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Multifunctional bioconjugation by Morita–Baylis–Hillman reaction in aqueous medium†

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An efficient approach for modular assembly of multifunctional bioconjugates from oligosaccharides, peptides and proteins with fluorescent probes/affinity tags based on Morita–Baylis–Hillman (MBH) reaction in aqueous medium has been developed.

Selective modification of biomolecules is an important strategy for modulating the function of biomolecules and studying their roles in complex biological systems.[1] However, selective biomolecule modification is difficult to achieve as the bioconjugation reactions need to be chemoselective and efficient in aqueous medium within a narrow pH and temperature range. Thus, the development of new chemical methods for selective bioconjugation has attracted great attention.[2] In particular, oligosaccharides exhibit important biological functions including cell signalling, host–pathogen interaction, and cancer cell metastasis[3] and are thus attractive targets for bioconjugation.[4]

Aldehydes have long been used as bioorthogonal handles for bioconjugation of oligosaccharides.[5] Through periodate or galactose oxidase-mediated alcohol oxidation, aldehyde moieties could be easily introduced into oligosaccharides,[1] and then converted into oximes and hydrazones *via* C=N double bond formation. However, the drawbacks including slow reaction kinetics, acidic (pH 5–6) reaction conditions, cross-reactivity with metabolites[2b] and hydrolysis of C=N double bonds[6] restrict their use in biological applications.

The assembly of multifunctional biomolecules through multiple ligations provides an easy access to novel bioconjugates with high structural complexity. These multifunctional bioconjugates may contain alkyne moieties for further modification *via* click reaction, fluorescent probes for molecular imaging, and/or biotin tags for streptavidin-based immobilization.[7] Along with our ongoing direction toward bioconjugation research,[8] we envisioned that amine-catalyzed Morita–Baylis–Hillman (MBH) reaction[9] could be developed as an efficient approach for multifunctional modification of aldehyde-based oligosaccharides owing to its high selectivity and mild aqueous reaction conditions.[10]

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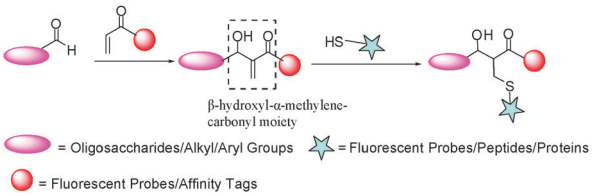
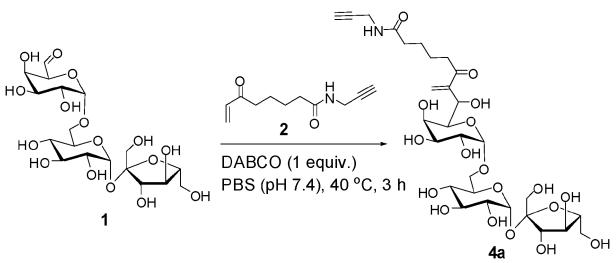


Scheme 1 MBH-based multifunctional bioconjugation.

Through the MBH reaction, the aldehyde moiety of oligosaccharides could be selectively modified by vinyl ketones *via* the formation of a β-hydroxyl-α-methylene-carbonyl moiety. Here we first report the use of the MBH reaction for multifunctional modification of oligosaccharides and demonstrate that the β-hydroxyl-α-methylene-carbonyl moiety could be further modified by thiol-based biophysical probes and cysteine-containing peptides and proteins. The present modular approach opens up a new direction for combinatorial assembly of multifunctional bioconjugates with high structural diversity (Scheme 1).

The MBH reaction of unprotected D-raffinose aldehyde **1** (10 mM) and vinyl ketone **2** (3 equiv.) in the presence of 1,4-diazabicyclo[2.2.2]octane (DABCO) (1 equiv.) in PBS buffer (50 mM, pH 7.4) at 40 °C for 3 h was conducted (Scheme 2). Vinyl ketone-modified D-raffinose **4a** with the hydroxyl groups remaining intact was obtained in 88% aldehyde conversion[11] by LC-MS analysis of the crude reaction mixture. In addition, our studies indicated that good to excellent conversion (65–97%) in the MBH coupling reaction of **1** and **2** could be achieved at pH 5.1–9.3 (Table S1 in ESI†). Apart from DABCO, a number of amines commonly used in catalyzing MBH reaction are also found to be effective.

We studied the time course of the MBH reaction of D-raffinose aldehyde **1** and vinyl ketone **2** in the presence of



Scheme 2 Modification of D-raffinose aldehyde **1** with vinyl ketone **2**.

