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# A highly effective bifunctional ligand for radioimmunotherapy applications†

**Hyun-Soon Chong,* Hyun A. Song, Chi Soo Kang, Thien Le, Xiang Sun, Mamta Dadwal, Hyunbeom Lee, Xiaoli Lan, Yunwei Chen and Anzhi Dai**

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**A novel bifunctional ligand (3p-*C*-NETA) for antibody-targeted radioimmunotherapy (RIT) of β-emitting radioisotopes $^{90}$Y and $^{177}$Lu was efficiently synthesized *via* an unexpected regiospecific ring opening of an aziridinium ion. 3p-*C*-NETA instantly formed a very stable complex with $^{90}$Y or $^{177}$Lu. 3p-*C*-NETA is an excellent bifunctional ligand for RIT.**

A tumor-targeting monoclonal antibody (mAb) has been linked to a cytotoxic radioisotope for antibody-targeted radiation cancer therapy (radioimmunotherapy, RIT) for selective delivery of a cytotoxic radioactive metal to cancer cells while minimizing radiation exposure to healthy cells.[1–3] RIT drugs for various cancers have been extensively tested in clinical trials.[4] Zevalin® is the first FDA-approved RIT drug for treatment of B-cell non-Hodgkins lymphoma (NHL) and consist of a mAb (anti-CD20), a pure beta-emitter $^{90}$Y ($t_{\frac{1}{2}}$ = 64.1 h, $E_{max}$ = 2.3 MeV), and a bifunctional ligand 1B4M-DTPA(2-(4-isothiocyanatobenzyl)-6-methyl-diethylene-triamine pentaacetic acid, Fig. 1).[5]

A successful RIT requires a bifunctional ligand that can effectively bind a radioisotope with clinically acceptable complexation kinetics and *in vivo* stability to minimize toxicity due to dissociation of metal complex or radiolytic damage resulted from extended exposure of the protein during radiolabeling.[6] Despite great potential of RIT as a cancer therapeutic modality proven by Zevalin®, less progress has been made on improvement of chelation chemistry. Among the bifunctional ligands available for RIT, *C*-DOTA (2-(4-nitrobenzyl)-1,4,7,10-tetraazacyclotetradecane-1,4,7,10-tetra-acetic acid, Fig. 1) and *C*-DTPA (2-(4-nitrobenzyl)-diethylenetriamine pentaacetic acid, Fig. 1) have been extensively investigated. The macrocyclic ligand *C*-DOTA is known to form a stable complex with many different radionuclides.[7] However, slow complexation kinetics of DOTA under mild reaction conditions remains an obstacle limiting the wide practical use of the ligand in RIT of relatively short half-lived α- and β-emitters such as $^{90}$Y, $^{212}$Bi ($t_{1/2}$ = 60.6 m), $^{213}$Bi ($t_{1/2}$ = 45.6 m), or $^{212}$Pb ($t_{1/2}$ = 10.64 h).[8] Acyclic *C*-DTPA instantly binds to the radionuclides, but the radiolabeled complexes of *C*-DTPA produced unfavorable *in vitro* and *in vivo* stability profiles.[9] The bifunctional ligands in the CHX-DTPA (Fig. 1, *trans*-cyclohexyl-*C*-DTPA) series containing a cyclohexyl ring were developed as efforts to improve stability of *C*-DTPA radiolabeled with α- or β-emitters.[10a] Among the CHX-DTPA-based ligands, enantiomerically pure CHX-A″-DTPA radiolabeled with $^{88}$Y (Fig. 1) was shown to produce more favorable *in vitro* and *in vivo* complex stability profiles relative to $^{88}$Y-1B4M-DTPA.[10] CHX-A″-DTPA or antibody conjugates of CHX-A″-DTPA were reported to rapidly form a complex with $^{88}$Y or $^{86}$Y. However, the *in vitro* serum stability data indicate that the Y(III) complexes of CHX-A″-DTPA were less stable than those of *C*-DOTA.[10b–d]

