Chemistry—Methods

Research Article
doi.org/10.1002/cmtd.202400053

Chemistry Europe
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An Efficient Solid-Phase Synthetic Approach to Prepare TACN-Functionalized Peptides

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1,4,7-triazacyclononane (TACN) derivatives play important roles in various metal-based biomedical applications. However, the unmanageable functionalization of TACN remains a long-standing challenge to yield useful partially substituted building blocks. Herein, by utilizing nitrobenzoxadiazole (NBD) as a thiol-liable protecting group for secondary amines, a *bis*-NBD-substituted TACN was obtained as the first example in the preparation of partially substituted TACN without strict stoichiometric control and column chromatography. Upon facile deprotection of NBD using solid-phase synthesis, a series of TACN-peptide conjugates with different bioactive peptides and chelating units were derivatized from the TACN building block, demonstrating the potential widespread application of this work.

Introduction

1,4,7-Triazacyclononane (TACN) and its derivatives have attracted significant research interest because of their versatile coordination property towards a wide span of metal ions for diagnostic and therapeutic applications. TACN-based chelators with carboxylic or phosphate substituents (e.g., NOTA, NOTP, TRAP) equipped with positron-emitting radionuclides are commonly utilized in positron emission tomography (PET) for the diagnosis of various diseases, including cancers.[1–4] Compared to other radionuclides that are prepared from costly cyclotrons, Gallium-68 (^{68}Ga) can be easily obtained from a ^{68}Ge/^{68}Ga generator, which advocates the study and application of ^{68}Ga in both research and clinical use.[5,6] TACN-based chelators are known to have better coordination kinetics than cyclen (1,4,7,10-tetraazacyclododecane) counterparts although the latter ones have been adopted in the FDA-approved PET agent ^{68}Ga-DOTATOC.[7,8] Other than ^{68}Ga, TACN-based chelators were also considered ideal for ^{64}Cu PET[9,10] and Al^{18}F PET.[11,12] As Fe(III)-based magnetic resonance imaging (MRI) contrast agents emerge, TACN-based chelators with hydroxyl group and other chelating units also gain more attention with their excellent coordination towards Fe(III) ion.[13,14] Through adjusting the substituents on TACN, different metals can be accommodated to support variable applications which hence have led to thriving investigations on the effective synthesis of variously substituted TACN-based complexes. Researchers have applied TACN-derivatives on different biomolecules (e.g., antibodies, affibodies, peptides, etc.) using respective building blocks with functional groups like maleimide,[15] isocyanine,[16] tetrazine,[17] and dibenzocyclooctyne (DBCO)[18] for site-specific conjugations.

Despite their relatively favourable coordinating conditions, the synthesis of partially substituted TACN building blocks is far more difficult compared to that of cyclen. For example, the *tris*-substituted cyclen building block, 1,4,7,10-tetraazacyclododecane-1,4,7-*tris*(*tert*-butyl acetate) (*tris*-^{t}Bu-DO3A), can be synthesized from cyclen and *tert*-butyl bromoacetate in high yields by a simple one-step reaction followed by extraction and precipitation,[19] while the TACN analogue 1,4,7-triazacyclononane-1,4-*bis*(*tert*-butyl acetate) (*bis*-^{t}Bu-NO2A) cannot be synthesized under similar conditions. The direct alkylation of TACN with haloacetate uncontrollably yields products with different degrees of substitutions which were difficult to isolate even by column chromatography. In most of the previous studies, carbamate-based protecting groups (e.g., Cbz, Boc) were usually deployed on TACN to occupy one or two nitrogen atoms in advance.[20,21] However, protection by carbamate-based protecting groups still gives products with different degrees of substitution uncontrollably.[21–23] Although the significant difference in polarity allows for chromatographic separation, these protecting groups show limited UV absorbance at 254 nm, hindering the identification of desired products in the process. In addition, the use of protecting groups lengthens the synthetic route of the desired compound. To the best of our knowledge, there is no report so far to yield partially substituted

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cmtd.202400053

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doi.org/10.1002/cmtd.202400053
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TACN without strict stoichiometric control and column chromatography.

