PAPER

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Synthesis and biological evaluation of lipid A derived from commensal *Bacteroides*†

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The inflammation-inducing properties of lipopolysaccharides (LPS) of Gram-negative bacteria reside in their lipid A moiety. *Bacillus fragilis*, which is a commensal Gram-negative bacterium, biosynthesises lipid A that is structurally distinct from that of *E. coli* and other enteric bacteria. It is composed of a β1,6-linked glucosamine (GlcN) disaccharide that is only phosphorylated at the anomeric center. The major species of *B. fragilis* has five fatty acids and the amine of the distal GlcN moiety carries the unusual (*R*)-3-(13-methyltetradecanoyloxy)-1.5-methylhexadecanoic acid. A recent study indicates that the LPS of *B. fragilis* has anti-viral activity by selective induction of interferon (IFN)-β and is protective in mouse models of vesicular stomatitis virus (VSV) and influenza A. Heterogeneity in the structures of LPS and lipid A and possible contamination with other inflammatory components make it difficult to unambiguously define the immune-modulatory properties of LPS or lipid A. Therefore, we developed a synthetic approach for the preparation of the unusual major lipid A species derived from *B. fragilis*, which includes a synthetic approach for (*R*)-3-(13-methyltetradecanoyloxy)-1.5-methylhexadecanoic acid by the Wittig olefination to install the terminal isopropyl moiety. The proinflammatory and antiviral responses of synthetic *B. fragilis* lipid A were investigated in several cell lines and primary human monocytes by examining the production of interleukin (IL)-6 and IFN-β. It was found that *B. fragilis* does not induce the production of IL-6 and IFN-β but can partially antagonize the production of pro-inflammatory cytokines induced by *E. coli* LPS and lipid A.

Introduction

Members of the genus *Bacteroides*, such as *B. thetaiotaomicron* and *B. fragilis*, are commensal Gram-negative bacteria that are abundant in the human ileum and large intestine.[1] They benefit the host by their ability to degrade indigestible plant polysaccharides, thereby providing cross feeding. Furthermore, several cell surface structures of *Bacteroidetes*, most notably lipopolysaccharide (LPS), exhibit immunomodulatory properties potentially beneficial for the host.[2,3]

LPS extracted from *B. fragilis* has low endotoxic activity and can antagonize proinflammatory responses induced by enteric LPS.[4] Furthermore, LPS from *B. vulgatus*, which is a commensal bacterium of the murine intestine, can restore intestinal homeostasis in mice in which colitis was induced.[5,6] A recent study has indicated that a heterogeneous LPS mixture of *B. fragilis* has anti-viral activity by selective induction of interferon (IFN)-β and is protective in mouse models of vesicular stomatitis virus (VSV) and influenza A virus.[4] Microbial compounds that induce IFN-β have the potential to function as therapeutics for various human diseases, making the LPS of *B. fragilis* an interesting target to pursue such opportunities.

The inflammation-inducing properties of LPS reside in its lipid A moiety.[7] Lipid A can be recognized by a heterodimeric complex of Toll-like receptor (TLR) 4 and myeloid differentiation factor 2 (MD-2) that is found on the surface of myeloid cells.[8] The binding of lipid A results in the formation of an m-shaped receptor multimer comprised of two TLR4–MD2–LPS complexes.[9] The resulting dimerization of the intracellular domains of two TLR4s creates binding sites for adapter proteins – myeloid differentiation primary response protein (MyD88) and Toll/IL-1R domain containing adaptor-inducing IFN-β (TRIF). MyD88-dependent cellular activation leads to the production of (pro)inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6. On the other hand, the TRIF-dependent pathway induces phosphorylation and dimerization of the transcription factor interferon regulatory factor 3 (IRF-3), which then activates the IFN

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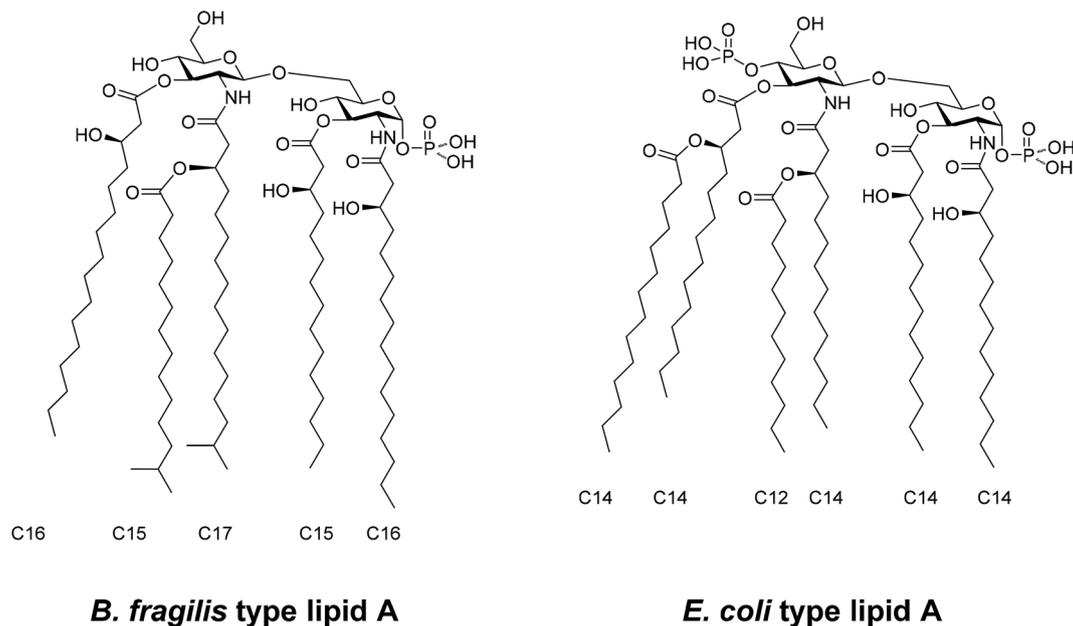*Org. Biomol. Chem.*, 2024, **22**, 8793–8800 | 8793

