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Ultrastable complexes for *in vivo* use: a bifunctional chelator incorporating a cross-bridged macrocycle†

Elizabeth A. Lewis, Ross W. Boyle and Stephen J. Archibald*

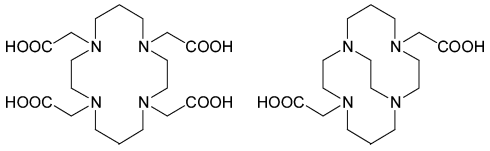
Department of Chemistry, University of Hull, Cottingham Road, Kingston upon Hull, UK HU6 7RX. E-mail: s.j.archibald@hull.ac.uk; Fax: +44(0)1482 466410; Tel: +44(0)1482 465488

Received (in Cambridge, UK) 10th May 2004, Accepted 8th July 2004
First published as an Advance Article on the web 20th August 2004

The synthesis, copper(II) complexation and biotin conjugation of a bifunctional chelator incorporating a cross-bridged macrocycle are described.

A bifunctional chelator (BFC) is a chelating ligand with a functional group that can be used for attachment to a bio-targeting vector. There is much current interest in the use of BFCs for medical imaging and therapy *e.g.* MRI contrast agents, radioimmunotherapy.[1,2] The ability of a BFC to bind and retain the appropriate metal ion (*e.g.* Gd, ^{90}Y, ^{64}Cu) *in vivo*, allowing its localisation at the target site (*e.g.* tumour or organ) and limiting its potentially harmful release, is crucial. Consequently, there is an ongoing requirement for the development of novel, high stability chelators for bioconjugation purposes.

Recent research efforts to develop BFCs have focused on the use of macrocyclic systems, which typically impart relatively high stability to their metal complexes compared to acyclic ones. In particular, systems based on tetraazacyclotetradecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (TETA) have shown potential as candidates for labelling biomolecules for diagnostic imaging and targeted radiotherapy, with clinical trials in progress.[3] However, there is evidence of transchelation from radiometal labelled TETA-bioconjugates *in vivo*. For example, Anderson and co-workers have demonstrated that ^{64}Cu dissociates from the peptide conjugate TETA-D-Phe^{1}-octreotide in the liver and binds to superoxide dismutase in high concentrations.[4] Similarly, Meares and co-workers have reported ^{67}Cu transfer from an antibody-TETA conjugate to ceruloplasmin in lymphoma patients.[5]



TETA, **1** CB-TE2A, **2**

An analogue of TETA in which two non-adjacent nitrogen atoms are linked by an ethylene bridge has been developed by Weisman and co-workers (so-called 'cross-bridged' macrocycle, CB-TE2A, **2**).[6] It exhibits far greater complex stability and overcomes these problems.‡[7] The ^{64}Cu complex of CB-TE2A is much less susceptible to transchelation *in vivo* than that of TETA: ^{64}Cu-CB-TE2A resulted in substantially lower values of protein-associated ^{64}Cu than observed for ^{64}Cu-TETA [13 ± 6% *vs.* 75 ± 9% at 4 h].[7b] Importantly, these findings indicate that a BFC based on CB-TE2A has the potential to be a powerful tool for medical imaging and therapy.[8] Herein, we present the synthesis and characterisation of the first BFC incorporating a cross-bridged macrocycle, 4,11-diacetic acid-6-(4-isothiocyanatobenzyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane (NCSBz-CB-TE2A).§ The reactivity of our functionalised cross-bridged systems towards Cu(II) and the biologically relevant molecule, biotin, has also been demonstrated.

