.96. ESI-MS (m/z) for $[M+H]^+$ (calculated): 273.09 (273.08).

2.3.3. TATE-aldehyde (19)

A solution of TATE-cysteine (**14**, 7.5 mg, 6.5 μmol) in a mixture of phosphate buffer (0.1 M, pH 6, 250 μL) and MeCN (250 μL) was added to *N*-(2-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)ethyl)-4-formylbenzamide (**18**, 2.2 mg, 8.1 μmol) and the pH of the mixture was adjusted to 7.2 by addition of phosphate buffer (0.5 M, pH 7.2, ~75 μL). After 5 min, the crude product was purified by semipreparative HPLC with 0–60% MeCN + 0.1% TFA in 8 min as the gradient (R_t = 3.9 min). The product was obtained as white solid after lyophilization (6.1 mg, 4.3 μmol, 66% yield).

ESI-MS (m/z) for $[M+H]^+$ (calculated): 1424.51 (1424.50); (m/z) for $[M+2H]^{2+}$ (calculated): 712.76 (712.75).

2.4. Chelator-peptide-conjugates

2.4.1. TATE-MT-DOTA (20)

A solution of maleimido-TATE (**13**, 3.0 mg, 2.4 μmol) in a mixture of phosphate buffer (0.1 M, pH 6, 100 μL) and MeCN (100 μL) was added to a solution of thiol-DOTA (**1**, 1.2 mg, 2.7 μmol) in phosphate buffer (0.1 M, pH 6, 100 μL) and the pH of the mixture was adjusted to 7.2 by addition of phosphate buffer (0.5 M, pH 7.2, 50–100 μL). After 5 min, the mixture was acidified using 1 M HCl (~25 μL) and the crude product was purified by semi-preparative HPLC with 0–40% MeCN + 0.1% TFA in 8 min as the gradient (R_t = 4.8 min) and obtained as white solid after lyophilization (2.9 mg, 1.7 μmol, 70% yield).

ESI-MS (m/z) for $[M+K+Na]^+$ (calculated): 1766.62 (1766.65).

2.4.2. TATE-TM$_2$-DOTA (21), TATE-TM$_3$-DOTA (22), and TATE-TM$_4$-DOTA (23)

A solution of TATE-cysteine (**14**, 3.0 mg, 2.6 μmol) in a mixture of phosphate buffer (0.1 M, pH 6, 100 μL) and MeCN (100 μL) was added to a solution of 1.1 eq. of the respective DOTA-maleimide (DOTA-M-(PEG)$_3$ (**2**), MDB-DOTA (**3**) or ME-DOTA (**4**)) in phosphate buffer (0.1 M, pH 6, 100 μL) and the pH of the mixture was adjusted to 7.2 by addition of phosphate buffer (0.5 M, pH 7.2, 50–100 μL). After 5 min, the mixture was acidified using 1 M HCl (~25 μL) and the crude products were purified by semi-preparative HPLC with 0–40% MeCN + 0.1% TFA in 8 min as the gradient and obtained as white solids after lyophilization.

2.4.2.1. TATE-TM$_2$ -DOTA (21). 64% Yield; $R_{t(purification)}$ = 4.6 min; ESI-MS (m/z) for $[M+K+Na]^{2+}$ (calculated): 1015.34 (1015.36); (m/z) for $[M-H]^-$ (calculated): 1965.75 (1965.76).

2.4.2.2. TATE-TM$_3$ -DOTA (22). 66% Yield; $R_{t(purification)}$ = 4.0 min; ESI-MS (m/z) for $[M-H]^-$ (calculated): 1894.70 (1894.70), (m/z) for $[M+Na+K]^-$ (calculated): 1956.61 (1956.66).

2.4.2.3. TATE-TM$_4$ -DOTA (23). 73% Yield; $R_{t(purification)}$ = 4.6 min; MALDI-MS (m/z) for $[M+H]^+$ (calculated): 1836.3 (1835.7).

2.4.3. TATE-AoA-DOTA (24)

A solution of aminooxy-TATE (**15**, 3.0 mg, 2.7 μmol) was dissolved in a mixture of phosphate buffer (0.1 M, pH 4, 400 μL) and MeCN (400 μL) and added to DOTA-aldehyde (**6**, 2.6 mg, 4.0 μmol). The pH of the mixture was adjusted to 4.0–5.0 using phosphate buffer (0.5 M, pH 5.0, 100–150 μL) and allowed to react for 5 min. The product was purified by semi-preparative HPLC with 0–60% MeCN + 0.1% TFA in 8 min as the gradient (R_t = 4.1 min) and obtained as white solid after lyophilization (3.4 mg, 1.9 μmol, 72% yield).

ESI-MS (m/z) for $[M+K]^+$ (calculated): 1796.68 (1796.69).

2.4.4. TATE-AAo-DOTA (25)

A solution of TATE-aldehyde (**19**, 3.0 mg, 2.1 μmol) was dissolved in a mixture of phosphate buffer (0.1 M, pH 4, 400 μL) and MeCN (400 μL) and added to aminooxy-DOTA (**7**, 1.4 mg, 2.3 μmol). The pH of the mixture was adjusted to 4.0–5.0 using phosphate buffer (0.5 M, pH 5.0, 100–150 μL) and allowed to react for 5 min. The product was purified by semi-preparative HPLC with 0–50% MeCN + 0.1% TFA in 8 min as the gradient (R_t = 4.3 min) and obtained as white solid after lyophilization (3.5 mg, 1.7 μmol, 83% yield).

ESI-MS (m/z) for $[M+K+Na]^{2+}$ (calculated): 1031.36 (1031.37).

2.4.5. TATE-HA-P-DOTA (26) and subsequent deprotection to TATE-HA-DOTA (28)

To a mixture of TATE-5-hexynoic acid (**16**, 5 mg, 4.4 μmol) and P-DOTA-azide (**10**, 3.8 mg, 4.4 μmol) in H_2O/THF (1:1, 750 μL) was added a $CuSO_4$ solution (0.1 M, 11 μmol, 110 μL) and copper powder (~10 mg). After 60 min reaction, the solids were removed by centrifugation and a solution of sodium sulfide ($Na_2S{\cdot}9H_2O$, 4.8 mg, 22 μmol) in water (100 μL) was added. The resulting precipitate was removed by centrifugation. After 10–15 min, the solvent was removed and the residue incubated with TFA/TIS/H_2O (95:2.5:2.5, 2 mL) for 2 h. After evaporation of the volatile components, the crude product was purified by semi-preparative HPLC with 0–50% MeCN + 0.1% TFA in 8 min as the gradient (R_t = 5.1 min). The product was obtained as white solid after lyophilization (1.6 mg, 0.84 μmol, 19% yield).