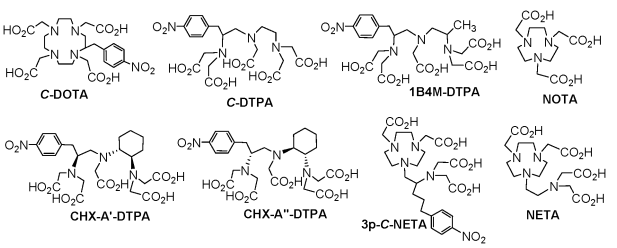


**Fig. 1** Ligands in preclinical or clinical use for RIT.

We reported that NETA ({4-[2-(bis-carboxymethyl-amino)-ethyl]-7-carboxymethyl-[1,4,7]triazonan-1-yl}-acetic acid, Fig. 1) is a promising chelator for RIT of α- and β-emitters including $^{90}$Y, $^{177}$Lu ($t_{1/2}$ = 6.7 d), $^{212}$Bi, $^{213}$Bi, and $^{212}$Pb.[11–13] NETA possesses both a parent macrocyclic NOTA (1,4,7-triazacyclononane-*N*,*N*′,*N*″-triacetic acid, Fig. 1) backbone and a flexible acyclic tridentate pendant arm. The idea of designing octadentate NETA was to integrate the advantage of both the macrocyclic NOTA and acyclic DTPA frameworks, *i.e.*, both high thermodynamic stability and favorable formation kinetics. Indeed, NETA was found to bind to the metals with favorable complexation kinetics, and the corresponding NETA–metal complexes were stable *in vitro* and *in vivo*.[11–13]

As part of our continued effort to develop an optimized bifunctional ligand for RIT, a bifunctional version of NETA, 3p-*C*-NETA ({4-[2-(bis-carboxy-methylamino)-5-(4-nitrophenyl)-pentyl]-7-carbo-xymethyl-[1,4,7]tri-azonan-1-yl} acetic acid, Fig. 1) is designed. 3p-*C*-NETA possesses a longer propyl chain that is used to connect the NETA backbone and the functional *p*-$NO_2$ benzyl group for conjugation to antibody. The extended alkyl spacer was proposed to reduce steric

*Chemistry Division, Biological, Chemical, and Physical Sciences Department, Illinois Institute of Technology, 3101 S. Dearborn St, LS 182, Chicago, IL, USA. E-mail: chong@iit.edu; Fax: 312-567-3494; Tel: 312-567-3235*

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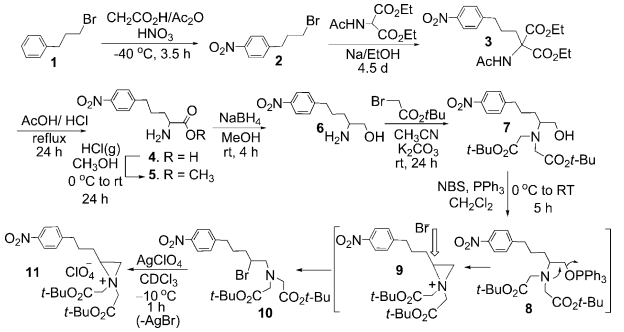
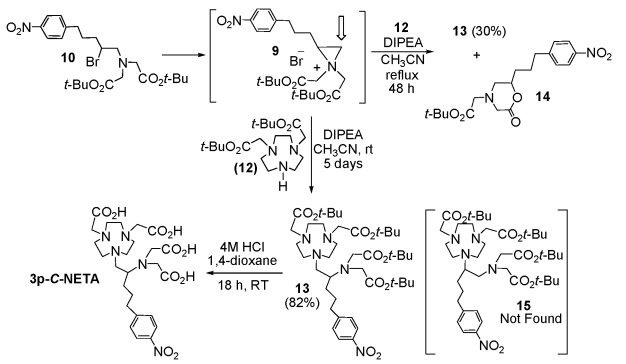
hindrance in complexation of the ligand with a metal resulting in improved complexation kinetics as compared to the standard methyl chain present in the known bifunctional ligands (Fig. 1). Herein, we report synthesis of a highly effective bifunctional ligand 3p-*C*-NETA based on an unexpected regiospecific ring opening of an aziridinium ion by nucleophilic bisubstituted TACN. 3p-*C*-NETA was evaluated for radiolabeling reaction kinetics with $^{90}$Y and $^{177}$Lu. The corresponding $^{90}$Y-3p-*C*-NETA and $^{177}$Lu-3p-*C*-NETA complexes were evaluated for *in vitro* serum stability. The *in vitro* complexation kinetics and stability data of 3p-*C*-NETA were compared to those of *C*-DOTA.

