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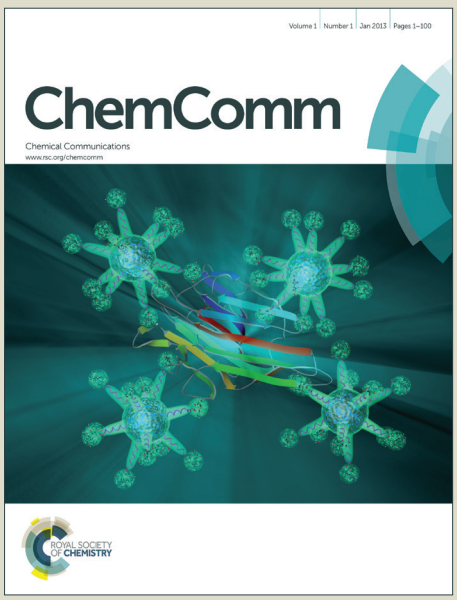

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COMMUNICATION

A multi-ligation strategy for the synthesis of heterofunctionalized glycosylated scaffolds

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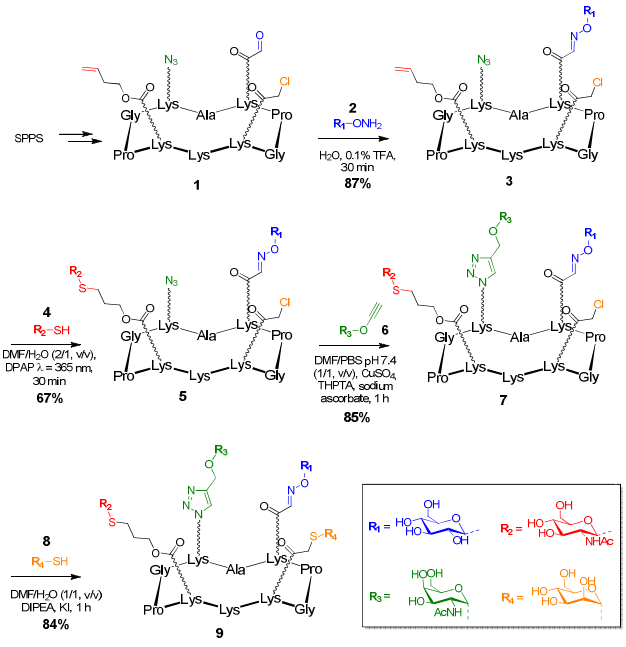
Well-defined heterofunctionalized glycosylated scaffolds with unprecedented molecular combinations have been prepared using up to five different bioorthogonal ligations. This approach opens up chemical access to a diversity of biomolecular structures with high biological potential.

The synthesis of multifunctional scaffolds decorated with diverse set of biomolecules such as carbohydrates, peptides and/or nucleic acids is of highest interest for therapeutic, diagnostic and theranostic applications.[1] The major synthetic hurdle to achieve this aim arises from the presence of diverse functional groups in these biomolecules which precludes the utilization of standard coupling chemistries based on successive – and often complex – protection/deprotection and activation protocols. A large panel of chemoselective ligations (also referred to as "click" chemistry) has been developed over the last decades to overcome these problems.[2] They now represent the methods of choice to synthesize diverse bioconjugates from unprotected building blocks in mild conditions with excellent yields and reproducibility. From the first bioactive molecules which have been prepared using single chemoselective reaction such as copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC),[3] thiol-ene coupling (TEC)[4] or oxime ligation (OL),[5] major progresses have been made towards the development of improved strategies based on either iterative assemblies[6] or dual[7] and triple[8] bioorthogonal ligations. However the synthesis of compounds with higher molecular diversity remains a tremendous challenge. Herein we demonstrate for this first time that a cascade methodology comprising up to five different bioorthogonal chemical reactions, *i.e.* OL, TEC, CuAAC, thiol-chloroacetyl coupling (TCC),[9] amide coupling and/or disulfide bond formation enables rapid access to unprecedented constructs combining carbohydrates, peptide, nucleic acid and labelling agents.

We focused our attention on a recent family of synthetic glycoconjugates that display different carbohydrate motifs, namely heteroglycoclusters (hGCs).[10] There is indeed a growing interest in the synthesis of these structures because they mimic the heterogeneous carbohydrates expression of biological systems. Therefore they represent ideal tools to deepen the current understanding of carbohydrate-protein interactions and to discover more active and selective ligands.[11] Owing to the advantageous structural features of cyclopeptides[12] and the large diversity of commercial protected amino acids, these scaffolds seems ideal systems to demonstrate the feasibility of our method.

The pentavalent scaffold **1** (Scheme 1) displaying five functional groups (*i.e.* glyoxoaldehyde, chloroacetyl, azide, alkene and amine) was prepared from a pre-functionalized, orthogonally protected linear peptide (see Electronic supplementary information). To avoid side reactions that may occur during the assembly process and to determine the best reaction sequence, we studied the chemical stability of these functional groups in conditions required for each bioorthogonal ligations. Because glyoxyaldehyde is the most sensitive group,[13] we decided to perform OL as the first step, followed by TEC, CuAAC and TCC in a stepwise strategy.