Fig. 1 Structures of *B. fragilis*-type lipid A and *E. coli*-type lipid A.

pathway, resulting in IFN-β production. Thus, it appears that the LPS of *B. fragilis* B. skews immune cell activation towards the TRIF pathway.[4]

Bacteroidetes species, such as *B. fragilis*, biosynthesise lipid As that are structurally distinct from that of *E. coli* and other enteric bacteria (Fig. 1).[10,11] They are composed of a β1,6-linked glucosamine (GlcN) disaccharide that is only phosphorylated at the reducing anomeric center. The major species of *B. fragilis* has five fatty acids. The amine of the proximal GlcN moiety is modified as (*R*)-3-hydroxyhexadecanoate and the C-3 hydroxyl as 3-(*R*)-hydroxylpentadecanoyl ester. The amine of the distal GlcN moiety carries the unusual (*R*)-3-(13-methyltetradecanoyloxy)-1.5-methylhexadecanoic acid and the C-3′ position is modified by an ester of (*R*)-3-hydroxyhexadecanoic acid. Lipid As from other *Bacteroidetes* species, including that of *B. thetaiotaomicron*, appear to have similar structures.[2] On the other hand, lipid A from *E. coli* is 1,4′-bis-phosphorylated and has (*R*)-3-hydroxymyristyl residues at the C-2, C-2′, C-3 and C-3′ positions (Fig. 1). The (3)-hydroxyacyl moieties of the distal GlcN moiety are further modified by lauric and myristic acids.

Human gut microbes, including *Bacteroidetes*, are in general resistant to inflammation-associated antimicrobial peptides.[12] Interestingly, a mutant of *Bacteroides thetaiotaomicron* that cannot remove the C-4′ phosphate from its lipid A was displaced from the microbiota during inflammation caused by infection.[13] Thus, it appears that the fine structural features of lipid A of *Bacteroides* contribute not only to immune-modulatory activity but also to maintenance of a healthy microbiota.

Heterogeneity in the structure of lipid A and possible contaminations with other inflammatory components make it difficult to unambiguously define the immune properties of

LPS or lipid A.[14,15] Chemical synthesis offers an attractive approach to obtain well-defined lipid A derivatives for structure–activity relationship studies.[7,16–18] Therefore, we set out to develop a synthetic approach to prepare the major lipid A derived from *B. fragilis*. It includes a synthetic approach for the unusual (*R*)-3-(13-methyltetradecanoyloxy)-1.5-methylhexadecanoic acid by the Wittig olefination to install the terminal isopropyl moiety.[19] The resulting synthetic lipid A was examined for its ability to induce the production of pro-inflammatory (IL-6) and anti-viral (IFN-β) cytokines and the results were compared with similar responses induced by LPS, lipid A and monophosphoryl lipid A from *E. coli*. In addition, the antagonistic properties of lipid A from *B. fragilis* to reduce proinflammatory responses induced by LPS and lipid A were investigated. It was found that lipid A from *B. fragilis* does not induce the production of IL-6 and IFN-β but can partially antagonize the production of pro-inflammatory cytokines induced by *E. coli* LPS and lipid A.

