

Triazole biotin: a tight-binding biotinidase-resistant conjugate†‡

Cite this: *Org. Biomol. Chem.*, 2013, **11**, 7700Anne I. Germeroth,^a Jill R. Hanna,^a Rehana Karim,^a Franziska Kundel,^a Jonathan Lowther,^a Peter G. N. Neate,^a Elizabeth A. Blackburn,^b Martin A. Wear,^b Dominic J. Campopiano^a and Alison N. Hulme^{*a}Received 18th July 2013,
Accepted 30th September 2013

DOI: 10.1039/c3ob41837e

www.rsc.org/obc

The natural amide bond found in all biotinylated proteins has been replaced with a triazole through CuAAC reaction of an alkynyl biotin derivative. The resultant triazole-linked adducts are shown to be highly resistant to the ubiquitous hydrolytic enzyme biotinidase and to bind avidin with dissociation constants in the low pM range. Application of this strategy to the production of a series of biotinidase-resistant biotin-Gd-DOTA contrast agents is demonstrated.

Introduction

The use of the triazole as an amide bond isostere was highlighted by Sharpless in his seminal review of the impact of “click” chemistry in 2003.¹ However, the application of this strategy to confer hydrolytic stability in amide linkages to the essential cofactor biotin **1a**, such as found in the biological carrier biocytin **2** (biotinyl- ϵ -lysine),² and recently suggested as a rare epigenetic modification of histone lysines,³ has not been explored. Functionalisation of (bio)molecules with biotin is employed across a wide array of separation, immobilisation, pre-targeted therapeutic and medical imaging technologies due to its extremely strong affinity for the proteins avidin⁴ and streptavidin.⁵ This “avidity” results from unusually strong hydrogen-bonding interactions within the binding site for the ureido moiety of biotin,⁶ and improved protein packing on binding the ligand.⁷

Regulation of histone biotinylation through formation and hydrolysis of the histone lysine-biotin amide bond is only partly understood,^{3,8} but maintenance of levels of biotin from dietary sources is known to be achieved through the biotinidase-induced cleavage of biotin from both biocytin **2**, and short biotinylated peptides.⁹ Indeed, biotinidase deficiency is an inherited genetic disorder that if left untreated can result in major disabilities.¹⁰ However, biotinidase has also been

shown to catalyze the hydrolysis of other esters and amides,^{11–13} and engineering in biotinidase resistance is important in the design of pre-targeted therapies and imaging agents.^{14–18} Although not yet explored, hydrolysis-resistant biotinylation agents for histone modification might allow recent advances in site-specific histone labelling using near-native labels to be exploited,¹⁹ enabling understanding of the epigenetic role of biotinylation. Additionally, the development of biotinylated, hydrolytically stable, drug conjugates which exploit the over-expression of the sodium-dependent multi-vitamin transporter,²⁰ which mediates the cell uptake of biotin, in several aggressive cancer cell lines is attractive.

Results and discussion

Design of biotinidase-resistant conjugates

Some resistance to cleavage by biotinidase has been achieved by blocking,^{14,15} reversing the sense,¹⁶ or even removing the carbonyl of the amide bond to biotin;^{15,17} however, each of these approaches has an impact on binding to the target protein avidin or streptavidin.¹⁷ We sought to design a biotin label which would be resistant to cleavage by biotinidase, but would retain a high binding affinity for avidin. Our hypothesis was that triazole **3**, prepared by copper(I)-catalysed azide alkyne cycloaddition (CuAAC) coupling of alkyne **4** to an azide (RN₃), would provide an optimum non-hydrolysable isostere of the amide bond (Fig. 1b). Correspondingly, triazole **5**, derived from coupling known azide **6** to a terminal alkyne (RC≡CH), would provide a non-hydrolysable isostere of the reversed amide bond (Fig. 1b).§ Novel alkyne **4** was readily prepared

^aEaStCHEM School of Chemistry, University of Edinburgh, West Mains Road, Edinburgh, EH9 3JJ, UK. E-mail: Alison.Hulme@ed.ac.uk; Fax: +44 (0)131 650 4743; Tel: +44 (0)131 650 4711

^bCentre for Translational and Chemical Biology, University of Edinburgh, Mayfield Road, Edinburgh, EH9 3JR, UK

† Celebrating 300 years of Chemistry at Edinburgh.

