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An amine-derivatized, DOTA-loaded polymeric support for Fmoc solid phase peptide synthesis

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ABSTRACT

An amine-derivatized DOTA has been used to modify the surface of a polymeric support for conventional solid phase peptide synthesis (SPPS) following standard Fmoc chemistry methods. This methodology was used to synthesize a peptide–DOTA conjugate that was demonstrated to be a PARACEST MRI contrast agent. Therefore, this synthesis methodology can facilitate Fmoc SPPS of molecular imaging contrast agents.

Molecular imaging has recently emerged as a powerful method to provide medical information at the molecular level.[1] Molecular imaging contrast agents have been conjugated to many types of peptides that target cell receptors and enzymes, in order to diagnose pathological tissues and assess early therapeutic responses.[2–5] Macrocyclic metal chelates, such as metalated 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (DOTA) have been conjugated to peptides and detected with MRI, PET, and SPECT imaging.[6–8] As a testament to their potential, 23% (187 of 820) of the journal publications that describe molecular imaging contrast agents with metal chelates contain one or more peptidyl ligands. As a testament to their demonstrated utility for biomedical imaging, 8% (46 of 594) of the entries in the molecular imaging contrast agent database (MICAD) consist of metal chelates with one or more peptidyl ligands.[9]

The facile synthesis of metal chelate contrast agents that include peptides is needed to accelerate the research and clinical translation of molecular imaging. Previously reported methods have conjugated the carboxylates of DOTA to the amines of peptides, including the N-terminus, the side chain of lysine, and unnatural amino acid derivatives.[10] DOTA derivatives of succinimide and isothiocyanate have also been conjugated to peptide amino groups.[11,12] However, coupling DOTA only to peptide amines can limit synthesis methodologies.[13,14]

To resolve these limitations in the synthesis of peptidyl DOTA conjugates, we previously developed an amine-derivatized DOTA and used this product to couple DOTA to the C-terminus of a peptide using solution-phase methods.[15] We have also loaded an amine-derivatized DOTA onto a polymeric support, and used this product to couple DOTA to the peptide C-terminus and within the peptide backbone using standard solid phase peptide synthesis (SPPS) methods.[16] These synthetic pathways used a CBZ group to protect the α-amino group of the DOTA derivative. Cleavage of the CBZ group required harsh conditions using diethylaluminum chloride/thioanisole at −78°.[17] These harsh conditions also cleaved the DOTA from the resin, which caused a lower concentration of DOTA on the polymeric support and consequently reduced the final yield of peptide–DOTA conjugates.

To address these limitations, we have converted the CBZ protecting group of an amine-derivatized DOTA to a Fmoc protecting group before loading DOTA onto the resin. This report describes the new synthetic pathway to prepare an Fmoc-protected, amine-derivatized, DOTA-loaded polymeric support that is applicable to conventional Fmoc-SPPS. This product was used to synthesize a peptide–DOTA conjugate with high yield and high purity. Finally, this peptide–DOTA contrast agent was shown to have utility as a PARACEST MRI contrast agent.

CBZ-Gly(Br)-OMe (methyl 2-(benzyloxycarbonylamino)-2-bromoacetate) was prepared with overall yield of 70% following a previously reported synthetic pathway.[18,19] Compound **2** was synthesized from **1** with *tert*-butyl bromoacetate in overall yield

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of 83%.[20,21] Compound **3** was synthesized from CBZ-Gly(Br)-OMe and **2** through exhaustive alkylation in the presence of K_2CO_3, yielding a pale yellowish solid (83%).[22] Subsequently, compound **4** was obtained quantitatively by the deprotection of the CBZ group from compound **3** through hydrogenolysis using 10%-Pd/C.[23] The hydrogenolysis reaction continued for 4 h until there was no change in H_2 pressure, yielding product quantitatively as a pale yellowish solid. Compound **5** was prepared from compound **4** by use of Fmoc-Cl with zinc dust.[24] In this step, a small portion of activated zinc dust was added until the reaction mixture attained neutral pH and the mixture of Fmoc-Cl and zinc dust was added to the reaction solution in one portion. The reaction time was 20 min. Unreacted Fulvene was washed with diethyl ether and the product was dried to yield a white solid (87%).[25] To prevent the formation of a zinc chelate during this step, zinc ions were removed from the zinc dust by thoroughly washing the dust with sequential treatments of distilled water, EtOH, acetone, and MC. Furthermore, the preparation of compound **5** was carried out in acetonitrile, which minimized dissolution of zinc ions from zinc dust that is insoluble in acetonitrile. The mass spectrum showed no evidence that a zinc chelate was formed during this step. Compound **6** was obtained from compound **5** (4.1 g, 5 mmol) by the deprotection of *tert*-butyl ester using 50% TFA in MC for 2 h, yielding a pale yellowish product quantitatively.[26]