Results and discussion

Chemical synthesis

It was envisaged that lipid A from *B. fragilis* (**1**) could be synthesised from glycosyl donor **2** and acceptor **3** and lipids **4**, **5**, and **6** (Fig. 2). 2-Methylnaphthyl ethers (Nap) was selected as permanent protecting group because it can be more readily removed by hydrogenation[20,21] compared to the conventionally employed benzyl ether. The C-3 hydroxyl groups of glycosyl donor **2** and acceptor **3** were modified by (*R*)-3-(2-naphthylmethoxy)hexadecanoic ester and (*R*)-3-(2-naphthylmethoxy) pentadecanoic ester, respectively, to minimize the number of
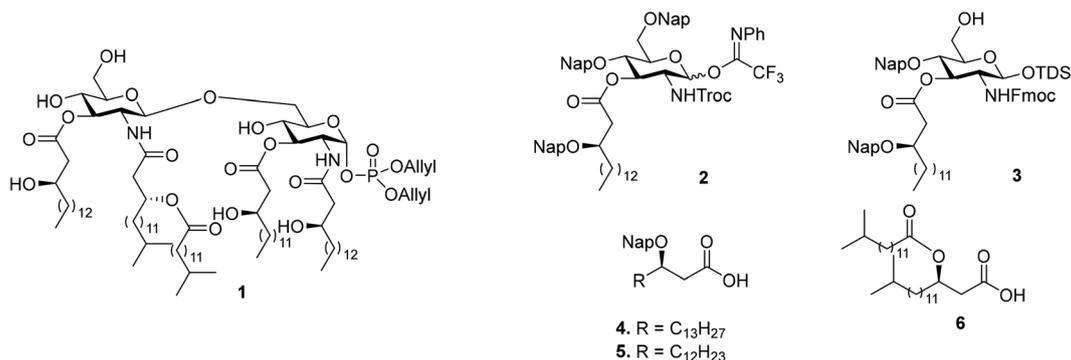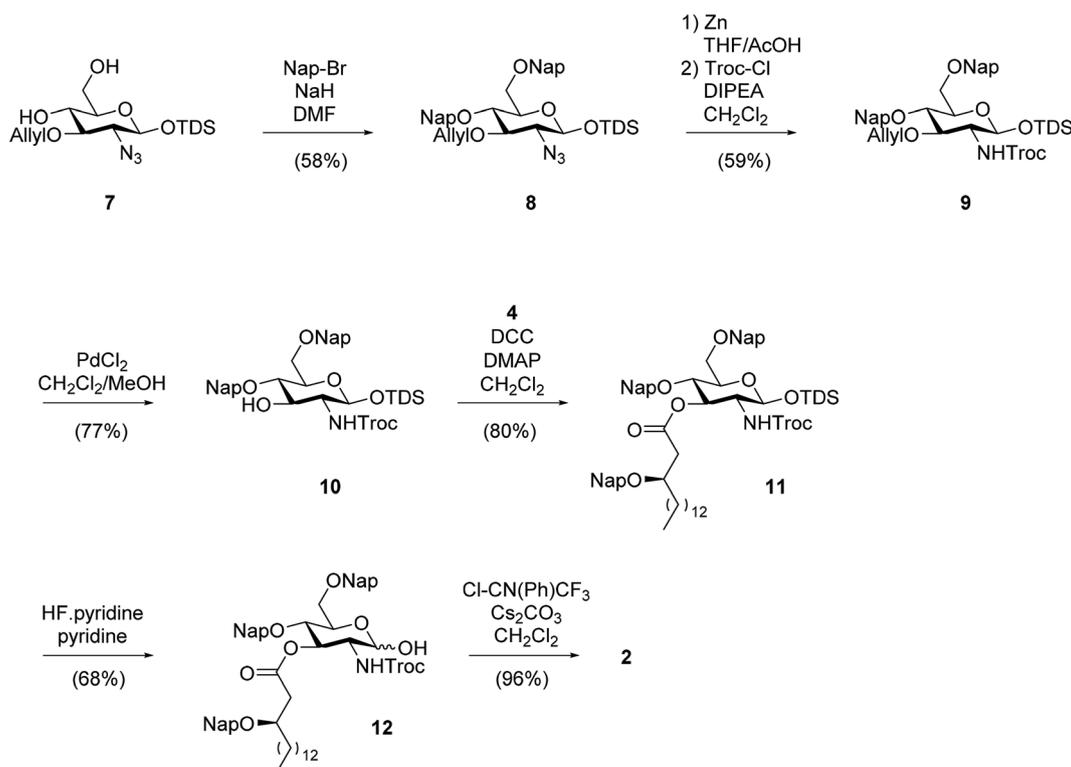
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Fig. 2 *B. fragilis* lipid A (**1**) and building blocks (**2**–**6**) for chemical synthesis.

synthetic steps that need to be performed at the disaccharide stage. The amine of the donor was protected as 2,2,2-trichloroethoxycarbonyl (Troc) carbamate because it can perform neighboring group participation during glycosylation, resulting in the selective formation of 1,2-*trans*-glycosides. It can be selectively cleaved by zinc in acetic acid to give a free amine that can be acylated with **6**. The amine of acceptor **3** was protected as 9-fluorenylmethyloxycarbonyl (Fmoc) carbonate because it can be removed under mild basic conditions without influencing other functionalities, allowing the introduction of **4**. The anomeric phosphate was installed at a late stage using diallyl diisopropylphosphoramidite and can be readily deprotected by PdCl$_2$.[22]

