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Application of tris-allyl-DOTA in the preparation of DOTA–peptide conjugates

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Abstract—The synthesis of tris-allyl-DOTA starting from cyclen and its application in the preparation of DOTA–peptide conjugates is reported. Clinically important conjugates such as DOTA–Tyr^3-octreotide (DOTA–TOC), DOTA–Tyr^3-octreotate (DOTA–TATE) as well as a DOTA–RGD peptide were synthesized in high yields with Fmoc solid phase peptide synthesis. The final, extremely reliable de-allylation was achieved on solid phase by different methods identifying morpholine/Pd(0) as the most suitable one obtaining all DOTA peptide conjugates in high yields. All DOTA–peptides were purified by reversed phase HPLC and structural identity was proved using MALDI-TOF mass spectrometry.

A variety of radiolabeled peptides such as octreotide and octreotate derivatives are used in clinical routine and clinical research, both for diagnosis and endoradiotherapy.[1] The use of peptides as targeting vectors is a promising approach to assess tumors expressing specific peptide-receptors on the cell surface.[2–4] Tyr^3-octreotate as well as Tyr^3-octreotide are somatostatin analogs with high affinity for the somatostatin receptor subtype-2. This subtype is over-expressed on the cell surface of several tumors of the neuroendocrine system, making somatostatin analogs eligible compounds for tumor targeting.[5–7] The $\alpha_v\beta_3$ integrin receptor was found to be overexpressed on both endothelial cells of neovasculature and tumor cells of various origins. Small cyclic peptides containing the Arg-Gly-Asp (RGD) sequence are potent antagonists for this receptor and are used in the form of monomers, dimers or tetramers for tumor imaging after derivatization with radionuclides.[8] The DOTA ligand is often used for complexation of diagnostic radiometals such as ^{64}Cu, ^{68}Ga, ^{86}Y, and ^{111}In and for therapeutic radionuclides such as ^{67}Cu and ^{90}Y.[9] To date only tris-*t*-butyl-DOTA is commercially available and has, therefore, gained widespread application. The synthesis of this compound has been described by Mäcke and co-workers.[10] It is still used although a series of efforts have been made to develop alternative strategies for the synthesis of DOTA derivatives.[11] One restriction of using tris-*t*-butyl-DOTA is the unreliable deprotection step using neat TFA or TFA/radical scavenger cocktails.[12] In the several cases observed, incomplete deprotection of the *t*-Bu groups leads at least to significantly reduced yields also involving a hampered purification step. In some cases the long deprotection time causes decomposition of the desired peptide conjugates.

In this contribution, tris-allyl-DOTA (**3**) is described which exhibits mildly removable allylester moieties using a Pd-catalyst. Under these conditions acid labile protecting groups are not affected.[13]

The synthesis of tris-allyl-DOTA (**3**) started from commercially available cyclen (**1**) (Fig. 1). The reaction conditions followed the method of Heppeler et al.[10] reacting **1** with *t*-butylbromoacetate in $CHCl_3$ yielding the corresponding *t*-butyl 2-(1,4,7,10-tetraazacyclododecan-1-yl) acetate (**2**) in 94% yield. It is, however, crucial to evaporate the $CHCl_3$ at ambient temperature to avoid the formation of an intramolecular acid amide (**4**) before purifying **3** by means of column chromatography.[14] The next step involved the coupling of three

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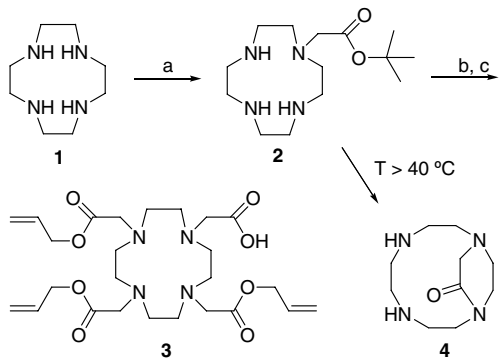

Figure 1. Synthesis of tris-allyl-DOTA (**3**) and formation of 4 at $T > 40$ °C: (a) cyclen, *t*-butylbromoacetate (0.5 equiv), $CHCl_3$, rt (b) K_2CO_3, allylchloroacetate, CH_3CN, rt (c) TFA, 0–25 °C, 24 h.