Scheme 1 Synthesis of the hGC **9**.

To synthesize hGC **9**, OL was performed with 1.2 equivalent of β-Glc hydroxylamine **2**[14] at room temperature in water containing 0.1% of TFA. After 30 minutes at room temperature, analytical HPLC indicated quantitative conversion of **1** into **3** (Fig. 1a). Pure compound **3** was obtained with 87% yield after purification by semi-preparative RP-HPLC. The photo-induced TEC was carried out in DMF/water in the presence of 3 equivalents of β-GlcNAc thiol **4**[14] and 2,2-dimethoxy-2-phenylacetophenone (DPAP) under UV irradiation (365 nm). The mixture was directly purified after 30 minutes to prevent addition

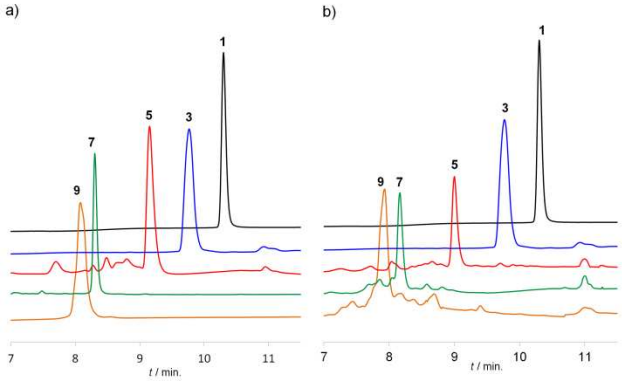
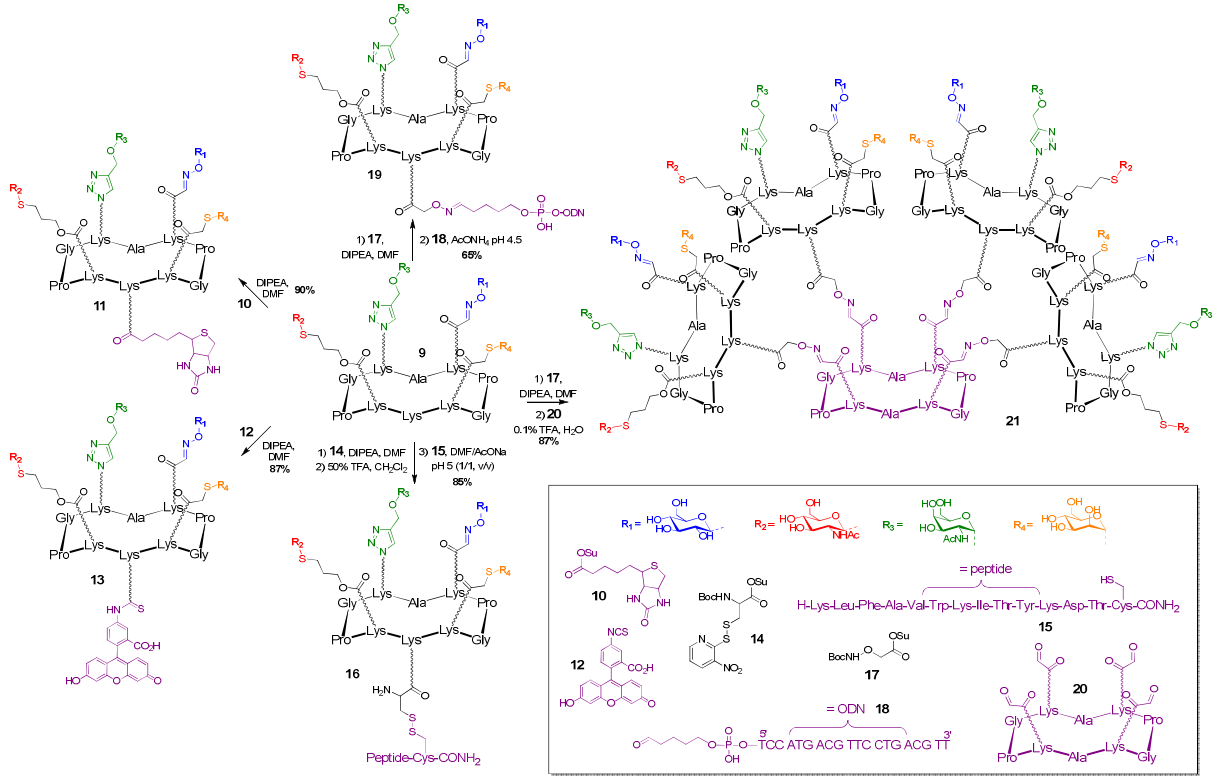


Fig. 1 Crude analytical RP-HPLC profile (λ = 214 nm) for each coupling reaction (*ie* blue: OL; red: TEC; green: CuAAC; orange: TCC) leading to compound **9**: a) Stepwise strategy (purification after each step); b) Sequential one-pot strategy (no intermediate purification).