The preparation of glycosyl donor **2** started from compound **7**[23] having an amino masking azido functionality at the C-2 position. The C-4 and C-6 hydroxyls of this compound can be readily protected by Nap ethers by Williamson ether synthesis using 2-methylnaphthyl bromide (NapBr) in the presence of NaH in DMF to give compound **8**. We also attempted to install Nap ethers on a similar compound having Troc at the C-2 position under acid conditions using *O*-(2-naphthylmethyl) trichloroacetamidate; however, these efforts were unsuccessful.[24] Next, the azido group of **8** was reduced using Zn in a mixture of THF and acetic acid and the resulting amine was immediately protected as Troc carbamate by reaction with 2,2,2-trichloroethoxycarbonyl chloride (TrocCl) in the presence of *N,N*-diiso-
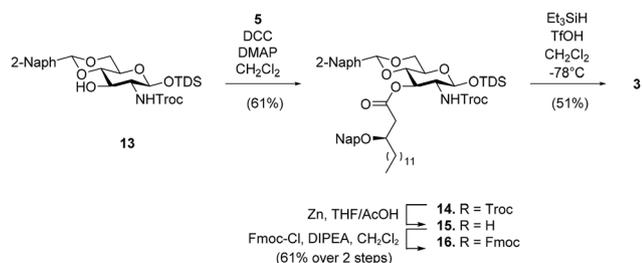

Scheme 1 Preparation of glycosyl donor **2**.

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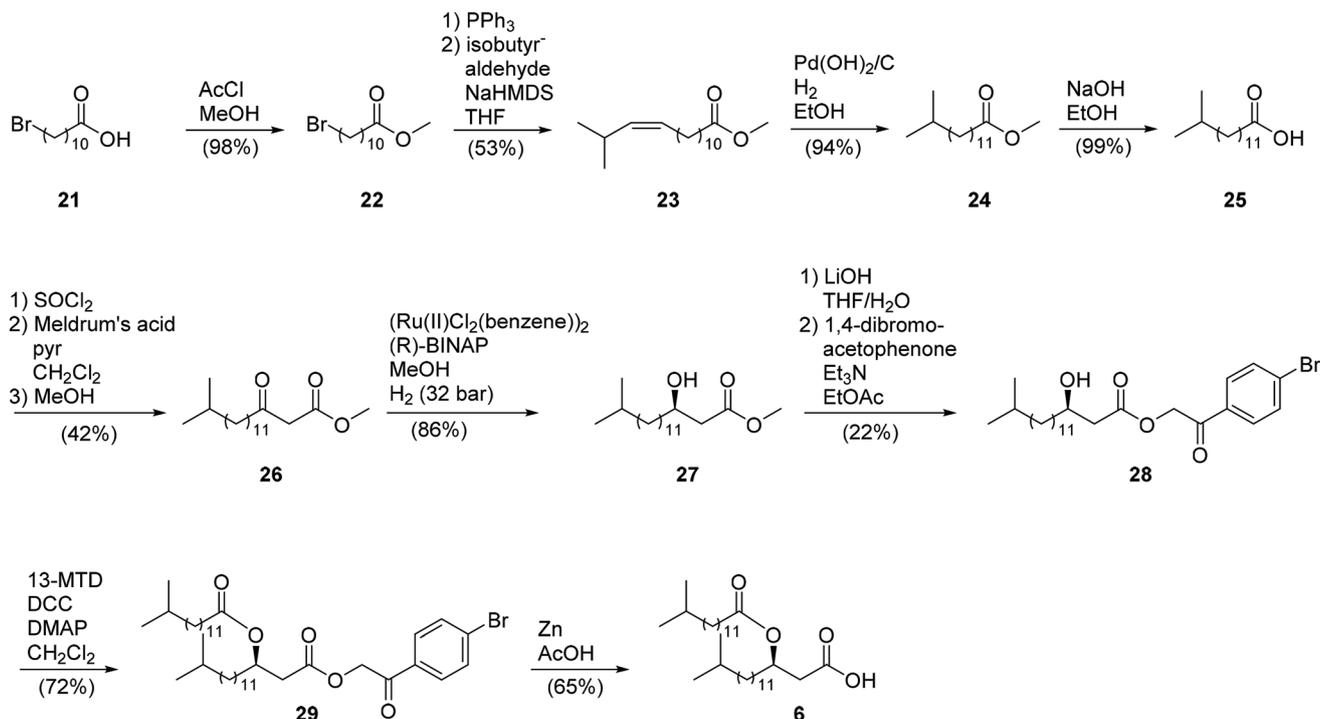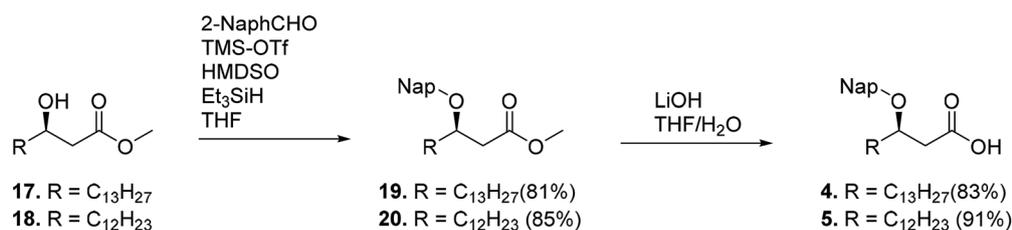*Org. Biomol. Chem.*, 2024, **22**, 8793–8800 | **8795**

propylethylamine (DIPEA). After silica gel column chromatography, **9** was obtained in a yield of 59% over two steps. The allyl ether at the C-3 position of **9** was removed using $PdCl_2$ in a mixture of CH_2Cl_2 and MeOH to give **10**. In the next step, the C-3 hydroxyl group of **10** was modified by the Steglich acylation

