Chemical synthesis of glycans up to a 128-mer relevant to the O-antigen of Bacteroides vulgatus

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Glycans are involved in various life processes and represent critical targets of biomedical developments. Nevertheless, the accessibility to long glycans with precise structures remains challenging. Here we report on the synthesis of glycans consisting of \( \alpha\text{-(1}\rightarrow\text{3})\beta\text{-Man}\) repeating unit, which are relevant to the O-antigen of Bacteroides vulgatus, a common component of gut microbiota. The optimal combination of assembly strategy, protecting group arrangement, and glycosylation reaction has enabled us to synthesize up to a 128-mer glycan. The synthetic glycans are accurately characterized by advanced NMR and MS approaches, the 3D structures are defined, and their potent binding activity with human DC-SIGN, a receptor associated with the gut lymphoid tissue, is disclosed.

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Glycans represent the most abundant biopolymers in nature, which constitute prominent parts of all living organisms and mediate fundamental biological processes. Short glycans (oligosaccharides) with less than 20 monosaccharide units, such as those being conjugated with lipids and proteins on the cell surfaces, are usually sufficient to fulfill various biological functions, including cell signaling, adhesion, and migration. Longer glycans, such as glycosaminoglycans, display both structural properties and various physiological and pathological functions. Polysaccharides, such as cellulose and starch/glycogen, serve for structural requirements or energy storage. The microbial glycans are structurally and functionally extremely diverse. For example, those on the microbe cell envelope mediate host–microbial cross-talk and play a critical role in the activation of the immune system. The development of robust strategies for the synthesis of well-defined carbohydrate structures is nowadays a top priority and has an enormous impact in various biomedical sectors; in this context, synthetic carbohydrate–vaccines, as well as glycoconjugates-based adjuvants, have been produced. Paradoxically, functional studies on long glycans with precise structures have been rarely performed, due to the extremely difficult acquisition of pure long glycans via either isolation from natural sources or via chemical synthesis from mono- and oligosaccharide units. Indeed, natural glycans occur as heterogeneous mixtures, or despite showing homogeneous repeating units exhibit a range of size distributions. The chemical synthesis of long glycans has been limited by the notorious low efficiency of the glycosidic coupling and protecting group manipulations. Consequently, glycans containing over 20 monosaccharide units have been synthesized only occasionally, contributing to the general idea that chemical synthesis of long glycans are not yet of practical usefulness. In this regard, a paradigm shift in the functional studies of long glycans with precise structures requires the advancement of feasible approaches to their preparation, bringing innovation to the conventional synthetic carbohydrate chemistry.

**Bacteroides vulgatus** mpk is a commensal strain occurring commonly in the gastrointestinal tract of American and Western European population. Recent studies demonstrated that this bacterium exerted strong immune-modulating properties leading to the prevention of colitis-induction in mouse models, and the Bacteroides lipopolysaccharide (LPS) induced hyporesponsiveness towards subsequent LPS-stimuli. Also, the administration of Bacteroides LPS re-established intestinal immune homeostasis in a mouse model for experimental colitis, thus correlating the health-promoting effects to the weak agonistic properties of this LPS.

Further, in the human innate immune system, we demonstrated a relevant capability of the LPS to induce anti-inflammatory cytokines. The α-(1→3)-β-Mann-(1→4)-Rha of this repeat unit is highlighted in red, and the β-(1→4) glycosidic bond between Man and Rha in blue. Source data are provided as a Source Data file.

**Results**

**Syntheses of the glycans.** The target glycans consist of two types of glycosidic linkages, i.e., Rha-(1α→3)-Man and Man-(1β→4)-Rha. The β-mannopyranoside linkages are known to be difficult to construct in a highly stereoselective manner, while the α-rhamnopyranoside linkages are among the easiest to synthesize. This knowledge forced us to fix the requisite β-mannopyranoside linkage at a disaccharide level and to extend the glycan resorting to α-rhamnopyranosylation. Such a disaccharide building block required orthogonal protection at the Rha 1-OH and Man 3-OH, which could be transformed into the corresponding donor and acceptor for a convergent glycan elongation. Disaccharide 82, bearing MP (p-methoxyphenyl) and TBS (tert-butyldimethylsilyl) group at the reducing and non-reducing end, respectively, was found to be effective in the present work, in which the 4,6′-O-benzylidene and 2′-O-benzyl group were used to facilitate the construction of the β-mannopyranoside linkage and the 2′-O-benzoyl group to secure the α-rhamnopyranosylation in the subsequent glycan assembly (Fig. 2).