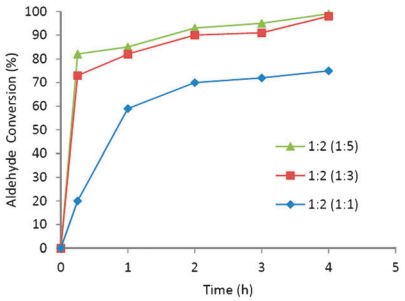
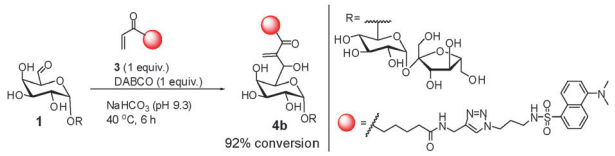


Fig. 1 Time course of the MBH reaction of D-raffinose aldehyde **1** with **2**.

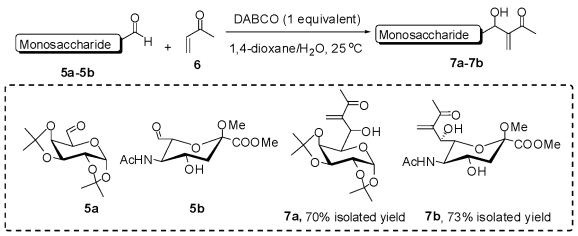


Scheme 3 Modification of D-raffinose aldehyde **1** with vinyl ketone **3**.

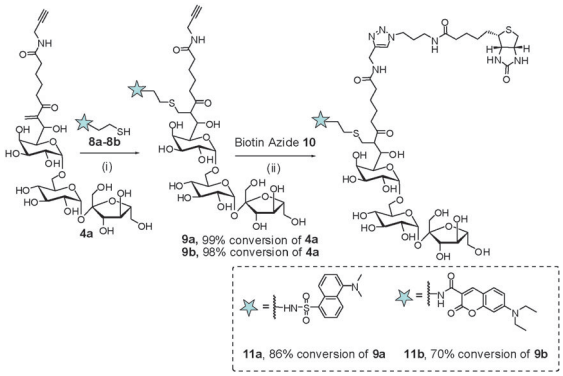
DABCO (1 equiv.) in $NaHCO_3$ solution (pH 9.3) at 40 °C (Fig. 1). When 3 equivalent of **2** was used, 73% aldehyde conversion was observed in 15 min. Up to 80% conversion was obtained in 1 h, and the conversion reached the maximum (98%) in 4 h. Using 5 equivalent of **2**, a slight increase in aldehyde conversion (82%) could be achieved in 15 min. Using 1 equivalent of **2**, good overall conversion (69%) was obtained in 4 h.

Next, we proceeded to study the MBH-based oligosaccharide bioconjugation using fluorescent vinyl ketone **3** (Scheme 3). Notably, using 1 equivalent of **3**, selective modification of D-raffinose aldehyde **1** with DABCO (1 equiv.) in $NaHCO_3$ solution (pH 9.3) gave fluorescent D-raffinose **4b** with 92% conversion at 40 °C in 6 h.

To provide support for the formation of the β-hydroxyl-α-methylene-carbonyl moiety *via* the MBH reaction, milligram scale reactions with monosaccharides **5a–5b** were performed. Reaction of protected galactose aldehyde **5a** and methyl vinyl ketone **6** (3 equiv.) with DABCO (1 equiv.) in 1,4-dioxane/water (1 : 1) at 25 °C for 12 h gave the MBH adduct **7a** in 70% isolated yield with a diastereomeric ratio (dr) of 86 : 14 by ^{1}H NMR analysis of the crude reaction mixture (Scheme 4).[12] Aldehyde-bearing sialic acid **5b** could be converted to the corresponding MBH adduct **7b** in 73% isolated yield with a dr of 88 : 12, and the absolute configuration of the major diastereomer of **7b** was determined by X-ray crystallographic analysis (Fig. S13, ESI†). The present MBH-based bioconjugation reaction features selective aldehyde modification *via* stable C–C bond formation that addresses the intrinsic problem of low hydrolytic stability of the C=N double bond in oxime-/hydrazone-based bioconjugation reactions.[6]



Scheme 4 MBH reaction of monosaccharide aldehydes **5a–5b** with **6**.



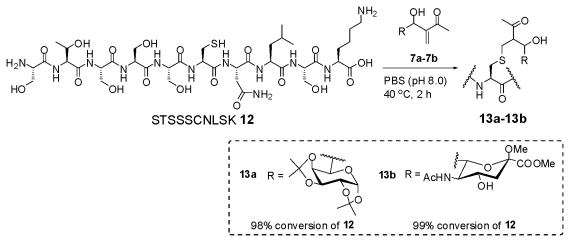
Scheme 5 Bifunctional modification of alkyne-bearing D-raffinose **4a** with thiol-based dansyl **8a** and coumarin **8b** followed by click reaction with biotin-azide **10**. Reaction conditions: (i) PBS buffer (pH 8.0), 40 °C, 2 h; (ii) $CuSO_4$, sodium L-ascorbate, 25 °C, 12 h.