‡ Electronic supplementary information (ESI) available: Synthesis and characterization of azide **6**, complexes **7–9**, biotinidase activity assay, enrichment of biotinidase from serum, biotinidase stability assay, HABA displacement and ITC binding assays. See DOI: 10.1039/c3ob41837e

§ For synthetic ease the azide **6** corresponding to the amide bond reversal of an amide of homobiotin was chosen for this investigation.



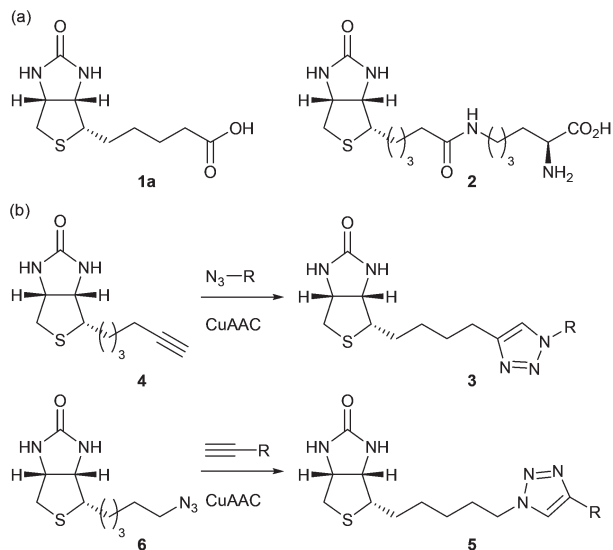


Fig. 1 (a) Biotin **1a** and biocytin **2**. (b) Non-hydrolysable triazoles (**3**, **5**) arising from CuAAC coupling of biotin-alkyne **4** and biotin-azide **6** (respectively).

from *D*-biotin methyl ester *via* reduction to the corresponding aldehyde and reaction with the Ohira–Bestmann reagent (47% overall yield);[‡] whilst azide **6** was prepared in 3 steps (62% overall yield) from the same starting material.²¹

To determine the efficacy of the triazole-linked biotin species, in terms of biotinidase resistance and avidin binding, a series of biotinylated Gd-DOTA complexes appropriate for pre-targeted magnetic resonance imaging were generated.²² Synthesis of MR imaging agents **7–9** was achieved through the CuAAC coupling of **4** and **6** with the Gd-complexes of three known DOTA “click” ligands (Fig. 2).^{23–25} These complexes allowed us to investigate both modes of CuAAC coupling (**7** vs. **8**), as well as to probe the effects of linker length between the triazole-linked biotin and bulky Gd-DOTA complex (**8** vs. **9**). The precursor Gd-complexes were readily prepared from the DOTA “click” ligands,[¶] and were coupled (CuSO₄, NaAsc, TBTA, **4** or **6**, ^tBuOH–H₂O, rt, 16 h) to give triazoles **7–9** which were purified by HPLC prior to biological assays.

Biotinidase resistance

Human biotinidase was fractionated from human serum,²⁶ and its activity was assessed by measuring the rate of hydrolysis of the commercially available substrate, *N*-biotinyl-*p*-amino-benzoic acid (*N*-biotinyl-PABA).²⁷ HPLC analysis revealed that all three triazole complexes (**7–9**) were resistant to cleavage by biotinidase up to 126 h, in marked contrast to *N*-biotinyl-PABA which was fully hydrolyzed under the same conditions within 6 h (Fig. 3).

¶ Attempted coupling of DOTA ligands in the absence of the lanthanide metal was found to be unsuccessful unless excess Cu(I) was employed.