ESI-MS (m/z) for $[M+Na+K]^{2+}$ (calculated): 925.87 (925.87) and (m/z) for $[M+2Na+K]^{2+}$ (calculated): 936.86 (936.87).

2.4.6. TATE-AH-P-DOTA (27) and subsequent deprotection to TATE-AH-DOTA (29)

To a mixture of TATE-5-azidopentanoic acid (**17**, 3 mg, 2.6 μmol) and P-DOTA-alkyne (**9**, 2.0 mg, 2.6 μmol) in H_2O:THF (1:1, 750 μL) was added a $CuSO_4$ solution (0.1 M, 6.5 μmol, 65 μL) and copper powder (~10 mg). After 60 min reaction, the solids were removed by centrifugation and a solution of sodium sulfide ($Na_2S{\cdot}9H_2O$, 2.8 mg, 13 μmol) in water (100 μL) was added. The resulting precipitate was removed by centrifugation. After 10–15 min, the solvent was removed and the residue incubated with TFA/TIS/H_2O (95:2.5:2.5, 2 mL) for 2 h. After evaporation of the volatile components, the crude product was purified by semi-preparative HPLC with 0–50% MeCN + 0.1% TFA in 8 min as the gradient (R_t = 5.0 min). The product was obtained as white solid after lyophilization (1.0 mg, 0.51 μmol, 20% yield).

ESI-MS (m/z) for $[M+Na+K]^-$ (calculated): 1850.87 (1850.75).

2.4.7. TATE-HA-DOTA (28)

To a mixture of TATE-5-hexynoic acid (**16**, 5 mg, 4.4 μmol) and DOTA-azide (**12**, 2.8 mg, 4.4 μmol) in H_2O/THF (1:1, 750 μL) was added a $CuSO_4$ solution (0.1 M, 11 μmol, 110 μL) and copper powder (~10 mg). After 60 min reaction, the solids were removed by centrifugation and a solution of sodium sulfide ($Na_2S{\cdot}9H_2O$, 4.8 mg, 22 μmol) in water (100 μL) was added. The resulting precipitate was removed by centrifugation and after 10–15 min, the mixture was acidified with 2 M HCl (100 μL). The crude product was purified by semi-preparative HPLC with 0–40% MeCN + 0.1% FA in 8 min as the gradient (R_t = 4.7 min). The product was obtained as white solid after lyophilization (1.8 mg, 1.0 μmol, 22% yield).

ESI-MS (m/z) for $[M+K]^+$ (calculated): 1828.76 (1828.76), (m/z) for $[M+H+K]^{2+}$ (calculated): 914.88 (914.88) and (m/z) for $[M+Na+K]^{2+}$ (calculated): 925.87 (925.87).

2.4.8. TATE-AH-DOTA (29)

To a mixture of TATE-5-azidopentanoic acid (**17**, 5.2 mg, 4.4 μmol) and DOTA-alkyne (**11**, 2.7 mg, 4.4 μmol) in H_2O/THF (1:1, 750 μL) was added a $CuSO_4$ solution (0.1 M, 11 μmol, 110 μL) and copper powder (~10 mg). After 60 min reaction, the solids were removed by centrifugation and a solution of sodium sulfide ($Na_2S{\cdot}9H_2O$, 4.8 mg, 22 μmol) in water (100 μL) was added. The resulting precipitate was removed by centrifugation and after 10–15 min, the mixture was acidified with 2 M HCl (100 μL). The crude product was purified by semi-preparative HPLC with 0–40% MeCN + 0.1% FA in 8 min as the gradient (R_t = 4.7 min). The product was obtained as white solid after lyophilization (1.9 mg, 1.1 μmol, 25% yield).

ESI-MS (m/z) for $[M+K]^+$ (calculated): 1828.76 (1828.76), (m/z) for $[M+Na+K]^{2+}$ (calculated): 925.87 (925.87).

2.5. Radiolabeling reactions with ^{68}Ga

A solution of the respective DOTA-peptide-conjugate (**20**–**25**, **28**, and **29**, 10–13 nmol) in HEPES buffer (0.025 M, pH 4.0, 100 μL) was added to 120–270 MBq of ^{68}Ga in sodium acetate solution obtained by fractioned elution of a ^{68}Ge/^{68}Ga generator (IGG100, Eckert & Ziegler, Berlin, Germany) with HCl (0.1 M, 1.2 mL) and subsequent titration to pH 3.5–4.0 by addition of sodium acetate solution (1.25 M, 90–95 μL). After reaction for 10 min at 99 °C, the reaction mixtures were analyzed by analytical radio-HPLC. The radiolabeled peptide conjugates were found to be 96–99% pure and obtained in specific activities of 12.7–20.3 GBq/μmol.