Although the bulky 3′-O-TBS group installed at the manno-pyranoside donors was known to be detrimental to the β-selectivity in mannosylation, we managed to prepare the desired disaccharide building block 8 in decagram scales with a convenient and inexpensive procedure (Supplementary Fig. 1 & Supplementary Table 1). Removal of the TBS group with TBAF (THF, rt) led to disaccharide acceptor 84 smoothly; at this stage, the β/α isomers resulted from the mannosylation step could be easily separated. The desired 6-hexynylbenzoate donor 8D was prepared from 8 via two steps, i.e., oxidative removal of the anomic MP group with CAN (CH2Cl2, MeCN, H2O, 0 °C) and condensation with 6-hexynylbenzoic acid (EDCI, DMAP, CH2Cl2, rt). As expected, the glycosidic coupling between disaccharide donor 8D and acceptor 8A proceeded smoothly under the standard gold(I)-catalyzed conditions (0.1 equiv. Ph3PAuNTf2, 5 Å MS, CH2Cl2, 0 °C)30, leading to tetrasaccharide 9 in 92% yield at a decagam scale, with the α-rhamnopyranoside linkage being exclusively formed.

The convenient synthesis of 4-mer 9 from 2-mer 8 established an effective approach toward assembly of the 2n+1-mer glycans via repetition of a cycle of three transformations (Fig. 2), including (1) selective cleavage of the anomeric MP group (with CAN) and condensation of the resulting hemiacetal with 6-hexynylbenzoic acid to provide the donor (steps a and b); (2) selective removal of the non-reducing end TBS ether (with TBAF) to provide the acceptor (step c); and (3) coupling of the donor and acceptor (under the catalysis of Ph3PAuNTf2) to furnish the 2n+1-mer glycan (step d). These transformations were found robust, so that the syntheses of 8-mer 10, 16-mer 11, and 32-mer 12 met with no incident in gram-scales.

Further transformations starting from 32-mer 12 (molecular weight (Mw) = 11,354) were challenged by the decreased solubility of the macromolecular substrates. Thus, removal of the TBS ether on 12 was carried out at a low concentration of 14.0 mM in THF; hydrolysis of the anomer MP group at 2.2 mM in CH2Cl2/MeCN/H2O and condensation with 6-hexynylbenzoic acid at 2.1 mM in CH2Cl2. These transformations were clean so that the desired 32-mer acceptor 12A (88%) and donor...
Two sets of glycosylation products were prepared via two iterative cycles to prepare glycans up to 128-mer (Fig. 2a). In the first cycle (Fig. 2b), the terminal reducing and nonreducing units were coincident and clearly distinguishable from the molecular size repeating units were coincident and clearly distinguishable from the preceding steps and avoided detrimental impurities from the proceeding steps and avoided degradation of the aldehyde. Nevertheless, hydrogenolysis of the longer glycans starting from 16-mer 13 was found difficult to complete, because the gradually releasing hydroxyl groups could cause an unwanted decrease of the solubility in the reaction solvents. Thus, repetitive hydrogenolysis was performed after the removal of the benzoates to cleave the remaining benzyl groups in a mixture solvent of MeOH/H2O/HOAc. Moreover, a combination of 10% Pd/C and 10% Pd(OH)2 was used as the catalyst. The complete and clean reactions enabled acquiring the free glycans with satisfactory purity via simple filtration through Sephadex G-25 (for 1–5) or LH-60 (for 6 and 7).

### Structural characterization.

The fully protected and free glycans were well characterized by a combination of NMR and MS analyses. As for the free glycans 1–7, the 1H-NMR spectra (Supplementary Figs. 3–7; Supplementary Table 6) were coincident and clearly distinguishable from the terminal repeating units and nonreducing units. The molecular size of the glycans was estimated by the integration of the 1H-NMR signals (ratio of internal and terminal units) and by the retention time on GPC (Supplementary Fig. 2); for the longer size