Cross reactivity with ketone-containing metabolites such as pyruvate and oxaloacetate is an inherent problem in the oxime-/hydrazone-based bioconjugation reactions especially in cell-based applications.[7] We are pleased to find that no MBH adduct was detected in the reaction of methyl vinyl ketone **6** with methyl pyruvate and oxaloacetic acid, respectively, as confirmed by ^{1}H NMR and ESI-MS analysis.

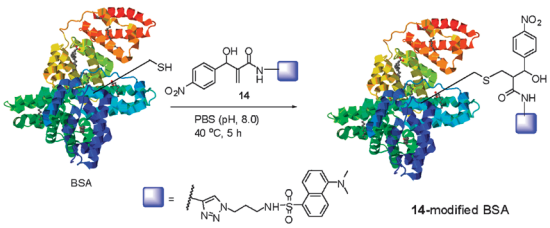
One unique advantage of the MBH-based bioconjugation reaction is that the β-hydroxyl-α-methylene-carbonyl moiety incorporated into oligosaccharides allows further ligation with thiol-based biophysical probes *via* conjugate addition. As illustrated in Scheme 5, alkyne-bearing D-raffinose **4a** was treated with thiol-based dansyl **8a** (1 equiv.) and coumarin **8b** (1 equiv.) at 40 °C for 2 h to give dansyl-D-raffinose **9a** and coumarin-D-raffinose **9b** with 99% and 98% conversions, respectively. Through click reaction with biotin azide **10**, **9a** and **9b** could be converted into the corresponding bifunctional biotin-dansyl-D-raffinose **11a** and biotin-coumarin-D-raffinose **11b** with 86% and 70% conversions. It is envisioned that the excellent versatility and compatibility of the MBH-based bioconjugation reaction sequence significantly expands the scope of multifunctional bioconjugate assembly.

The successful modification of the β-hydroxyl-α-methylene-carbonyl moiety of the MBH adducts with thiol-based biophysical probes encouraged us to explore the possibility of a more challenging ligation with cysteine-containing peptides. Gratefully, the coupling reaction of an unprotected cysteine-containing peptide STSSSCNLSK **12** (0.1 mM) with β-hydroxyl-α-methylene-carbonyl-bearing MBH adducts **7a** and **7b** (5 equiv.) in PBS buffer (50 mM, pH 8.0) at 40 °C for 2 h gave the corresponding products **13a** and **13b** with 98% and 99% conversions, respectively (Scheme 6). As confirmed by LC-MS/MS analysis, **7a** and **7b** were selectively ligated to the cysteine sulfhydryl group of **12** without modification of the N-terminal α-amino group or the side chains of serine and lysine.

Note that the ligation of the single, surface exposed cysteine residue of bovine serum albumin (BSA) to fluorescent dansyl-linked MBH adduct **14** (50 equiv.) was confirmed by SDS-PAGE and ESI-MS analysis (Scheme 7). It was found that **14**-modified BSA gave a fluorescent signal under UV excitation in SDS-PAGE protein analysis (Fig. 2). No modification was observed with lysozyme, which contains no free cysteine residue.



Scheme 6 Selective modification of cysteine-containing peptide STSSSCNLSK **12** with β-hydroxyl-α-methylene-carbonyl-bearing MBH adducts **7a–7b**.



Scheme 7 Selective modification of free cysteine-containing protein BSA with fluorescent dansyl-linked MBH adduct **14**.

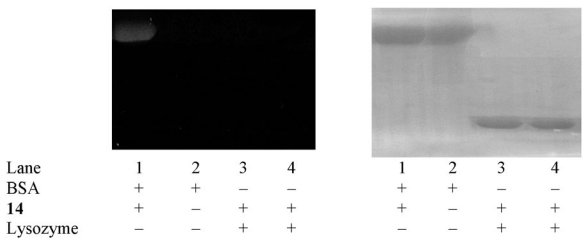


Fig. 2 SDS-PAGE of BSA, **14**-modified BSA and lysozyme: fluorescence visualization (left) and Coomassie staining (right).

The peaks at 66 547 Da (BSA) and 67 140 Da (**14**-modified BSA) revealed incorporation of one molecule of **14** per BSA.

In conclusion, we have developed an efficient MBH-based aldehyde bioconjugation reaction for multifunctional modification of oligosaccharides, peptides and proteins with fluorescent probes/biotin tags. The excellent compatibility of the MBH-based bioconjugation reaction with thiol-based bioconjugation and click reaction allows modular assembly of multifunctional bioconjugates that would have widespread applications.

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