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Synthesis of Type 1 Lewis b hexasaccharide antigen structures featuring flexible incorporation of L-[U-¹³C₆]-fucose for NMR binding studies

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While ¹³C-labelled proteins are common tools in NMR studies, lack of access to ¹³C-labelled carbohydrate structures has restricted their use. L-fucose is involved in a wide range of physiological and pathophysiological processes in mammalian organisms. Here, L-[U-¹³C₆]-Fuc labelled type I Lewis b (Le^b) structures have been synthesised for use in NMR binding studies with the Blood-group Antigen Binding Adhesin (BabA), a membrane-bound protein from the bacterium *Helicobacter pylori*. As part of this work, an efficient synthesis of a benzylated L-[U-¹³C₆]-Fuc thioglycoside donor from L-[U-¹³C₆]-Gal has been developed. The design and synthesis of an orthogonally protected tetrasaccharide precursor enabled controlled introduction of one or two ¹³C-labelled or non-labelled fucosyl residues prior to global deprotection. NMR analysis showed that it is straightforward to assign the anomeric centres as well as the H-5 positions to the individual fucosyl residues which are relevant for NMR binding studies.

Introduction

Helicobacter pylori is a spiral-shaped Gram-negative bacterium, which causes gastric and duodenal ulcers and is a major cause of stomach cancer. The bacterium selectively colonises the gastric epithelium. 1-5 Unlike other pathogens, Helicobacter pylori has evolved to survive the highly acidic environment by metabolising urea to ammonia and carbon dioxide by a nickel depending urease.⁶ ^{7, 8} The bacterium expresses at least five types of adhesins9 which enable adherence to the stomach epithelium and concomitant production of several cytotoxins that destroy stomach epithelial cells, creating painful ulcers. The resulting chronic inflammation promotes cell proliferation, and thus predisposes the host to stomach cancer. One of the five adhesins expressed by Helicobacter pylori is the membranebound protein "Blood-group Antigen Binding Adhesin" (BabA). BabA binds to type I Lewis b (Le^b) antigens expressed on the surface of gastric epithelial cells. Analyses of binding specificities of Helicobacter pylori strains from across the world suggest that BabA has evolved in response to host mucosal glycosylation patterns which permits *Helicobacter pylori* to adapt to its host and to maintain persistent colonisation.¹⁰⁻¹² A crystal structure of the carbohydrate-binding domain of BabA has been obtained while bound to a synthetic Lewis b structure. Analysis has revealed that the anchoring point for the binding of the glycan is the terminal disaccharide D-Gal α (1 \rightarrow 2) L-Fuc motif of the Le^b hexasaccharide.^{13, 14}

Detailed knowledge of these carbohydrate-protein interactions of Helicobacter pylori is crucial for understanding the structural and molecular basis for resulting diseases. While ¹³C-labelled proteins are common tools in NMR studies, lack of access to ¹³Clabelled carbohydrate structures has limited their use. Despite the difficulties arising from the need to synthesise the ¹³Clabelled material, ¹³C-enriched glycans are gaining traction as structure determination¹⁵ valuable tools for and conformational analysis¹⁶. Conformational analysis of a ligand bound to a lectin can be carried out by Saturation Transfer Difference (STD) and Transfer Nuclear Overhauser Effect (NOE) NMR experiments and do not require isotopically enriched material.17 Intramolecular NOE uses a combination of unlabelled carbohydrates and labelled proteins to extrapolate ligand-protein interactions^{18, 19}, while ¹³C-filtered NOESY can be used for identifying the conformation of bound ligands.^{20, 21} More recently, a combination of ¹³C-labelled carbohydrates and ¹⁵N (or ¹³C/¹⁵N) labelled proteins has been used to detail contact sites on the carbohydrate and the protein simultaneously.22 Availability of ¹³C-labelled Le^b structures would provide access to valuable tools for probing the interactions with BabA and other Le^b binding proteins.