To circumvent the above-mentioned difficulties of preparing partially substituted TACN, many studies have been conducted to give alternative synthetic approaches for TACN derivatives. Several previous works constructed substituted TACN *ab initio*, including the reduction of substituted 1,4,7-tribenzyl-1,4,7-triazonane-2,6-dione,[24,25] bicyclic ammonium salt,[26,27] or cyclic tripeptoids.[28,29] Nonetheless, it is tedious to reconstruct the polyamine ring and change the substituents to form various TACN-based complexes. On the other hand, Guérin et al. developed a total solid-phase approach that installed a bromoacetate to the peptide chain followed by adding excess unprotected TACN to conduct an S_N2 reaction.[30] Although this approach was conducted on peptides with long PEG-based spacer successfully without preparing functionalized TACN building blocks, certain degree of cross-link between peptide chains was observed in our trials (Figure S1–2), and thus cannot be regarded as a universal solution for the convenient synthesis of TACN-peptide conjugates.

As mentioned above, protecting groups are critical for the synthesis of partially protected building blocks. When it comes to solid-phase synthesis, Fmoc and Boc are the common examples to protect α-amines. Orthogonal amine protecting groups, like Dde/ivDde, Mtt/Mmt, and Alloc, offer another direction for modification as they can be selectively removed while keeping other protecting groups (e.g., Boc, Pbf) and the peptide on the solid support for further elaborations. These protecting groups are widely-applied in primary amines to enable the facile synthesis of branched peptides, multi-functionalized peptides, and cyclic peptides *via* solid-phase synthesis.[31,32] However, the report of orthogonal protecting groups for secondary amines is rather limited. Alloc is currently the only reported candidate for secondary amine protection on solid phase for the synthesis of peptidomimetics.[32–34] Nevertheless, deprotection of Alloc suffers from the drawbacks of difficult deprotection, requisite of inert atmosphere in Pd catalysis, gas generation during the reaction and the inability to visualize the completeness of deprotection. Regarding the disadvantages of Alloc and the surging demand for the synthesis of more complicated molecules with diverse functions, we would like to develop new orthogonal protecting groups for secondary amines (such as TACN) to offer more available choices.

Nitrobenzoxadiazoles (NBD) are a series of small fluorescent dyes with high sensitivity to the environment. NBD on primary amines is stable and thus it has been reported within dyes and sensors.[35–40] The covalent bonding between NBD and secondary amines is vulnerable to sulfide which gives violet NBD-SH.[41] Taking advantage of the bond-breaking mechanism between NBD and sulfides, which induced a dramatic change in absorbance and fluorescence upon the formation of NBD-SH, researchers exploited NBD as sensors for H_2S.[42–44] Based on our previous experience, NBD motifs have a large influence on the solubility of compounds in different solvents. Bridging the needs of developing new secondary amine protecting groups, solubility change upon NBD conjugation and the vivid reaction of NBD on secondary amines with sulfides, we considered it feasible to functionalize TACN with NBD as a protecting group for secondary amines. We presumed that TACN with different degrees of substitution can be separated by their large difference in polarity and solubility, while the distinctive absorption of NBD can help monitor the reaction. Moreover, the reaction between NBD and sulfides invoked us to use NBD as a protecting group, as in thiol labile protecting groups for cysteines[45] where the residue gives characteristic colour change during deprotection. Hence, we attempted to prepare NBD-substituted TACN building blocks for solid-phase synthesis.

