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Total Solid-Phase Synthesis of NOTA-Functionalized Peptides for PET Imaging

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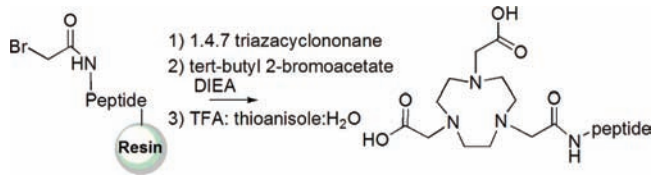
ABSTRACT



A convenient approach to functionalize peptides either at the *N*-terminal or on a lysine side chain with 1,4,7-triazacyclononane-*N*,*N*′,*N*″-triacetic acid (NOTA) chelating unit has been developed on solid support. The chelate was assembled in a two-step process starting with bromo-acetylated peptides. Deprotection and cleavage of the resin-bound NOTA peptides were performed with use of trifluoroacetic acid (TFA) in the presence of thioanisole and water to give free NOTA peptides.

Several bifunctional chelates (BFCs) have been investigated as carriers of radiometals for Positron Emission Tomography (PET) imaging. 1,4,7,10-Tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tretraacetic acid (DOTA) is the most widely used macrocycle in radiopharmaceutical research and development as a chelator for metal radioisotopes.[1] However, DOTA is not optimal for radiolabeling some radiometals such as Cu-64 due to the moderate stability of the complex *in vivo*, resulting in demetalation and subsequent accumulation of the radiometal in nontarget tissues. Cross-bridged cyclam-based chelates[2] and SarAr[3] offer improved kinetic stability to *in vivo transmetalation in comparison with DOTA. Nevertheless, chelates of this type are difficult to prepare and a large excess of these chelating units is often required for their attachment to peptides*,[3] limiting their general use. Previous studies have described the potential use of NOTA as BFC for divalent and trivalent metal ions. When coupled to peptides, this chelate may present higher resistance to transmetalation reactions *in vivo* as compared with other larger polyaminocarboxylate chelators like DOTA.[4] However, a large excess of NOTA is required for its conjugation to purified peptides.[4] For these reasons, there is a growing demand for chelate derivatives properly functionalized to facilitate their attachment to peptides, ideally during peptide synthesis.

In the current study, we report a convenient synthetic approach for the preparation of NOTA peptides directly on solid support. The NOTA unit was selectively introduced

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(1) De Leon-Rodriguez, L. M.; Kovacs, Z. *Bioconjugate Chem.* **2008**, *19*, 391–402.
(2) (a) Sprague, J. E.; Peng, Y.; Sun, X.; Weisman, G. R.; Wong, E. H.; Achilefu, S.; Anderson, C. J. *Clin. Cancer Res.* **2004**, *10*, 8674–8682. (b) Sun, X.; Wuest, M.; Weisman, G. R.; Wong, E. H.; Reed, D. P.; Boswell, C. A.; Motekaitis, R.; Martell, A. E.; Welch, M. J.; Anderson, C. J. *J. Med. Chem.* **2002**, *45*, 469–477.
(3) Di Bartolo, N.; Sargeson, A. M.; Smith, S. V. *Org. Biomol. Chem.* **2006**, *4*, 3350–3357.
(4) Prasanphanich, A. F.; Nanda, P. K.; Rold, T. L.; Ma, L.; Lewis, M. R.; Garrison, J. C.; Hoffman, T. J.; Sieckman, G. L.; Figueroa, S. D.; Smith, C. J. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 12462–12467.

