ChemComm

RSC Publishing

COMMUNICATION

View Article Online
View Journal

A tetrazine templated method for the synthesis of ternary conjugates†‡

Cite this: DOI: 10.1039/c3cc46634e

Received 29th August 2013,
Accepted 25th September 2013

DOI: 10.1039/c3cc46634e

www.rsc.org/chemcomm

Boddu Venkateswara Rao,[a] Snehal Dhokale,[b] Pattuparambil R. Rajamohanan[b] and Srinivas Hotha*[a]

Conjugation is an important reaction that enables coupling of molecules. Many protocols exist for the synthesis of binary conjugates from two different molecules or for the polyvalent display of a single molecule. There aren't many methods for the synthesis of ternary conjugates. However, methods for ternary conjugation are important for understanding the interplay of interactions between three biomolecules (or any three molecules *per se*). A strategy for ternary bioconjugation using inverse electron demand Diels–Alder reaction with tetrazine is studied. Ternary conjugation was demonstrated by the reaction of a model glyco-peptide binary conjugate with a fluorescent tagged olefin.

A chemical reaction wherein two individual molecules are coupled together covalently in the presence or absence of a reagent is known as a conjugation reaction and if the reacting partners are biomolecules then the process is known as bioconjugation.[1,2] Bioconjugation of molecules is important for studying various cellular events such as protein–protein interactions, imaging of cells, for the measurement of distances between epitopes, for modification of materials, *etc.*[1] Any conjugation method is known as bioorthogonal if the reaction conditions do not interfere with the functional groups, which frequently occurs in the highly heterogeneous biological samples.[3a] Such bioorthogonal reactions are significant for a range of applications comprising cell surface modification,[3b] protein engineering,[3c] and immunoassay development.[3d] Amidation,[3e] native chemical ligation,[3f] Staudinger ligation,[3b] azide–alkyne [3+2] cycloaddition ('click' reaction),[3g,h] olefin metathesis,[3i] imine formation,[3a] tetrazine-based ligation,[3j] and Michael addition[3k] are some of the bioconjugation methods;[3] among these, Staudinger ligation[3b] and click reaction[3g,h] between azide and alkyne are the most popular bioorthogonal reactions.[3j] Tetrazine-ligations were probed independently by Fox[4a] and Hilderbrand[4b] and since then, many reports have highlighted applications in biological[4c–f] and material science.[4g,h]

Most of the above methods are useful to study binary complexes as only two partners participate in the conjugation. In contrast, methods for multi- and poly-valent display of a single molecule exploiting dendrimers,[5a] nanoparticles,[5b] calixarenes,[5c] *etc.* as templates are known.[5] However, stepwise conjugation of molecules to get ternary complexes in a modular fashion is still a formidable challenge though many applications of such ternary conjugates can be envisioned (Fig. 1).[6,7] In this study, synthesis of ternary bioconjugates using dichlorotetrazine (**1**)[8] as a templating scaffold has been investigated.

Chlorine atoms in the dichlorotetrazine (**1**) moiety can undergo S_NAr reaction with nucleophiles whereas the tetrazine nucleus can participate in the inverse electron demand Diels–Alder (IEDDA) reaction.[4,9] Binary glycoconjugates resulting from S_NAr reaction of the tetrazine nucleus can be valuable probes for measuring

