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Supporting Information

The diagram shows a branched oligosaccharide structure. The left side is labeled "inner core" and "Chemical synthesis". It features a central mannose unit (blue circle) linked to three glucose units (green hexagons). One of these glucose units is further linked to a galactose unit (yellow hexagon). The right side is labeled "Enzymatic extension" and shows three parallel chains of sugar units (yellow hexagons, blue circles, green hexagons) extending from the inner core. The units are linked by α and β glycosidic bonds, with labels $\alpha 3$, $\beta 4$, $\alpha 3$, $\alpha 6$, and $\alpha 5$ indicating the linkage types. The chains terminate in a CO_2H group and a $\text{O}(\text{CH}_2)_3\text{NH}_2$ group.

Although there is strong scientific support for the involvement of anti-ganglioside antibodies in the pathogenesis of GBS,² molecular mechanisms by which immunotolerance is broken leading to autoimmune-like responses are not well

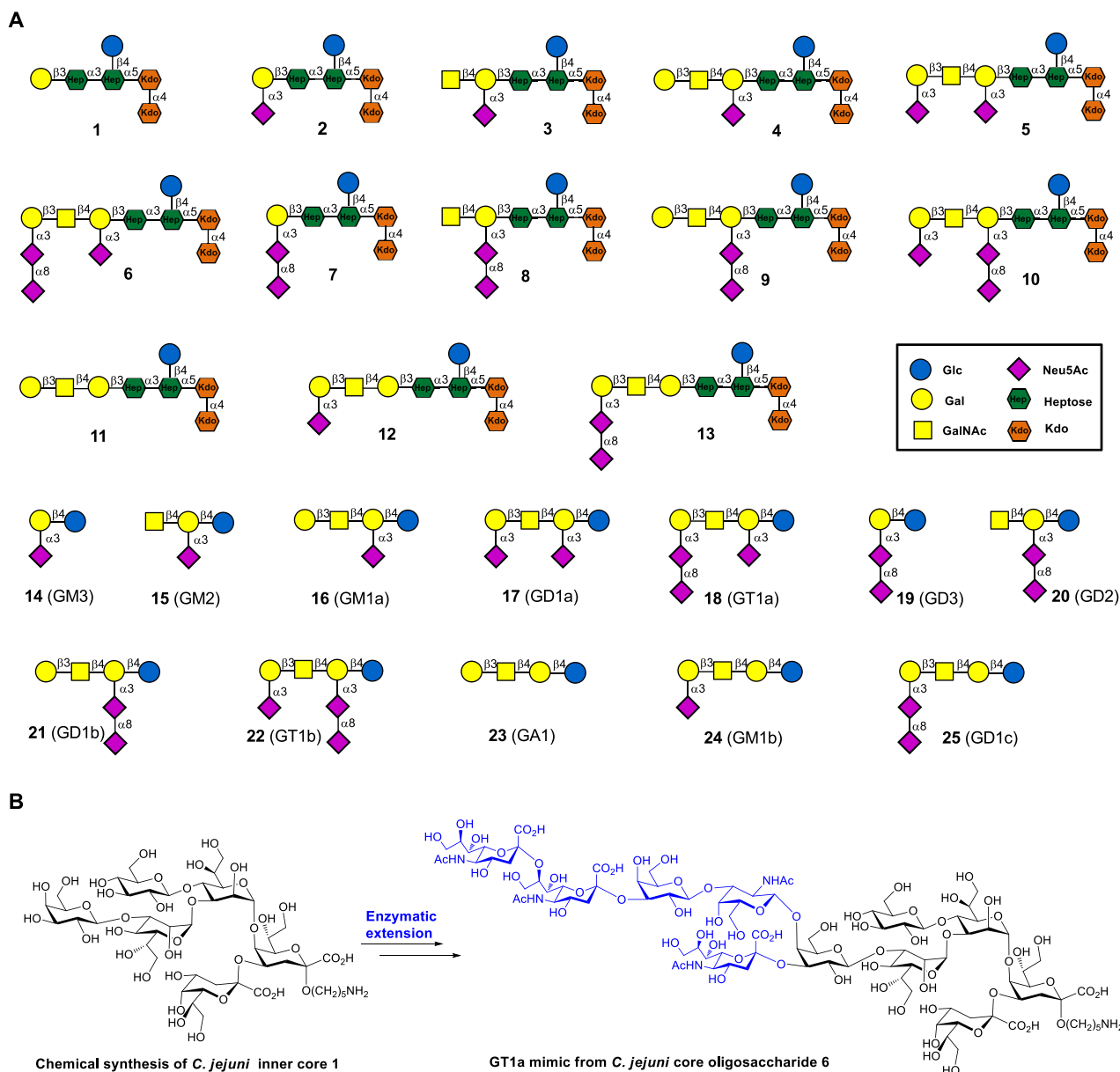
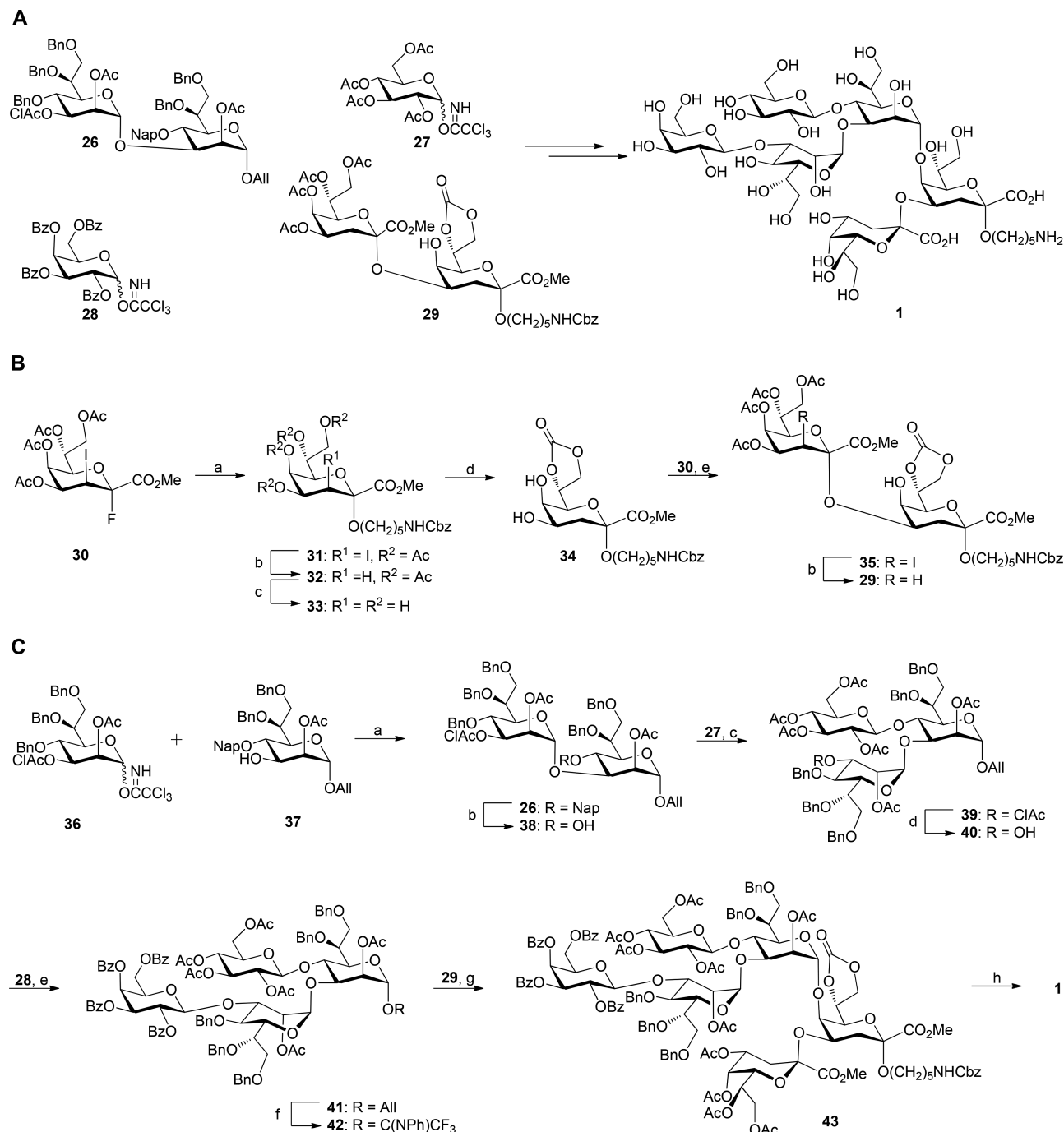