using lipid **4** in the presence of *N,N*-dicyclohexylcarbodiimide (DCC) and a catalytic amount of 4-(dimethylamino)pyridine (DMAP) to give **11**. The anomeric thexyldimethylsilyl (TDS) group of **11** was removed by HF in pyridine and the resulting **12** could be isolated by precipitation from water (Scheme 1). Finally, conversion of the anomeric hydroxyl group of **12** into *N*-phenyl trifluoroimidate[25,26] using 2,2,2-trifluoro-*N*-phenyl acetimidoyl chloride in the presence of caesium carbonate in CH_2Cl_2 afforded donor **2** in a yield of 96%.

Glycosyl acceptor **3** could easily be prepared from **13**[27] and **5** (Scheme 2). Thus, the carbohydrate building block **13** was acylated with **5** using DCC in the presence of a catalytic amount of DMAP to give **14**. Next, Troc carbamate of **14** was replaced by Fmoc by a two-step procedure entailing the reductive removal of Troc by Zn in THF and acetic acid to give the free amine **15** that was reacted with 9-fluorenylmethyloxycarbonyl chloride to give **16** in an overall yield of 61%. Reductive opening of the 4,6-acetal of **16** using dichlorophenyl-

Scheme 2 Preparation of glycosyl acceptor **3**.

**Scheme 3** Preparation of lipids **4** and **5** (top) and bi-antennary lipid **6** (bottom).

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borane and triethylsilane[26,28] in CH_2Cl_2 at $-78\ ^\circ C$ afforded acceptor **3** in a yield of 51%.

The preparation of 3-(*R*)-2-naphthylmethoxyhexadecanoic acid (**4**) and 3-(*R*)-(2-naphthylmethoxy)pentadecanoic acid (**5**) started from **17**[29,30] and **18**,[31] respectively. Reductive conditions were employed to install the Nap ether[32] to prevent epimerization of the chiral center under basic conditions, resulting in **19** and **20**. Saponification of the methyl ester afforded the protected acids **4** and **5**. 13-Methyltetradecanoic acid (13-MTD; **25**) was prepared by a five-step procedure starting from commercially available 11-bromoundecanoic acid (Scheme 3). Esterification of this compound using acetyl chloride in methanol gave **22**. The latter compound was treated with triphenylphosphine (neat) at 140 °C to give the corresponding triphenylphosphonium bromide salt that was used without purification in a Wittig reaction with isobutyraldehyde to provide methyl 14-methyltetradecano-11-enate **2** mainly as the *cis*-isomer. Hydrogenation of the double bond of **23** using $Pd(OH)_2$ gave **24** that was treated with NaOH to saponify the ester to provide **25**.

Having **25** in hand, attention was focused on the preparation of lipid **6**. Thus, **25** was converted into an acyl chloride by reflux in thionyl chloride and then coupled with Meldrum's acid (2,2-dimethyl-1,3-dioxane-4,6-dione) in a mixture of pyridine and dichloromethane to give an intermediate malonate that was subjected to decarboxylation in methanol to provide β-ketoester **26**. The ketone of the latter compound was enantioselectively reduced by catalytic hydrogenation in the presence

of freshly prepared (*R*)-BINAP-RuCl₂ to give 3-hydroxyl fatty acid **27**.[33] The enantiomeric excess was determined to be 99% by optical rotation measurement. The methyl ester was saponified and the resulting carboxylic acid was protected as a 2-(4-bromophenyl)-2-oxoethyl ester by reaction with 4-bromoacetophenone to give **28**. Esterification of the C-3 hydroxyl of **28** with **25** using DCC in the presence of catalytic DMAP resulted in the formation of **29**. The 2-(4-bromophenyl)-2-oxoethyl ester could be selectively cleaved by activated Zn in AcOH to give, after purification by silica gel chromatography, compound **6**.