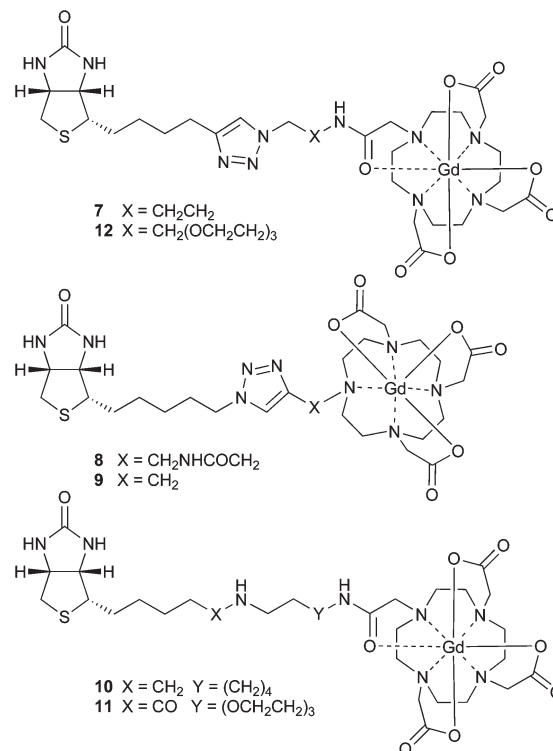


Fig. 2 Biotin Gd-DOTA complexes for pre-targeted MRI.

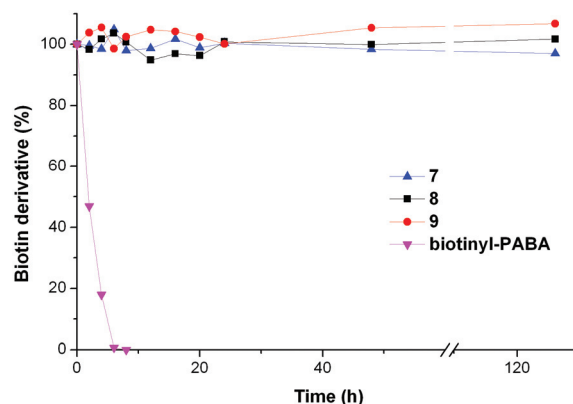


Fig. 3 Stability of **7–9** to cleavage by partially-purified biotinidase contrasted with cleavage of *N*-biotinyl-PABA under the same conditions. Samples were incubated with biotinidase (0.57 AU) in phosphate buffer (0.05 M, pH 6) for up to 126 h; the percentage of the complex remaining was determined by HPLC against an internal standard (resorcinol).

Binding affinity for avidin

The binding affinity of complexes **7–9** to avidin was probed using spectrophotometric competition experiments against the known ligand 4-hydroxy-azobenzene-2-carboxylic acid (HABA, $K_D = 5.8 \times 10^{-6}$ M).²⁸ All of the triazoles **7–9** were shown to inhibit the formation of the HABA:avidin complex in a dose-dependent manner (Fig. 4), and comparison with three additional ligands of known binding affinity (biotin **1a** $K_D = 10^{-15}$ M; desthiobiotin **1b** $K_D = 10^{-13}$ M; and diaminobiotin **1c** $K_D = 1.2 \times 10^{-7}$ M)^{4,29} suggested binding affinities for



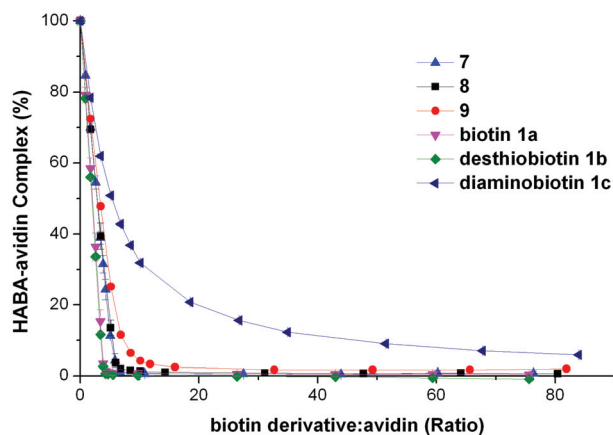


Fig. 4 Binding of MRI agents 7–9 to avidin assessed spectrophotometrically by displacement of HABA from the HABA–avidin complex at pH 7.5. [Diaminobiotin displacement assay conducted at pH 10.]

complexes 7–9 in the nM to pM range. Both the amide bond and reversed amide bond triazole isosteres bound to avidin strongly; but complex 9 bound least strongly, indicating that longer linker lengths to the bulky DOTA complex were more favourable.