Several strategies for the synthesis of type 1 Lewis structures have been reported. Danishefsky and co-workers have synthesised the Le^b tetrasaccharide and hexasaccharide using a polymer-based oligosaccharide preparation method with

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glycals as glycosyl donors.^{23, 24} Chernyak *et al.* have also reported the synthesis of the Le^b hexasaccharide using a convergent synthesis involving a tetrasaccharide thioglycoside donor and a spacer-equipped 3',4'-diol lactoside acceptor.²⁵ Furthermore, Lahmann *et al.* have reported the synthesis of the Le^b hexasaccharide, forming a tetrasaccharide acceptor *via* a [2+2] glycosylation which was then di-fucosylated.²⁶ Fournière *et al.* reported the synthesis of the Le^b pentasaccharide by first preparing the trisaccharide backbone, followed by difucosylation.²⁷ The Le^b tetrasaccharide was synthesised in addition to the Le^a trisaccharides by Ryzhov *et al.*²⁸ and Yan *et al.*²⁹ An enzymatic approach was also adopted by Chen and coworkers towards the truncated Le^b and Le^a structures.³⁰

Herein, we report the synthesis of a uniformly ¹³C labelled fucosyl donor as a general building block and an improved preparation of Lewis b structures *via* a linear approach. The flexibility of this approach allows access to novel mono and di L- $[U^{-13}C_6]$ -fucose labelled Le^b hexasaccharide structures, exemplified by the mono L- $[U^{-13}C_6]$ -Fuc-labelled Le^b hexasaccharide **1** and the di L- $[U^{-13}C_6]$ -Fuc-labelled Le^b hexasaccharide **2** (Scheme 1).

labelled material when the carbon source in the growth media 26J is ¹³C labelled.³¹

In previous syntheses of Le^b hexasaccharide structures, a per-Obenzylated fucosyl thioglycoside donor has been used successfully, and therefore the analogous L-[U-13C6]-Fuc 4 (Scheme 1) was envisioned as a suitable labelled donor. Since L-[U-13C₆]-Fuc is prohibitively expensive, the less costly L-[U-13C₆]-Gal was selected as starting material. The synthetic route was elaborated using unlabelled D-Gal (S-24-S-29, supplementary material) and then applied to $L-[U^{-13}C_6]$ -Gal (Scheme 2). Thus, after a near quantitative conversion of L-[U-13C₆]-Gal into its pentaacetate 932, 33, a thiotolyl group was introduced at the anomeric position using p-thiocresol in the presence of BF₃·Et₂O (98% yield).³⁴ Deacetylation of **10**, followed by protection of the 6-position as TBDMS silvl ether and subsequent benzylation of the remaining free hydroxy groups, furnished L-galactoside 11 in a 75% yield over 3 steps. The silyl ether was removed with TBAF (12, 96% yield) and the 6-position was tosylated, producing a 94% yield of compound 13. Reduction with LiAlH₄ in THF (61% yield) gave the desired L-[U-¹³C₆]-fucosyl donor 4 in an overall yield of 41% from $L-[U-^{13}C_6]$ -Gal.



Scheme 1. The target structures and the building blocks shown in a simplified retrosynthesis scheme: mono L-[U-¹³C₆]-Fuclabelled Le^b hexasaccharide **1**, di L-[U-¹³C₆]-Fuc-labelled Le^b hexasaccharide **2** and the unlabelled Le^b hexasaccharide **3** were prepared from the labelled and unlabelled building blocks **4** – **8**.

Results and discussion

The easiest access to isotopically enriched monosaccharides is via biosynthesis, which provides 100% uniformly [U-13C]



Scheme 2. Reagents and conditions: a) 1. NaOAc, Ac₂O, 140 °C, 30 min, 99 % (9); 2. MePhSH, BF₃·Et₂O, CH₂Cl₂, rt, 2 h, 98% (10); b) 1. NaOMe, MeOH, rt, 30 min, 99%; 2. TBDMSCI, pyridine, 0 °C to rt, 4 h, 95%; 3. BnBr, NaH, DMF, 0 °C to rt, 2 h, 79% (11); c) Bu₄NF, THF, rt, 16 h, 96%; d) TsCI, pyridine, rt, 3 h, 94%; e) LiAlH₄, THF, reflux, 61%.