Figure 1. Synthetic ganglioside mimics from *C. jejuni* core oligosaccharides and normal ganglio-oligosaccharide library by a chemoenzymatic approach: (A) synthetic ganglioside mimic and ganglioside oligosaccharide library; (B) chemically synthesized inner core hexasaccharide 1 and enzymatic extension of 1 to afford compounds such as GT1a ganglioside mimic 6.

understood. Furthermore, the role of anti-ganglioside antibodies in diagnosis is fraught with difficulties, and in particular the frequency and specificity of anti-ganglioside antibodies are low leading to false negative results. The positive predictive value of anti-ganglioside antibodies, especially those of the IgM class, is also compromised because these can occur in other diseases. Detection of anti-ganglioside antibodies is mainly performed by ELISA using gangliosides,¹⁸ usually obtained by isolation from natural sources. These compounds are often not homogeneous, and only a limited number of structures are readily available, which is impeding comprehensive analysis of structure–binding relationships and mechanisms by which they promote nerve damage.

During natural infections, the immune system is primed by LOS of *C. jejuni* strains,¹⁹ and thus we hypothesized that anti-ganglioside antibodies elicited during *C. jejuni* infection are directed to epitopes that straddle the inner core region of LOS

and the ganglioside structural analogs. To test this mode of immune recognition, we synthesized a large panel of oligosaccharides composed of the inner core oligosaccharide of the LOS of *C. jejuni* extended by various ganglioside mimics. Compound 1 resembles the inner core oligosaccharide of *C. jejuni*, and compounds 2–13 are derived from this structure but extended by the oligosaccharide mimics of GM1 (2), GM2 (3), GM1a (4), GD1a (5), GT1a (6), GD3 (7), GD2 (8), GD1b (9), GT1b (10), GA1 (11), GM1b (12), and GD1c (13). We also prepared the corresponding ganglioside oligosaccharides 14–25 (Figure 1). Compounds 2–13 were obtained by a chemoenzymatic strategy in which inner core oligosaccharide 1 was prepared chemically and then extended using appropriate microbially derived glycosyltransferases to install ganglio-mimetics. The ganglioside oligosaccharides 14–25 were prepared starting from spacer modified lactose which was enzymatically extended to provide the target compounds.

Scheme 1. (A) Chemical Synthesis of Inner Core Hexasaccharide **1** and (B) Synthesis of Kdo2 Glycosyl Acceptor **29**⁴⁴

⁴⁴Reagents and conditions: (a) $\text{HO}(\text{CH}_2)_3\text{NHCbz}$, $\text{BF}_3 \cdot \text{Et}_2\text{O}$, CH_2Cl_2 , 3 Å molecular sieves, 0 °C to rt, 2.5 h, 75%; (b) lauroyl peroxide, $\text{ClCH}_2\text{CH}_2\text{Cl}$ /cyclohexane 1:7, reflux at 90 °C, 2 h, 83% for **32**, 87% for **29**; (c) $\text{NaOCH}_3/\text{CH}_3\text{OH}$, rt, 4 h, 92%; (d) diphosgene, *sym*-collidine, THF, −25 °C, 30 min, 76%; (e) $\text{BF}_3 \cdot \text{Et}_2\text{O}$, CH_2Cl_2 , 3 Å molecular sieves, 0 to 15 °C, 2 h, 80%. (C) Assembly of hexasaccharide **1**, reagents and conditions: (a) TMSOTf , CH_2Cl_2 , 4 Å molecular sieves, −20 to −10 °C, 30 min, 89%; (b) DDQ, CH_2Cl_2 /PBS buffer (100 mM, pH 7.4) 10:1, rt, 1.5 h, 78%; (c) TMSOTf , CH_2Cl_2 , 4 Å molecular sieves, 0 to 5 °C, 70 min, 80%; (d) thiourea, *sym*-collidine, $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ 2:3, 70 °C, 24 h, 85%; (e) TMSOTf , CH_2Cl_2 , 4 Å molecular sieves, −10 to 0 °C, 60 min, 90%; (f) (i) PdCl_2 , $\text{CH}_2\text{Cl}_2/\text{MeOH}$ 1:5, rt, 2.5 h, 86%; (ii) $\text{CF}_3\text{C}(\text{NPh})\text{Cl}$, Cs_2CO_3 , CH_2Cl_2 , rt, 4 h, 85%; (g) TMSOTf , CH_2Cl_2 , 5 Å molecular sieves, rt, 2 h, 40%; (h) (i) 1 M $\text{NaOH}(\text{aq})/\text{CH}_3\text{OH}/\text{dioxane}$ 1:1:3, rt, 16 h, (ii) H_2 , $\text{Pd}(\text{OH})_2/\text{C}$, *t*-BuOH/ $\text{H}_2\text{O}/\text{HOAc}$ 3:2:0.02, rt, 18 h, 42% over 2 steps.