We have recently reported that bromination of *N*,*N*-bisubstituted β-amino alcohols provided aziridinium salts that promptly underwent regiospecific ring opening by the counter-anion bromide to produce secondary *N*,*N*-bisubstituted β-amino bromides.[14] We wanted to apply aziridinium ion chemistry to the synthesis of 3p-*C*-NETA. The key reaction steps in the synthesis of 3p-*C*-NETA are the formation and the regiospecific ring opening of **9** by bisubstituted TACN **12**[13] to provide **13** (Schemes 1 and 2).



**Scheme 1** Synthesis of secondary β-amino bromide **10** and aziridindium ion **11**.

Secondary β-amino bromide **10** was prepared by a practical, reproducible, and readily scalable synthetic route as shown in Scheme 1. The starting material *p*-nitrophenylpropyl bromide **2** was prepared *via* modification of a known procedure[15] and reacted with sodium salt of diethyl acetamido malonate to afford compound **3**. Racemic *p*-nitrophenylpropylalanine **4** that was prepared from decarboxylation and removal of the acetyl protection group in **3** was converted to amino methyl ester **5**. Reduction of **5** with $NaBH_4$ followed by alkylation of **6** with *t*-butyl bromoacetate provided **7**. Bromination of **7** in $CH_2Cl_2$ using NBS and $PPh_3$ provided the secondary β-amino bromide **10** in 66% isolated yield. Compound **10** was fully characterized by $^1$H and $^{13}$C NMR and CHN and HRMS analysis (supporting information†). A proposed reaction mechanism for the transformation of **7** to **10** is shown in Scheme 1. Aziridinium salt **9** containing counter anion bromide was initially formed from intramolecular rearrangement of **8** *via* attack of the nucleophilic nitrogen atom followed by removal of triphenylphosphine oxide. Regiospecific ring opening of **9** at the more hindered carbon provided secondary β-amino bromide **10**. Aziridinium ion **11** was isolated from the reaction of **10** with silver perchlorate and characterized by $^1$H and $^{13}$C NMR analysis (the Supporting Information†).