Compounds **3**–**6** have a geminal amine structure after coupling CBZ-Gly(Br)-OMe to **2** in Step b (Fig. 1 and Scheme 1). To confirm the successful synthesis of compounds **3–6,** the chemical shifts of the α-proton and α-carbon, Hα and Cα, were tracked for each synthetic step. From 1D ^{1}H and ^{13}C NMR spectra, the peaks from Hα and Cα were assigned to resonances at 5.53 ppm and 92.59 ppm for compound **3**, 4.23 ppm and 85.12 ppm for compound **4**, 5.25 ppm and 84.83 ppm for compound **5,** and 5.27 ppm, 84.56 ppm for compound **6**, respectively. The ^{1}H NMR spectra of Hα are shown in Figure 2.

Two equivalents of compound **6** (1.86 mmol) relative to the hydroxyl group content on the resin were used to modify the surface of a Wang resin (0.93 mmol/g) with HBTU (4 equiv)/HOBt (1 equiv)/DIEA (10 equiv) for 1 day. After washing the resin with NMP, the resin was treated with 50% *tert*-butanol in MC for 4 h.[27,28] To cap un-reacted hydroxyl groups on the resin, the resin was dispersed in MC and treated with acetic anhydride (Ac_2O). The concentration of the amino group was quantified as 0.41 mmol/g using a Fmoc titration method[29] and 0.44 mmol/g using picric acid titration[30] after correcting for the effect of tertiary amines on DOTA from the results of Fmoc-NH-DOTA resin. These quantifications of resin loading were based on the 1.61 g weight of the product **7**, and not the 1.00 g weight of the initial Wang resin, following the convention in SPPS methodology. Compared with the initial concentration of functional group of Wang resin, the loading efficiency was 71–76%.

To demonstrate the synthesis of a peptide–DOTA conjugate, a Wang resin **7** was then used to synthesize a peptide, CBZ-Gly-Gly-Arg (ZGGR). This peptide is a substrate for urokinase-type plasminogenequivvator (uPA), and the ability of the peptide–DOTA conjugate to detect uPA is the focus of ongoing research studies in our laboratory.[31] Compound **7** was treated with Fmoc-Arg(pbf)-OH/HBTU/HOBt/DIEA for 1 h and washed with NMP. The same reaction was conducted a second time to double couple the arginine amino acid to **7**. The Fmoc group was deprotected with 20% piperidine in NMP for 40 min. Following the same procedures, Fmoc-Gly and CBZ-Gly were double coupled onto the resin. The peptide–DOTA conjugate was cleaved with 95% TFA in MC. The final product was precipitated in diethyl ether and dried in vacuo, yielding white solids (88% as determined by weight, relative to the original concentration of the amine on **7**). The identity of the peptide–DOTA conjugate was confirmed with mass spectrometry.

To chelate a lanthanide ion with this product, Z-Gly-Gly-Arg-DOTA was first dissolved in acetonitrile. The solution was filtered with a 0.2 μm PTFE membrane filter to remove undissolved solids such as TFA salts. Tm-triflate was added to the solution and the solution was stirred at 50 °C for 12 h. The pH was monitored for 4 h and DIEA was added as an acid scavenger to maintain a neutral or weakly basic condition when a decrease in pH was detected. The reaction mixture was concentrated and re-dissolved in water at pH 9 to remove excess Tm(III) that formed a $Tm(OH)_3$ precipitate. The pH was adjusted to 7 and the solution was lyophilized to yield **8** as a white fluffy powder.