The oligosaccharides were printed as a glycan microarray, which was used to investigate the binding reactivities of a range of lectins, anti-ganglioside antibodies, and serum antibodies of normal and GBS patients. Although the lectins and anti-ganglioside antibodies recognized a ganglio-oligosaccharide

and corresponding mimic equally well, the patient serum samples bound much more strongly to the mimics. This observation indicates that GBS patients have not generated primary autoreactive antibodies but cross-reactive antibodies, and most likely epitopes targeted by serum antibodies include

a heptosyl residue unique of bacterial LOS. Such an epitope will be perceived as foreign for mammalian immune cells, thereby providing a rationale for breaking immune-tolerance. It was also observed that the microarray platform detects with greater sensitivity anti-GM1a antibodies compared to traditional ELISA using bovine extracted gangliosides, and this will be attractive for diagnosis, disease monitoring, and immunological research.

RESULTS AND DISCUSSION

Chemical Synthesis of Inner Core Oligosaccharide

(1). The chemical synthesis of the inner core hexasaccharide **1** is challenging due to the low acceptor reactivity of the C5-hydroxyl of Kdo- α -(2 \rightarrow 4)-Kdo acceptor **29**^{20–22} and the need to install glycosidic bonds to form a highly crowded 3,4-branched heptoside.^{23,24} We envisaged that fully protected hexasaccharide **1** could be synthesized by a convergent and stereocontrolled [4 + 2] approach (Scheme 1) relying on the use of flexible disaccharide intermediate **26**, which is modified by the orthogonal protecting groups 2-naphthylmethyl (Nap) ether, chloroacetyl (ClAc) ester, and allyl (All) ether. The ability to remove the Nap ether and ClAc ester protecting groups in different orders made it possible to establish the optimal glycosylation sequence, using glycosyl **27** and **28**, to prepare tetrasaccharide **41** having a crowded 3,4-branched heptoside. Removal of the anomeric allyl ether of **41** and conversion of the resulting lactol into a donor followed by glycosylation with **29** would install the challenging α (1 \rightarrow 5)-linked Hep-Kdo glycosidic linkage affording the protected hexasaccharide **43**. Global deprotection of the latter compound would provide the required inner core hexasaccharide **1**.

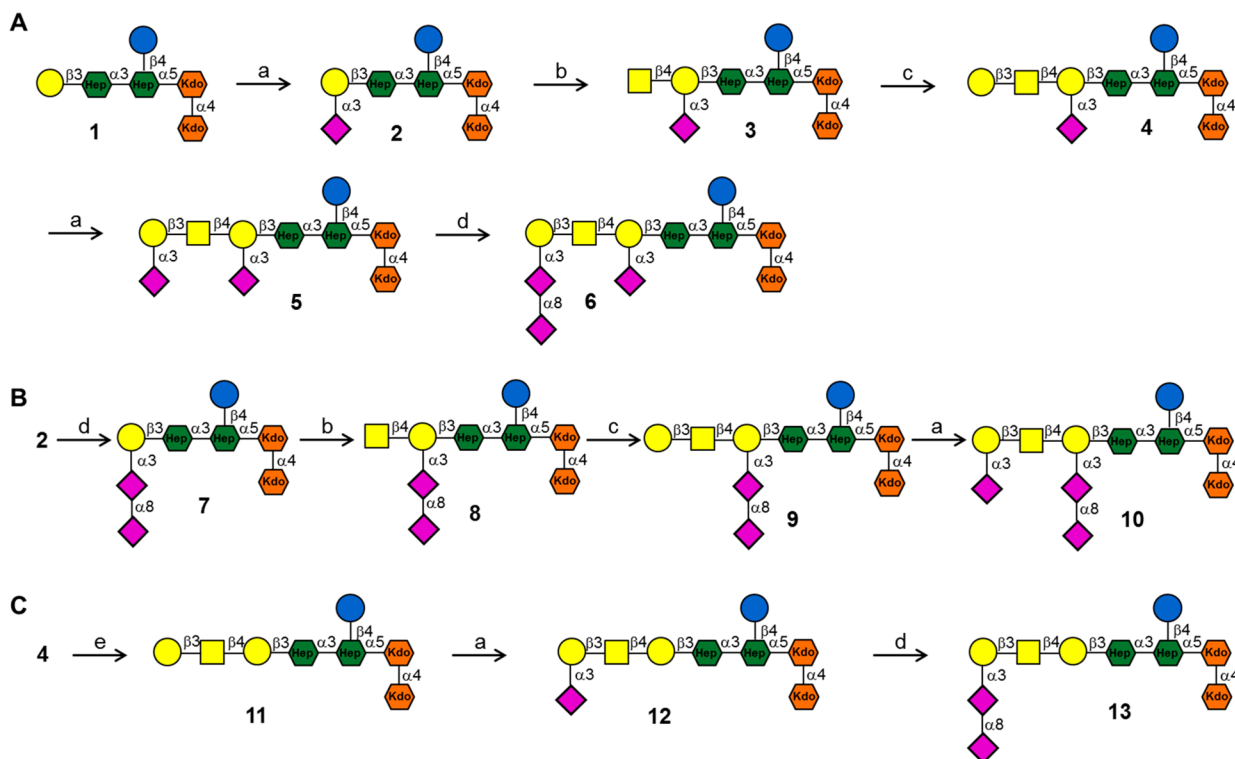
First, α (2 \rightarrow 4)-linked Kdo dimer **29** was prepared which has a free hydroxyl at C5 for further glycosylation at this position (Scheme 1B). Glycosylation using a Kdo donor with an iodide at C3 as a stereodirecting group is attractive because it provides only α -anomeric products.²⁵ Thus, coupling of donor **30** with benzyloxycarbonyl protected 5-aminopentanol in the presence of $\text{BF}_3 \cdot \text{Et}_2\text{O}$ (2 equiv) as the promoter gave ketoside **31** in a yield of 75% as only the 2,3-*trans*-isomer. Only a small amount of the glycal byproduct (8%) was isolated due to a competing elimination reaction. The 3-iodo-substituent of **31** was readily removed by hydrogen atom transfer from cyclohexane induced by lauroyl peroxide in 1,2-dichloroethane to afford spacer modified α -linked Kdo **32**.²⁶ ^1H NMR analysis of **32** showed a low-field shift of H-4 (δ = 5.26 ppm), which was typical for an α -anomeric Kdo linkage in 4-O-acylated Kdo derivatives.^{27,28} Treatment of **32** with a catalytic amount of sodium methoxide in methanol resulted in removal of the acetyl esters to give tetraol **33**. The heteronuclear coupling constant of C-1 with the axial proton at C-3 position ($^3J_{\text{C1-H3ax}}$ < 1 Hz) further confirmed the α -anomeric configuration of Kdo.²⁷ The 7,8-diol of **33** was selectively protected as a cyclic carbonate by treating with diphosgene (1 equiv) in the presence of *sym*-collidine at -25°C to give **34** having a free diol at C-4,5. Careful control of the reaction conditions including the equivalence of diphosgene and temperature was important to avoid further reaction of the 4,5-O-diol. The equatorial C4 hydroxyl of **34** has higher glycosyl acceptor reactivity compared to the axial hydroxyl at C5, and thus it was expected that **34** is an appropriate acceptor for glycosylation at C4 without a need for further protecting group manipulations. Indeed, a $\text{BF}_3 \cdot \text{Et}_2\text{O}$ promoted glycosylation of **30** with **34** resulted in the selective formation of α (1,4)-linked ketoside **35**