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**Fig. 2 Iterative assembly to the preparation of glycans up to 128-mer (7).** a CAN, CH2Cl2, MeCN, H2O, 0 °C; b o-hexynylbenzoic acid, EDCI, DMAP, CH2Cl2, rt; c TBAF, HOAc, THF, rt; d Ph3PAuNTf2 (0.1 or 0.2 equiv.), 5 Å MS, CH2Cl2, 0 °C; e BzCl, Et3N, CH2Cl2, rt; f H2, 10% Pd/C (and 20% Pd(OH)2), solvent, rt; g NaOCH3, CH3OH, rt. The experimental procedures varied slightly for substrates of different sizes (Supplementary Tables 2–5), and the hydrogenolysis (step f) was repeated a couple of times after step g until the benzyl groups were fully removed. CAN cerium (IV) diaminonitrinate, DMAP 4,4-dimethylaminopyridine, EDCI 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, MS molecular sieves, rt room temperature, TBAF tetrabutylammonium fluoride, THF tetrahydrofuran. The newly synthesized glycosidic bond is highlighted in red, and reacting functional groups in blue. Source data are provided as a Source Data file.
glycans, a combination of DOSY NMR and advanced MALDI-MS analyses was applied. In particular, DOSY-NMR measurements, capable of determining the molecular self-diffusion coefficients, were successfully applied to estimate the molecular weights of the longest glycans (Fig. 3c; Supplementary Fig. 13). In particular, DOSY-derived Mw for 64-mer was well consistent with the theoretical value; the average molecular mass of 128-mer, complicated by its size and lability during ionization, was instead determined by MALDI-FT-ICR-MS (Fig. 3b; Supplementary Fig. 14).

Conformation and immunological activities. Together with the complete NMR assignments (Supplementary Table 6; Supplementary Figs. 3–7), molecular mechanics and dynamic simulation were carried out to assess the conformational behavior of the free glycans (Supplementary Figs. 8–12; Supplementary Table 7). The glycosidic linkages adopted Φ values in accordance with the exo-syn anomic conformation and were characterized by a certain degree of flexibility. The oligomers tend to form extended, flexible structures, as confirmed by the inter-residue NOE contacts, diagnostic of the absence of a compact disposition of the sugar backbone (Fig. 4a). On longer glycans (i.e., 64- and 128-mer), this may lead to a tendency to pack into supramolecular assemblies, this latter also accounting for the low solubility of the longer glycans and the low sensitivity of MALDI technique for the high Mw 128-mer.

The synthetic approach allowed us to deploy a full set of pure synthetic different sized O-antigen fragments, which were tested for immunological activity. Given the presence of rhamnose and mannose in B. vulgatus O-antigen and considering the niche and the physiological roles of these commensal bacteria, we used three immune receptors of the gut-associated lymphoid tissues in the study. Thus, B. vulgatus LPS and their fragments were examined on a direct ELISA with three human C-type lectins (i.e., DC-SIGN, Langerin, and Dectin-1), showing a qualitative binding to DC-SIGN and Langerin (Supplementary Fig. 15a). Subsequently, the binding of B. vulgatus LPS to DC-SIGN and Langerin was tested in a quantitative ELISA competition experiment; a better binding to DC-SIGN was observed (Supplementary Fig. 15b). Following up, the synthetic glycans were tested on a competition ELISA experiment against DC-SIGN, adding unreported information that could explain the tolerogenic signaling induced by the commensal B. vulgatus in gut-associated lymphoid tissues.

Fig. 3 Characterization of the glycans. | a Overlaid 1H-NMR spectra (acquired on Bruker 600 MHz with cryogenic probe and analyzed with Bruker Topspin) of the free glycans 1–7. b MALDI-FT-ICR-MS spectrum of 128-mer. The observed average m/z of 128-mer, detected as [M + Na]+, is in agreement with the calculated theoretical value (m/z 19771.1). c 2D diffusion ordered spectroscopy (DOSY) NMR spectra of glycans 1–7. The x-axis represents the 1H-NMR dimension; the y-axis represents the diffusion dimension; the double-logarithmic plot of D against Mw (Supplementary Fig. 13) provided a calibration curve described by the least-squares fitted linear equation Log D = −7873 − (0.486 Log Mw). Source data are provided as a Source Data file.
Discussion