A synthetic route has been developed that affords **10** in seven steps (Scheme 1) in reasonable overall yield (*ca.* 20%). We have previously described the efficient synthesis of the starting bisaminal, **3**, in which a 4-nitrobenzyl group is appended at the 6-position of the tetraazacyclotetradecane ring.[9] Highly regioselective di-substitution at non-adjacent *N*-positions of **3** is achieved by stirring an acetonitrile solution with an excess of benzyl bromide (*ca.* 10 equiv.) for 8 d to give **4** in good yield (75%).¶ Treatment of **4** with a large excess (*ca.* 50 equiv.) of sodium borohydride in methanol results in double reductive ring expansion and formation of **5** (78%).‖ On hydrogenolysis of **5**, debenzylation and nitro reduction occur efficiently in a single step to yield **6** (79%). The primary amine site is selectively protected over the two secondary amine ones in **6** using one equivalent of benzaldehyde in methanol. After stirring at RT overnight **7** is isolated in excellent yield (93%). **7** can be considered a useful precursor to a range of di-*N*-substituted cross-bridged systems by choice of appropriate electrophile. In the current synthetic route, addition of two equivalents of *tert*-butylbromoacetate to **7** in the presence of anhydrous potassium carbonate in dry acetonitrile leads to the formation of **8** (50%) on stirring at RT for 3 d. Treatment of **8** with an aqueous 6 M HCl solution heated to 100 °C for 3 d allows both imine and ester hydrolysis to be achieved quantitatively, to afford **9**.5HCl. For bioconjugation purposes, **9** can be converted to the isothiocyanate derivative **10** by reaction with thiophosgene under biphasic conditions (aqueous solution, pH 8/$CHCl_3$, >95%).

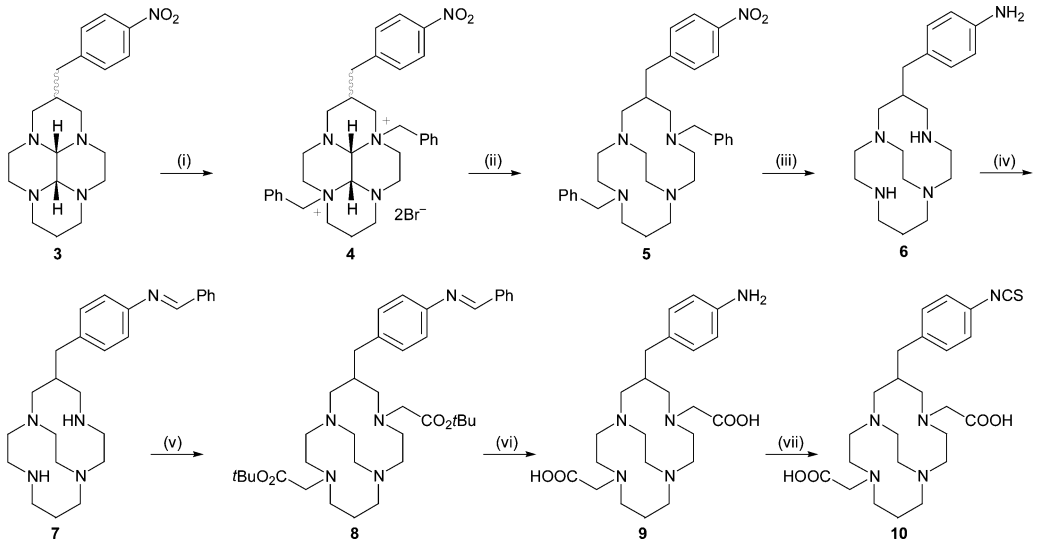
The coordination chemistry of cross-bridged macrocycles has been studied in detail by a number of groups.‡[6,10] In order to confirm that we observed similar reactivity towards Cu(II) as reported by Weisman and co-workers for CB-TE2A,[6] we performed a complexation reaction with ligand **9**. Combination of an equimolar amount of copper(II) nitrate trihydrate and **9**.5HCl in a 1:5 mixture of methanol and aqueous sodium hydroxide (5 equiv.) affords the neutral species Cu(**9**) as a dark purple solid, in good yield (69%) [single peak observed by ES^+ corresponding to Cu(**9**)Na^+]. Radiolabelling of the BFC is often carried out after a protein or antibody conjugate has been formed, necessitating the use of mild complexation conditions.[2] It is noteworthy therefore, that Cu(**9**) is formed easily at RT in aqueous solution.