equivalents of allylchloroacetate with **2** under basic conditions in acetonitrile. The resulting *t*-Bu and allyl protected DOTA intermediate was not further purified. To prove product formation, an analytical sample was purified via HPLC and analyzed with MALDI-TOF spectroscopy.[15] To finally obtain **3**, the crude oily residue was treated with ice cold TFA which was allowed to warm to ambient temperature until HPLC analysis showed the absence of starting material (ca. 24 h). Final purification of **3** involved a multiple gradient elution starting with pure ethanol.[16] After elution of lipophilic impurities the solvent was changed to acetonitrile/0.1% TFA to elute more polar compounds. Compound **3** was obtained using a 1:1 mixture of acetonitrile/0.1% TFA and water/0.1% TFA which appeared after solvent removal as a white solid (yield 42%) (Table 1).

The application of compound **3** as a useful DOTA synthon in solid phase peptide synthesis (SPPS) was proven with Tyr^3-octreotate (TATE), Tyr^3-octreotide (TOC), and a cyclic RGD-peptide (cyclo(fKRGD)). The syntheses were performed using standard Fmoc SPPS via a batch-wise procedure. Tyr^3-octreotate and Tyr^3-octreotide were synthesized according to published procedures starting from commercially available preloaded resins.[17,18] For the synthesis of the RGD peptide, Fmoc-Asp-OAll was loaded onto a NovaSyn TGA resin (90 μm) using *N*-methylimidazole (MeIm) and 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT). The RGD peptide was synthesized with standard Fmoc SPPS, that is, 5.0 equiv AA, 4.9 equiv HBTU, and 10.0 equiv DIPEA in DMF at 25 °C. The allylester of Asp was cleaved by the standard method with morpholine and Pd(0) catalyst in DCM. Cyclization of the RGD peptide was achieved on solid phase using 10.0 equiv diisopropylcarbodiimide/10.0 equiv HOBT. Subsequently the Mtt protecting group of Lys was removed with TFA (2%) in DCM for the final coupling with **3**. The DOTA derivative **3** was conjugated to all solid phase bound peptides similar to an amino acid (4.0 equiv tris-allyl DOTA, 3.9 equiv HBTU, 15.0 equiv DIPEA) and the allylester groups were removed following three different procedures:[19] (1) morpholine/$[PPh_3]_4Pd(0)$/DCM, (2) dimedone/$[PPh_3]_4Pd(0)$ DCM, (3) sulfinic acid/$[PPh_3]_4Pd(0)$/DCM. Procedure 1 proved as the most effective method removing the allyl-ester groups quantitatively whereas in procedures 2 and 3 allyl-protected peptides could still be detected even after several hours by HPLC.

To cleave the peptide conjugates DOTA–TATE, DOTA–TOC, and cyclo(fK(DOTA)RGD) (DOTA–RGD) from the solid phase and to remove all protecting groups, neat TFA as well as TFA/TIS/H_2O 95:2.5:2.5 were used yielding the peptide–DOTA conjugates in 17–22% after preparative HPLC purification.[20] The identity of the peptides was proven by MALDI-TOF mass spectrometry.[21] To confirm the reliability of this synthetic pathway, all syntheses were performed at least three times and were found reproducible. To finally prove the advantage of **3** in comparison to tris-*t*-Bu-DOTA, we synthesized a batch of TOC on solid phase, and divided the batch into two lots which were reacted with tris-allyl-DOTA and tris-*t*-Bu-DOTA, respectively. In the case of tris-allyl-DOTA the resin was then incubated with morpholine/$[PPh_3]_4Pd(0)$/DCM to cleave the allyl moieties. Samples of 10 mg of the resins were reacted with neat TFA as well as TFA/TIS/H_2O 95:2.5:2.5 to yield the desired peptide. Typical HPLC-chromatograms of the crude peptide are shown in Figure 2, exemplifying the superiority of tris-allyl-DOTA in the SPPS of DOTA–TOC.