3. Results and discussion

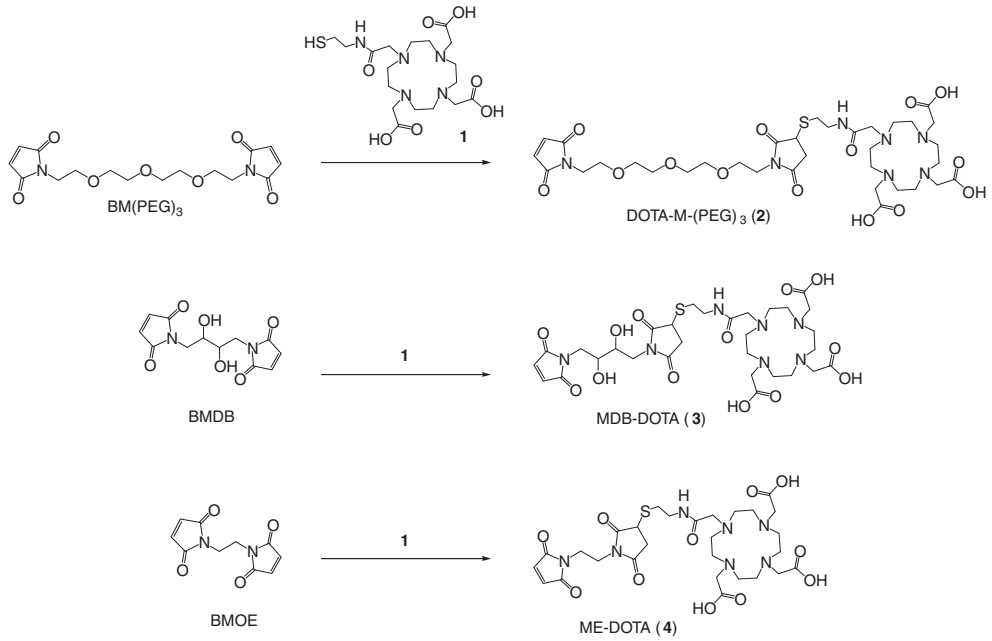
3.1. Synthesis of DOTA derivatives

As click chemistry reactions show a high chemoselectivity under mild reaction conditions, we aimed at finding a straightforward synthesis route to obtain a full set of DOTA derivatives comprising functional groups suitable for the application in the presently most often used click chemistry reactions: (1) thiol-maleimide-, (2) aldehyde-aminooxy-, and (3) alkyne-azide coupling. Subsequently, the different reaction types should be compared regarding the achievable coupling yields and efficiencies.

3.1.1. Synthesis of DOTA derivatives for application in Michael addition reactions

To make use of this reaction which proceeds under mild physiological conditions, we first synthesized a DOTA derivative comprising a thiol (**1**, 2,2′,2″-(10-(2-(2-Mercaptoethylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid, Scheme 1).[27] Starting from this synthon, DOTA derivatives comprising maleimide moieties together with varying spacer lengths could conveniently be synthesized by a one-step reaction with the respective commercially available bis-maleimides (Scheme 1).

This favorable strategy allows the synthesis of DOTA-maleimides variable in spacer length, influencing the biochemical



Scheme 1. Reaction pathway for the syntheses of the DOTA-maleimides DOTA-M-$(PEG)_3$ (**2**), MDB-DOTA (**3**), and ME-DOTA (**4**) obtained by reacting $BM(PEG)_3$, BMDB, and BMOE with thiol-DOTA (**1**). Reagents and conditions: phosphate buffer, pH 7.2, 5 min, 57%, 40%, and 48% yields.

properties of the resulting conjugates which is an important factor in molecular imaging or therapy.[15,29]

As model compounds for this synthesis strategy, three different DOTA derivatives were synthesized by reacting **1** in aqueous solution at pH 7.2 with bis-maleimido-PEG_3 ($BM(PEG)_3$), 1,4-bismaleimidyl-2,3-dihydroxybutane (BMDB), and bis-maleimidoethane (BMOE). The reactions proceeded quantitatively within 5 min and without the formation of any side-products giving the DOTA-maleimides DOTA-M-$(PEG)_3$ (**2**),[26] MDB-DOTA (**3**), and ME-DOTA (**4**) after HPLC purification in 57%, 40%, and 48% preparative yields, respectively.

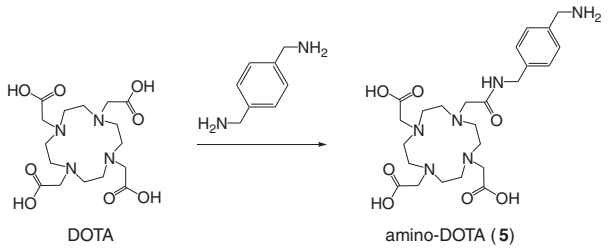
Thus, maleimide-containing DOTA derivatives with varying properties can conveniently be synthesized using this strategy.

3.1.2. Synthesis of DOTA derivatives for application in oxime formation reactions

DOTA chelators suitable for this reaction type have to comprise either an aminooxy or aldehyde functionality. Although ketone-derivatized compounds can also be applied, aldehydes are preferable as they show much higher reactivities towards hydroxylamines.

For the introduction of an aldehyde or aminooxy-functionality, some protected aldehyde- and aminooxy-containing amine-reactive building blocks are commercially available.

Thus, in a first step, a DOTA derivative comprising an amino functionality and a chromophore for a simplified HPLC-purification was synthesized (amino-DOTA, **5**) by reacting DOTA with 1,4-bis(aminomethyl)benzene (Scheme 2).



Scheme 2. Synthesis route for amino-DOTA (**5**). Reagents and conditions: DIC, H_2O/MeCN/pyridine, 16 h, 75% yield.

In order to introduce an aldehyde functionality, amino-DOTA (**5**) was reacted with 3,3-dimethoxy-propionic acid giving the protected DOTA-aldehyde in moderate yields after HPLC-purification. Surprisingly, this intermediate could not be deprotected by subsequent treatment with acids yielding the desired aldehyde. Instead, the respective alcohol was formed quantitatively. The same result was found when applying an aldehyde-releasing H-Val-H NovaSyn® TG resin for solid phase syntheses. Again, only the alcohol could be isolated instead of the aldehyde after cleavage from the solid support.

Finally, the DOTA-aldehyde derivative **6** could be obtained by reacting amino-DOTA (**5**) with *p*-formylbenzoic acid *N*-hydroxysuccinimide ester (SFB) in aqueous solution at pH 7.2 (Scheme 3). Under these conditions, the amino group of **5** selectively reacts with the active ester of SFB giving **6** in a moderate yield of 37%.

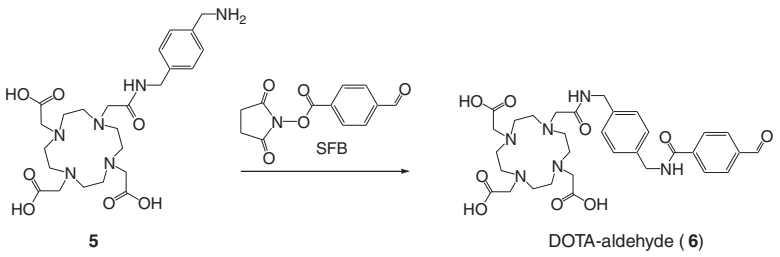
This two-step reaction pathway starting from commercially available DOTA means a considerable reduction of synthesis complexity and reaction steps for a DOTA-aldehyde compared to the literature.[17]