Comparison with other biotinidase-resistant conjugates

To compare this isostere-based strategy against other biotinidase-resistant approaches we took arguably the most studied DOTA ligand, the reduced biotinamidoethylamine–DOTA conjugate referred to as r-BHD^{17b} or ST2210³⁰ (Gd-complex 10, Fig. 2); for which an avidin dissociation constant ($K_D = 2.9 \times 10^{-7}$ M) has recently been determined by isothermal calorimetry (ITC).³¹ Ligand ST2210 was synthesised as previously described,^{17b} and both it and the conjugate of biotin-PEG3-amine with the mono-activated DOTA NHS ester (DO3A-NHS) were treated with $GdCl_3$ to effect the formation of amine-linked complex 10 and amide-linked complex 11 respectively. Synthesis of the isostere-based, biotinidase-resistant complex 12 was achieved through CuAAC coupling of alkyne 4 with a commercial bi-functional PEG3-aminoazide; followed by coupling to DO3A-NHS and Gd-complex formation as for complex 11. Each of the complexes (10–12) was purified by HPLC prior to the determination of avidin dissociation constants by ITC.³²

Since the binding of complexes 10–12 to avidin was predicted to be very strong, we determined K_D values in a series of competition experiments using amine-linked complex 10 as a reference.³³ Calorimetric titration of 10 was determined to be close to the limit of the experimental range ($K_D = 4.1 \pm 1.5 \times 10^{-9}$ M); the binding was assumed to be non-cooperative across the avidin tetramer and the ligand was shown to saturate each of the four binding sites. This measured binding affinity is $\sim 10^2$ fold greater than previously reported,³¹ but we attribute this to the rigorous purification of both the complex (by HPLC) and protein (by size exclusion) prior to

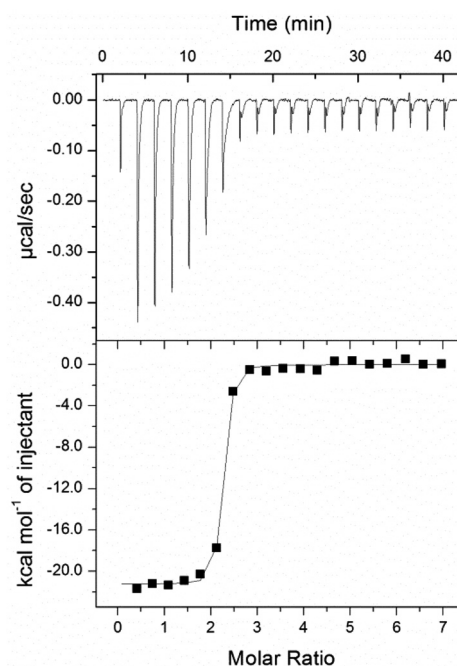


Fig. 5 Calorimetric titration of 12 into avidin in competition with 10 in sodium acetate buffer (100 mM; pH 5.5). Top panel: individual injections of a solution of 12 (and 10) into a solution of avidin (and 10). Bottom panel: titration curve, enthalpy/(mole of 12 injected) versus 12/(avidin) molar ratio.

measurement. To determine the binding affinities of amide-linked complex 11 and the natural biotin carrier biocytin 2, and compare them with the triazole-linked complex 12, the amine-linked complex 10 was added to bring the apparent equilibrium dissociation constant (K_D) closer to the range of the instrument (Fig. 5). The binding affinities of these amide- and triazole-linked ligands were determined to be in the low picomolar to femtomolar range ($K_D \leq 10 \times 10^{-12}$ M), and of equivalent strength to each other suggesting that the triazole-linked biotin mimics the natural biotin amide bond very closely in its binding to avidin.

Conclusions

We have demonstrated that a triazole-linked biotin conjugate derived either from alkynyl biotin 4, or from the related biotin azide 6, provides an excellent biotinidase-resistant isostere of the biotin-amide bond. Derivatives of biotin alkyne 4 linked to appropriate spacers have equilibrium dissociation constants which are equivalent to their amide counterparts, in contrast to other biotinidase-resistant linkages. Hydrolase-resistant biotin conjugates based on the triazole isostere will lead to efficient pre-targeting agents for therapeutic and imaging applications, and an enhanced understanding of the role of protein biotinylation where hydrolytic cleavage (*e.g.* by biotinidase, holocarboxylase synthetase *etc.*) might make this otherwise difficult to address.