Results and Discussion

To start, we stirred TACN (trihydrochloride, 1 equiv.) and NBD-Cl (2 equiv.) in H_2O/MeCN (v/v, 1/1) with DIPEA (5 equiv.) simply in one portion in room temperature (Figure 1). Surprisingly, the *bis*-NBD-TACN (**1**) was observed as the dominant product after 3 h reaction, while *mono*- and *tris*- substituted products were of neglectable amounts. The brick-red precipitate **1** merely dissolved in either dichloromethane (DCM) or the reaction mixture, which was purified easily by washing the crude product with DCM/hexane (v/v, 2/1) to remove the solvent, residual DIPEA and NBD-Cl. We then attempted to attach **1** to the peptide directly by an S_N2 reaction with pre-installed bromoacetate on the peptide chain at room temperature overnight but it failed. We therefore tried to install *tert*-butyl acetate to **1** in advance but there was still no reaction when the mixture with DIPEA in DMF added, was stirred at room temperature overnight. These two failures indicated that the last nitrogen atom on TACN became less reactive when the other two nitrogen atoms were substituted by NBD, which also explained the selective formation of *bis*-substituted product in the first step. This result echoes with the *ab initio* calculations on the TACN and compound **1**: the frontier highest occupying molecular orbital (HOMO) is spreading among the nitrogen atoms in TACN, but it is delocalizing in the electron-rich NBD

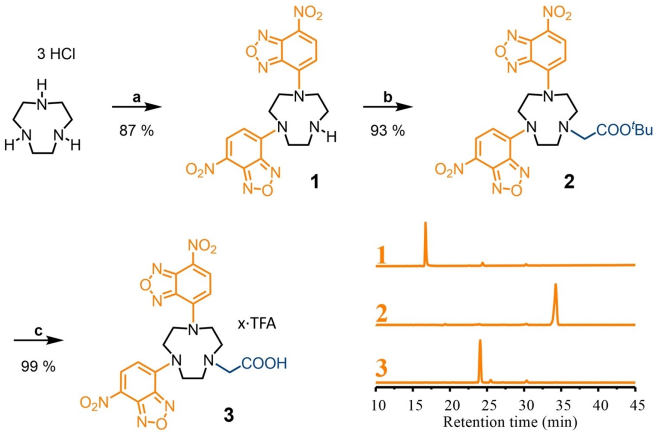

Figure 1. Synthesis of *bis*-NBD protected TACN building block. Reagents and condition: a) NBD-Cl, DIPEA, H_2O/MeCN, r.t., 3 h; b) *tert*-butyl bromoacetate, DIPEA, DMF, 120 °C, 3 h; c) TFA/DCM/TIPS, v/v/v, 50/50/2.5, r.t., 16 h.

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moiety in **1**, explaining the reduced reactivity of **1** after substituted by NBD. The delocalization on the third nitrogen atom of **4a** supports the effect of electron-withdrawing property of NBD-moiety affecting the substitution (Figure S7). We therefore heated the mixture to 120 °C to promote the installation of *tert*-butyl acetate, and complete reaction was achieved in 3 h. Interestingly, the desired product **2** showed good solubility in DCM due to the introduction of one more hydrophobic moiety; it was purified through liquid-liquid extraction followed by precipitation with hexane. The *tert*-butyl group of **2** was removed with 50% TFA and 2.5% TIPS in DCM (v/v) to obtain **3** as a brick-red powder by precipitating in diethyl ether. An 80% overall yield was achieved in the above three-step synthesis without any column chromatography involved, and the product of each step was well-characterized by ^{1}H NMR and ^{13}C NMR.