As expected, complex coupling patterns due to ${}^{13}C{}^{-1}H$ and ${}^{13}C{}^{-1}$ couplings were observed for the labelled L-Fuc donor **4** in the ${}^{1}H$ and ${}^{13}C$ NMR spectra respectively but decoupling produced the matching spectra for the literature known unlabelled donor L-Fuc **5**³⁵ (Supplementary Figure 1).

The corresponding unlabelled L-Fuc 5^{35} , the D-GlcNH₂ 7^{36} , the D-Gal 8^{37} thioglycoside donors, and the D-Lac acceptor 6^{38} (Scheme 1) were prepared according to literature procedures. The assembly of the tetrasaccharide backbone 15 started with the NIS/AgOTf-mediated glycosylation at room temperature with D-Lac acceptor 6 and D-GlcNH₂ donor 7 to give the corresponding 1,2-*trans* linked trisaccharide 14^{38} (58%) (Scheme 3). The phthalimido and acetyl protecting groups were **Organic & Biomolecular Chemistry Accepted Manuscrip**

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removed simultaneously with EDA in EtOH. Subsequent selective *N*-acetylation was performed with Ac₂O in MeOH or toluene/MeOH (2:1, v/v), furnishing 3"-OH acceptor **15** (76%). In previous studies, we observed orthoester formation as a common side product during the glycosylation of acceptor **15** with the 2-*O*-acetyl analogue of donor **8**. 2-Benzoate protected glycosyl donors are less prone to orthoester formation.³⁹,⁴⁰ The 2-*O*Bz thioglycoside donor **8** was successfully trialled and underwent glycosylation with acceptor **15** promoted by NIS and AgOTf (cat.) to furnish tetrasaccharide **16** in an 81% yield.



Scheme 3. Reagents and conditions: a) 7, NIS, AgOTf (cat.), 4 Å molecular sieves, CH_2Cl_2 , rt, 20 min, 58%; b) 1. EDA, EtOH, reflux, 16 h; 2. Ac₂O, MeOH/toluene (2:1, v/v), rt, 30 min; 76% over 2 steps; c) 8, NIS, AgOTf (cat.), 4 Å molecular sieves, CH_2Cl_2 , rt, 20 min – 2 h, 81%.

The orthogonal protecting group pattern of tetrasaccharide **16** permits the preparation of three different acceptors, and thus provides flexibility with respect to the position and number of L-[U- $^{13}C_6$]-Fuc residues to be incorporated. One step deprotection exposes either the 4"-OH group when using reductive ring opening conditions (acceptor **17**) or the 2"'-OH position under Zemplén conditions (acceptor **18**), whilst sequential deprotection of both positions provides the corresponding diol acceptor **19**.

For the synthesis of the fully protected 2'"-mono L-[U-13C6]-Fuc labelled Le^b hexasaccharide 22 (Scheme 3), tetrasaccharide 16 was subjected to reductive ring-opening conditions with NaBH₃CN and HCI/Et₂O in THF to reveal the 4"-OH, giving acceptor 17 (85%).^{41,42} Acceptor 17 underwent glycosylation with L-Fuc donor 5 using Lemieux's halide-assisted methodology to ensure α -selectivity. ⁴³ This produced pentasaccharide 20 in a moderate yield of 45%. The removal of the 2"-benzoyl group under Zemplén conditions required extended reaction times and a slightly elevated temperature in order to unmasked the second fucosylation site (21, 93%). The L-[U-¹³C₆]-Fuc residue was installed using donor **4** and Lemieux's halide-assisted glycosylation conditions, and the 2'''-mono L-[U-¹³C₆]-Fuc labelled Le^b hexasaccharide 22 was obtained in a 79% yield. A similar reaction sequence with stepwise introduction of the fucosyl residues was also carried out with acceptor 18 to give the unlabelled 2"-fucosylated pentasaccharide (73%).