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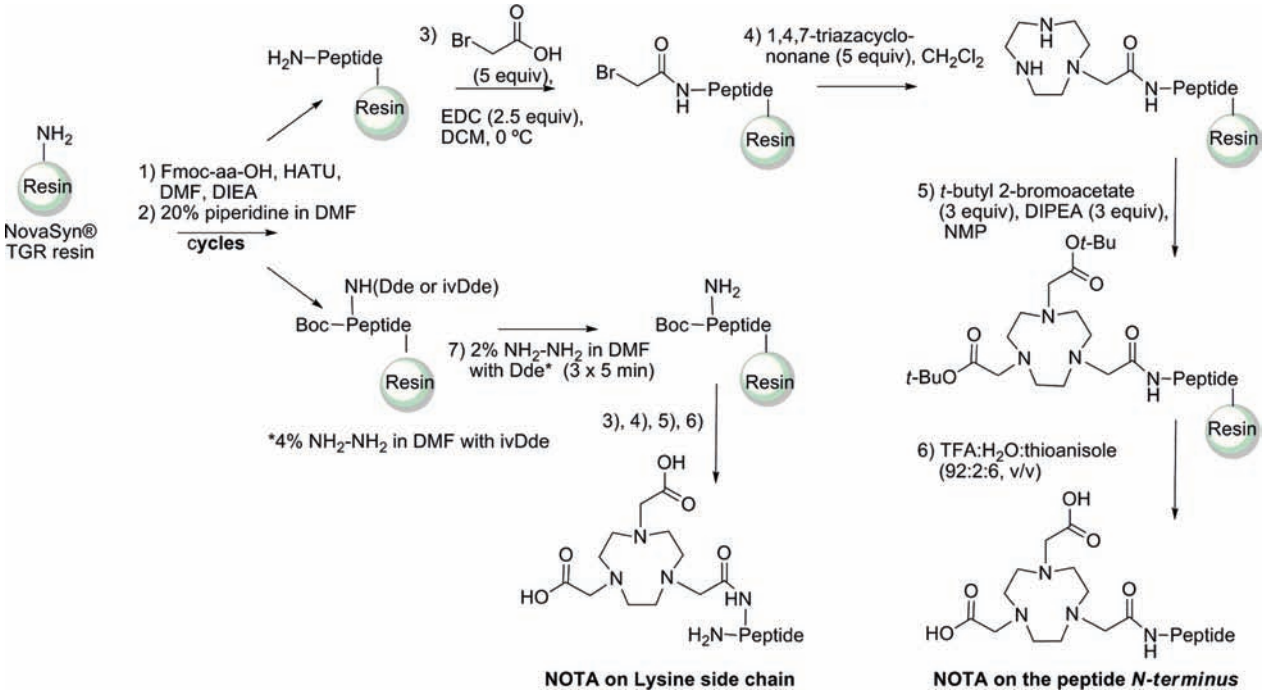
Scheme 1. Synthesis of NOTA Peptides



either at the *N*-terminal position or on a lysine side chain. In the later approach, *N*-terminal BOC-amino acid and 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde)[5] or 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde)[6] protected lysine are essential to ensure the effectiveness of the orthogonal NOTA functionalization (Scheme 1). This paper also described in detail the synthesis and characterization of three NOTA conjugate peptides, NOTA-Aoc-BBN(6-14), (NOTA)Lys^{4}BVD15, and (NOTA-Ahx)Lys^{4}BVD15 (Figure 1), that respectively bind with high affinity to gastrin releasing peptide receptors (GRPR) and neuropeptide Y Y1 receptors (NPY1R) frequently overexpressed on human breast and prostate cancer cells.[7] A comparison between DOTA and NOTA peptide series is also presented, DOTA peptides being prepared with commercially available tris-*tert*-butyl-DOTA or Fmoc-(tris-*tert*-butyl-DOTA)lysine (Macrocyclics, Dallas, TX, USA). Peptides were synthesized by a continuous flow method on a Pioneer Peptide Synthesis System (PerSeptive Biosystems) with the Fmoc (fluorenylmethyloxycarbonyl) strategy (Scheme 1).

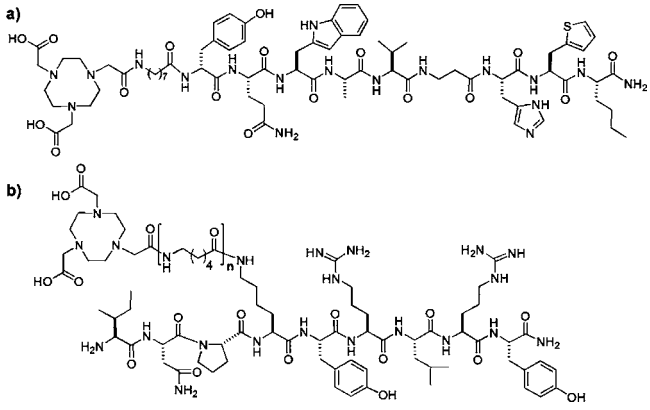


Figure 1. Structures of (a) NOTA-Aoc-BBN(6-14) and (b) (NOTA)-Lys^{4}BVD15 ($n = 0$) or (NOTA-Ahx)Lys^{4}BVD15 ($n = 1$).

Using NovaSyn TGR resin, a 2-fold excess of Fmoc-protected amino acids over the resin substitution rate was utilized for coupling. Fmoc-protected amino acids were activated for coupling with an equimolar amount of HATU (2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and 2-fold excess of DIEA (diisopropylethylamine). Fmoc deprotection was performed in 20% piperidine in DMF. After peptide assembly, the NOTA moiety was synthesized manually on the solid phase under mechanic agitation. Reaction conditions presented herein have been optimized by monitoring each step (2 to 7, Scheme 1) on analytical reversed-phase HPLC after cleavage of a small amount (10 mg) of the resin-bound peptide with 95% aqueous TFA. Functionalization of the amino terminus of the peptide resin was provided through the use of the appropriate α-bromocarboxylic acid activated as the sym-

(5) Bycroft, B. W.; Cahn, W. C.; Chhabra, S. R.; Hone, N. D. *J. Chem. Soc., Chem. Commun.* **1993**, 778–779.