A trifluoromethanesulfonic acid (TfOH) catalyzed glycosylation of glycosyl acceptor **3** and donor **2** resulted in the selective formation of β-glycoside **30** in a yield of 71% (Scheme 4). Next, the Troc group of **30** was removed by activated Zn to give a free amine. Multiple reaction conditions were explored to acylate the amine with **6** to give **31**. Low yields were obtained using DCC in combination with K-OxymaPure or hydroxybenzotriazole (HOBt), which in part was due to coupling of the secondary carboxylate to the C-2 amine. Gratifyingly, the desired compound **31** was obtained in a yield of 38% using DCC alone and under these conditions, no side product formation was observed. The Fmoc group of **31** was deprotected using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and the resulting amine was acylated with **4** using DCC and K-OxymaPure to afford **32** in a yield of 72%. The anomeric TDS group was removed using HF in a mixture of pyridine and THF to give pyranose **33**. Treatment of the latter compound with di-allyl *N,N*-diisopropylphosphoramidite in the presence of 1*H*-tetrazole
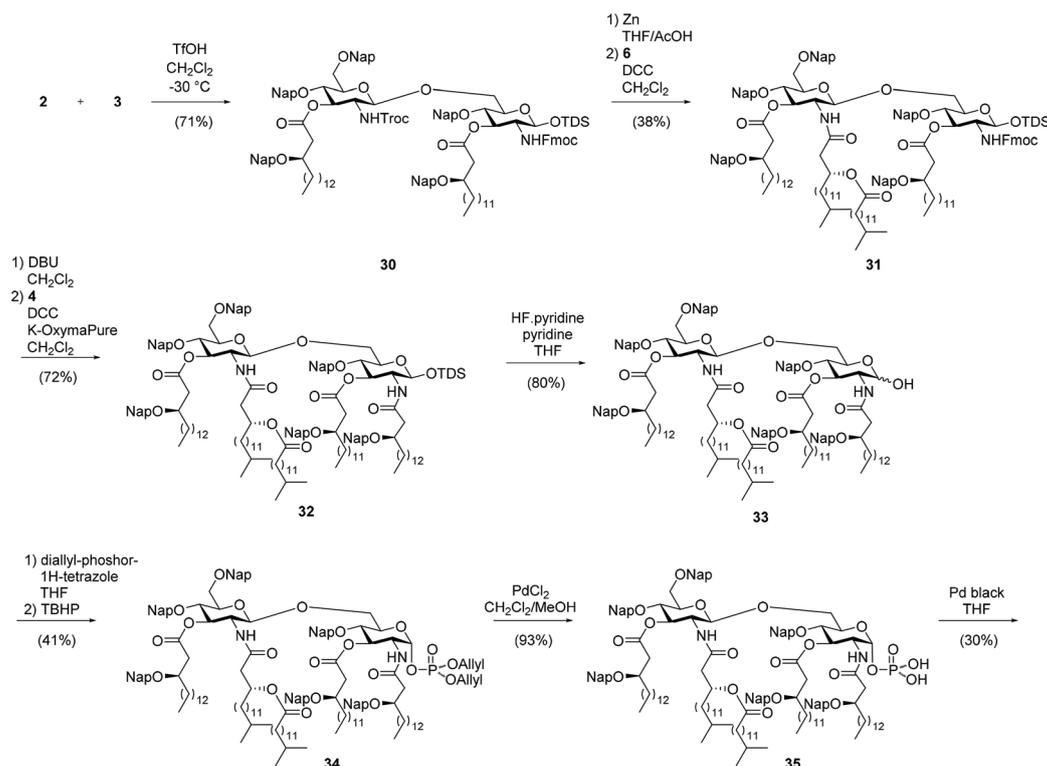
Scheme 4 Assembly and deprotection of *B. fragilis* lipid A (**1**).

resulted in the stereoselective installation of an alpha-anomeric phosphite that was *in situ* oxidized using the mild oxidant *tert*-butyl hydroperoxide to give α-anomeric phosphotriester **34**. The complete deprotection of **34** was carried out in two steps, from which the first was cleavage of the allyl esters of the phosphotriester using PdCl$_2$ in a mixture of CH$_2$Cl$_2$ and MeOH. In the second step, the obtained anomeric phosphate **35** was subjected to catalytic hydrogenation over Pd black in THF for cleavage of the Nap protecting groups. Initial attempts at room temperature and low pressure H$_2$ (g) (hydrogenation balloon) were not successful, leading to only partial deprotection. After varying the reaction temperature and pressure, the optimal conditions were found to be at 50 °C and 50 bar of H$_2$. Target compound **1** was obtained in a yield of 30% after purification by Sephadex LH-20 size exclusion column chromatography and trituration from petroleum ether. The anomeric phosphate was found to be prone to cleavage

when a 1 : 1 mixture of dichloromethane and methanol was used as the eluent for Sephadex LH-20 column chromatography. NMR analysis indicated that at least 60% of the phosphate was cleaved. Fortunately, when employing a mixture of CHCl$_3$/methanol/H$_2$O (2/3/1), no cleavage of the anomeric phosphate was observed, even after 5 days. The stability of an anomeric phosphate in this solvent mixture was found to be comparable with other anomeric phosphates in the literature.[34] The structure was confirmed by NMR (^{1}H, ^{31}P and 2D NMR) and MALDI-TOF MS analysis.