The utility of the peptide–DOTA chelate for molecular imaging studies of uPA was investigated by measuring the agent's PARA-

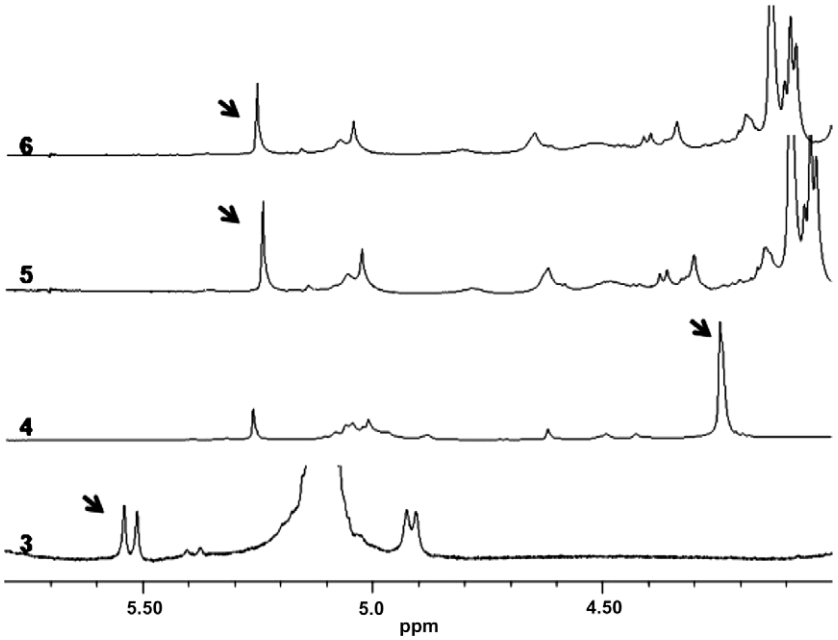
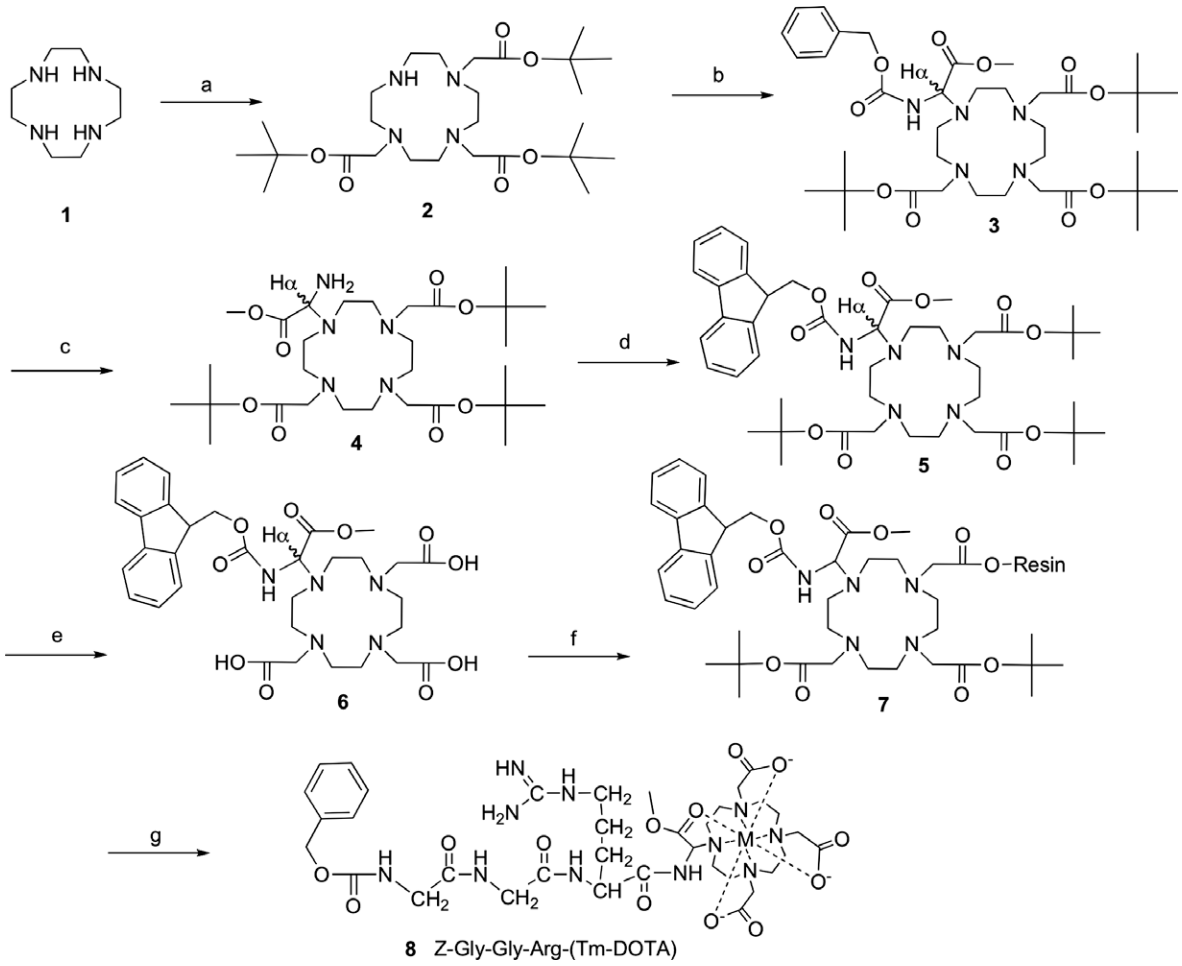