For the synthesis of an aminooxy-derivatized DOTA synthon, amino-DOTA (**5**) was reacted with Boc-aminooxy-acetic acid. The resulting intermediate was subsequently deprotected with neat trifluoroacetic acid (TFA) within 15 min, giving the desired product **7** in 28% overall yield after HPLC purification (Scheme 4). This two-step procedure means a considerable decrease of the synthesis complexity and number of necessary reaction steps for an aminooxy-DOTA derivative.[22]

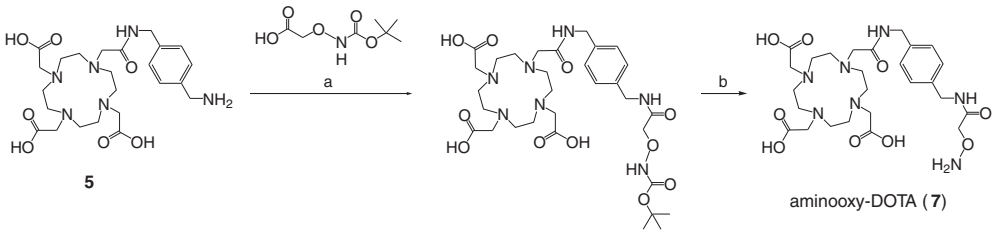
Noteworthy, the corresponding synthesis route using bis-Boc-aminooxy-acetic acid gave the product **7** in only low yields due to an incomplete removal of both Boc-protecting groups. The prolongation of the deprotection reaction time resulted in the formation of decomposition products, limiting the reaction yields and impairing the HPLC-purification.

3.1.3. Synthesis of DOTA derivatives for application in 1,3-dipolar cycloaddition reactions

In a first approach, as it was anticipated that free carboxylic functionalities of unprotected DOTA derivatives might prevent a 1,3-dipolar cycloaddition between azides and alkynes by



Scheme 3. Reaction pathway for the synthesis of DOTA-aldehyde (**6**) starting from amino-DOTA (**5**). Reagents and conditions: phosphate buffer, MeCN, pH 7.2, 1 h, 37% yield.



Scheme 4. Synthesis route for aminooxy-DOTA (**7**). Reagents and conditions: (a) HBTU, DIPEA, DMF, 30 min (not isolated); (b) TFA, TIS, 15 min, overall yield: 28% yield.

interfering with the copper catalyst, *tert*-butyl-protected DOTA derivatives comprising an azide and alkyne functionality were synthesized.

Following the synthesis route of amino-DOTA (**5**), a fully *tert*-butyl-protected DOTA-amine (P-amino-DOTA, **8**) was synthesized from tris-*t*Bu-DOTA and 1,4-bis(aminomethyl)benzene (Scheme 5). P-amino-DOTA was subsequently reacted with 5-hexynoic acid and 5-azidopentanoic acid giving the fully protected alkyne- and azide-comprising chelators *tert*-butyl-protected P-DOTA-alkyne (**9**) and *tert*-butyl-protected P-DOTA-azide (**10**) (Scheme 5) in yields of 11% and 39%, respectively, after HPLC-purification.

Following a similar approach, the respective unprotected azide- and alkyne-functionalized DOTA derivatives were synthesized to investigate their suitability in a copper-catalyzed cycloaddition reaction with appropriately functionalized Tyr^3-octreotate derivatives in addition to the *tert*-butyl-protected derivatives. These synthons would be more advantageous than the protected compounds **9** and **10**, as they would make a final *tert*-butyl-deprotection of the peptide-DOTA-coupling products unnecessary.

The unprotected alkyne- and azide-comprising DOTA derivatives **11** and **12** were synthesized by reacting amino-DOTA (**5**) with 5-hexynoic acid and 5-azidopentanoic acid in a straightforward one-step reaction (Scheme 5), giving both compounds in 29% and 32% yields after HPLC-purification, meaning a considerable reduction of synthesis steps and complexity compared to other methods.[23,24]

3.2. Synthesis of functionalized peptide derivatives based on Tyr^3-octreotate

In order to show the applicability of the synthesized DOTA derivatives for efficient and site-specific chelator-introduction into biomolecules and to directly compare the reactivities and coupling efficiencies using the different click chemistry reactions, Tyr^3-octreotate was chosen as bioactive model compound. It contains several side chain functionalities which have to prove to not cross-react during the site-specific DOTA synthon conjugation reactions.

Tyr^3-octreotate was synthesized using the standard solid-phase Fmoc peptide synthesis strategy.[28] For the derivatization of the peptide with functional groups applicable in the described click chemistry reactions, the fully protected peptide was modified on resin with maleimido hexanoic acid, cysteine, bis-Boc-aminooxy acetic acid, 5-hexynoic acid and 5-azidopentanoic acid under standard reaction conditions. The respective peptide derivatives (**13**–**17**, Fig. 1) were obtained after cleavage from resin, simultaneous deprotection and HPLC-purification.

The synthesis of the aldehyde-comprising peptide proved to be intricate, as the same problem of quantitative alcohol over aldehyde formation as described in 3.1.2. was observed. The synthesis strategy of reacting the peptide liquid-phase with SFB—as it was successful for the synthesis of **6**—was not applicable since Tyr^3-octreotate contains two amino functionalities giving three possible reaction products that would be difficult to distinguish and separate.

To circumvent this problem, a novel crosslinker, *N*-(2-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)ethyl)-4-formyl-benzamide (MEFB), was developed to site-specifically introduce the aldehyde functionality into the peptide. The maleimido group of this crosslinker chemoselectively reacts with a thiol-functionality at pH 7.2 to give an aldehyde-derivatized compound. MEFB (**18**) was synthesized from *N*-(2-aminoethyl)maleimide trifluoroacetate salt and SFB according to Scheme 6 in a good yield of 55% after HPLC-purification.