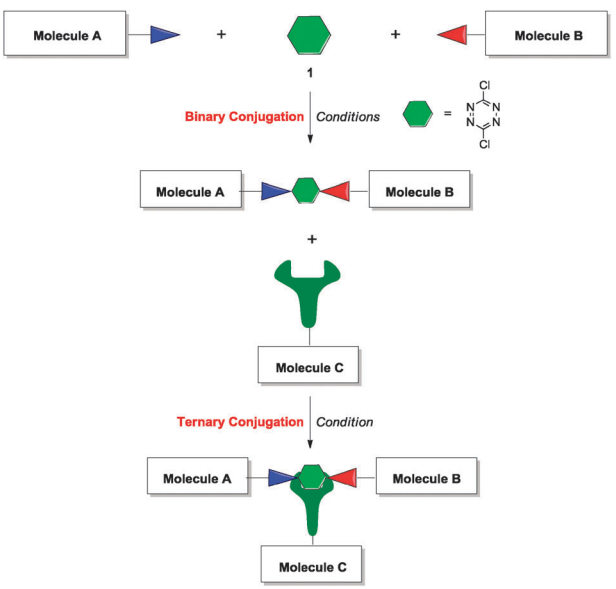


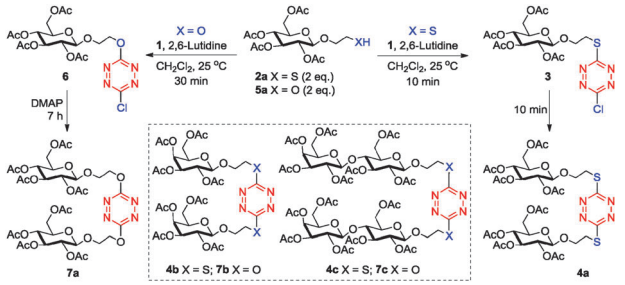
Fig. 1 Cartoon representation of the ternary conjugation.

[a] *Department of Chemistry, Indian Institute of Science Education and Research, Pune, India. E-mail: s.hotha@iiserpune.ac.in; Fax: +91 20 2589 9790; Tel: +91 20 2590 8015*

[b] *Central NMR Facility, National Chemical Laboratory, Pune 411 008, India*

† Dedicated to Prof. Krishna N. Ganesh on his 60th birthday.

‡ Electronic supplementary information (ESI) available: General experimental procedures and spectral charts of all compounds. See DOI: 10.1039/c3cc46634e



Scheme 1 Homodimeric binary glycoconjugates.

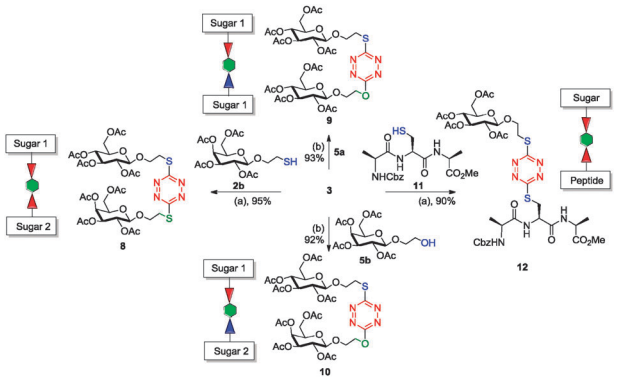
distances between carbohydrate binding sites and as reversible cross-linking agents. Further, binary conjugates can be extrapolated to ternary conjugates by IEDDA reaction.

To begin our investigation, 1-mercaptoethyl per-*O*-acetyl glucopyranoside (**2a**) was treated with tetrazine **1** and 2,6-lutidine in CH_2Cl_2 at 25 °C for 10 min to observe the formation of the monoaddition product (**3**) that further got converted to the binary glycoconjugate (**4a**) in 97% yield (Scheme 1).[10]

Similar conditions afforded *pseudo*-di- (**4b**) and tetra- (**4c**) saccharides in 98% and 95% yields respectively (Scheme 1). Glucoside **5a** was observed to give the monoaddition product **6** in 0.5 h and did not get converted to the binary conjugate. Addition of one molar equivalent of more nucleophilic DMAP led to the formation of binary conjugate **7a** in 95% yield.[8] A similar reaction with hydroxyethyl glycosides of protected galactose and lactose resulted in the formation of binary conjugates **7b** and **7c** in 92 and 90% yield respectively (Scheme 1). It is interesting to note that the tetrazine nucleus can be photochemically cleaved to release the glycan.[11]