Figure 1. NMR spectra of Hα in compounds **3**–**6**. The peak from Hα is marked with an arrow.



Scheme 1. Synthesis of α-amino-DOTA and a peptide–DOTA PARACEST MRI contrast agent. (a) *tert*-butylbromoacetate, K_2CO_3 (6 equiv), acetonitrile, 70 °C, 6 h, 83%; (b) CBZ-Gly(Br)-OMe, K_2CO_3 (6 equiv), acetonitrile, 70 °C, 6 h, 83%; (c) H_2, 10-Pd/C, ethanol; 2 h, quantitative; (d) Fmoc-Cl, zinc dust, acetonitrile, 87%; (e) 50% TFA in CH_2Cl_2, quantitative; (f) (i) Wang resin, HBTU (4 equiv), HOBt (1 equiv), DIEA (10 equiv), NMP, rt, overnight; (ii) 50% *tert*-BuOH in CH_2Cl_2, 70%; (g) (i) 20% piperidine, NMP; (ii) SPPS of Z-Gly-Gly-Arg(pbf) following standard Fmoc-chemistry (iii) 95% TFA, CH_2Cl_2, 4 h; (iv) Tm-triflate, acetonitrile, 50 °C, overnight.

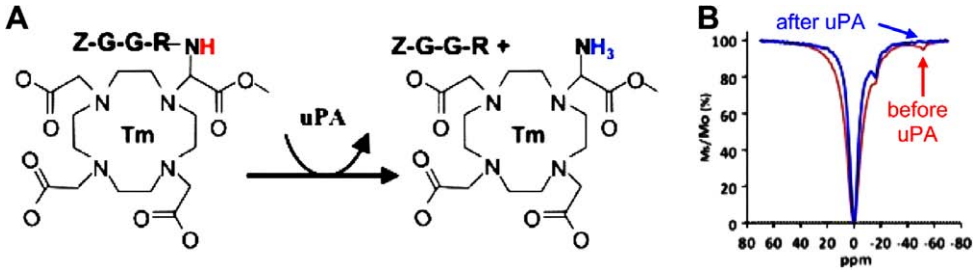


Figure 2. Detection of urokinase Plasminogen Activator (uPA) with **8**. (A) The proposed mechanism of uPA cleavage of **8** and the PARACEST spectra of **8** shows that an amide proton (red) is converted to an amine (blue) after uPA cleaves the ZGGR peptide ligand from the agent. (B) The CEST spectrum showed a CEST effect from the amide at −52 ppm before uPA was added (red). The disappearance of this CEST effect after uPA was added (blue) was used to detect uPA. An enzyme-unresponsive agent, Yb-DOTA-Gly, shows a CEST effect at −16 ppm before and after uPA was added, which served as an internal control.