in high yield. Dehalogenation of the latter compound was achieved using standard conditions providing the desired dimeric Kdo acceptor **29**.

Next, attention was focused on the preparation of 3,4-branched heptosyl donor **42** (Scheme 1C). Thus, a TMSOTf promoted glycosylation of **36** with **37** gave disaccharide **26** as only α -anomer ($J_{\text{C1-H1}}$ = 176 Hz) due to neighboring group participation by the acetyl ester at C-2 of the donor. To give **38**, the Nap ether of **26** was oxidatively removed using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ)²⁹ in a mixture of CH_2Cl_2 and phosphate buffer (pH 7.4) without affecting any other protecting groups. A glycosylation of the latter acceptor with glycosyl donor **27**, using TMSOTf (1.3 equiv) as the activator, afforded the 3,4-branched trisaccharide **39**. A relatively large quantity of TMSOTf was required to avoid the formation of an orthoester byproduct (Figure S1). Furthermore, the use of per-benzyolated glucosyl trichloroacetimidate as donor did not provide the corresponding trisaccharide probably due to steric hindrance.²³ Next, the chloroacetyl (ClAc) ester^{30,31} of **39** was selectively cleaved by treatment with thiourea in the presence of *sym*-collidine to give trisaccharide **40**, which was glycosylated with glycosyl donor **28** using a catalytic amount of TMSOTf as promotor to afford tetrasaccharide **41** in 90% yield. Trifluoro-*N*-phenylacetimidate **42** was prepared by a two-step procedure involving selective removal of the allyl ether of **41** using PdCl_2 to give a lactol, which was reacted with *N*-phenyltrifluoroacetimidoyl chloride in the presence of Cs_2CO_3 .^{32,33}

Having glycosyl acceptor **29** and donor **42** in hand, attention was focused on the preparation of inner core hexasaccharide **1**. Preactivated glycosyl acceptor **29** using a catalytic amount of TMSOTf followed by slow addition of glycosyl donor **42** at room temperature resulted in the formation of hexasaccharide **43**. Inverse glycosylation^{34,35} at room temperature was essential, and standard glycosylation conditions gave only trace amounts of **43**, and in this case mainly decomposition of the glycosyl donor and acceptor was observed. The low reactivity of the sterically hindered C-5 hydroxyl of **29** probably causes a mismatch in reactivity with glycosyl donor **42** resulting in a challenging glycosylation. Finally, global deprotection of **43** to give hexasaccharide **1** was accomplished by saponification of the esters and carbonate using sodium hydroxide, followed by catalytic hydrogenolysis over $\text{Pd}(\text{OH})_2/\text{C}$ to remove the benzyl ethers. The NMR data of **1** confirmed the anomeric configurations of all glycosidic linkages.

Previously, we³⁶ and others^{23,37,38} have employed a 7,8-O-isopropylidene protected Kdo acceptor for glycosylations to increase the reactivity of the axial C5-hydroxyl. However, the use of such an acceptor proved incompatible with $\text{BF}_3 \cdot \text{Et}_2\text{O}$ mediated glycosylation and resulted in the formation of a complex mixture of products probably due to isopropylidene cleavage (Figure S2). Our studies demonstrate that a 7,8-carbonate is an attractive protecting group for Kdo containing oligosaccharides being extended at the anomeric center and C-4 and C-5. We also explored an alternative glycosylation sequence for the preparation of **1** by constructing the challenging α -Hep-(1 \rightarrow 5)-Kdo glycosidic linkage to give α -Hep-(1 \rightarrow 5)-Kdo- α -(2 \rightarrow 4)-Kdo trisaccharide that was converted into a glycosyl acceptor for coupling with a β -Gal-(1 \rightarrow 3)-Hep donor to form a properly protected pentasaccharide for further glucosylation (Figure S3). Although the pentasaccharide was detected by MALDI-MS, it was difficult to purify and

Scheme 2. Enzymatic Extension of 1 to Install Various Outer Core Ganglioside Epitopes^a

^aReagents and conditions: (a) PmST1, CMP-Neu5Ac, 100 mM Tris-HCl (pH 8.0), 37 °C, 81% for 2, 75% for 5, 74% for 10, 76% for 12; (b) CgtA, UDP-GalNAc, 10 mM MgCl₂, 50 mM Tris-HCl (pH 7.5), 37 °C, 92% for 3, 88% for 8; (c) CgtB, UDP-Gal, 10 mM MgCl₂, 50 mM Tris-HCl (pH 7.5), 37 °C, 83% for 4, 80% for 9; (d) CstII, CMP-Neu5Ac, 100 mM Tris-HCl (pH 8.0), 37 °C, 76% for 6, 77% for 7, 74% for 13; (e) α 2,3,6,8,9 neuraminidase A from *Arthrobacter ureafaciens*, 5 mM CaCl₂, 50 mM sodium acetate (pH 5.5), 37 °C, 94%.

the yield was low (<10%). Probably steric hindrance at the C-3'' hydroxyl of α -Hep-(1 \rightarrow 5)-Kdo- α -(2 \rightarrow 4)-Kdo trisaccharide led to failure of the glycosylation.