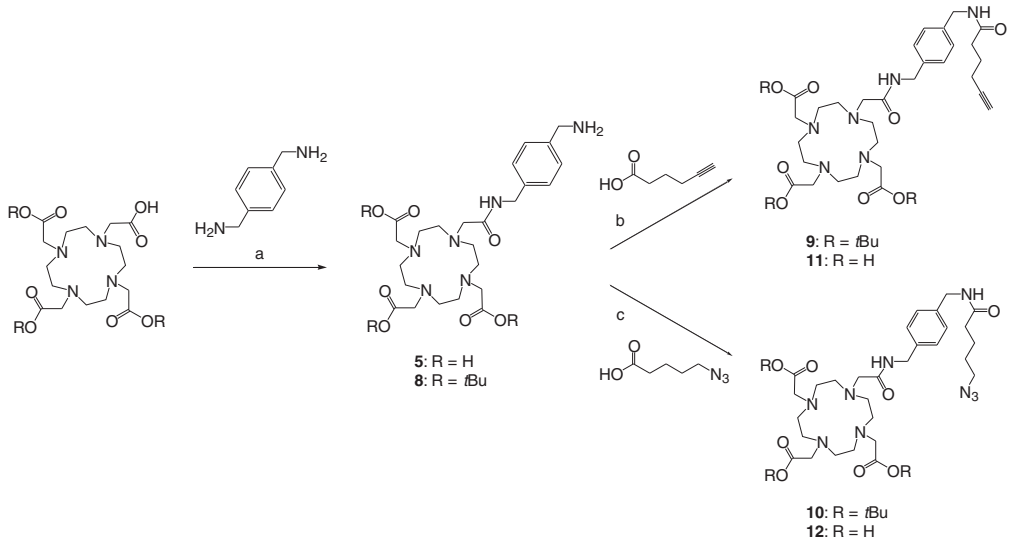
This crosslinker was reacted with TATE-cysteine (**14**) in aqueous medium giving the *N*-terminally modified peptide TATE-aldehyde (**19**, Fig. 1).

Figure 1 gives the structures of the Tyr^3-octreotate derivatives used for the subsequent click chemistry coupling reactions with the DOTA derivatives **1**–**4**, **6**, **7**, and **9**–**12**.

3.3. Conjugation reactions of the 'clickable' DOTA building blocks with the Tyr^3-octreotate-derivatives

3.3.1. Conjugation of thiol-DOTA (1) and DOTA-maleimides 2, 3, and 4 to TATE-maleimide (13) and TATE-cysteine (14)

The chemoselective addition reaction of **1** to **13** and **14** to **2**, **3**, and **4** (using 1.1 eq. of the chelator synthon) was carried out in a mixture of phosphate puffer together with MeCN at pH 7.2 and was completed after a reaction time of less than 5 min. Moreover, the reaction proceeded not only fast, but also without side-reactions (Fig. 2A) and gave the products TATE-MT-DOTA (TATE-maleimide-thiol-DOTA, **20**) and TATE-TM$_2$-DOTA (**21**), TATE-TM$_3$-DOTA



Scheme 5. Schematic depiction of the synthesis of P-amino-DOTA (**8**), P-DOTA-alkyne (**9**), P-DOTA-azide (**10**), DOTA-alkyne (**11**), and DOTA-azide (**12**). Reagents and conditions: (a) HBTU, DIPEA, DMF, 30 min, (75% yield for **5**, 68% yield for **8**); (b) HBTU, DIPEA, DMF, 60 min, (11% yield for **9**, 29% yield for **11**); (c) HBTU, DIPEA, DMF, 60 min, (39% yield for **10**, 32% yield for **12**).

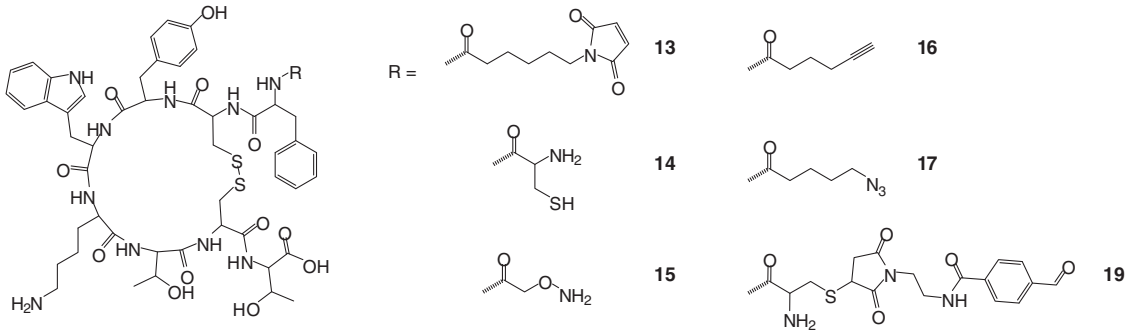


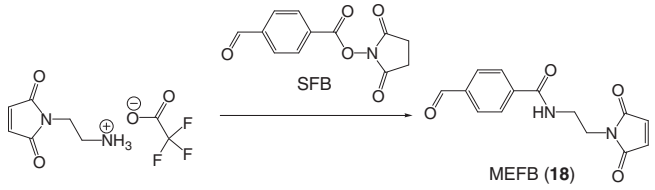
Figure 1. Structures of the peptide derivatives **13**–**17** and **19** that were used in the coupling reactions with the respective chelator derivatives.

(**22**), and TATE-TM_4-DOTA (**23**) (TATE-thiol-maleimide$_{(x)}$-DOTA) in 70%, 64%, 66% and 73% yields after HPLC-purification, respectively.

3.3.2. Conjugation of DOTA-aldehyde (6) and aminooxy-DOTA (7) to aminooxy-TATE (15) and TATE-aldehyde (19)

The oxime formation between **6** and **15** and **7** and **19** was performed in a pH-range between 4.0 and 5.0 using a mixture of phosphate buffer and MeCN as solvent system. As described for the Michael addition reactions yielding the conjugates **20**–**23**, the oxime formation was completed within reaction times of less than 5 min and also proceeded without side-reactions facilitating the purification of the products (Fig. 2B).

This is a considerable improvement in terms of reaction time compared to the DOTA-ketone-to-aminooxy coupling reaction described earlier, requiring a reaction time of 18 h.[23] This improvement can be attributed to the higher reactivity and thus high suitability of aldehydes compared to ketones for this reaction type. The peptide–DOTA-conjugates TATE-AoA-DOTA (TATE-aminooxy-aldehyde-DOTA, **24**) and TATE-AAo-DOTA (TATE-aldehyde-aminooxy-DOTA, **25**) were obtained in 72% and 83% yield after HPLC-purification, respectively.



Scheme 6. Schematic depiction of the synthesis of the crosslinker MEFB (**18**). Reagents and conditions: phosphate buffer, MeCN, pH 7.2, 60 min, 55% yield.