Experimental

Preparation of biotin alkyne 4

(3*aS*,4*S*,6*aR*)-4-(Hex-5-yn-1-yl)tetrahydro-thieno[3,4-*d*]imidazole-2-one **4**. Biotin methyl ester³⁴ (0.320 g, 1.24 mmol) was dissolved in anhydrous DCM (8 mL), and the solution was cooled to -78 °C. DIBAL-H (2.09 mL, 2.30 mmol, 1.1 M solution in cyclohexane) was added over 10 min, and the mixture stirred at -78 °C for 4 h. The excess DIBAL-H was quenched with anhydrous methanol (5 mL), and the reaction mixture was allowed to warm to 0 °C. Potassium carbonate (0.340 g, 2.46 mmol), Ohira-Bestmann reagent (0.290 g, 1.51 mmol), and anhydrous methanol (5 mL) were added and the reaction stirred for 24 h. Further Ohira-Bestmann reagent (0.290 g, 1.51 mmol) was added and the reaction was stirred for a further 18 h. Rochelle's salt (10 mL) and ethyl acetate (20 mL) were added and the mixture stirred vigorously for 1 h. The organic layer was separated, washed with brine (3 × 20 mL), dried over magnesium sulfate, and the solvent was removed under reduced pressure. The crude mixture was purified by column chromatography (DCM-MeOH, 90:10) to give terminal biotin alkyne **4** as a colourless solid (0.13 g, 0.58 mmol, 47%). *R*_f (DCM-MeOH, 90:10) = 0.5; mp 158–162 °C; IR 3242 (NH), 2108 (C≡CH), 1703 (C=O); ¹H NMR δ (500 MHz, CDCl₃) 4.97 (1H, s, NHCHCH), 4.89 (1H, s, NHCHCH₂), 4.55 (1H, dd, *J* = 7.7, 4.6 Hz, NHCHCH), 4.37 (1H, dd, *J* = 7.7, 4.6 Hz, NHCHCH₂), 3.21 (1H, ddd, *J* = 8.0, 6.6, 4.6 Hz, SCH), 2.97 (1H, dd, *J* = 12.8, 5.0 Hz, CH_AH_BS), 2.77 (1H, d, *J* = 12.8 Hz, CH_AH_BS), 2.26 (2H, td, *J* = 6.5, 2.5 Hz, CH₂C≡CH), 2.00 (1H, t, *J* = 2.5 Hz, C≡CH), 1.75–1.65 (2H, m, CH₂), 1.62–1.55 (4H, m, 2 × CH₂); ¹³C NMR δ (126 MHz, CDCl₃) 162.9 (CO), 84.1 (C), 68.8 (CH), 61.9 (CH), 60.1 (CH), 55.3 (CH), 40.6 (CH₂), 28.1 (CH₂), 28.0 (CH₂), 27.9 (CH₂), 18.1 (CH₂); *m/z* (ESI+, MeOH) 471 ([2M + Na]⁺, 100%), 247 ([M + Na]⁺, 21), 225 ([M + H]⁺, 11); HRMS (ESI+, MeOH) [2M + Na]⁺ found 471.1864, C₁₁H₁₆N₂OS requires 471.1859.

Preparation of biotinidase-resistant complex 12

Triazole-linked biotin PEG3 amine 13. Biotin alkyne **4** (0.059 g, 0.263 mmol) and TBTA (0.1 eq.) were dissolved in *t*-BuOH-H₂O (2:1, 30 mL) and allowed to stir for 15 min. Sodium ascorbate (0.009 g, 0.044 mmol) was then added and allowed to stir for 15 min followed by copper(II) sulfate (0.006 g, 0.022 mmol). After a further 15 min 1-amino-11-azido-3,6,9-trioxoundecane (2.19 mL, 0.219 mmol; 0.1 M in H₂O) was added and the solution stirred under nitrogen at room temperature for 16 h. QuadraPure-IDA metal scavenger resin was added and the reaction mixture was gently shaken overnight. The resin was removed by filtration, and the aqueous solution extracted with EtOAc (2 × 20 mL). The aqueous solution was then lyophilised to afford crude triazole as a colourless solid. The crude product was purified by preparative RP-HPLC using a C18 column and H₂O-acetonitrile + 0.1% TFA as mobile phase to give triazole-linked biotin amine **13** as a colourless solid (76 mg, 65%). HPLC *R*_t = 38 min (SI Method 2); ¹H NMR δ (500 MHz, D₂O) 7.92 (1H, NCCHN),