The synthesis of glycans up to a 128-mer (7) consisting of disaccharide repeating unit of \(-4\)-\(\alpha\)-Rha-(1→3)-\(\beta\)-Man-(1→n) have been achieved. The present success is attributable to the following aspects: (1) The strategic \(2^n + 2^n\) glycosylation enables exponential growth of the glycan sizes, leading to 128-mer via only six repetitive cycles of transformations, and ready separation of the double-sized products from the remaining and decomposed substrates. The heroic syntheses of a linear 50-mer and a 100-mer via step-wise glycosylation demanded exhaustive reactions (glycosylation and capping on a solid support) at each step; while the synthesis of the 151-mer and 92-mer took advantage of multifold glycosylations to install the identical branches. (2) The overall protecting group arrangement enables robust transformations into donors and acceptors, provides appropriate solubility, and allows global deprotection at the macromolecular level. It is noteworthy that the 15% yield achieved for the deprotection of 128-mer (14→7) corresponds to an average ~99.4% yield for each cleavage of the 322 C–O bonds. Thus far, hydrogenolysis of benzyl ethers has been employed as the final step in all the synthesis of long glycans over 50-mer, however, cautiousness remains for this transformation which could require tedious exploration of the reaction conditions. (3) The gold(I)-catalyzed glycosylation reaction, involving \(\alpha\)-rhamnopyranosylation, is both stereospecific (in the presence of a neighboring-participating benzoyl group) and high-yielding. The 64-mer donor (13\(^D\)) represents the longest glycosyl donor that has ever been employed for successful glycosylation; this might be attributed to the merits of glycosyl ortho-alkynylbenzoates, including easy preparation, stability, and selective activation under mild conditions.

Besides the synthetic achievement, the availability of pure homogeneous glycans has allowed us to test the limits of analytical tools for the characterization of polysaccharides. Thus, the accurate confirmation of the molecular size of 64-mer (6) represents one of the longest glycans determined so far by DOSY-NMR, and the average molecular mass of 128-mer (7) represents the highest mass of a synthetic linear polysaccharide being measured by MS. Moreover, the synthetic O-antigen showed a selective affinity with human lectin DC-SIGN and this might be relevant information for therapeutic use against different gut-associated inflammatory diseases. We expect that the present report can inspire researches on the syntheses and thus functional studies on pure and homogenous polysaccharides for different therapeutic uses.

Methods

General methods for the syntheses. Reactions were carried out in glassware. Crushed 4 or 5 Å molecular sieves were activated through flame-drying under high vacuum immediately prior to use. All chemicals were purchased as reagent grade and used without further purification, unless otherwise noted. Analytical thin-layer chromatography was performed using Merck precoated silica gel 60 F-254 plates. Compound spots were visualized by UV light (254 nm) and immersion into a solution of 5% H\(_2\)SO\(_4\) in ethanol, followed by hot air gun heating. Column chromatography was performed on silica gel (200–300 mesh). Gel filtration was performed on Sephadex LH-60, LH-20, or G25 (8%K). Optical rotations were obtained on Anton Paar MCP 5500 polarimeter at 589 nm (Na).

Synthesis of 64-mer donor 13\(^D\). A solution of 64-mer 13 (320 mg, 14.2 \(\mu\)mol) in CH\(_2\)Cl\(_2\)/MeCN (10 mL/4 mL) was stirred at 0 °C. Ammonium ceric nitrate (281 mg, 0.51 mmol) was dissolved in H\(_2\)O (1 mL) at 0 °C. The latter solution was added into the former via syringe, and the mixture was stirred at 0 °C for 2 h. The mixture was diluted with CH\(_2\)Cl\(_2\) and washed with sat. aq. NaHCO\(_3\) and brine, respectively. The organic layer was dried over anhydrous Na\(_2\)SO\(_4\) and concentrated in vacuo. The residue was purified by silica gel column chromatography (petroleum ether/EtOAc/CH\(_2\)Cl\(_2\), 4:1:3) to give the corresponding crude hemiacetal (301 mg) as a yellowish solid.

To a mixture of the crude hemiacetal (301 mg, ~13.5 \(\mu\)mol), EDCI (32.1 mg, 0.17 mmol), DMAP (20.6 mg, 0.170 mmol), and ortho-hexynylbenzoic acid (20.4 mg, 0.10 mmol), was added CH\(_2\)Cl\(_2\) (10 mL). The mixture was stirred at rt for 10 h, and was then diluted with CH\(_2\)Cl\(_2\) and washed with sat. aq. NaHCO\(_3\) and brine, respectively. The organic layer was dried over anhydrous Na\(_2\)SO\(_4\) and concentrated in vacuo. The residue was purified by silica gel column chromatography (petroleum ether/EtOAc/CH\(_2\)Cl\(_2\), 12:1:5:3) as a white solid.

