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Development of Bifunctional Chelates Bearing Hydroxamate Arms for Highly Efficient ^{64}Cu Radiolabeling

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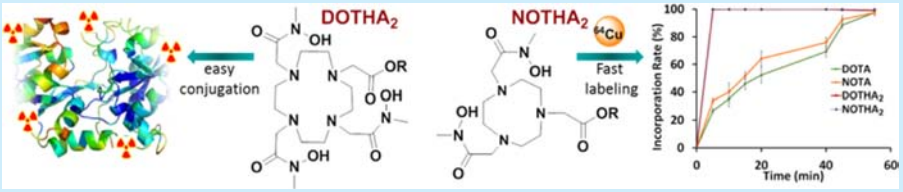
Supporting Information



ABSTRACT: Convenient approaches for the synthesis of $DOTHA_2$ and $NOTHA_2$, two cyclic bifunctional chelates (BFCs) bearing hydroxamate arms, have been developed. These novel BFCs coordinate ^{64}Cu with fast kinetics at room temperature in a wide range of concentrations and pH. The corresponding radiochemical complexes showed high stability, low residual activity in various tissues, and fast clearance in normal mice. The ability to conjugate $DOTHA_2$ to both a small peptide and a large protein is also reported.

Copper has five radionuclides (^{60}Cu, ^{61}Cu, ^{62}Cu, ^{64}Cu, and ^{67}Cu) that can be used in copper radiopharmaceuticals. However, because of its availability, the research efforts in radiocopper chemistry are mainly concentrated on the use of ^{64}Cu ($T_{1/2}$ = 12.7 h; 17.4% β^+, 43% EC, 39% β^-), a radioisotope with low positron energy (E β^+_{max} = 0.656 MeV) that is ideal for positron emission tomography (PET) imaging quantification and β^- emissions, along with Auger electrons for radiotherapy.[1] Several bifunctional chelates (BFCs) bearing carboxylate pendant arms were investigated as carriers of ^{64}Cu for PET imaging; DOTA is among the most widely used.[2] However, ^{64}Cu−DOTA complexes were reported to have poor in vivo stability, resulting in demetalation and subsequent accumulation of the radiometal in nontarget tissues.[3] The stability of the ^{64}Cu complex in vivo is a critical factor for optimal radiopharmaceutical design.[4]

Previous studies have described the potential of NOTA, PCTA, NE3TA, CB-TE2A, CB-TETA, SarAr, and more recently Pycup derivatives as BFCs for ^{64}Cu.[3b,4,5] Although their corresponding chelates present high resistance to transmetalation reactions in vivo when conjugated to peptide-based biomolecules, the available BFCs are limited by either challenging synthesis or harsh and/or slow radiolabeling chemistry, which may cause proteolysis and/or radiolysis of natural peptides and proteins.[4b,5]

While the metal chelation of the hydroxamic acids has been well established, their use in the development of BFCs is limited. Only a few examples of BFCs bearing hydroxamic acid pendant arms were found in the literature. Among them are trisuccin,[6] diethylenetriamine pentahydroxamate (DTPH),[7] and desferrioxamine B (Df),[8] a siderophore mostly used for ^{89}Zr complexation. These ligands are acyclic, and to our knowledge, none of them were investigated in radiocopper chemistry.

Herein we report the synthesis of *N*-methylhydroxamates derived from tetraaza- and triazamacrocycles, named $DOTHA_2$ and $NOTHA_2$, for the labeling of peptide-based biomolecules (Scheme 1). Fast labeling kinetics as well as an increased stability of the ^{64}Cu(II) complexes can be expected for $DOTHA_2$ and $NOTHA_2$ due to the advantage of the macrocyclic effect[9] and the increased basicity of the hydroxamate group (pK_a = 8.5) versus carboxylate group (pK_a = 4.75). The radiolabeling efficiency, the stability, and the in vivo behavior of ^{64}Cu-labeled $DOTHA_2$ and $NOTHA_2$ were examined and compared with those of ^{64}Cu−DOTA and NOTA analogues. Because of the importance of the conjugation step and its effect on the subsequent applications of the conjugates, we developed a protocol in which the activated ester of partially protected $DOTHA_2$ is conveniently and efficiently conjugated by way of an amide bond formation to H_2N-PEG-[D-Tyr^6,βAla^{11},Thi^{13},Nle^{14}]−BBN(6−14), a bombesin (BBN) peptide. This BBN derivative acts as an agonist[10] and binds with high affinity to the gastrin-releasing peptide receptor (GRPR), which is overexpressed in prostate,[11] breast,[12] and other cancers.[13] $DOTHA_2$−BBN conjugate was also examined with respect to GRPR affinity, ^{64}Cu-labeling efficacy, and stability. Finally, we report a procedure utilizing an activated ester of unprotected $DOTHA_2$ for its conjugation to