Table 1. Analytical data for compounds **2** and **3** (^1H, ^{13}C NMR; MALDI-TOF mass spectroscopy (MS)/ESI MS, elemental analysis)

Compound	^1H NMR (400 MHz)	^{13}C NMR (100.6 MHz)	MS[a]	Elemental analysis (%)
2	δ (DMSO-d_6) = 3.36 (s, 2H), 2.90–3.05 (m, 16H), 1.41 (s, 9H)	δ (DMSO-d_6) = 171.57, 81.95, 54.57, 49.29, 44.77, 42.85, 42.71, 28.43	286.19 $[M]^+$	Calcd:[b] C, 38.22; H, 5.29; N, 8.91. Found: C, 38.70; H, 5.52; N, 9.35
3	δ (DMSO-d_6) = 2.95–3.35 (m, 16H), 3.72 (br s, 4H), 3.96 (br s, 2H), 4.05 (br s, 2H), 4.54 (d, 4H, J = 5.47 Hz), 4.60 (d, 2H, J = 5.47 Hz), 5.15–5.35 (m, 6H, J = 1.17 Hz, J = 1.56 Hz), 5.81–5.87 (m, 3H)	δ (DMSO-d_6) = 170.20, 158.57, 158.23, 132.50, 132.21, 118.77, 118.41, 118.13, 115.20, 65.31, 53.92, 53.75, 53.18, 50.83, 48.65	525.4 $[M]^+$	Calcd:[c] C, 45.20; H, 5.75; N, 7.27. Found: C, 44.67; H, 5.86; N, 7.34

[a] MALDI-TOF (Kratos Kompact MALDI-III) and ESI (TSQ-7000, Thermo Electron, Bremen) mass spectrometry was used.

[b] Trace amounts of di-substituted cyclen (*tert*-butyl 2,2′-(1,4,7,10-tetraazacyclododecane-1,4-diyl)diacetate) could not be removed by column chromatography. Therefore 10 mg (25 μmol) of **2** was purified by HPLC (Column: Chromolith® Performance RP-18e 100 × 4.6 (Merck Darmstadt, Germany), gradient eluent: 100% H_2O + 0.1% TFA after 5 min 100% acetonitrile + 0.1% TFA; flow: 4.0 mL/min; retention time: t_R (**2**) = 1.38 min) to obtain **2** as a TFA salt (**2** + 3*x*TFA).

[c] Compound **3** was obtained as a TFA salt (**3** + 3*x*TFA + H_2O).

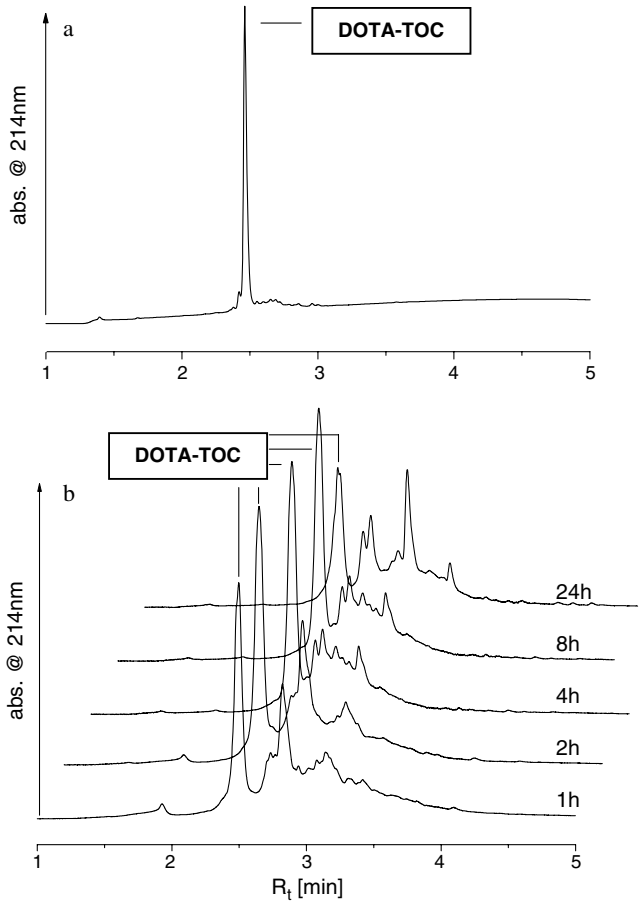

Figure 2. (a) Typical HPLC-chromatogram of crude DOTA–TOC synthesized with tris-allyl-DOTA after deprotection of the allyl moieties and a cleavage time of 90 min applying TFA/TIS/H_2O 95:2.5:2.5 (b) Typical HPLC-chromatograms of crude DOTA–TOC synthesized with tris-*t*-Bu-DOTA after a cleavage time of 1 h, 2 h, 4 h, 8 h and 24 h.[22]

In conclusion, we synthesized tris-allyl-DOTA for the synthesis of DOTA conjugated peptides such as DOTA–TATE, DOTA–TOC, and a DOTA–RGD peptide offering an alternative and reliable synthesis protocol. In direct comparison to the usually applied tris-*t*-Bu-DOTA, the allyl protecting groups of the solid phase bound DOTA–peptide conjugates could be removed quantitatively under mild conditions. Tris-allyl-DOTA is a valuable new synthon for the improved synthesis of DOTA–peptide conjugates resulting in significantly higher yields.