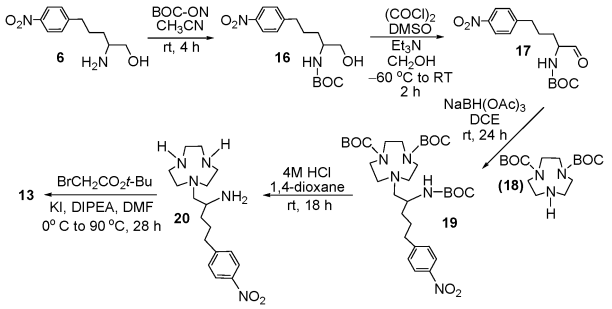


**Scheme 2** Synthesis of 3p-C-NETA *via* regiospecific ring opening of aziridinium ion **9**.

Reaction of **10** with bisubstituted TACN (**12**) in the presence of DIPEA as a base provided **13** as the exclusive product in an excellent isolated yield (82%). It is reasonably proposed that **10** first rearranged to form aziridinium ion **9** wherein the less hindered methylene carbon was attacked by the bulky nucleophile **12** to provide the regioisomer **13**. We initially attempted the synthesis of **13** by the reaction of **10** with **12** in $CH_3CN$ under reflux. However, the reaction provided **13** in a very poor yield (30%) due to the formation of an oxomorpholine derivative **14** as the major product from an intramolecular rearrangement of **9**. To avoid the formation of **14**, the reaction was repeated at room temperature and provided only **13** in a significantly improved isolated yield along with the unreacted starting materials **10** and **12**. The regioisomer **15** that could be formed by nucleophilic attack of **12** at the more hindered methylene carbon in **9** was not isolated from either of the reactions. The *t*-butyl groups in **13** were removed by the treatment of **13** with 4M HCl/1,4-dioxane, and 3p-*C*-NETA in the nitro form was obtained in a quantitative yield (Scheme 2).

To confirm the regiochemistry observed in the nucleophilic ring opening of the aziridinium ion **9**, compound **13** was separately prepared based on the reaction route starting from compound **6** (Scheme 3). Swern oxidation of *N*-BOC protected amino alcohol **16** that was prepared by reaction of **6** with BOC-ON provided compound **17**. Reductive amination of **17** with **18** using sodium triacetoxyborohydride provided **19** which was subsequently treated with HCl (g) in 1,4-dioxane to afford **20**. A base-promoted reaction of **20** with *tert*-butyl bromoacetate produced compound **13** in a very poor isolated yield (<5%) due to the formation of the partially- or poly-alkylated byproducts in the reaction mixture and resultant low purification yield. The isolated yield of **20** may be improved through optimization of alkylation reaction condition. The low-yield alkylation reaction of **20** clearly highlights the value of the efficient synthetic method for polar macrocyclic bifunctional ligands based on regiospecific ring opening of labile aziridinium ion (Scheme 2) reported herein. Finally, regiochemistry in the ring opening of **9** was confirmed by the identical $^1$H and $^{13}$C NMR data of **13** that was separately prepared *via* the two synthetic routes (Schemes 2 and 3).

The new ligand 3p-*C*-NETA was evaluated for radiolabeling efficiency with the β-emitting radioisotopes, $^{90}$Y and $^{177}$Lu. Radiolabeling reaction kinetics of 3p-*C*-NETA with $^{90}$Y or $^{177}$Lu was performed at room temperature and various pH