To prepare NOTA-peptide conjugates, **3** was coupled onto the YRGD peptide *via* its -COOH motif following routine coupling protocol quantitatively to give crimson resins. We then attempted to remove NBD with different sources of sulfides (Na_2S, NaHS, K_2S, ZnS); however, most of these sulfide salts had limited solubility in DMF, a preferred solvent in SPPS. Among the metal sulfides, sodium sulfide (Na_2S) and sodium hydrosulfide (NaHS) caught our attention as they dissolved gradually in DMF to give the respective sulfide solution for resin rinsing. Colourless Na_2S solution turned purple upon washing with the resins. The resin sample was cleaved to be analyzed by HPLC and ESI-MS to investigate the deprotection efficiency but only partial deprotection of NBD was observed in such condition. Then, we switched to NaHS which has better solubility. The yellow-green NaHS solution turned deep violet immediately when shaken with the crimson NBD-containing resin (Video S1). Interestingly, NBD can be removed vividly and completely by simply washing with NaHS. After several trials, we found that repetitive washing by 1% NaHS in DMF (w/v) could remove NBD from resin effectively. The resin was washed alternately with NaHS solution and DMF until the filtrate only gave the green colour of NaHS solution. The complete deprotection of NBD was also supported by HPLC and MS. After the removal of the NBD protecting group, the two amines on TACN were revealed for further elaborations with alkyl halides. We attempted to synthesize NOTA-peptide conjugates by functionalizing the amines with *tert*-butyl acetates. However, overalkylation was observed when *tert*-butyl bromoacetate was used for the S_N2 reaction which echoed with previous study.[30] This problem was resolved using *tert*-butyl chloroacetate, a less reactive analogue of *tert*-butyl bromoacetate. The alkylation by *tert*-butyl bromoacetate gave a mixture of products with different degrees of substitution, while the trial carried out with *tert*-butyl chloroacetate gave distinct peak of desired product **4a** (Figure S4). Therefore, we adopted alkyl chlorides in the derivatization of resin-bound TACN. These optimal protocols were established to give NOTA-YRGD conjugates (**4a**) with a 42% overall yield. Apart from haloalkanes, we also attempted to modify the TACN-peptide conjugate with propylene oxide to yield **4b**, which was also capable of metal chelation.

To prove the synthesized products are ideal precursors for ^{68}Ga PET agents, the coordination of non-radioactive Ga^{3+} with **4a** was investigated. The reaction was done by adding 1 mM $GaCl_3$ solution to a 25 μM solution of **4a** in acetate buffer (pH 3.6) at room temperature without stirring. After equilibrating for 10 minutes, the solution was analyzed by HPLC and ESI-MS. A single peak with shorter retention time, together with the m/z ratio of labelled compound, suggested that **4a** was completely converted to the desire product **4a-Ga** rapidly without heating (Figure 2b). To better understand the chelation