Subsequent reductive ring-opening of the benzylider acetal 26J (66%) and a repeated fucosylation with unlabelled L-Fuc donor 4 provided the fully protected, unlabelled Le^b hexasaccharide 24, demonstrating the feasibility of this approach to access also the 4''- mono L-[U-¹³C₆]-Fuc labelled Le^b hexasaccharide.



Scheme 4. Reagents and conditions: a) NaBH₃CN, 2 M HCI/Et₂O, 3 Å molecular sieves, THF, rt, 40 min, 85%; b) Br₂, Et₄NBr, 4 Å molecular sieves, CH₂Cl₂/DMF (9:1, v/v), rt, 16 h (donor 5: 20, 45%; donor 4: 22, 79%; 4: 23, 52%; 5: 24, 80%); c) 1 M NaOMe/MeOH, MeOH, rt to 40 °C (21, 26 h, 93%) and 1 M NaOMe/MeOH, MeOH/CH₂Cl₂ 7:1, rt to 40 °C (18, 48 h, 73%) and (19, 64 h, 88%).

The di-L-[U-¹³C₆]-Fuc labelled hexasaccharide **23** and unlabelled Le^b hexasaccharide **24** are also accessible from diol acceptor **19** obtained from tetrasaccharide **17** by debenzoylation (88%, Scheme 4). Interestingly, this transesterification was even slower than the corresponding reaction on the mono fucosylated pentasaccharide **21**. As above, the unlabelled L-Fuc donor **5** was converted into the corresponding bromide in the presence of acceptor diol **19**, with the glycosylation facilitated by Et₄NBr to give the protected unlabelled hexasaccharide **24** in an 80% yield (Scheme 4). The same halide-assisted glycosylation conditions were applied to the L-[U-¹³C₆]-Fuc donor **4** and diol acceptor **19** to produce the di-L-[U-¹³C₆]-Fuc labelled hexasaccharide **23** in a moderate yield (52%).

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Scheme 5. Reagents and conditions: a) H₂, Pd/C, 0.1 M aq. HCl, 1,4-dioxane/H₂O (3:1, v/v), rt, 2 d, (**1**, 87%; **2**, 30%; **3**, 96%).

The three hexasaccharides **22**, **23** and **24** were globally deprotected by Pd-catalysed hydrogenolysis to give targets **1**, **2** and **3** respectively. A variety of hydrogenolysis conditions were attempted. The presence of aqueous HCI was found to be crucial for efficient progression of the reaction in all cases, while a range of solvents were tolerated. The use of a mixture of heterogeneous catalysts (Pd/C and Pd(OH)₂/C) was found to be beneficial in similar unpublished work but not for the compounds presented here. Applying optimised conditions, the deprotections proceeded very well for both the 2^{'''}-mono L-[U-¹³C₆]-Fuc hexasaccharide **22** and the unlabelled hexasaccharide **24**, giving the mono-labelled (**1**, 87%) and unlabelled (**3**, 96%) Le^b hexasaccharides in excellent yields. After deprotection of **23**, a low yield of the di-L-[U-¹³C₆]-Fuc labelled hexasaccharide **2** (30%) was recorded.

¹H NMR and ¹H-¹³C-HSQC was used to assign most of the signals and a CLIP-HSQC experiment confirmed the presence of two α -Fuc linkages and β -linkages for the remaining four glycosidic bonds of the known unlabelled Leb hexasaccharide 3 (Figure 1 A and B). 1D TOCSY spectra were generated by selective excitation of each of the six H-1 signals individually (Figure 1 C). In combination with the NMR data obtained for the monolabelled hexasaccharide 1 (vide infra), this enabled us, to assign undoubtedly the anomeric and the H-5 signals - which appeared in the same region occupied by the anomeric signals, to the individual 2" and the 4" fucosyl residues. There was no substantial difference in the chemical shift between the methyl groups of the two fucosyl residues which are otherwise distinct signals. The ability to distinguish between the individual fucosyl residues is of importance for NMR binding experiments with the labelled material.