(6) Chhabra, S. R.; Hothi, B.; Evans, D. J.; White, P. D.; Bycroft, B. W.; Chan, W. C. *Tetrahedron Lett.* **1998**, *39*, 1603–1606.

(7) (a) Markwalder, R.; Reubi, J. C. *Cancer Res.* **1999**, *59*, 1152–1159. (b) Gugger, M.; Reubi, J. C. *Am. J. Pathol.* **1999**, *155*, 2067–2076. (c) Ruscica, M.; Dozio, E.; Boghossian, S.; Bovo, G.; Martos Riaño, V.; Motta, M.; Magni, P. *Endocrinology* **2006**, *147*, 1466–1473. (d) Reubi, J. C.; Gugger, M.; Waser, B. *Eur. J. Nucl. Med.* **2002**, *29*, 855–862.

metric anhydride.[8] The bromide was then displaced by the amine function of the 1,4,7-triazacyclononane (5 equiv) in anhydrous CH_2Cl_2 following the method of Meares.[9] Mechanical agitation was maintained for 3 h at room temperature to ensure complete consumption of the starting material. The cyclen peptide was then alkylated with *tert*-butyl 2-bromoacetate (3 equiv) in the presence of DIPEA in dry NMP for 2 h. During the cyclen alkylation step, a considerable amount of overalkylation (i.e., three groups instead of two) was observed with longer reaction time and/or with more than 3 equiv of *tert*-butyl 2-bromoacetate.

For the selective NOTA functionalization on the lysine side chain, we used an *N*-terminal Boc/(Dde)Lys protecting group strategy since Fmoc is readily deprotected under conditions used for Dde cleavage. After coupling of the Boc-amino acid at the *N*-terminus, the Dde group was cleaved according to the standard protocole of 2% hydrazine hydrate in DMF.[5] However, the best results in terms of selectivity were achieved with use of the ivDde group, a Dde hindered variant for orthogonal protection of lysine. Compared to Dde this protecting group showed not only complete stability toward 20% piperidine in DMF but also considerable hindrance of $N \rightarrow N'$-migration.[6] Cleavage of the ivDde group required an increase in hydrazine concentration to 4% (v/v) and a longer reaction time (3 × 5 min). Once orthogonal deprotection of lysine was achieved, (NOTA)Lys peptide was prepared following bromoacetylation, cyclen introduction, and alkylation with *tert*-butyl 2-bromoacetate procedures described above (Scheme 1). Finally, peptides were deprotected and cleaved from the polymer support by treatment with a cocktail of TFA:H_2O:thioanisole (92:2:6) for 3 h. The resin was removed by filtration and washed with TFA. Combined filtrates were added dropwise to ethyl ether. The precipitated crude peptides were centrifuged, and the ether solution was decanted. Crude peptides were purified by flash chromatography on a Biotage SP4 system, using a C18 cartridge. Purity of the peptides was verified by analytical reversed-phase HPLC. Peptide identity was confirmed by MALDI-TOF MS or API 3000 LC/MS/MS.

Figure 2 displays the HPLC chromatograms of crude DOTA and NOTA peptide amides. Comparable crude yields were obtained with the commercially available DOTA chelate and the NOTA chelate directly assembled on the solid phase, showing the efficacy of our synthetic approach. All peptides were prepared with overall yields of 11−36% based on the substitution rate of the resin, determined photometrically from the amount of Fmoc chromophore released upon treatment of the resin with piperidine/DMF. According to analytical HPLC, the purity was 93% for DOTA peptides and greater than 95% for the NOTA series. Characterizations of the peptides are summarized in Table 1.