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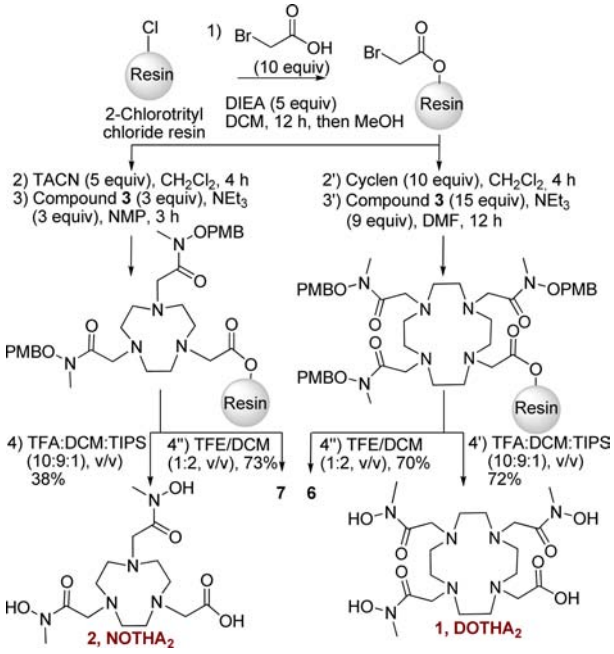
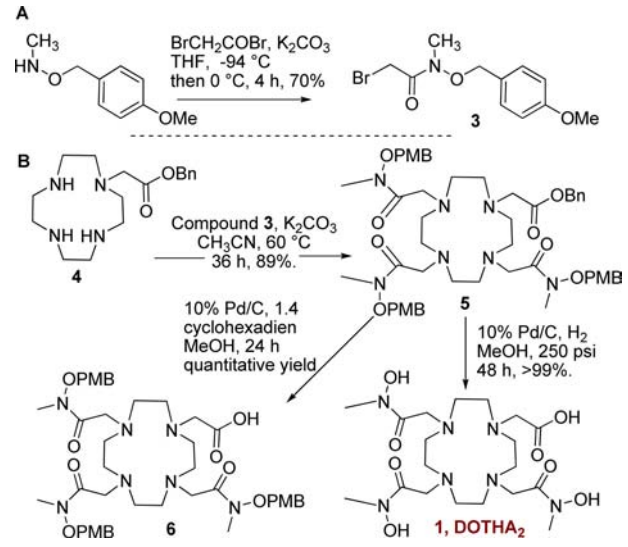
Scheme 1. Synthesis of $DOTHA_2$ (1) and $NOTHA_2$ (2) on Solid Phase



Scheme 2. Synthesis of 2-Bromo-*N*-methyl-*O*-(*p*-methoxybenzyl)hydroxylamineacetamide 3 Pendant Arm (A); Synthesis of $DOTHA_2$ (1) and Partially Protected $DOTHA_2$ 6 in Solution (B)



bovine serum albumin (BSA) as a representative for larger biomolecules (proteins and antibodies).

$DOTHA_2$ and $NOTHA_2$ were synthesized on the solid phase in a three-step process starting with bromo-acetylated resin (Scheme 1).[17] Reaction conditions have been optimized by monitoring each step on analytical reversed-phase high-performance liquid chromatography (HPLC) after cleavage of a small amount (10 mg) of the resin-bound peptide with 95% trifluoroacetic acid (TFA) in dichloromethane (DCM). Functionalization of the resin was provided through the use of α-bromocarboxylic acid in the presence of *N,N*-diisopropylethylamine (DIEA). For the preparation of $NOTHA_2$, the bromide was then displaced by the amine function of the 1,4,7-triazacyclononane (TACN) in anhydrous DCM following the method of Meares.[18] A mechanical shaking was maintained for 4 h at room temperature to ensure complete consumption of the starting material. The TACN was then alkylated with 2-bromo-*N*-methylbenzylhydroxylamine−acetamide **3** (Scheme 2) in the presence of triethylamine in dry *N*-methylpyrrolidinone (NMP) for 3 h. During the TACN alkylation step, a considerable amount of overalkylation (i.e., three groups instead of two) was observed with longer reaction time and/or more than 3 equiv of compound **3**. Similar procedures were followed for the preparation of $DOTHA_2$ on the solid phase with a prolonged reaction time for step 3′ to ensure complete alkylation of the cyclen (Scheme 1). Both BFCs were cleaved from the resin with 90% TFA in DCM using triisopropylsilane (TIPS) as a scavenger. $NOTHA_2$ and $DOTHA_2$ were prepared with overall yields of 38% and 72%, respectively, based on the substitution rate of the resin. Selective cleavage of BFCs was also performed using trifluoroethanol (TFE) in DCM to afford the OPMB-protected trihydroxamates **6** and **7** in overall yields of 70% and 73%, respectively (Scheme 1).