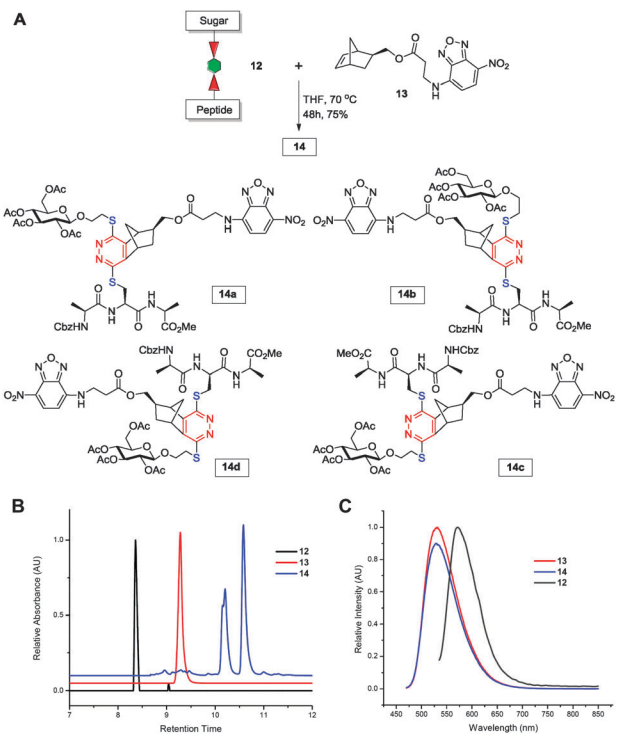
There can be two types of binary conjugates *viz.* homo- and hetero-linked conjugates. Further, heteroconjugates differ in the point of attachment or the type of molecule that is participating in the conjugation step. Successful identification of conditions for binary conjugation prompted the investigation of tetrazine chemistry for the synthesis of hetero binary conjugates. The homodimerization can be arrested at the monoconjugation level (*e.g.* **3**) and the tetrazine can be subjected to the heterodimerization. Accordingly, the chlorotetrazine **3** was isolated and subjected to the conjugation reaction with (i) another galactose-derived mercaptan **2b** to form tetrazine-linked gluco-galacto hybrid molecule **8**; (ii) compound **5a** to give tetrazine-linked gluco–gluco hybrid molecule **9**; (iii) galactose derivative **5b** to result in gluco-galacto hybrid molecule **10**. Heterodimers **8** and **10** differ in the point of attachment, dimers **9** and **10** differ in the type of sugar and dimers **8** and **9** differ in both the type of sugar and the point of attachment as well. Also, the chlorotetrazine **3** was treated with a model Cbz.Ala.Cys.Ala.OMe tripeptide (**11**) and 2,6-lutidine in CH_2Cl_2 at 25 °C for 10 min to obtain the sugar-peptide binary conjugate **12** in 90% yield (Scheme 2).[10]

Further conjugate **12** could serve as a model substrate for the IEDDA reaction to investigate ternary conjugation. Among the suite of molecules available for IEDDA reaction, fluorescently labelled alkene (**13**) was considered since the ternary conjugate can be identified by fluorescence spectroscopy.



Scheme 2 Heterodimeric binary glycoconjugates. Reagents: (a) 2,6-lutidine, 25 °C, 10 min; (b) DMAP (1 eq.), 25 °C, 7 h.