Peptide derivatives were also tested for receptor binding affinity on different cell lines overexpressing GRPR (PC3 human prostate carcinoma) or NPY1R (MCF-7 human breast carcinoma). As the DOTA chelator is commercially available,

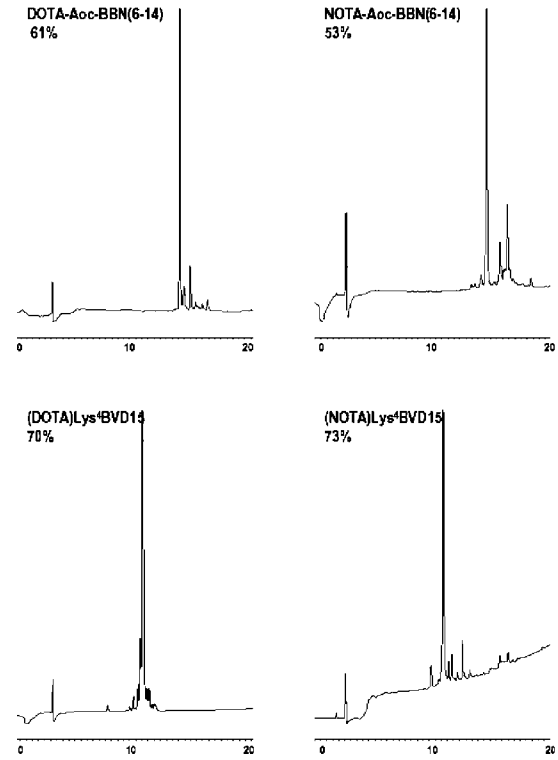


Figure 2. HPLC chromatograms of crude DOTA and NOTA peptide amides on an analytical C_{18} column.

it was first selected to optimize the design of our peptides. Both DOTA-Aoc-BBN(6-14) and (DOTA)Lys^{4}BVD15[10] peptides displayed good binding affinities to GRPR and NPY1R (Table 1), respectively. Preliminary results indicate

Table 1. Analytical Data for DOTA and NOTA Peptides

peptide	mass calcd	mass found[a]	yield (%)	purity (%)[b]	K_i (nM)
DOTA-Aoc-BBN(6-14)	1667	1668	11	93	23 ± 7[c]
NOTA-Aoc-BBN(6-14)	1566	1567	16	93	2.2 ± 1.4[c]
(DOTA)Lys^{4}BVD15	1608	1608	12	98	63 ± 25[d]
(NOTA)Lys^{4}BVD15	1507	1508	30	97	23 ± 13[d]
(NOTA-Ahx)Lys^{4}BVD15	1620	1621	36	95	5.6 ± 4.4[d]

[a] Masses were measured via MALDI mass spectrometry with Micromass Tof Spec 2F. [b] Purity was determined by HPLC analysis. [c] Affinities for GRPR were determined with [^{125}I-Tyr^4]Bombesin in PC3 cells. [d] Affinities for Y1 receptors were determined with ^{125}I-NPY in MCF-7 cells.

that all NOTA peptides had inhibition constant (K_i) values slighly lower than their DOTA peptide counterparts. This suggests that the introduction of a smaller chelating unit at the *N*-terminus of the peptide or on a lysine side chain

(8) Robey, F. A.; Fields, R. L. *Anal. Biochem.* **1989**, *177*, 373–377.
(9) Peterson, J. J.; Pak, R. H.; Meares, C. F. *Bioconjugate Chem.* **1999**, *10*, 316–320.
(10) Guérin, B.; Dumulon-Perreault, V.; Tremblay, M.-C.; Ait-Mohand, S.; Fournier, P.; Dubuc, C.; Authier, S.; Bénard, F. [(DOTA)Lys^4]BVD15, a Novel and Potent Neuropeptide Y Analog Designed for Y1 Receptor-Targeted Breast Tumor Imaging. In *Bioorg. Med. Chem. Lett.* Accepted for publication.

allowed the BBN or BVD15 peptide to interact more efficiently with their respective receptors. Increasing the distance between the NOTA and the peptide backbone contributed to improve the NPY1R binding affinity. Indeed, the K_i value of the (NOTA-Ahx)Lys^{4}BVD15 was more than four times lower than the (NOTA)Lys^{4}BVD15 when tested in the MCF-7 human breast cancer cell line.

In conclusion, we have described the preparation and the *in vitro* activity of three NOTA conjugate peptides, NOTA-Aoc-BBN(6-14), (NOTA)Lys^{4}BVD15, and (NOTA-Ahx)-Lys^{4}BVD15 (Figure 1). NOTA peptides are readily synthesized and purified on the milligram scale. This successful synthetic strategy can be applied to the synthesis of various peptide conjugates. Our *in vitro* results indicate that NOTA-Aoc-BBN(6-14) and (NOTA)Lys^{4}BVD15 derivatives are potent GRP and NPY analogues. Further *in vitro*/*in vivo* experiments with ^{64}Cu and ^{68}Ga for the evaluation of the labeled NOTA peptides as potential breast and/or prostate cancer PET imaging agents are warranted.

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Supporting Information Available: Experimental procedures, characterization data, competition assay procedure, and K_i determination for DOTA and NOTA peptide derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

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