In order to demonstrate the high reactivity of **10** towards biologically relevant molecules, the biotin ethylenediamine- conjugate has been formed.** A solution of **10** was stirred with biotin ethylenediamine (*ca.* 0.9 equiv.) in an aqueous sodium carbonate (*ca.* pH 10) solution for 4 h at RT. Negative ion electrospray mass spectrometry (Fig. 1) clearly shows loss of the isothiocyanate-functionalised macrocycle (**10**) and the formation of the biotin-conjugate (**11**). A three step procedure whereby a patient is administered first with biotinylated anti-tumor antibodies followed by avidin or streptavidin and finally a biotinylated chelator-conjugate has been used successfully to image target sites.[11] Experiments to investigate the potential of **11** for use in pretargeted diagnosis and therapy are underway.

In summary, we report the efficient synthesis of a BFC that incorporates a cross-bridged macrocycle and is an ideal candidate for use in medical imaging and therapy. The ability of the system to

† Electronic supplementary information (ESI) available: Further experimental details and characterisation for compounds **9–11** and Cu(**9**); See http://www.rsc.org/suppdata/cc/b4/b406906d/

DOI: 10.1039/b406906d



Scheme 1 Reagents and conditions: (i) 10 equiv. benzyl bromide, CH_3CN, N_2, RT, 8 d; (ii) 50 equiv. $NaBH_4$, EtOH, N_2, RT, 14 d, neutral alumina chromatography; (iii) H_2, 10% Pd/C, HOAc, RT, 3 d; (iv) 1 equiv. benzaldehyde, CH_3OH, N_2, RT, o/n; (v) 2 equiv. *tert*-butylbromoacetate, K_2CO_3, CH_3CN, N_2, RT, 3 d, neutral alumina chromatography; (vi) 6 M HCl, 100 °C, 2 d; (vii) 1.1 equiv. Cl_2CS, $CHCl_3/H_2O$, pH 8, RT, o/n.

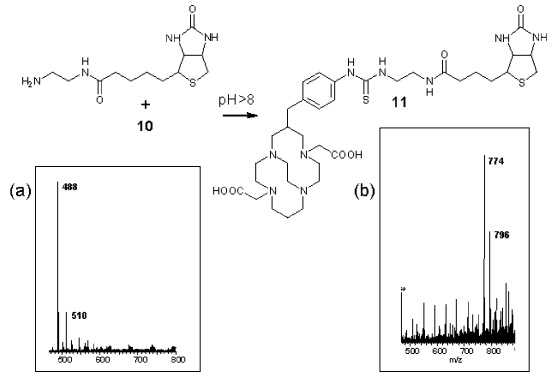


Fig. 1 Biotin conjugate synthesis inset ESMS$^-$ of (a) **10**, *m/z* 510 (25, M + Na − 2)$^-$, 488 (100, M − 1)$^-$; (b) **11**, *m/z* 796 (60, M + Na − 2)$^-$, 774 (100, M − 1)$^-$ (for full MS see ESI).

bind Cu(II) ions has been demonstrated and the biologically relevant biotin-conjugate formed.

We acknowledge the Wellcome Trust (E. A. L., grant no. 069719) for funding. We also thank Dr Trevor Dransfield (University of York) and the EPSRC National Service (Swansea) for electrospray mass spectrometry.

Notes and references

† Electronic supplementary information (ESI) available: Further experimental details and characterisation for compounds **9–11** and Cu(**9**); See http://www.rsc.org/suppdata/cc/b4/b406906d/

‡ On binding to metal ions all four nitrogen lone pairs of the cross-bridged ligand converge upon a cleft that encapsulates the cation with an enforced *cis*-V^6 ligand conformation; this conformational fixing leads to the remarkable kinetic stability.[10b]

§ The high reactivity of the isothiocyanate functionality toward free amine sites of biomolecules is well established making **10** the BFC of choice.[12]

¶ Highly regioselective bisquaternisation is a result of macrocycle conformation;[6] two diastereoisomers are formed in 1:1 ratio as previously described.[9]

‖ Ring inversion, whereby the ethylene bridge passes through the plane of the 14-membered ring, is slow on the NMR timescale and so there are two possible conformational isomers in solution observed by ^1H and ^{13}C NMR spectroscopy arising from the relative positions of the ethylene bridge and 4-substituted phenyl group in the free ligand.

** An ethylenediamine spacer has been introduced in the biotin-conjugate of **10** to minimise interference from the chelator on binding to avidin/streptavidin; longer spacers can also be introduced.

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