Enzymatic Synthesis of Ganglioside Oligosaccharides and Ganglioside Mimics. Having inner core hexasaccharide 1 in hand, attention was focused on enzymatic extension to give ganglioside mimics 2–13 (Scheme 2). Glycosyltransferases from microbes such as *C. jejuni* and *Pasteurella multocida* have been identified that can assemble ganglio-series oligosaccharides. These include the α 2,3-sialyltransferase PmST1³⁹ from *Pasteurella multocida* that can form GM3; the β 1,4-N-acetylgalactosaminyltransferase (CgtA) from *C. jejuni* which can synthesize GM2; β 1,3-galactosyltransferase (CgtB)⁴⁰ from *C. jejuni* that can prepare GM1; and the bifunctional sialyltransferase CstII⁴¹ from *C. jejuni* that has both α 2,3-sialyltransferase (GM3 oligosaccharide synthase) and α 2,8-sialyltransferase (GD3 oligosaccharide synthase) activity. Starting from lactose, these enzymes have been used to prepare several oligosaccharide moieties of gangliosides including GM3, GM2, GM1a, GD3, GD2, and GD1b.⁴² The enzyme toolbox has, however, not made it possible to prepare a number of other ganglio-oligosaccharides including GA1, GM1b, and GD1c. Here, we introduce a synthetic strategy that addressed the latter deficiency and made it possible to prepare an unprecedented collection of ganglio-oligosaccharides (14–25). It was expected that the galactosyl moiety of inner-core oligosaccharide 1 would provide a proper starting structure for extension by the glycosyltransferases^{24,42–44} and was amenable to our strategy to give access to ganglioside mimics 2–12.

First, inner-core hexasaccharide 1 was sialylated with PmST1 and cytidine-5'-monophospho-N-acetylneuraminic acid (CMP-Neu5Ac) to give GM3 mimic 2. The progress of the reaction was carefully monitored by ESI-MS because PmST1 has residual hydrolytic activity and, after consumption of CMP-NeuAc, can hydrolyze the product.⁴⁵ Oligosaccharide 2 was smoothly converted into GM2 mimic 3 using CgtA and uridine 5'-diphospho-N-acetylgalactosamine (UDP-GalNAc). Next, the synthesis of GM1a mimic 4 was accomplished by treatment of 3 with CgtB in the presence of uridine 5'-diphosphogalactose (UDP-Gal). To accomplish a high yield, it was critical to employ only 1 equiv of UDP-Gal because an excess of this reagent resulted in further galactosylation of 4. α 2,3-Sialylation of 4 with PmST1 and CMP-Neu5Ac resulted in the formation of GD1a mimic 5, which was further sialylated using α (2,8)-sialyltransferase CstII in the presence of CMP-Neu5Ac and calf intestine alkaline phosphatase (CIAP) to provide GT1a mimic 6 (Scheme 2A). ¹H NMR analysis of 6 showed a downfield shift of the H-8 (from 3.73 to 4.02 ppm) compared to the same proton of the terminal α (2,3)-Neu5Ac-II residue of 5. In addition, a NOESY spectrum revealed a correlation between the H-3ax (δ = 1.580) of terminal α (2,8)-Neu5Ac-III and H-8 (δ = 4.016) of α (2,3)-Neu5Ac-II⁴³ (Figure S16), further confirming that the α (2,8)-Neu5Ac moiety was attached to the terminal α (2,3)-Neu5Ac. Thus, the results demonstrate that CstII only extends the terminal α 2,3-linked sialoside.

In parallel, 2 was converted into GD3 mimic 7 using CstII and CMP-Neu5Ac (Scheme 2B). The amount of CMP-Neu5Ac was carefully controlled to avoid further α (2,8)-

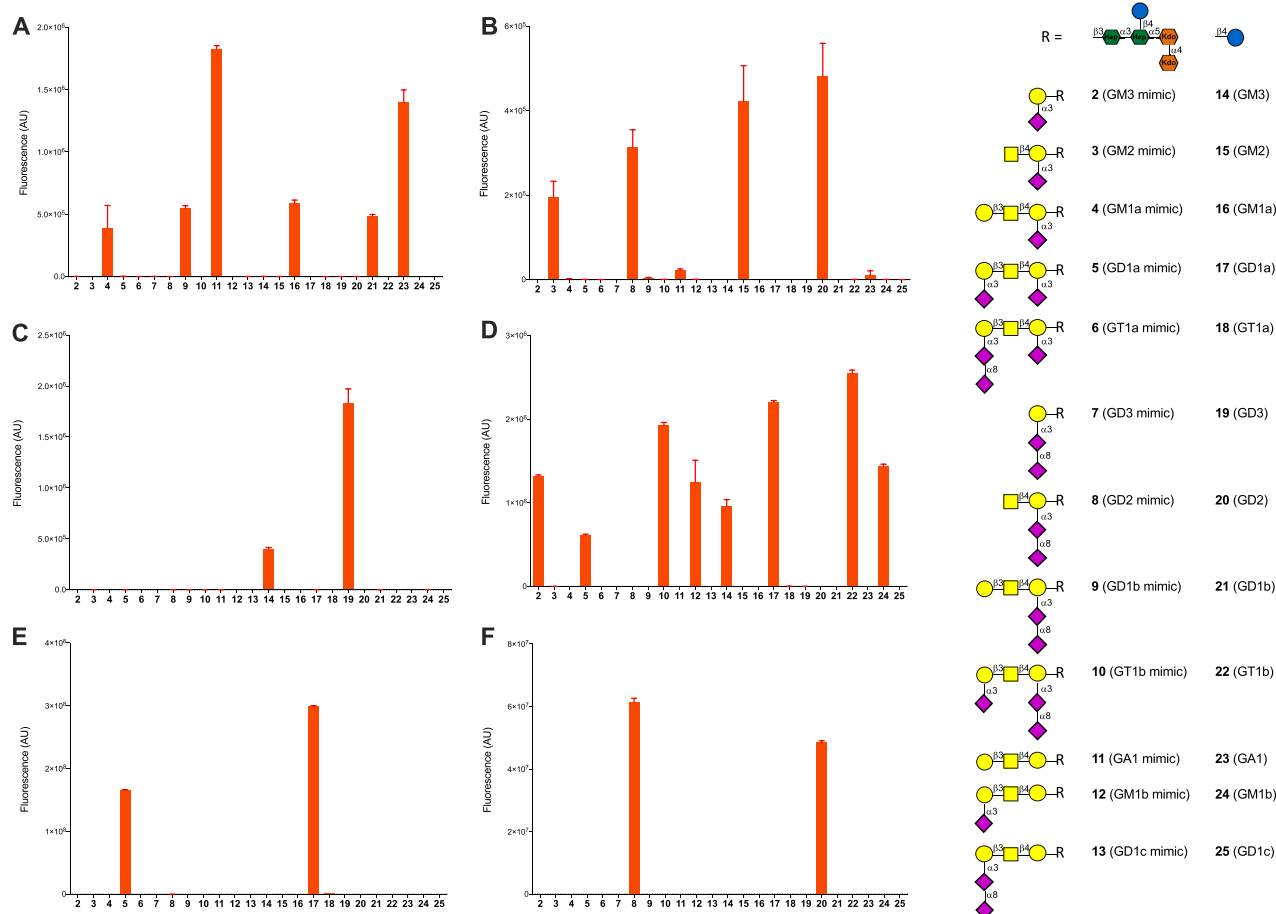


Figure 2. Microarray results of the synthetic ganglioside library at 100 μM with (A) RCA I (20 $\mu\text{g/mL}$), (B) SBA (20 $\mu\text{g/mL}$), (C) MAL II (20 $\mu\text{g/mL}$), (D) WGA (10 $\mu\text{g/mL}$), (E) anti-GD1a mAb, clone GD1a-1 (2 $\mu\text{g/mL}$), and (F) anti-GD2 mAb, clone 14.G2a (2 $\mu\text{g/mL}$). Bars represent the mean \pm SD.