As described in Scheme 2, hydroxylamine pendant arm **3** was obtained in good yield by treating *N*-methyl-OPMB-hydroxylamine[14] with 2-bromoacetyl bromide and potassium carbonate in tetrahydrofuran (THF) at −94 °C (Scheme 2A). $DOTHA_2$ was also synthesized in solution in a two-step process starting from 1,4,7,10-tetraazacyclododecane-1-benzyl acetate trihydrochloride salt (**4**)[15] as shown in Scheme 2B. Briefly, the fully protected $DOTHA_2$ was prepared by the addition of 2-bromo-*N*-methyl-*O*-(*p*-methoxybenzyl)hydroxylamine acetamide **3** to the monoalkylated cyclen **4**. After workup, the residue was purified via column chromatography to afford pure **5** as a beige solid. Deprotection of the benzyl group of **5** was carried out selectively by a treatment with 10% palladium on-charcoal in the presence of 1,4-cyclohexadiene.[16] After filtration and evaporation, the crude oil was dissolved in methanol and precipitated with diethyl ether to quantitatively yield **6** as a white fluffy solid. Complete deprotection of **5** was achieved by catalytic hydrogenation with activated palladium-on-charcoal at high pressure (260 psi) to afford with an excellent yield $DOTHA_2$ as an oil, which precipitated as a white fluffy solid upon addition of cold diethyl ether. Although $DOTHA_2$ can be obtained in larger amounts when prepared in solution, the approach on the solid phase is straightforward and does not require isolation and purification of the intermediates.

$DOTHA_2$ and $NOTHA_2$ were successfully labeled at room temperature after 5 min either by incubation of $^{64}Cu(OAc)_2$ in 0.1 M ammonium acetate buffer or $^{64}CuCl_2$ in 1 M HEPES buffer at pH 7 (Figure 1a). Hydroxamate BFCs were labeled rapidly at room temperature while their analogues DOTA and NOTA required longer reaction times (1 h) under the same conditions (Figure 1b). Radiolabeling conditions for $DOTHA_2$ and $NOTHA_2$ with ^{64}Cu can range from a pH of 5.5 to 8.5 (Figure 1c,d). However, we observed only 20−25% radiolabeling yields at pH 3.5 and 10.5 for our hydroxamate BFCs. It appears that deprotonation or exchange of the metal ion becomes more difficult at pH 3.5 for the hydroxamic pendant arms. The poor yield at elevated pH can be explained by the formation of colloidal $Cu(OH)_2$, since we used $^{64}Cu(OAc)_2$ for the radiolabeling.[5h] Specific activities of the corresponding radiolabeled-BFCs were excellent and varied from 75 to 100 TBq/mmol. It was found that the labeling efficiency of several chelators dropped significantly when the chelator concentration was lower than 1 μM.[19] $DOTHA_2$ can be radiolabeled

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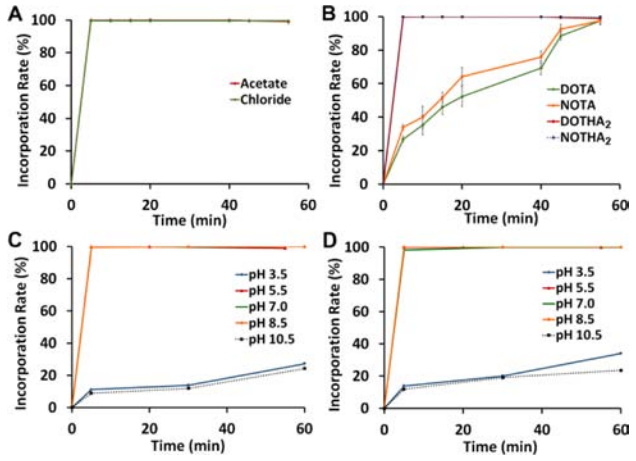