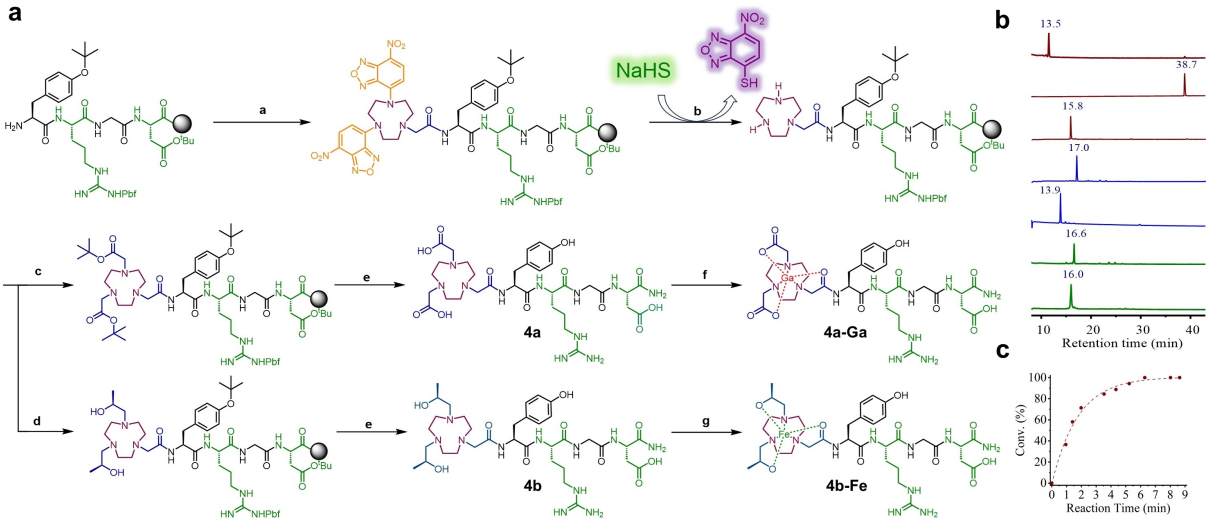

Figure 2. a. Synthesis of TACN-YRGD (**4a&b**) and the corresponding Ga complex (**4a-Ga**) and Fe complex (**4b-Fe**) using building block **3**. Reagents and condition: a) **3**, PyBOP, DIPEA, DMF, r.t., 3 h; b) NaHS/DMF (1% w/v); c) *tert*-butyl chloroacetate, DIPEA, DMF, r.t., 36 h; d) (S)-(−)-Propylene oxide, r.t., overnight; e) TFA/TIPS/H_2O, v/v/v, 95/2.5/2.5; r.t., 3 h; f) 1 mM $GaCl_3$, pH 3.6, 10 minutes; g) $FeCl_2$, 1 equiv., pH 3.6, 1 h. **b**. Step-by-step reaction monitoring of the synthesis. Top to bottom: Marron line: unmodified YRGD peptide, crude sample cleaved after step a, crude sample cleaved after step b; Blue line: post cleavage **4a** crude, **4a-Ga** reaction mixture; Green line: post cleavage **4b** crude, purified **4b-Fe. c**. Gallium chelation efficiency of 1 μM **4a** with 100 equiv. $GaCl_3$ solution in acetate buffer (pH 3.6).

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efficiency of **4a** with Ga^{3+}, the reaction was repeated by adding 100 equiv. of $GaCl_3$ to a 1 μM solution of **4a** in acetate buffer and monitored by the ratio between the peak area of **4a** and **4a-Ga** in HPLC chromatogram, **4a** was found to be fully labelled within 6.3 minutes, as summarized in Figure 2c. The result supported that the gallium labelling efficiency of **4a** is high and showed the potential of the synthesized NOTA-peptide conjugate as a promising precursor of targeting PET agents. Moreover, we illustrated the possibility of preparing Fe(III)-based MRI agents with *bis*-NBD-NO1A modified peptide conjugates. Stoichiometric amount of $FeCl_2$ was added to **4b** in acetate buffer to react for 1 h at room temperature. Analysis by HPLC and ESI-MS confirmed that **4b** had been completely converted to **4b-Fe** (Figure 2b) and its T_2 relaxivity had been measured (Figure S6). Both trials suggested that *bis*-NBD-NO1A is a versatile building block for the derivatization of TACN-peptide conjugates.

We further extended this synthetic approach to a series of resin-bound peptides with different bioactivities, which included nuclear localization sequence (NLS, lead to **5**), mitochondrial localization sequence (MLS, lead to **6**), HIV-1 Tat peptide (lead to **7**), SSTR-targeting peptide (derived from the FDA approved drug, octreotide, lead to **8**) and LMP1-targeting peptide (lead to **9**), as shown in Figure 3. The compatibility of NBD deprotection with other generic orthogonal protecting groups (e.g. Alloc and Dde) in solid-phase peptide synthesis was studied. We incorporated Fmoc-Lys(Alloc)-OH and Fmoc-Lys(Dde)-OH onto NLS peptide beforehand. Following the above synthetic approach, the product was found with NBD completely removed and substituted by an alkyl chloride antenna derived from carbostyril, while the Alloc and Dde groups were kept intact on the respective lysine side chains. Alternately, the compatibility of Alloc- & Dde-modified NLS peptide with **3** was examined under Alloc and Dde deprotection conditions. NBD groups on *bis*-NBD-NO1 A withstood the Alloc deprotection while it was partially removed in the Dde deprotection, which may be attributed to the nucleophilic attack of hydrazine on the NBD-NR_2 (summarized in Figure S5). The result proved that the propagation of our TACN building block, deprotection of NBD, and the installation of chelating arms had no influence on these orthogonal protecting groups, yet it should be noted that the deprotection of NBD should be arranged prior to Dde deprotection. The high versatility and convenience together with good compatibility ensure the further applications of *bis*-NBD-NO1A on other bioactive peptides to facilitate the development of targeting PET agents.

Conclusions

In this study, NBD was utilized as an easy-to-remove, orthogonal, monitorable protecting group for secondary amine in solid-phase synthesis, which provides an alternative for the diversification of secondary amine during SPPS. Also, *bis*-NBD protected TACN was synthesized as the first partially substituted building block without column chromatography and strict stoichiometric control. The synthesized TACN building block can be used to prepare TACN-peptide conjugates with a high degree of freedom in both peptides and TACN substituents, which paves the way for various biomedical applications.