3.3.3. Conjugation of P-DOTA-alkyne (9), P-DOTA-azide (10), DOTA-alkyne (11), and DOTA-azide (12) to TATE-5-hexynoic acid (16) and TATE-5-azidopentanoic acid (17)

A challenge using the 1,3-dipolar cycloaddition of azides to alkynes for the coupling of a chelator is the required copper catalyst since the generated Cu^{2+}-ions become chelated by unprotected but also fully carboxyl-protected DOTA which prevents a subsequent radiolabeling of the substance. As a result of the necessary quantitative removal of the copper catalyst and—if applied—the removal of DOTA carboxyl-protecting groups, the overall yields decrease using this click chemistry reaction.

Nevertheless, the application of this reaction type could be advantageous in cases when this particular conjugation system is desired to have a complementary chemoselective reaction at hand or to obtain a compound profiting from the special properties of the formed triazole.

For the conjugation of the protected DOTA derivatives **9** and **10** to the peptides **16** and **17**, several different reaction conditions were applied without success.[30–32] Solely reaction conditions based on those described by Knör et al.[23] resulted in the formation

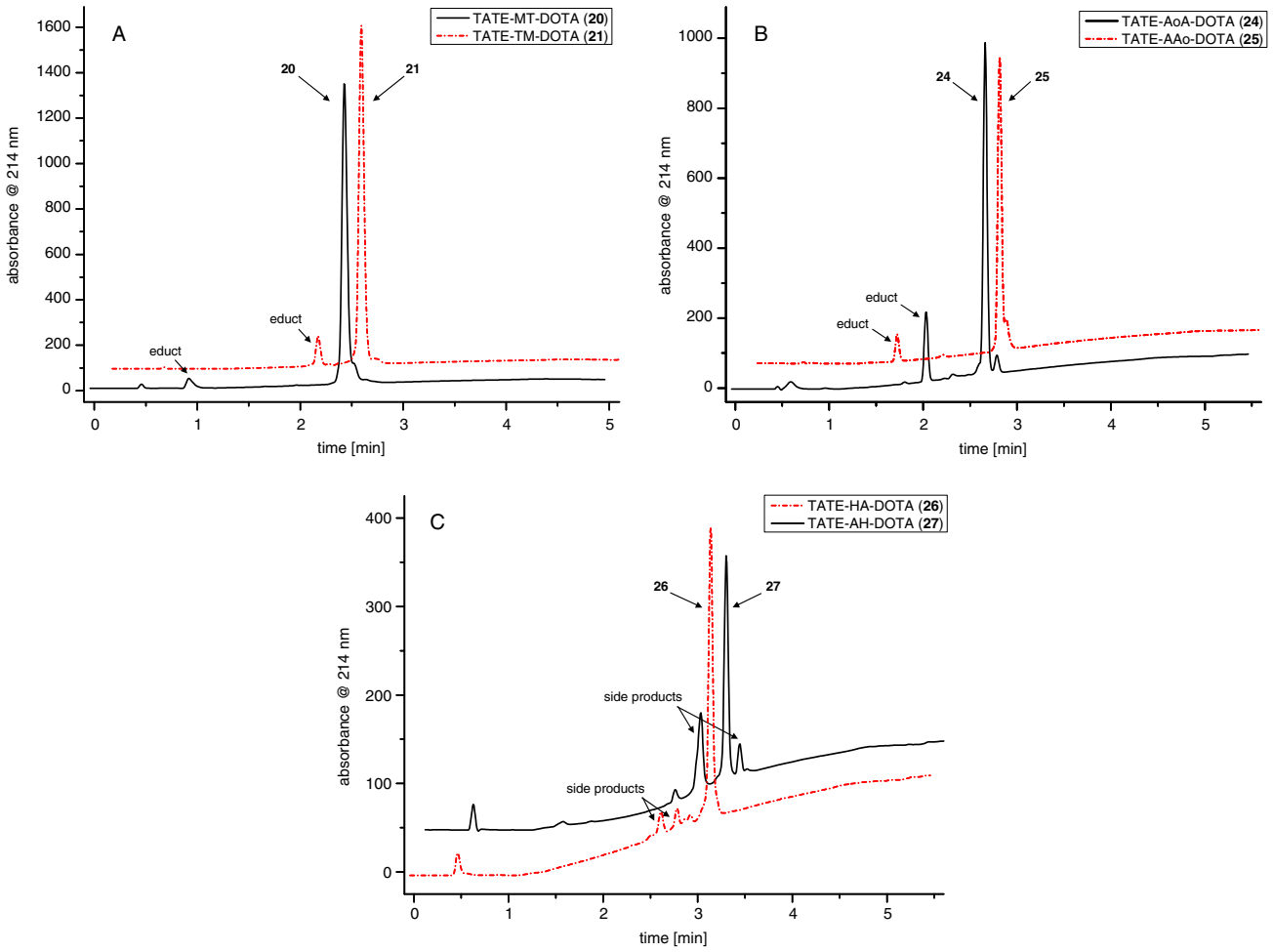


Figure 2. Analytical HPLC-chromatograms of conjugation reaction mixtures of (A) **1** with **13** (solid line) and **14** with **2** (dashed line) after 5 min reaction time; (B) **6** with **15** (solid line) and **7** with **19** (dashed line) after 5 min reaction time; (C) **9** with **17** (solid line) and **10** with **16** (dashed line) after 60 min reaction time.

of coupling products and gave the final *t*Bu-deprotected conjugates free from chelated copper. These conditions apply 1.2 equiv of copper sulfate together with copper powder as catalyst and water/THF (1:1) as solvent system. Following the cycloaddition reaction and the removal of the DOTA protecting groups, sodium sulfide is used to remove copper ions from the mixture as well as from the chelator by precipitation.

When adapting these reaction conditions, we obtained the best results applying 2.5 equiv of copper sulfate and observed that the reactions proceeded far more efficiently than anticipated, resulting in complete coupling reactions after 1 h of synthesis time instead of 18. Furthermore, we found no reductive cleavage of the dithiol bonds occurring due to the copper catalyst precipitation with sodium sulfide as it was described before.[23] This can be attributed to an optimal ratio of Na_2S and oxidized reductant (NaS–SNa) and the strongly basic pH of the reaction mixture, resulting in the recyclization of the peptides[33,34] which makes an additional time-consuming recyclization step dispensable.

Thus, these modifications of reaction conditions reduce the number of synthesis steps and the overall reaction time significantly.