sialylation.⁴⁴ Furthermore, it was observed that the rate of $\alpha(2,8)$ -sialylation of **2** is faster than for **6**. Next, **7** was subjected to CgtA in the presence of UDP-GalNAc to form GD2 mimic **8**. Treatment of the latter compound with CgtB and 1 equiv UDP-Gal resulted in the formation of GD1b mimic **9**, which was further sialylated by PmST1 to provide GT1b mimic **10**.

The synthesis of GA1, GM1b, and GD1c mimics **11**, **12**, and **13** is challenging because previous studies have shown that CgtA⁴⁶ requires an $\alpha(2,3)$ -linked sialic acid at the terminal galactoside to install a $\beta(1,4)$ -GalNAc moiety, and thus compound **1** cannot be extended by this enzyme and the enzyme toolbox does not provide entry into **11–13**. To address this difficulty, we opted to treat **4** with a sialidase to give **11** which can then be further elaborated with the glycosyltransferases to provide **12** and **13**. Commercially available $\alpha(2,3,6,8)$ neuraminidases from *Clostridium perfringens* and *Vibrio cholerae* were not able to cleave the internal and branched Neu5Ac moiety. Gratifyingly, $\alpha(2,3,6,8,9)$ neuraminidase A from *Arthrobacter ureafaciens* could remove the Neu5Ac residue of **4** to give GA1 mimic **11**. A relatively high concentration of this neuraminidase and a prolonged incubation time were required to cleave the internal Neu5Ac residue. Sialylation of **11** using PmST1 and CMP-Neu5Ac afforded GM1b mimic **12**, which could be further sialylated using CstII to provide GD1c mimic **13** (Scheme 2C).

The corresponding ganglioside oligosaccharides **14–25** (Figure S11) were prepared by a similar strategy starting from lactose modified by an anomeric aminopropyl linker. The preparation of GA1, GM1b, and GD1c did not require expensive $\alpha(2,3,6,8,9)$ neuraminidase, and in this case, treatment of **16** with 2 M acetic acid resulted in clean removal of the internal Neu5Ac to give GA1 oligosaccharide **23** (Figure S11),⁴⁷ which was further extended using appropriate glycosyltransferases to provide GM1b and GD1c oligosaccharides **24** and **25**, respectively. It is important to note that treatment of **4** with acetic acid results in cleavage of the Kdo glycosidic linkages.

The enzymatic conversions were monitored by ESI-MS, and products were purified by Bio-Gel P-2 or P-4 size exclusion chromatography and HPLC using a HILIC column (XBridge Amide 5 μm , 10 mm \times 250 mm, Waters), and the structural identity of each compound was confirmed by NMR spectroscopy (1D and 2D) and HR-MS.

Glycan Recognition of Lectins, mAbs, and Serum Antibodies Using a Glycan Microarray Platform. The synthetic oligosaccharides **1–25** were employed to examine specificities of serum antibodies of patients in the acute stage of GBS following a *C. jejuni* infection, and the results were compared to healthy control serum samples. For this purpose, a glycan microarray was constructed by piezoelectric non-contact printing of the aminopentyl containing glycans onto *N*-hydroxysuccinimide (NHS) ester activated glass in replicates