**Scheme 3** Structural determination of **13** for confirmation of regiochemistry in ring opening of **9**.

compared to that of *C*-DOTA (Table 1 and the Supporting Information†). 3p-*C*-NETA instantly formed a complex with $^{90}$Y at pH 5.5 with the excellent radiolabeling efficiency (97.4 ± 0.7%) in 1 min (Table 1). Radiolabeling of 3p-*C*-NETA with $^{177}$Lu was essentially complete in 1 min at all the range of pH studied. *C*-DOTA was significantly slower in binding $^{90}$Y at pH 5.5 (83.5 ± 8.13%, 1 h) relative to 3p-*C*-NETA, and radiolabeling of *C*-DOTA with $^{90}$Y was incomplete even at 1 h time point. Formation of $^{177}$Lu-*C*-DOTA was relatively faster than that of $^{90}$Y-*C*-DOTA at all the range of pH. *In vitro* serum stability of the radiolabeled complexes was performed to determine if 3p-*C*-NETA or *C*-DOTA radiolabeled with $^{90}$Y or $^{177}$Lu remained stable without loss of $^{90}$Y or $^{177}$Lu in human serum ITLC and radio size-exclusion HPLC (Supporting Information†). $^{90}$Y-3p-*C*-NETA and $^{177}$Lu-3p-*C*-NETA were readily prepared from the reactions of 3p-*C*-NETA with $^{90}$Y or $^{177}$Lu at room temperature and directly used for serum stability studies. *C*-DOTA is reported to form less stable intermediate metal complexes with Lanthanides under mild reaction condition based on a three step complexation mechanism.[16] To ensure complete radiolabeling and formation of the most stable complexes of *C*-DOTA, $^{90}$Y-*C*-DOTA and $^{177}$Lu-*C*-DOTA were prepared by the reaction of *C*-DOTA with $^{90}$Y or $^{177}$Lu at either room temperature ($^{177}$Lu) or 37 °C ($^{90}$Y) over an extended period of time. Radiolabeling of *C*-DOTA with $^{90}$Y was determined to be incomplete even at 30 h time point by SE-HPLC. $^{90}$Y-*C*-DOTA was separated from a small amount of unbound $^{90}$Y present in the reaction mixture through a Chelex-100 column. $^{177}$Lu-3p-*C*-NETA, $^{90}$Y-3p-*C*-NETA, and $^{177}$Lu-*C*-DOTA were used without further purification. Both $^{90}$Y-3p-*C*-NETA and $^{177}$Lu-3p-*C*-NETA were stable in human serum for 2 weeks as evidenced by SE-HPLC and ITLC analysis, and no measurable radioactivity dissociated from the complexes was observed (Supporting information†). $^{90}$Y-*C*-DOTA and $^{177}$Lu-*C*-DOTA were quite stable in serum over the time period of measurements by ITLC and HPLC (Supporting information†). However, both $^{90}$Y-*C*-DOTA and $^{177}$Lu-*C*-DOTA in serum produced an unbound $^{90}$Y or $^{177}$Lu-related impurity that appeared as a very small peak in radio SE-HPLC chromatograms ($t_R$ = ~14 min).

**Table 1** Radiolabling efficiency (%) of 3p-*C*-NETA or *C*-DOTA with $^{90}$Y or $^{177}$Lu (RT, 0.25M $NH_4OAC$, pH 5.5)[a]

| | 3p-*C*-NETA | | *C*-DOTA | |
|---|---|---|---|---|
| Time (min) | $^{90}$Y | $^{177}$Lu | $^{90}$Y | $^{177}$Lu |
| 1 | 97.4 ± 0.7 | 100.0 ± 0.0 | 77.1 ± 3.7 | 94.5 ± 3.9 |
| 5 | 98.1 ± 1.1 | 100.0 ± 0.0 | 78.8 ± 5.1 | 98.8 ± 1.1 |
| 10 | 98.7 ± 1.6 | 100.0 ± 0.0 | 69.4 ± 10.6 | 99.5 ± 0.5 |
| 20 | 98.7 ± 2.2 | 100.0 ± 0.0 | 71.2 ± 11.2 | 99.9 ± 0.1 |
| 30 | 99.4 ± 0.9 | 100.0 ± 0.0 | 76.1 ± 9.52 | 99.9 ± 0.1 |
| 60 | 99.5 ± 1.0 | 100.0 ± 0.0 | 83.5 ± 8.13 | 100.0 ± 0.0 |

[a] ITLC ($CH_3CN$:$H_2O$ = 3:2); Radiolabeling efficiency (mean ± standard deviation %) was measured in triplicate ($n$ = 3).

In summary, the new bifunctional ligand was efficiently prepared by the new synthetic method centered on the regiospecific ring opening of the aziridinium ion. Radiolabeling of 3p-*C*-NETA with $^{90}$Y or $^{177}$Lu was found to be highly efficient under mild conditions. $^{90}$Y-3p-*C*-NETA and $^{177}$Lu-3p-*C*-NETA were stable in serum for at least 14 days. The radiolabeling kinetics and *in vitro* serum stability data suggest that 3p-*C*-NETA is a very promising bifunctional ligand that can be directly used for RIT applications of various cancers and may overcome the limitations associated with the currently available ligands *C*-DOTA and *C*-DTPA for RIT. 3p-*C*-NETA conjugated to an antibody is being investigated for RIT applications of α or β-emitting radioisotopes.

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