Accordingly, binary conjugate **12** and alkene **13** were refluxed in THF for 48 h to obtain a mixture of ternary conjugates **14a–d** in 75% yield (Scheme 3A). Formation of ternary conjugates **14** can be attributed to the fact that the Diels–Alder reaction occurred in an *exo*- or *endo*-manner, additionally releasing N_2 and oxidizing to the aromatic nucleus.[9,10] Attempted purification by preparative HPLC using a C18 column gave two fractions containing ternary conjugates which in turn were observed to contain two compounds each (Scheme 3B). The outcome of the ternary conjugation is confirmed by HRMS and many NMR experiments. The NMR spectra of **14** revealed resonances corresponding to all the three conjugating partners and the molecular weight of those fractions was found to be identical [calcd *m/z* $C_{53}H_{63}N_9O_{21}S_2$ + H: 1226.3658 and found: 1226.3662].[10]



Scheme 3 (A) Inverse electron demand Diels–Alder reaction for the synthesis of ternary conjugates, normalized overlay LC traces (B) and fluorescence emission spectra (C) of **12** (λ_{exc} = 412 nm), **13** (λ_{exc} = 460 nm) and **14** (λ_{exc} = 460 nm).

In the ^{1}H NMR spectrum of compound **14**, resonances due to acetyl groups of the sugar moiety were observed as three singlets around 2.10 ppm, methyl ester of the peptide was noticed as a singlet around 3.75 ppm and those of the NBD nucleus were identified around 6.28 and 8.53 ppm.[10] Furthermore, ternary conjugates **14** showed fluorescence emission λ_{max} at 528 nm matching with that of the NBD-ester **13**. It is appealing to observe that the fluorescence emission λ_{max} at 572 nm corresponding to the tetrazine moiety of binary conjugate **12** disappeared and hence confirming the formation of ternary conjugates **14** (Scheme 3C).

In summary, salient features of tetrazine chemistry were exploited for the synthesis of ternary conjugates. Binary homo- and hetero-dimeric glycoconjugates are prepared by reaction of mercaptoethyl or hydroxyethyl saccharides and the ternary conjugation was performed by inverse electron demand Diels–Alder reaction of the tetrazine nucleus. The method is modular and thus a suite of molecules can participate in ternary conjugation.

S.H. thanks the DST, New Delhi, for the SwarnaJayanthi Fellowship and B.V.R. thanks CSIR for the financial support.