However, contrary to the addition and oxime formation reactions described in Sections 3.3.1. and 3.3.2., some unidentified side-products are formed in the course of the triazole formation reactions (Fig. 2C). Interestingly, comparing the coupling reactions of both DOTA-alkynes + TATE-azide and both DOTA-azides + TATE-alkyne, more side-products were formed during the reactions of the DOTA-alkynes **9** and **11** with the peptidic azide **17**.

The peptide–DOTA-conjugates TATE-HA-DOTA (TATE-hexynoic-acid-azide-DOTA, **28**) and TATE-AH-DOTA (TATE-azide-hexynoic-acid-DOTA, **29**) were obtained in three reactions steps (coupling of the DOTA synthon, precipitation of copper ions and *tert*-butyl-deprotection) in overall yields of 19 and 20% after HPLC-purification, respectively.

For the conjugation of the unprotected DOTA derivatives **11** and **12** to the peptides **17** and **16**, the same modified reaction conditions were applied, directly yielding TATE-HA-DOTA (**28**) and TATE-AH-DOTA (**29**), making a final *tert*-butyl-deprotection dispensable and resulting in shorter overall reaction times and higher yields.

This also renders this synthesis strategy better suited for the conjugation of DOTA to molecules sensitive to harsh deprotection steps. The products in this case were obtained in overall yields of 22% and 25%, respectively.

However, the overall coupling yields using the triazole formation were significantly lower compared to those obtained using the alternative click chemistry reaction types (Sections 3.3.1. and 3.3.2.), although the coupling reaction itself proceeded quite efficiently. This suggests a possible co-precipitation of the products with the copper catalyst, diminishing the overall obtainable yields.

3.4. Radiolabeling of peptide–DOTA-conjugates 20–25, 28, and 29 with ^{68}Ga

In order to demonstrate that the obtained DOTA-peptide-conjugates can be labeled with radiometal nuclides in radiochemical

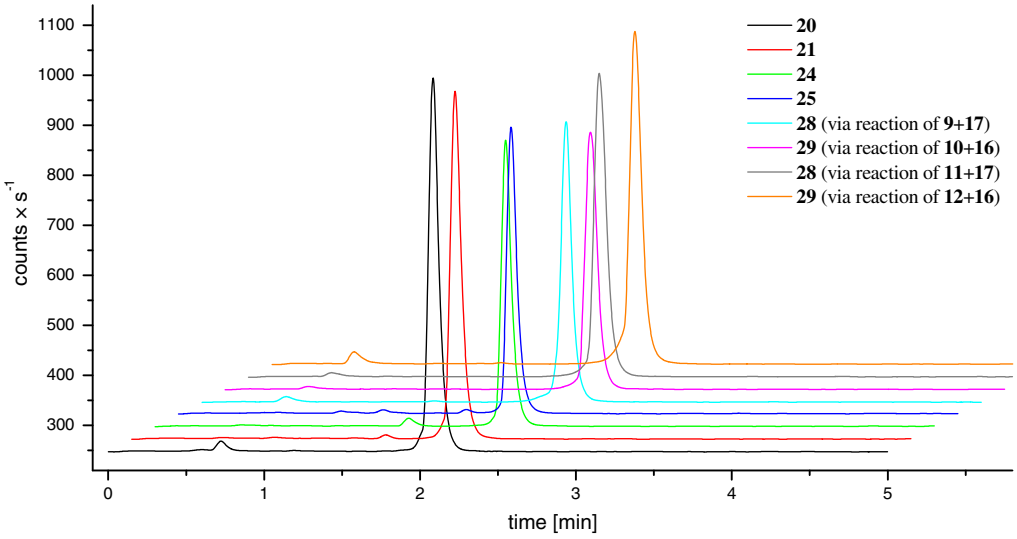


Figure 3. Analytical radio-HPLC chromatograms of the reaction mixtures of the DOTA–peptide-conjugates **20**, **21**, **24**, **25**, **28**, and **29** with ^{68}Ga.

yields and specific activities comparable to products obtained via application of tris-*t*Bu-DOTA, they were radiolabeled with ^{68}Ga applying the standard labeling procedure for ^{68}Ga-peptides used in clinical trials.

For the radiolabeling reactions, 10–13 nmol of the chelator-conjugated peptides were reacted with 120–270 MBq of ^{68}Ga for 10 min at 99 °C and subsequently analyzed by radio-HPLC. The radiolabeled products were obtained in radiochemical purities of 96–99% as determined by radio-HPLC (Fig. 3) and in specific activities between 12.7 and 20.3 GBq/μmol.

These results show that all DOTA-peptide-conjugates can be radiolabeled with ^{68}Ga in high radiochemical purities and specific activities.

In case of the radiolabeling of **28** and **29** (obtained via both reaction pathways 2.4.5.–2.4.8.), the high labeling yield with ^{68}Ga provides further proof that the copper catalyst was completely removed from the complex which otherwise would result in only low radiolabeling yields.

4. Conclusion

Several different DOTA derivatives—DOTA-maleimides, DOTA-thiol, DOTA-aldehyde, aminooxy-DOTA, DOTA-alkyne, DOTA-azide, protected DOTA-alkyne and protected DOTA-azide—were synthesized for site-specific peptide-derivatization via click chemistry reactions. All chelator derivatives were accessible in straightforward syntheses starting from commercially available synthons within one or few steps which means for most of these derivatives a considerable improvement regarding synthesis time and complexity compared to already known procedures.

For each of these synthons, the chemoselective and efficient introduction into an appropriately derivatized model peptide based on Tyr^3-octreotate via the respective click chemistry reaction could be demonstrated and some considerably improved conjugation reaction conditions could be found. Among the coupling methods used, the addition reaction of thiols to maleimides and the oxime formation between aldehydes and hydroxylamines proceeded equally well within few minutes without forming side-products. The triazole formations between alkynes and azides, however, required slightly longer reaction times and gave some side-products, but also yielded the desired products in reasonable yields. Due to the denaturing conditions required for the removal of the copper catalyst, this reaction type should be well-suited for the modification of peptides and other insensitive biomolecules but might be of limited applicability for the derivatization of susceptible biomolecules such as proteins.

The synthesized peptide–DOTA-conjugates were labeled with ^{68}Ga in almost quantitative yields within 10 min, demonstrating the suitability of the developed DOTA synthons for derivatization of bioactive molecules intended for radiometal labeling.