4.66–4.59 (3H, m, NHCHCH, CHNCH₂), 4.42 (1H, dd, *J* = 7.9, 4.5 Hz, NHCHCH₂), 3.99 (2H, t, *J* = 4.9 Hz, CH₂O), 3.79–3.73 (2H, m, CH₂O), 3.70–3.60 (8H, m, 4 × OCH₂), 3.34 (1H, dt, *J* = 9.7, 5.1 Hz, SCH), 3.27–3.17 (2H, m, CH₂NH₂), 2.99 (1H, dd, *J* = 13.0, 5.0 Hz, CH_AH_BS), 2.79 (1H, d, *J* = 13.0 Hz, CH_AH_BS), 2.77 (2H, t, *J* = 7.3 Hz, CH₂CCH), 1.82–1.52 (4H, m, 2 × CH₂), 1.49–1.36 (2H, m, CH₂); ¹³C NMR δ (126 MHz, D₂O) 165.33 (CO), 148.13 (C), 123.57 (CH), 69.64 (CH₂), 69.58 (CH₂), 69.50 (CH₂), 69.45 (CH₂), 68.83 (CH₂), 66.35 (CH₂), 62.08 (CH), 60.23 (CH), 55.44 (CH), 49.90 (CH₂), 39.64 (CH₂), 39.09 (CH₂), 28.23 (CH₂), 27.65 (CH₂), 27.63 (CH₂), 24.13 (CH₂); *m/z* (ESI+, MeOH) 465 ([M + Na]⁺, 5%), 443 ([M + H]⁺, 100); HRMS (ESI+, MeOH) [M + H]⁺ C₁₉H₃₅O₄N₆S requires 443.2435, found 443.2438.

Triazole-linked biotin PEG3 Gd-DOTA complex 12. Triazole-linked biotin amine **13** (0.020 g, 0.045 mmol) was dissolved in a minimum volume borax solution (~0.5 mL; 0.08 M aq.) and cooled to 0 °C. To this was added a solution of the NHS-DOTA-ester (0.052 mg, 0.068 mmol) in borax solution (0.5 mL; 0.08 M aq.). The solution was adjusted to pH 7 with NaOH (1.0 M aq.) then warmed to room temperature and stirred for 24 h. The crude product was lyophilised to give a colourless solid that was purified by preparative RP-HPLC using a C18 column and H₂O-acetonitrile + 0.1% TFA as mobile phase to give the triazole-linked DOTA ligand as a colourless oil (0.010 g, 27%); *R*_t = 37 min (SI Method 2). The triazole-linked DOTA ligand (0.010 g, 0.012 mmol) was then dissolved in H₂O (1.0 mL) and the solution was adjusted to pH 7 using KOH (0.1 M aq.). GdCl₃·6H₂O (0.0045 g, 0.012 mmol) was dissolved in H₂O (1.0 mL) and then added to the ligand solution. After stirring for 15 minutes the solution was readjusted to pH 6 using KOH (0.1 M aq.) and then the reaction mixture was stirred at 60 °C for 4 h. The solution was cooled to room temperature and again adjusted to pH 6 using KOH (0.1 M aq.). Lyophilisation gave a colourless solid that was purified by preparative RP-HPLC using a C18 column and H₂O-acetonitrile + 0.1% TFA as mobile phase, to give triazole-linked biotin PEG3 Gd-DOTA complex **12** as a colourless solid (0.0077 g, 65%). HPLC *R*_t = 38 min (SI Method 2); *m/z* (ESI+, MeOH-NH₄OAc) 1006 ([¹⁵⁸GdM + Na]⁺, 46%), 984 ([¹⁵⁸GdM + H]⁺, 57), 595 (100). The appropriate isotope pattern was observed.

Acknowledgements

We thank the Wellcome Trust, University of Edinburgh (IKTF) and EC FP6 (Marie Curie Fellowship to JRH; contract MEST-CT-2005-020744) for financial support of this work. JL and DJC thank the BBSRC for funding (BB/I013687/1).

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