Biological evaluation

Next, we evaluated the immunomodulatory properties of lipid A from *B. fragilis* and compared them with the similar properties of *E. coli* 055:B5 LPS, and lipid A and monophosphoryl lipid A from *E. coli*.[35] Release of IL-6 was evaluated in primary human monocytes by applying a wide concentration range
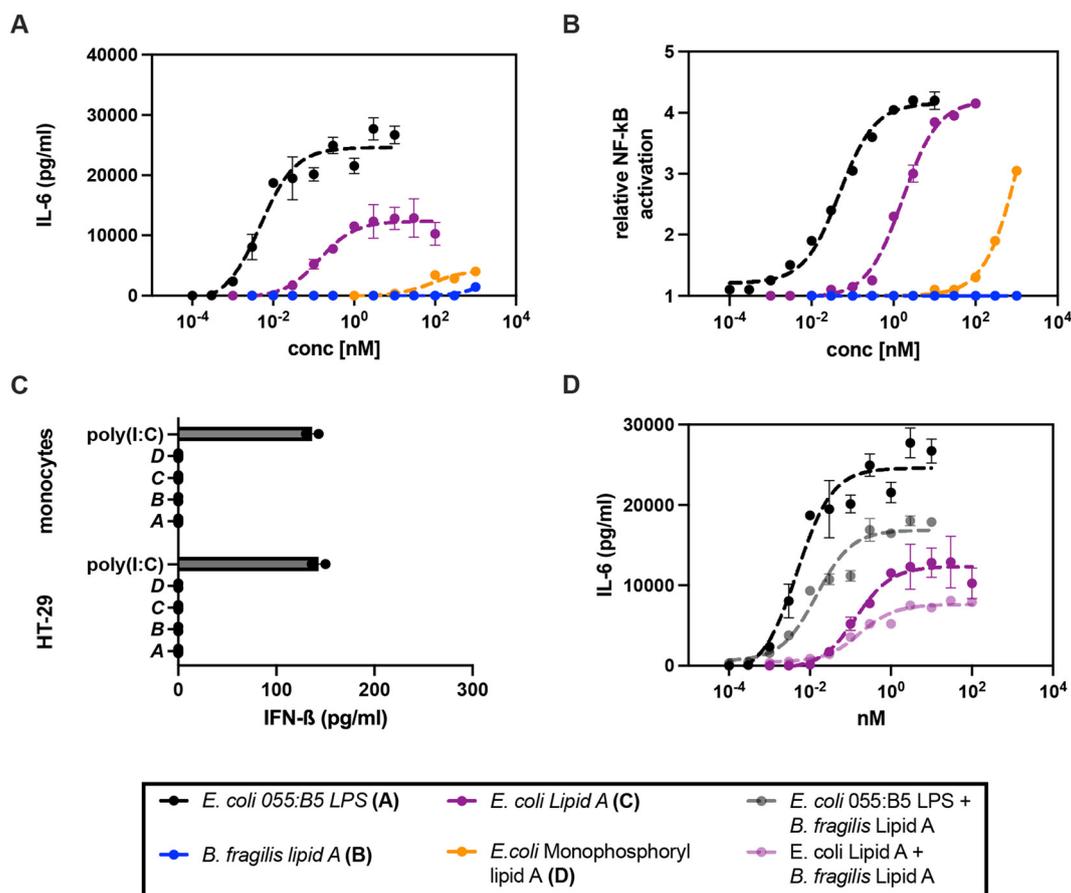

Fig. 3 Biological activity of lipid A from *B. fragilis* to induce pro- and anti-viral cytokine secretion. (a) *E. coli* 055:B5 LPS, (b) *B. fragilis* lipid A, (c) *E. coli* lipid A, and (d) *E. coli* monophosphoryl lipid A were administered to (A) human primary monocytes (*n* = 4, 16 h) and (B) the hTLR4 SEAP HEK-Blue reporter cell line (control: Null2 SEAP HEK-Blue, *n* = 2, 24 h) using a wide concentration range (0.0001 nM–1000 nM); 100 ng mL^{-1} LPS corresponds to 10 nM in accordance with an average molecular mass of 10 kDa.[36,37] Representative data, expressed as relative NF-κB activation, are shown as the mean of two biological replicates. EC$_{50}$ values were calculated by sigmoidal curve fitting with a constrained hill slope. (C) The human colon epithelial cancer HT29 cell line (*n* = 3) and human primary monocytes (*n* = 2) were exposed to (a) *E. coli* 055:B5 LPS (10 nM), (b) *B. fragilis* lipid A (1000 nM), (c) *E. coli* lipid A (100 nM), and (d) *E. coli* monophosphoryl lipid A (1000 nM) and poly(I:C) HMW LyoVec (10 μg mL^{-1}) for 24 and 16 h, respectively. IFN-β release is shown as the means of biological duplicates. (D) Human primary monocytes (*n* = 2) were pre-incubated with 300 nM *B. fragilis* lipid A for 30 min and subsequently exposed to a wide concentration range (0.0001–100 nM) of *E. coli* 055:B5 LPS and *E. coli* lipid A. The data are shown as the mean of biological duplicates from one representative donor.

(0.01 nM–1000 nM) of the compounds for 16 h and unstimulated cells served as the negative control. As expected, *E. coli* 055:B5 LPS showed the highest potency with an EC$_{50}$ (concentration producing 50% activity) of 0.005 nM. Lipid A from *E. coli* showed a somewhat lower potency (EC$_{50}$ of 0.14 nM) and efficacy (maximum or plateau level). On the other hand, monophosphoryl lipid A from *E. coli* and lipid A from *B. fragilis* (**1**) only triggered low levels of pro-inflammatory cytokine production at a concentration of 10 nM and 300 nM, respectively (Fig. 3A) and unstimulated human monocytes did not release any IL-6.