Acknowledgments

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References and notes

1. Wadas, T. J.; Wong, E. H.; Weisman, G. R.; Anderson, C. J. *Chem. Rev.* **2010**, *110*, 2858.
2. Anderegg, G.; Arnaud-Neu, F.; Delgado, R.; Felcman, J.; Popov, K. *Pure Appl. Chem.* **2008**, *77*, 1445.
3. Al-Nahhas, A.; Win, Z.; Szyszko, T.; Singh, A.; Nanni, C.; Fanti, S.; Rubello, D. *Anticancer Res.* **2007**, *27*, 4087.
4. Reubi, J. C.; Maecke, H. R. *J. Nucl. Med.* **2008**, *49*, 1735.
5. Maecke, H. R.; Hofmann, M.; Haberkorn, U. *J. Nucl. Med.* **2005**, *46*, 172S.
6. Basu, S.; Kumar, R.; Rubello, D.; Fanti, S.; Alavi, A. *Minerva Endocrinol.* **2008**, *33*, 257.
7. Heppeler, A.; Froidevaux, S.; Mäcke, H. R.; Jerman, E.; Behe, M.; Powell, P.; Hennig, M. *Chem. Eur. J.* **1999**, *5*, 1974.
8. Mier, W.; Hoffend, J.; Krämer, S.; Schuhmacher, J.; Hull, W. E.; Eisenhut, M.; Haberkorn, U. *Bioconjugate Chem.* **2005**, *16*, 237.
9. Schlesinger, J.; Koezle, I.; Bergmann, R.; Tamburini, S.; Bolzati, C.; Tisato, F.; Noll, B.; Klussmann, S.; Vonhoff, S.; Wuest, F.; Pietzsch, H. J.; Steinbach, J. *Bioconjugate Chem.* **2008**, *19*, 928.
10. Martin, S. M.; O'Donnell, R. T.; Kukis, D. L.; Abbey, C. K.; McKnight, H.; Sutcliffe, J. L.; Tuscano, J. M. *Mol. Imaging Biol.* **2009**, *11*, 79.
11. Wängler, C.; Wängler, B.; Eisenhut, M.; Haberkorn, U.; Mier, W. *Bioorg. Med. Chem.* **2008**, *16*, 2606.
12. Wängler, B.; Beck, C.; Wagner-Utermann, U.; Schirrmacher, E.; Bauer, C.; Rosch, F.; Schirrmacher, R.; Eisenhut, M. *Tetrahedron Lett.* **2006**, *47*, 5985.
13. De Leon-Rodriguez, L. M.; Kovacs, Z.; Dieckmann, G. R.; Sherry, A. D. *Chemistry* **2004**, *10*, 1149.
14. Wängler, C.; Schirrmacher, R.; Bartenstein, P.; Wängler, B. *Curr. Med. Chem.* **2010**, *17*, 1092.
15. Glaser, M.; Morrison, M.; Solbakken, M.; Arukwe, J.; Karlsen, H.; Wiggen, U.; Champion, S.; Kindberg, G. M.; Cuthbertson, A. *Bioconjugate Chem.* **2008**, *19*, 951.
16. Li, L.; Olafsen, T.; Anderson, A. L.; Wu, A.; Raubitschek, A. A.; Shively, J. E. *Bioconjugate Chem.* **2002**, *13*, 985.
17. Barge, A.; Tei, L.; Upadhyaya, D.; Fedeli, F.; Beltrami, L.; Stefania, R.; Aime, S.; Cravotto, G. *Org. Biomol. Chem.* **2008**, *6*, 1176.
18. Yim, C. B.; Dijkgraaf, I.; Merkx, R.; Versluis, C.; Eek, A.; Mulder, G. E.; Rijkers, D. T.; Boerman, O. C.; Liskamp, R. M. *J. Med. Chem.* **2010**, *53*, 3944.

19. Wängler, C.; Schirrmacher, R.; Bartenstein, P.; Wängler, B. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1926.
20. Schlesinger, J.; Fischer, C.; Koezle, I.; Vonhoff, S.; Klussmann, S.; Bergmann, R.; Pietzsch, H. J.; Steinbach, J. *Bioconjugate Chem.* **2009**, *20*, 1340.
21. Thonon, D.; Jacques, V.; Desreux, J. F. *Contrast Media Mol. Imaging* **2007**, *2*, 24.
22. Hovinen, J. *Chem. Biodivers.* **2006**, *3*, 296.
23. Knör, S.; Modlinger, A.; Poethko, T.; Schottelius, M.; Wester, H. J.; Kessler, H. *Chemistry* **2007**, *13*, 6082.
24. Prasuhn, D. E., Jr.; Yeh, R. M.; Obenaus, A.; Manchester, M.; Finn, M. G. *Chem. Commun. (Camb.)* **2007**, 1269.
25. Schultz, M. K.; Parameswarappa, S. G.; Pigge, F. C. *Org. Lett.* **2010**, *12*, 2398.
26. Wängler, C.; Maschauer, S.; Prante, O.; Schafer, M.; Schirrmacher, R.; Bartenstein, P.; Eisenhut, M.; Wängler, B. *ChemBioChem* **2010**, *11*, 2168.
27. Wängler, C.; Wängler, B.; Eisenhut, M.; Haberkorn, U.; Mier, W. *Bioorg. Med. Chem.* **2008**, *16*, 2606.
28. Wellings, D. A.; Atherton, E. *Methods Enzymol.* **1997**, *289*, 44.
29. Tolmachev, V.; Orlova, A. *Curr. Med. Chem.* **2010**.
30. Yim, C. B.; Boerman, O. C.; de Visser, M.; de Jong, M.; Dechesne, A. C.; Rijkers, D. T.; Liskamp, R. M. *Bioconjugate Chem.* **2009**, *20*, 1323.
31. Schirrmacher, R.; Lakhrissi, Y.; Jolly, D.; Goldstein, J.; Lucas, P.; Schirrmacher, E. *Tetrahedron Lett.* **2008**, *49*, 4824.
32. Dijkgraaf, I.; Rijnders, A. Y.; Soede, A.; Dechesne, A. C.; van Esse, G. W.; Brouwer, A. J.; Corstens, F. H.; Boerman, O. C.; Rijkers, D. T.; Liskamp, R. M. *Org. Biomol. Chem.* **2007**, *5*, 935.
33. Classz, M. *Ber. Dtsch. Chem. Ges.* **1912**, *45*, 2424.
34. Chen, L.; Annis, I.; Barany, G. *Curr. Protoc. Protein Sci.* Chapter 18, 2001, Unit18 16.