Figure 1. Radiolabeling efficiencies of (A) $DOTHA_2$ with ^{64}Cu and different counterions at pH 5.5; (B) $DOTHA_2$, $NOTHA_2$, DOTA, and NOTA with $^{64}Cu(OAc)_2$ in ammonium acetate buffer at pH 5.5 and ambient temperature; (C) $DOTHA_2$ and $^{64}Cu(OAc)_2$ at different pH; (D) $NOTHA_2$ and $^{64}Cu(OAc)_2$ at different pH.

efficiently (>95%) at a 10 nM chelator concentration in 5 min at room temperature when radiolabeled with 75 MBq of ^{64}Cu (Figure S1, Supporting Information).

The stability of $DOTHA_2$, $NOTHA_2$, and NOTA ^{64}Cu-labeled complexes was first measured at various pH (3−8.5) at 37 °C over 20 h. Over this pH range, both ^{64}Cu−BFC $DOTHA_2$ and $NOTHA_2$ displayed higher stabilities than NOTA. This difference in the decomplexation percentages is more noticeable at low pH, for which only ∼40% of ^{64}Cu−NOTA remained intact in solution at pH 3 after 20 h, compared to ∼80% for our hydroxamate BFCs (Figure S2, Supporting Information). The three ^{64}Cu−BFCs demonstrated a similar resistance to metabolic processes in plasma (20 h) and in vivo (4 h) with stability greater than 97%, (Table S1, Supporting Information).

Biodistribution and μPET imaging studies were done on normal female balb/c mice. We observed low residual activity in various tissues and fast hepatic and renal clearance for ^{64}Cu−$DOTHA_2$ and ^{64}Cu−$NOTHA_2$ (Figures 2 and S3, Supporting Information); the biodistribution profile of the two radiolabeled BFCs being very similar at each time point (Figure 2). While ^{64}Cu−NOTA displayed the lowest overall nonspecific uptake, our ^{64}Cu−BFCs still exhibited a percentage of ID/g lower than 1 in most organs at 4 and 20 h postinjection. The liver uptake of our BFCs was slightly higher than for ^{64}Cu/NOTA due to the increased lipophilicity of the hydroxamate group versus carboxylate group.[20] The increased hepatic clearance could complicate the interpretation of upper abdomen images. However, this issue may be resolved by the use of a hydrophilic linker when the chelator is used for the development of ^{64}Cu-labeled bioconjugate.[21]

A method for the conjugation of $DOTHA_2$ to a BBN peptide by way of an amide bond formation was developed. In this approach, the OPMB-protected trihydroxamate **6** was first activated by 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) in the presence of DIEA and then coupled at the *N*-terminal position of the partially protected BBN on resin, followed by the peptide's deprotection and cleavage. The conjugation was complete within 4 h and showed high coupling efficiencies with an overall yield of 35%. Purity of the $DOTHA_2$−BBN was confirmed to