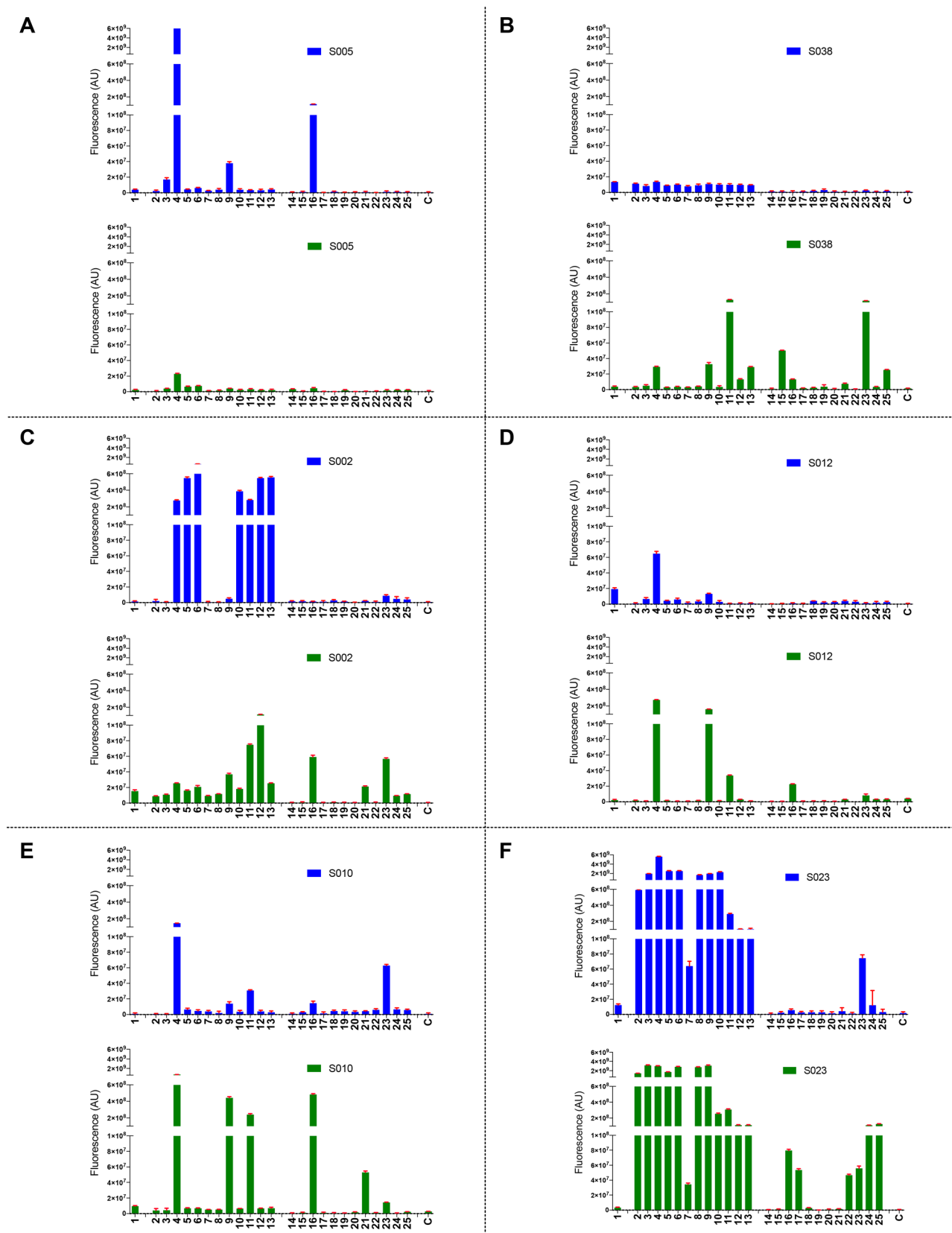


Figure 3. Microarray results of the synthetic ganglioside library (1–25) at 100 μ M with serum samples (1:500) (A) S005, (B) S038, (C) S002, (D) S012, (E) S010, and (F) S023. For each sample, the graph at the top shows in blue IgG responses and the graph at the bottom in green IgM responses. Bars represent the mean \pm SD. C indicates blank control. Assays are performed in the same session, and results are depicted at the same scale.

of six. To validate the array, the slides were examined for binding by the plant lectins RCA I, SBA, MAL II, and WGA. The subarrays were also probed for binding by a number of commercially available anti-ganglioside antibodies.

RCA I binds galactose, and as expected, all compounds bearing such a terminal residue (**16/4** GM1a/GM1a mimic; **21/9** GD1b/GD1b mimic; **23/11** GA1/GA1 mimic) were recognized (Figure 2A). SBA preferentially binds GalNAc but also recognizes Gal residues although with much lower affinity.⁴⁸ Binding to SBA was observed for compounds having a terminal GalNAc including compounds **15** (GM2), **20** (GD2), and their corresponding mimics **3** and **8** (Figure 2B). Weak binding was detected for compounds **23/11** (GA1/GA1 mimic) having a terminal Gal residue, and no binding was observed for **16/4** (GM1a/GM1a mimic) and **21/9** (GD1b/GD1b mimic) due to the inhibitory effect of Neu5Ac.⁴⁹ MAL-II binds Neu5Ac(α 2–3)Gal(β 1–4)GlcNAc/Glc moieties,⁵⁰ and as anticipated, compounds **14** and **19**, having this epitope, were bound by this lectin (Figure 2C).⁴⁹ The corresponding ganglioside mimics **2** and **7**, respectively, were not recognized most likely because the glucose moiety of the binding epitope is replaced by heptose. Finally, we examined WGA, which preferentially binds GlcNAc moieties and also interacts with glycoproteins via terminal sialic acid residues. As expected, only the ganglioside oligosaccharides having a terminal α (2,3)Neu5Ac moiety (**14**, **17**, **22**, **24**) and the corresponding mimics (**2**, **5**, **10**, **12**) showed binding (Figure 2D).

Next, binding profiles of several anti-ganglioside antibodies were determined. As expected, an anti-GD1a mAb (Figure 2E) only showed binding to **17** (GD1a) and **5** (GD1a mimic), and an anti-GD2 mAb (Figure 2F) only bound to **20** (GD2) and **8** (GD2 mimic). A polyclonal GM1 antibody (Figure S12A) exhibited some promiscuity and recognized compounds **16/4** (GM1a/GM1a mimic), **21/9** (GD1b/GD1b mimic), and **23/11** (GA1/GA1 mimic), indicating the terminal Gal(β 1,3)-GalNAc moiety of these compounds is recognized.⁵¹ The subarrays were also treated with neuraminidase A (N-A), which cleaves terminal α 2,3-, α 2,6-, and α 2,8-linked sialic acid residues, and then reprobed by this antibody, showing the expected binding patterns (Figure S12B). Importantly, the antibodies recognized the corresponding ganglio-oligosaccharides and ganglio-mimics with similar intensities indicating that the underlying inner-core oligosaccharide of the mimics does not influence recognition.

Having established the glyco-microarray is appropriate for examining specificities of anti-ganglioside antibodies, attention was focused on determining IgG and IgM antibodies binding profiles of 12 serum samples from patients that suffered from Guillain–Barré syndrome and had an antecedent *C. jejuni* infection (Table S2) and showed positive serological responses in a traditional ELISA for anti-ganglioside antibodies (Table S3).

Representative array results are presented in Figure 3, and the data for all patient serum samples are shown in Figure S13A. In each sample, IgM and/or IgG antibodies were observed that bound one or more of the ganglioside oligosaccharides (**14**–**25**). Anti-GM1a (**16**), anti-GM1b (**24**), and anti-GD1a (**17**) antibodies have been associated with the motor form of GBS after *C. jejuni* infection.^{11,12,52} The array data showed that the majority of the patients had elicited IgM and/or IgG antibodies targeting GM1a. Anti-GM1b and GD1a antibody responses were observed in only a few serum samples. Interestingly, many patients had elicited antibodies

against GA1 (**23**); however, the clinical significance of this observation needs validation. As expected, on slide treatment of **14** and **19** with neuraminidase to create lactose abolished binding confirming lactose is not recognized by the serum antibodies.

It was observed that there is a significant correlation between the recognition of specific ganglio-oligosaccharides and the corresponding mimic indicating cross-reactivity (see Figure S14 for **4** vs **16**). However, for almost all of the examined samples, the binding of the ganglioside mimics (**2**–**13**) was much stronger than that of the corresponding ganglioside oligosaccharides (**14**–**25**). Furthermore, in most cases, no or a low response was observed for the inner-core oligosaccharide (**1**). These observations indicate that the GBS patients had elicited antibodies mainly to the outer core region of LOS of *C. jejuni*. The finding that the ganglioside oligosaccharides are not as well recognized as the mimics indicates that gangliosides are suboptimal ligands for the serum antibodies. In this respect, the ganglio-oligosaccharides have a lactosyl (Gal β (1,4)Glc) moiety at the reducing end, whereas the mimics have a Gal β (1,3)Hep epitope at this position. Thus, it is likely that the serum antibodies recognize an epitope that encompasses the heptosyl residue of the inner-core and the ganglio-oligosaccharide of the outer core. A heptoside poorly mimics the glucosyl moiety of gangliosides, thereby providing a rationale for the differences in responsiveness of ganglio-oligosaccharides and mimics. Thus, it appears that antibodies elicited during *C. jejuni* infection leading to GBS are directed to epitopes that straddle the inner- and outer-core region of LOS that can cross-react with autologous gangliosides. This finding is in accordance with the clinical observation that GBS has a monophasic course in which patients tend to recover after clearing of the infection.