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14. Cyclen (3.0 g, 17.4 mmol) was dissolved in $CHCl_3$ (20 mL) for 30 min and *t*-butylbromoacetate (1.7 g, 8.7 mmol) was added dropwise within 1 h. The solution was stirred for additional 60 min, the solvent was removed under vacuo at ambient temperature and the crude product purified by column chromatography (8:9:4, $CHCl_3$/EtOH/NH_4OH). The solvent of the fractions containing product ($R_f = 0.7$) was removed in vacuo at rt to yield **2** (2.2 g, 8.2 mmol) in 94% yield.
15. Column: Chromolith® Performance RP-18e 100×4.6 (Merck Darmstadt, Germany), gradient eluent: 100% H_2O + 0.1% TFA after 5 min 100% acetonitrile + 0.1% TFA; flow: 4.0 mL/min; retention time: R_t (Intermediate) = 3.22 min, $m/z = 580.5$ $[M]^+$ (calcd 580.4).
16. To a suspension of **2** (3.1 g, 11 mmol) and K_2CO_3 (6.4 g, 47 mmol) in CH_3CN (60 mL) was added allylchloroacetate (6.0 g, 44 mmoL) in CH_3CN (15 mL) within 30 min and stirred at rt for 16 h. After removal of the solvent, the crude product was treated with TFA (10 mL) at 0 °C, allowed to warm to rt and stirred until **2** could not be detected by HPLC (ca. 24 h). After removing the solvent in vacuo, the residue was dissolved in EtOH, subjected to a silicagel column (ca. 500 g) and eluted (1) with EtOH

(1 L), (2) $CH_3CN + 0.1\%$ TFA (ca. 1.5 L), (3) CH_3CN + TFA 0.1%/$H_2O + 0.1\%$ TFA 1:1 to obtain **3** as a white solid (2.4 g, 4.6 mmol, 42%).

17. Fmoc-Thr-(*t*-Bu)-Wang resin (100–200 mesh) for DOTA–TATE; *O*-*t*-Butylthreoninol 2-chlorotrityl resin for DOTA–TOC (Merck Darmstadt, Germany).
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20. For DOTA–TATE and DOTA–TOC: Column: LiChrosorb® RPselectB 250 × 10 (Merck Darmstadt, Germany), gradient eluent: 100% $H_2O + 0.1\%$ TFA after 30 min 100% acetonitrile + 0.1% TFA; flow: 4.0 mL/min; retention time: t_R (DOTA–TATE) = 14.1 min and t_R (DOTA–TOC) = 13.3 min. DOTA–TATE and DOTA–TOC were white solids after lyophilization and the overall yield of the purified peptide was between 17–18%; for DOTA–RGD: Columns: 2 × Chromolith® SemiPrep RP-18e 100 × 10 (Merck Darmstadt, Germany), gradient eluent: 100% $H_2O + 0.1\%$ TFA after 15 min 100% acetonitrile + 0.1% TFA; flow: 8.0 mL/min; retention time: t_R (DOTA–RGD) = 6.55 min. DOTA–RGD was a white solid after lyophilization and the overall yield of the purified peptide was between 20–22%.
21. MALDI-TOF molecular weight determination was carried out using a Kratos Kompact MALDI-III apparatus: DOTA–TATE $m/z = 1434.6$ $[M]^+$ (calcd 1434.6); DOTA–TOC $m/z = 1420.6$ $[M]^+$ (calcd 1420.6); DOTA–RGD $m/z = 989.6$ $[M]^+$ (calcd 989.5).
22. Column: Chromolith® Performance RP-18e 100 × 4.6 (Merck Darmstadt, Germany), gradient eluent: 100% $H_2O + 0.1\%$ TFA after 5 min 100% acetonitrile + 0.1% TFA; flow: 4.0 mL/min; retention time: t_R (DOTA–TOC) = 2.46 min, $m/z = 1420.8$ $[M]^+$ (calcd 1420.6).