magnetic chemical exchange saturation transfer (PARACEST) effect. A sample was prepared that consisted of 50 mM of **8** in HEPES buffer at pH 7.2 and with 5% D_2O, and was incubated at 37 °C overnight with 100 U of uPA enzyme (EMD Biosciences Inc.) (Figure 2). A PARACEST agent that is unresponsive to uPA, Yb-DOTA-Gly_4 was also included in the sample at 20 mM concentration as a control. Two CEST spectra were obtained before and after uPA incubation by acquiring a series of 1D NMR spectra with a 300 MHz Varian NMR spectrometer at 37 °C and with frequency-selective presaturation applied in 1 ppm increments between 70 ppm and −70 ppm. This selective presaturation was performed with a continuous wave pulse applied for 4 s at 21 μT. Before incubation with uPA, the CEST spectrum showed PARACEST effects at −16 ppm and −52 ppm relative to water (which was referenced to 0 ppm to follow the convention used for MRI studies).[32] The PARACEST effect at −16 ppm was assigned to Yb-DOTA-Gly_4 and the PARACEST effect at −52 ppm was assigned to **8** based on previous reports of the PARACEST effects of Yb and Tm chelates of a peptide-(Tm-DOTA) conjugate and Yb-DOTA-Gly_4.[33,34] The PARACEST effect at −52 ppm disappeared after incubation with uPA, while the PARACEST effect at −16 ppm was unchanged. Thus, **8** can be detected using PARACEST MRI and can be used to detect uPA. However,

additional kinetics studies are warranted to prove that this change in the PARACEST effect is due to the catalysis mechanism of the uPA enzyme.

In conclusion, a new synthetic method has been demonstrated that prepares an Fmoc-protected, amine-derivatized, DOTA-loaded polymeric support for standard Fmoc SPPS. This polymeric support can be used to conjugate DOTA to the C-terminus of a peptide with high yield and high purity using conventional Fmoc-SPPS. Considering that a fully compatible Fmoc-SPPS method can facilitate scale-up of a synthesis methodology, this method may be more practical for commercial applications. Furthermore, Fmoc SPPS is applicable to building peptide or chemical libraries, which may further facilitate the development of diagnostic molecular imaging contrast agents.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2009.05.061.

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22. Compound **3**: *tert*-butyl 2,2′,2″-(10-(1-(benzyloxycarbonylamino)-2-methoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (CBZ-NH-DOTA-*t*Bu); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.35 (s, 27H), 2.75 (t, 16H), 3.2 (s, 6H), 3.50 (s, 3H), 5.00 (s, 2H), 5.35 (d, 1H), 7.13 (m, 5H), 6.40 (d, 1H); ^{13}C (125 MHz, DMSO-d_6) δ 172.38, 170.19, 169.35, 156.68, 136.21, 128.08, 127.56, 126.45, 92.59, 81.15, 66.49, 69.72, 57.37, 55.19, 52.81, 52.24, 51.11, 50.77, 49.94, 47.70, 27.65; MS-ESI *m/z* : 758.43 $[M+Na]^+$ (calcd. $C_{37}H_{61}N_5O_{10}$ 735.91).
23. Compound **4**: *tert*-butyl 2,2′,2″-(10-(1-amino-2-methoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (NH_2-DOTA-*t*Bu); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.37 (s, 27H), 2.75 (t, 16H), 3.30 (s, 6H), 3.55 (s, 3H), 4.55 (s, 1H); ^{13}C (125 MHz, DMSO-d_6) δ 170.24, 169.39, 163.21, 85.12, 81.98, 81.49, 57.86, 56.36, 55.44, 54.83, 52.72, 51.10, 49.03, 28.05, 27.92; MS-ESI *m/z* : 602.43 $[M+H]^+$ (calcd. $C_{29}H_{55}N_5O_8$ 601.41).
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26. Compound **6**: 2,2′,2″-(10-(1-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)-2-methoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (Fmoc-NH-DOTA); ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.85 (t, 16H), 3.55 (s, 6H), 3.90 (s, 3H), 4.15 (s, 2H), 4.4 (d, 1H), 5.35 (s, 1H), 7.5 (m, 8H), ^{13}C (125 MHz, $CDCl_3$) δ 172.38, 170.19, 169.45, 169.35, 169.32, 156.68, 136.21, 128.08, 127.73, 84.56, 81.58, 80.99, 66.49, 61.71, 57.37, 55.19, 52.81, 48.94, 47.70, 46.70, 27.65, 17.94, ESI-Mass *m/z*: 677.23 $[M+Na]^+$ (calcd for $C_{32}H_{41}N_5O_{10}$ 655.29).
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