Although commonalities were observed in the antibody responses, also differences were noted, which probably is due to the clinical heterogeneity of GBS. First, the magnitude of the antibody responses differed considerably between the various serum samples. For one patient, mainly IgG antibodies were observed (S005), whereas for another one predominantly IgM antibodies were detected (S038). However, most patients had elicited IgM and IgG antibodies, but in these cases also differences in binding patterns were observed. For several patients (S010, S014, S023, S035, S039) IgM and IgG antibodies were detected that bound to the ganglio-oligosaccharides as well as to the mimics. Other samples (S002, S007, S012) showed strong IgG antibody responses to the ganglioside mimics (**2**–**14**) with little- or no binding to the corresponding ganglio-oligosaccharides. In these cases, IgM antibodies were present that bound the ganglioside-oligosaccharides. Probably, class switching and antibody affinity maturation resulted in IgG antibodies with high affinity for the mimics but a low affinity for the gangliosides. It can, however, not be excluded that antibodies binding potently to gangliosides have been scavenged by nerves leaving antibodies to the mimics behind.⁵³

We also examined five serum samples (S017, S019, S024, S033, S037) of patients that had suffered GBS but for whom no anti-ganglioside antibodies had been detected by traditional ELISA (Table S3). In three of these samples (S017, S024, S033), the microarray detected anti-GM1a IgM antibody indicating the new platform is more sensitive to detect such antibodies (Figures S13B and S15). In addition, we analyzed antibody responses of 10 control serum samples, and as

expected, no or very low responses were measured (Figure S13C).

CONCLUSIONS

It is widely accepted that antibodies elicited to the outer core oligosaccharide of the LOS of *C. jejuni*, which structurally resembles the oligosaccharide moiety of gangliosides, cause neural injury and clinical symptoms of GBS.^{1–3} Despite their importance in the pathogenesis of GBS, the fine specificities of anti-ganglioside serum antibodies have been difficult to examine, and for example it is not known if elements of the inner core are part of antigenic epitopes. It is the expectation that knowledge of the fine specificities of anti-ganglioside antibodies elicited by GBS patients will provide a more comprehensive understanding of the microbial factors important for the pathogenesis of GBS and give opportunities to develop more robust platforms for diagnosis and monitoring of disease activity. Such investigations require a comprehensive set of well-defined and pure lipo-oligosaccharides of *C. jejuni* in which the conserved inner core is extended by various ganglioside mimetics. It also necessitates a complementary range of ganglio-oligosaccharides to systematically compare antigenic responses with the ganglio-mimics. To obtain a comprehensive range of ganglioside mimics, we drew inspiration from enzymatic approaches to prepare ganglio-oligosaccharides by extension of lactose by various microbial glycosyl transferases^{41,42,44,54} and envisaged that an inner core terminating in galactose would be also an appropriate precursor for these enzymes. We devised an efficient synthetic approach for *C. jejuni* inner core hexasaccharide based on a convergent and stereo-controlled [4 + 2] oligosaccharide assembly. It employed orthogonally protected building blocks that offered flexibility in the assembly of the hexasaccharide. Enzymatic extension of the hexasaccharide by a panel of glycosyltransferases afforded core oligosaccharides having various outer core ganglioside mimics. In parallel, we developed a chemoenzymatic approach for the gangliosides including the previously inaccessible GA1, GM1b, and GD1c by chemical or enzymatic removal of the sialoside of GM1a followed by enzymatic extension. The synthetic efforts provide an unprecedented collection of ganglio-oligosaccharides and their mimics including GM1, GM2, GM1a, GD1a, GT1a, GD3, GD2, GD1b, GT1b, GA1, GM1b, and GD1c.

The oligosaccharides were printed as a microarray to examine fine specificities of serum antibodies of GBS patients that had suffered an antecedent *C. jejuni* infection. The results showed that the majority of GBS patients had elicited IgM and/or IgG antibodies targeting GM1a (16). Interestingly, in almost all cases the GM1a mimic (4) was bound much more strongly indicating that in addition to the outer core, which mimics GM1a, part of the inner core is also recognized. The inner core (1) by itself gave low or no responsiveness highlighting that the outer core contains the critical antigenic component. Collectively, these observations indicate that antibodies elicited during the preceding *C. jejuni* infection leading to GBS are primarily raised to an epitope on the pathogen (LOS) that cross-react to the autologous gangliosides. Thus, although the outer core resembles a self-structure, it is attached to heptosyl residue thereby creating a foreign antigen for mammalian immune cells. It was also observed that the microarray platform can more sensitively detect anti-GM1a antibodies compared to a traditional ELISA format using bovine extracted gangliosides, which was especially the case for

IgM antibodies targeting GM1a. This may be due to the covalent binding of the antigens allowing the use of detergents during washing steps reducing nonspecific binding. Furthermore, a good correlation was found between the responsiveness of GM1a and its mimic (Figure S14), and thus the ganglioside mimics, which were recognized much more potently, may be attractive for the detection of serum antibodies associated with GBS. A glycan microarray has many advantages for the development of the next generation platform to examine the presence of anti-ganglioside antibodies to facilitate GBS diagnoses and disease monitoring. It requires minute quantities of precious oligosaccharides that can be printed in a format that allows in one operation the examination of a large number of compounds and samples. The assay is robust because compounds are chemically immobilized, and it is more sensitive and has a greater dynamic range compared to traditional ELISA. Antibodies that recognize ganglioside complexes have been observed in GBS patients.⁵⁵ A microarray platform makes it possible to print many different combinations of ganglio-oligosaccharides allowing the detection of such antibodies. To establish it as a platform for GBS diagnoses and monitoring of disease activity, future efforts will focus on the preparation of additional ganglioside mimics to determine the minimal epitope for binding by serum antibodies. In particular, attention will be focused on compounds having truncated inner core oligosaccharides to determine to what extent it is recognized by the serum antibodies. In addition, a large number of serum samples will be screened to establish threshold values of clinically relevant antibody responses.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.0c08583>.

Synthetic protocols, microarray procedures, compound characterization, and NMR and LC–MS spectra (PDF)

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Notes

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