Synthesis of 64-mer acceptor 13\(^A\). To a solution of 64-mer 13 (190 mg, 8.5 \(\mu\)mol) in anhydrous THF (5 mL), were added HOAc (2 \(\mu\)L, 34.9 \(\mu\)mol) and TBAF (1.0 M in THF, 86.0 \(\mu\)L, 86.0 \(\mu\)mol). The mixture was stirred at rt for 48 h, and then was diluted with CH\(_2\)Cl\(_2\) and with sat. aq. NH\(_4\)Cl and brine, respectively. The organic layer was dried over anhydrous Na\(_2\)SO\(_4\) and concentrated in vacuo. The residue was purified by silica gel column chromatography (petroleum ether/EtOAc/CH\(_2\)Cl\(_2\), 4:1:5:3) to give compound 13\(^A\) (283 mg, 88% for two steps, \(\alpha\)/\(\beta\)= 2.3:1) as a white solid.

Synthesis of 128-mer 7. To a solution of 128-mer 14 (45 mg, 1.0 \(\mu\)mol) in anhydrous THF (5 mL), were added HOAc (2 \(\mu\)L, 34.9 \(\mu\)mol) and TBAF (1.0 M in THF, 86.2 \(\mu\)L, 86.2 \(\mu\)mol). The mixture was stirred at rt for 46 h, and was then

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**Fig. 4 Conformation and immunological activities.** a A representative solution conformation of the 32-mer as determined by NMR and molecular simulation (Maestro Schrödinger), prepared with SweetUnitMol; b ELISA analysis of the competition binding of the glycanps and LPS from \(B\). \(vulgatus\) to the human C-type lectin DC-SIGN, with LPS from \(Shigella flexneri\) as a negative control. The competition experiments have been performed three times showing similar results; the graph shows data of one of these experiments. Source data are provided as a Source Data file.
diluted with CH₂Cl₂ and washed with sat. aq. NH₄Cl and brine, respectively. The organic layer was washed over anhydrous Na₂SO₄ and concentrated in vacuo. The residue was used in the next step without further purification.

To a mixture of the residue above (45 mg), DMAP (20 mg, 0.16 mmol), and Et₃N (30 μL, 0.21 mmol) in CH₂Cl₂ (2 mL), was added BzCl (12 μL, 104 μmol). The mixture was stirred at rt overnight, and was then diluted with CH₂Cl₂ and washed with sat. aq. NaHCO₃ and brine, respectively. The organic layer was dried over anhydrous Na₂SO₄ and concentrated in vacuo. The residue was then subjected to an equilibration time of 300 ps, then a 10,000 ps molecular dynamic simulation with SHAKE protocol to the hydrogen bonds. Trajectory coordinates were sampled every 2 ps, and a total of 3000 structures were collected for every simulation. Ensemble-average-interproton distances were calculated using the NOEPRM program by applying the isolated spin pair approximation as described. Solvent-accessible surfaces were calculated with the Surface utility of Maestro. Conformers were visualized with Maestro, Discovery Studio Visualizer and SmallMol.

Conformational studies: Molecular mechanics calculations were performed using the MM3* force field, a dielectric constant of 80 was used. For the disaccharide structure, both Φ and Ψ were varied incrementally using a grid step of 18°, each (Φ, Ψ) point of the map was optimized using 2000 PJE conjugate gradients. Molecular dynamic simulations were run by using the MM3* force field, bulk water solvation was simulated by using MacroModel generalized Born GB/SA continuum solvent model. Molecular dynamics simulations were performed at 298 K. Structures were initially subjected to an equilibration time of 300 ps, then a 10,000 ps molecular dynamic simulation was performed with a dynamic time-step of 1.5 fs, a bath constant of 0.2 ps and the

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Author contributions

B.Y. and A.M. designed the research and experiments. Q.Z. and Z.S. conducted the synthetic work. A.S. conducted the NMR and computational studies. F.C. performed the bioassays. S.N. measured the MS of long glycans. B.Y. and A.S. wrote the paper. All authors discussed the results and commented on the paper.

Competing interests

The authors declare no competing interests.

Additional information

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