of **4** to the chloroacetyl group even though such side reaction is pH dependant and unfavoured under these conditions.[15] The CuAAC ligation was next performed using α-GalNAc propargyl **6**[16] in the presence of $CuSO_4$, 3[tris(3-hydroxypropyltriazolylmethyl) amine (THPTA) and sodium ascorbate to provide **7** in 85% yield. The TCC reaction was finally carried out with α-Man thiol **8**[14] in a mixture of DMF/water in the presence of KI and *N*,*N*-diisopropylethylamine (DIPEA).[9] The pure tetravalent hGC **9** was isolated after purification in 84% yield, which corresponds to an overall yield of 41% after four successive bioorthogonal chemical reactions. Alternatively, we next investigated whether the attachment of carbohydrates could be realized using a sequential one-pot process to avoid requirement of intermediate purifications, to accelerate the assembly process and to increase the overall yield. For comparison, each reaction was thus performed in the same order and in conditions described above by simple adjustments of both pH and solvent in the crude mixture. It is noteworthy that whatever the concentration used (*ie* 2-20 mM), neither difference of reactivity nor loss of efficiency was observed. In all cases, similar reaction times were required to achieve complete conjugation (*i.e.* 3 hours for the total assembly) and clean crude mixture was observed for each step despite the accumulation of diverse reagents (Fig. 1b). More interestingly, final purification by RP-HPLC provided **9** in 47% overall yield, which clearly confirms that full assembly of tetra-heterovalent glycoclusters can be realized one-pot with higher efficiency than the stepwise strategy, and with a single purification.



Scheme 2 Conjugation of the hGC **9** with biotin (**10**), fluorescein (**12**), PV peptide (**15**), CpG oligonucleotide (**18**) and onto the cyclopeptide **20**.

With this tetravalent hGC in hands, we next evaluated the possibility to conjugate diverse range of biologically relevant structures to the remaining lysine side chain using different methods (Scheme 2). We first coupled labelling agent, *i.e.* biotin and fluorescein to the free amine function using biotin-succinimidyl ester **10** and fluorescein isothioscyanate **12**. After stirring 30 minutes at room temperature and purification, both conjugates **11** and **13** were obtained in 90 and 85% yields, respectively. Moreover, we introduced an antigenic peptide fragment from the type I-poliovirus protein containing a *C*-terminal cysteine (**15**). To this end, the cysteine **14** activated with *S*-3-nitro-2-pyridinesulfenyl (NPys) group was first coupled to the scaffold **9**. The conjugation was then performed in a mixture of DMF/AcONa (pH 5) and was found complete within one hour

to provide the conjugate **16** with 85% isolated yield. We next investigated whether OL can be used to prepare additional series of bioconjugates. Boc-aminooxy acetic acid **17**[17] was coupled to the scaffold **9** and the aminooxy group was subsequently deprotected by acidolysis. The resulting compound was reacted with a toll-like receptor 9 ligand, the CpG oligodeoxynucleotide **18** bearing an aldehyde at the 5'-end. The reaction occurred at room temperature in ammonium acetate buffer (pH 4.5) and was found complete within 2 hours. After purification, the CpG-hGC conjugate **19** was obtained in 65% yield. Finally, we reported recently that homo-hexadecavalent structures can be prepared with high efficiency using a divergent protocol including the self-condensation of cyclopeptides and carbohydrates by OL.[6e,18] We thus followed a convergent process to synthesize hetero-hexadecavalent structures from the aminooxylated hGC and the tetravalent cyclopeptide **20** displaying glyoxoaldehyde functions. The expected structure **21** was obtained in short reaction time (*i.e.* 1 hour) and excellent isolated yield (87%), which demonstrated the high versatility of this approach.

In conclusion, we described the first strategy comprising up to five different bioorthogonal conjugations to synthesize well-defined heteromultifunctional scaffolds. The strategy starts with either a stepwise or a sequential one-pot assembly of a tetravalent hGC displaying four different carbohydrate units by using fully compatible and orthogonal OL, TEC, CuAAC and TCC reactions. The resulting scaffold **9** which can be isolated in a total of 3 hours with a single purification step was further functionalized with diverse range of structures, *i.e.* biotin (**11**) and fluorescein (**13**) (by amide coupling), a poliovirus peptide fragment (**16**) (by disulfide bond formation) and the CpG oligodeoxynucleotide (**19**) (by OL). Moreover, a hexadecavalent hGC **21** was also synthesized by employing a convergent strategy using OL. Due to its modularity and versatility and the compatibility with carbohydrates, peptides and nucleic acids, we believe that this strategy might be applied to the construction of a larger variety of biomacromolecular systems. In particular, this strategy is currently used in our group to prepare fully synthetic multiantigenic synthetic vaccine candidates against tumors.[11a,19]

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Notes and references

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† Electronic Supplementary Information (ESI) available: Detailed description of synthesis of all compounds, RP-HPLC and MS analysis. See DOI: 10.1039/b000000x/

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