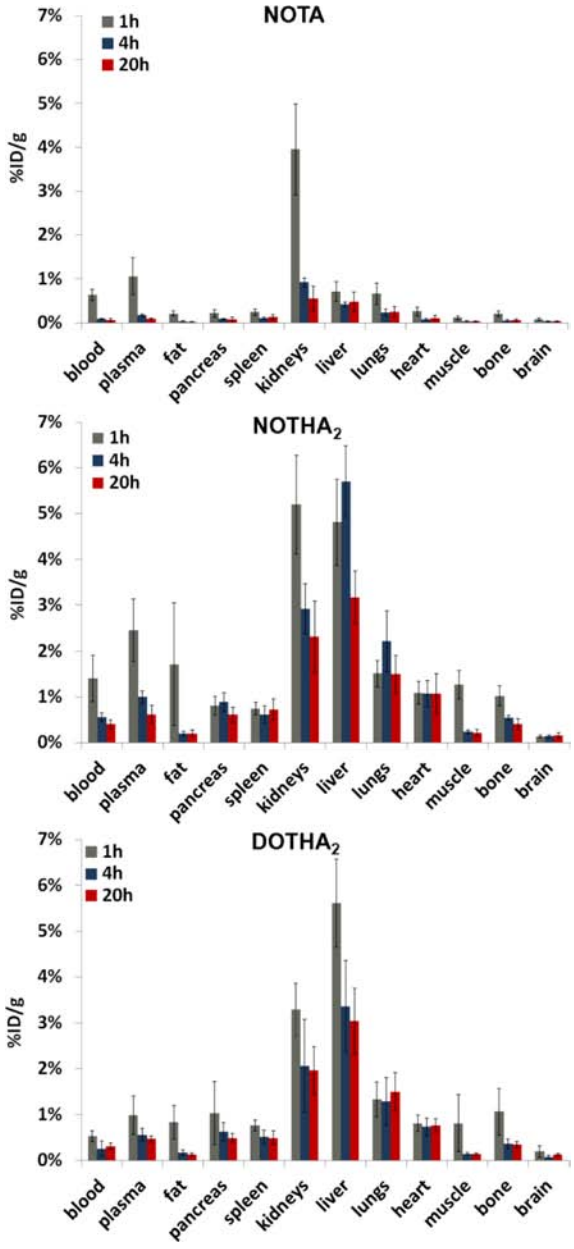

Figure 2. Biodistribution of ^{64}Cu−NOTA, ^{64}Cu−$NOTHA_2$, and ^{64}Cu−$DOTHA_2$ in female balb/c mice. The animals (n = 4−5 per group) were injected with ^{64}Cu-labeled chelators and were sacrificed after 1 (red bars), 4 (blue bars), and 20 (green bars) h postinjection. The organs were harvested, weighed, and counted in the gamma counter. The percent injected dose per gram of tissue (% ID/g) was determined by decay correction of each sample normalized to weight. The data for each group represents the mean ± SD.

be >94% by HPLC. The incorporation of the $DOTHA_2$ seems to have a minimal effect on the binding affinity of BBN since Cu−$DOTHA_2$−BBN gave an inhibition constant (K_i) value of 0.13 ± 0.41 nM, which is comparable to that of BBN in the low nanomolar range (0.59 ± 0.32 nM).[22] $DOTHA_2$−BBN was also labeled with high efficiency (>95%) using $^{64}Cu(OAc)_2$ in 0.1 M ammonium acetate buffer at pH 7 in less than 5 min at a concentration of 3−5 μM and without heating. ^{64}Cu−$DOTHA_2$−BBN is also stable after a 20 h incubation in mouse plasma and 4 h in vivo, since no trace of free ^{64}Cu nor of

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any metabolite was detected by radio-thin-layer chromatography (Figure S25, Supporting Information).

We also developed a more direct method involving the conjugation to BSA of a fully deprotected $DOTHA_2$ activated in situ by *N*-hydroxysuccinimide (NHS). This chemical step was quite challenging due to the interference of the hydroxamate moieties with the coupling chemistry used.[7] A molar ratio of 50:1 chelate/BSA was used for conjugation of $DOTHA_2$–NHS ester to surface lysine residues. Nonconjugated BFC and other low molecular weight impurities were removed by centricon using a spin-column filter. The average number of BFCs per BSA was determined to be 9 ± 0.75. The latter protocol is more direct for conjugation of our BFCs to proteins and antibodies, since it limits the number of steps postconjugation. Radiolabeling of $[DOTHA_2]_9$–BSA was accomplished at room temperature through incubation of $^{64}Cu(OAc)_2$ with the $DOTHA_2$-conjugated protein and showed a 99% radiolabeling yield.

Our results indicate that ^{64}Cu-labeled $DOTHA_2$ and $NOTHA_2$ offered very fast labeling kinetics at room temperature in a wide range of concentrations and pH. They are promising BFCs facilitating further exploration of ^{64}Cu–peptide based tracers for PET imaging. The high binding affinity and stability of ^{64}Cu–$DOTHA_2$–BBN make it suitable as a tracer for the fine-tuning of biological properties of labeled-BBN conjugates. Further in vitro/in vivo evaluation of this tracer as a potential cancer PET imaging agent is justified.

■ ASSOCIATED CONTENT

Supporting Information

Complete description of materials and methods, supporting figures (S1–S3), Table S1, NMR spectra of compounds **2**–**7**, and HPLC trace and mass spectra of $DOTHA_2$–BBN, Cu/$DOTHA_2$–BBN, and $[DOTHA_2]_n$–BSA. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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