To confirm that the observed activities were induced by binding to the TLR4/MD2 complex followed by NF-κB activation, the secreted embryonic alkaline phosphatase (SEAP) HEK-Blue reporter cell line expressing the human TLR4 (hTLR4) receptor in combination with MD2 and CD14 was stimulated with LPS and the lipid A derivatives and compared with the Null2 SEAP HEK-Blue reporter cell line lacking the hTLR4/MD2/CD14 complex (Fig. 3B). Indeed, the hTLR4 SEAP HEK-Blue cell line responded strongly to *E. coli* 055:B5 LPS (EC$_{50}$ of 0.048 nM) and lipid A (EC$_{50}$ of 1.7 nM). Monophosphoryl lipid A from *E. coli* only gave a response at high concentration (100 nM) while *B. fragilis* did not show any responsiveness. The stimulated Null2 SEAP HEK-Blue reporter cell line and unstimulated cells did not induce SEAP activity.

It has been reported that lipid As derived from commensal bacteria including *B. fragilis* can induce antiviral responses (*i.e.* IFN-β secretion).[4] Therefore, the human colon epithelial cancer HT29 cell line that is capable of producing and secreting IFN-β was exposed to a wide concentration range of lipid A from *B. fragilis* and LPS and lipid A derivatives from *E. coli* for 24 h. Encapsulated high molecular weight (HMW) poly(I:C) (LyoVec), which triggers IFN-β secretion through TLR3 activation, was employed as a positive control. HT29 cells responded in a dose-dependent manner to these stimuli – in the same manner as primary human monocytes – as shown by the quantification of the pro-inflammatory cytokine IL-8 (Fig. S1†). *B. fragilis* lipid A was not capable of inducing IFN-β secretion even at a concentration of 1000 nM, while HMW poly (I:C) (10 μg mL^{-1}) treatment resulted in a secretion of 137 pg mL^{-1} IFN-β (Fig. 3C). A similar experiment was performed in primary human monocytes and in this case, encapsulated HMW poly(I:C) also induced the production of IFN-β, whereas no response was measured for lipid A from *B. fragilis* (Fig. 3C) without any effect on viability (Fig. S2†). To further underpin this observation, the mouse macrophage cell line RAW 264.7 (NO-) was stimulated in the same manner. While LPS and lipid A derived from *E. coli* were able to induce IFN-β secretion, the synthesised lipid A from *B. fragilis* was not able to induce IFN-β secretion (Fig. S3†). In all experiments, unstimulated cells did not show any cytokine secretion.

To further elucidate the effect of lipid A from *B. fragilis* on TLR4 activation, primary human monocytes were pre-incubated for 30 min with a high concentration of lipid A from *B. fragilis* (300 nM) and subsequently stimulated with a wide concentration range (0.0001–10 nM) of *E. coli* 055:B5 LPS or *E. coli* lipid A. It was found that *B. fragilis* lipid A was able to inhibit the efficacy of IL-6 induced by *E. coli* 055:B5 LPS and lipid A by 31% and 38%, respectively (Fig. 3D). Application of lipid A from *B. fragilis* at a lower concentration did not result in inhibition and thus *B. fragilis* lipid A is a rather weak antagonist.

Conclusions

We have developed a synthetic approach for the preparation of the major lipid A derivative from *B. fragilis*. It includes an efficient synthetic approach for the unusual (R)-3-(13-methyltetradecanoyloxy)-1,5-methylhexadecanoic acid by the Wittig olefination to install a terminal isopropyl moiety. Furthermore, 2-methylnaphthyl ethers (Nap) were employed as permanent protecting groups because they can be more readily removed by hydrogenation compared to the conventionally employed benzyl ether. By protection of the C-4 hydroxyl of the acceptor and the C-4 and C-6 hydroxyls of the donor by Nap ethers, a disaccharide could be prepared that after protecting group manipulations and installation of fatty acids could be subjected to selective anomeric phosphorylation. Furthermore, Troc and Fmoc were employed as orthogonal amino protecting groups to selectively acetylate the amine of the proximal and distal glucosamine moieties. Cell biological experiments indicate that this synthetic lipid A derivative from *B. fragilis* does not induce the production of pro-inflammatory (IL-6) and antiviral (IFN-β) cytokines. It can, however, partially antagonize the production of pro-inflammatory cytokines induced by *E. coli* LPS or lipid A. The lack of induction of proinflammatory cytokines and antagonizing properties may contribute to a gut homeostasis. In this study, we prepared the major species of lipid A derived from *B. fragilis* and it cannot be excluded that minor lipid A derivatives of heterogeneous native preparations were responsible for inducing the detected production of IFN-β. Thus, future studies will focus on the preparation of other lipid A derivatives from *B. fragilis* to evaluate the lipid A derivative responsible for anti-viral cytokine (IFN-β) production. Earlier studies examined the responsiveness of mouse dendritic cell populations (cDC2, CD103$^+$CD11b$^+$ and CD103CD11b$^+$ cells) that reside in the colon to examine the induction of IFN-β.[4] It is possible that the ability to induce this cytokine in a TLR4/MD2-dependent manner relies on this cell type.

Data availability

The data underlying this study are available in the article and its ESI.†

Conflicts of interest

The authors declare no conflict of interest.

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