Figure 1. – **A**: ¹H NMR spectrum of the unlabelled Le^b hexasaccharide **3**; **B**: CLIP-HSQC of **3**; **C**: 1D TOCSY experiments for **3**. The anomeric signals were selectively excitated to generate spectra for each individual residue and then compared to the overall ¹H NMR spectrum (bottom).

The ¹H NMR spectrum of di-¹³C-labelled hexasaccharide **2** shows the expected additional ¹³C-¹H couplings corresponding to the labelled fucosyl residues (Figure 2A) which collapsed in the ¹³C-¹H decoupled experiments, reassembling the corresponding spectrum of the unlabelled hexasaccharide **3**. A standard ¹³C NMR experiment displays only the ¹³C enriched fucosyl residues, clearly showing the ¹³C-¹³C couplings, and makes it a

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convenient experiment to verify the incorporation of labelled material (Figure 2B).



Figure 2. – A: ¹H NMR spectrum of di- α -L-[U-¹³C₆] fucosylated **2** with ¹³C-¹H couplings visible (bottom, red), ¹³C-¹H decoupled spectrum of **2** (middle, blue), and for comparison the ¹H NMR spectrum of the unlabelled hexasaccharide **3** (top, green); **B**: ¹³C NMR spectrum of the di- α -L-[U-¹³C₆] fucosylated hexasaccharide **2**. Only the signals from the uniformly labelled fucosyl residues are visible; **C**: Direct comparison of the ¹³C NMR spectrum of mono 2^{'''}- α -L-[U-¹³C₆] fucosylated hexasaccharide **1** (bottom, red) with the ¹³C NMR spectrum of the di labelled hexasaccharide **2** (top, blue) allows unambiguous identification of the anomeric carbons of the fucosyl residues. There is no significant difference in the C-6 shifts between the fucosyl residues; **D**: The coupled (bottom, red) and ¹³C-¹H decoupled (top, blue) ¹H NMR spectra of the 2^{'''}- α -L-[U-¹³C₆] fucosylated hexasaccharide **1**. The stacked view allows unambiguous assignment of the anomeric and H-5 signals for the individual fucosyl residues.

The ¹H NMR spectrum of the mono-labelled hexasaccharide **1** shows again the expected ¹³C-¹H couplings from the 2^{'''}- α -L-[U-¹³C₆] Fuc residue, while the ¹³C-¹H decoupled spectrum overlays with the unlabelled hexasaccharide **3**. The presence of the ¹³C-¹H couplings makes the identification of the anomeric proton and the H-5 for the 2^{'''}- α -1 connected Fuc residue straightforward (Figure 2D). This information can also be obtained by comparing the ¹³C NMR data of di-labelled compound **2** to the ¹³C NMR data for the 2^{'''} mono labelled compound **1**, which shows the anomeric carbon of the α -L-[U-

¹³C₆] Fuc-(1 \rightarrow 2)-β-D-Gal linkage to appear downfield to the α-L-[U-¹³C₆] Fuc-(1 \rightarrow 4)-β-D-GlcNAc bond (Figure 2C).

Experimental

All experimental data are available in the supplementary file.

Conclusions

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In this work, an efficient synthesis of a ¹³C-labelled L-fucosyl donor was developed from L-[U-13C₆]-Gal. The synthesis of the L-[U-¹³C₆]-fucose donor **4** was carried out on a 500 mg scale in a yield of 41% over 8 steps. This uniformly ¹³C labelled fucosyl donor is a valuable building block for the preparation of labelled oligosaccharides for NMR binding studies. Thus, L-[U-13C6]-Fuc labelled type I Lewis b (Le^b) structures have been synthesised for use in NMR binding studies with BabA, a membrane-bound protein from the bacterium Helicobacter pylori. An orthogonally protected tetrasaccharide 16 allowed the regioselective introduction of one or two ¹³C labelled fucose residues producing the protected ¹³C labelled hexasaccharides 22 and 23. Subsequent hydrogenolysis afforded the differently ¹³C labelled hexasaccharides 1-3. NMR analysis showed that it is straightforward to distinguish between the differently positioned fucosyl residues in the oligosaccharides. Making those and related structures a valuable tool for NMR proteincarbohydrate binding studies.

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