References

1 C. I. Schilling, N. Jung, M. Biskup, U. Schepers and S. Brase, *Chem. Soc. Rev.*, 2011, **40**, 4840–4871.
2 (*a*) T. Clackson, in *Chemical Biology: From Small Molecules to Systems Biology and Drug Design*, ed. S. L. Schreiber, T. M. Kapoor and G. Wess, Wiley-VCH Verlag GmbH, Weinheim, Germany, 2008, ch. 4b; (*b*) P. A. Hessian and L. Fisher, *Eur. J. Biochem.*, 2001, **268**, 353–363; (*c*) A. Rutkowska and C. Schultz, *Angew. Chem., Int. Ed.*, 2012, **51**, 8166–8176; (*d*) R. Roy, in *Carbohydrate Chemistry*, ed. G. J. Boons, Chapman and Hall, UK, 1998, pp. 243–321.
3 (*a*) E. M. Sletten and C. R. Bertozzi, *Angew. Chem., Int. Ed.*, 2009, **48**, 6974–6998; (*b*) E. Saxon and C. R. Bertozzi, *Science*, 2000, **287**, 2007–2010; (*c*) J. A. Link, M. L. Mock and D. A. Tirrell, *Curr. Opin. Biotechnol.*, 2003, **14**, 603–609; (*d*) E. P. Diamandis and T. K. Christopoulos, *Clin. Chem.*, 1991, **37**, 625–636; (*e*) F. Albericio, *Curr. Opin. Chem. Biol.*, 2004, **8**, 211–221; (*f*) R. J. Clark and D. J. Craik, *Biopolymers*, 2010, **94**, 414–422; (*g*) C. W. Tornøe, C. Christensen and M. Meldal, *J. Org. Chem.*, 2002, **67**, 3057–3064; (*h*) Z. P. Demko and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2002, **41**, 2110–2113; (*i*) R. Dominique, S. K. Das and R. Roy, *Chem. Commun.*, 1998, 2437–2438; (*j*) R. K. V. Lim and Q. Lin, *Chem. Commun.*, 2010, **46**, 1589–1600; (*k*) C. E. Hoyle and C. N. Bowman, *Angew. Chem., Int. Ed.*, 2010, **49**, 1540–1573.
4 (*a*) M. L. Blackman, M. Royzen and J. M. Fox, *J. Am. Chem. Soc.*, 2008, **130**, 13518–13519; (*b*) N. K. Devaraj, R. Weissleder and S. A. Hilderbrand, *Bioconjugate Chem.*, 2008, **19**, 2297–2299; (*c*) C. M. Cole, J. Yang, J. Šečkutec and N. D. Devaraj, *ChemBioChem*, 2013, **14**, 205–208; (*d*) J. D. Thomas, H. Cui, P. J. North, T. Hofer, C. Rader and T. R. Burke, *Bioconjugate Chem.*, 2012, **23**, 2007–2013; (*e*) J. Schoch, M. Staudt, A. Samanta, M. Wiessler and A. Jäschke, *Bioconjugate Chem.*, 2012, **23**, 1382–1386; (*f*) E. Kaya, M. Vrabel, C. Deiml, S. Prill, V. S. Fluxa and T. Carell, *Angew Chem., Int. Ed.*, 2012, **51**, 4466–4469; (*g*) C. F. Hansell, P. Espeel and M. M. Stamenović, *J. Am. Chem. Soc.*, 2011, **133**, 13828–13831; (*h*) J. Malinge, C. Allain, L. Galmiche, F. Miomandre and P. Audebert, *Chem. Mater.*, 2011, **23**, 4599–4605.
5 (*a*) T. C. Shiao and R. Roy, *New J. Chem.*, 2012, **36**, 324–339; (*b*) J. Sebestik, P. Niederhafner and J. Jezek, *Amino Acids*, 2011, **40**, 301–370; (*c*) A. Dondoni and A. Marra, *Chem. Rev.*, 2010, **110**, 4949–4977.
6 (*a*) P. H. Seeberger, *Chem. Commun.*, 2003, 1115–1121; (*b*) R. A. Dwek, *Chem. Rev.*, 1996, **96**, 683–720; (*c*) P. M. Rudd, T. Elliott, P. Cresswell, I. A. Wilson and R. A. Dwek, *Science*, 2001, **291**, 2370–2376.
7 (*a*) W. R. Algar, D. E. Prasuhn, M. H. Stewart, T. J. Jennings, J. B. Blanco-Canosa, P. E. Dawson and I. L. Medintz, *Bioconjugate Chem.*, 2011, **22**, 825–858; (*b*) I. K. Guttilla and B. A. White, *J. Biol. Chem.*, 2009, **284**, 23204–23216; (*c*) C. L. Choi and A. P. Alivisatos, *Annu. Rev. Phys. Chem.*, 2010, **61**, 369–389; (*d*) L. I. Willems, N. Li, B. I. Florea, M. Ruben, G. A. Van der Marel and H. S. Overkleeft, *Angew. Chem., Int. Ed.*, 2012, **51**, 4431–4434.
8 (*a*) M. D. Coburn, G. A. Buntain, B. W. Harris, M. A. Hiskey, K. Y. Lee and D. G. Ott, *J. Heterocycl. Chem.*, 1991, **28**, 2049–2050; (*b*) D. E. Chavez and M. A. Hiskey, *J. Heterocycl. Chem.*, 1998, **35**, 1329–1332; (*c*) M. D. Helm, A. Plant and J. P. A. Harrity, *Org. Biomol. Chem.*, 2006, **4**, 4278–4280.
9 G. Clavier and P. Audebert, *Chem. Rev.*, 2010, **110**, 3299–3314.
10 See ESI‡.
11 M. J. Tucker, J. R. Courter, J. Chen, O. Atasoylu, A. B. Smith III and R. M. Hochstrasser, *Angew. Chem., Int. Ed.*, 2010, **49**, 3612–3616.