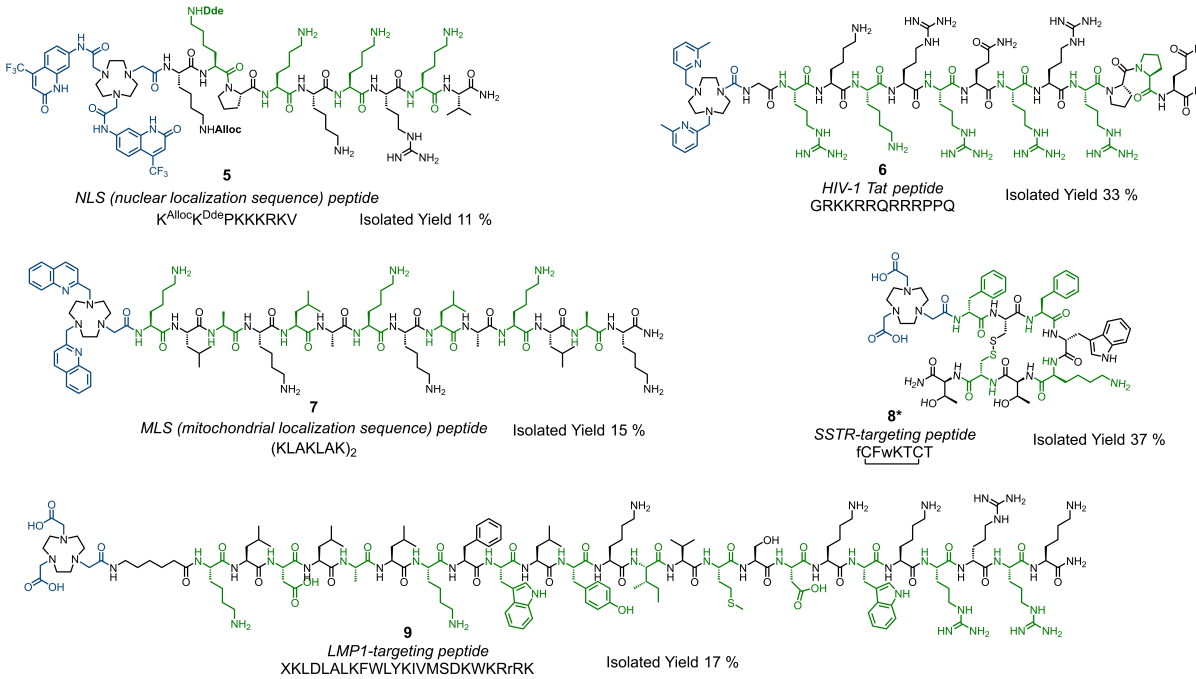

Figure 3. Synthesized TACN-peptide conjugates with potential biomedical applications (**5**–**9**). * The post-cleavage crude product was further cyclized by disulfide bond between two Cys.

Acknowledgements

K.-L. W. gratefully acknowledges the financial assistance from the Hong Kong Research Grants Council Grant No. 12300021, NSFC/RGC Joint Research Scheme (N_PolyU209/21), the Centre for Medical Engineering of Molecular and Biological Probes (AoE/M-401/20) and the Innovation and Technology Fund – Partnership Research Programme (PRP/040/23FX). G.-L. L. gratefully acknowledges the financial assistance from the Hong Kong Research Grant Council Grant No. 15301120. The precious advice in writing provided by Dr. Wai-Sum Lo from the Department of Applied Biology and Chemical Technology (ABCT) of The Hong Kong Polytechnic University (HKPolyU) is greatly appreciated. We also appreciate the service of high-resolution mass spectrometry provided by The University Research Facility in Life Sciences (ULS) of HKPolyU.

Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: 1,4,7-Triazacyclononane (TACN) · nitrobenzoxadiazole (NBD) · peptide conjugates · protecting group · Positron Emission Tomography (PET)

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Manuscript received: October 9, 2024
Version of record online: ■■, ■■