How to cite: *Angew. Chem. Int. Ed.* **2024**, e202402922
doi.org/10.1002/anie.202402922

Glycolipids Hot Paper

Chemical Synthesis of *Acetobacter pasteurianus* Lipid A with a Unique Tetrasaccharide Backbone and Evaluation of Its Immunological Functions

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Abstract: Lipopolysaccharide (LPS), a cell surface component of Gram-negative bacteria, activates innate immunity. Its active principle is the terminal glycolipid lipid A. *Acetobacter pasteurianus* is a Gram-negative bacterium used in the fermentation of traditional Japanese black rice vinegar (kurozu). In this study, we focused on *A. pasteurianus* lipid A, which is a potential immunostimulatory component of kurozu. The active principle structure of *A. pasteurianus* lipid A has not yet been identified. Herein, we first systematically synthesized three types of *A. pasteurianus* lipid As containing a common and unique tetrasaccharide backbone. We developed an efficient method for constructing the 2-trehalosamine skeleton utilizing borinic acid-catalyzed glycosylation to afford 1,1'-α,α-glycoside in high yield and stereoselectivity. A common tetrasaccharide intermediate with an orthogonal protecting group pattern was constructed via [2+2] glycosylation. After introducing various fatty acids, all protecting groups were removed to achieve the first chemical synthesis of three distinct types of *A. pasteurianus* lipid As. After evaluating their immunological function using both human and murine cell lines, we identified the active principles of *A. pasteurianus* LPS. We also found the unique anomeric structure of *A. pasteurianus* lipid A contributes to its high chemical stability.

Introduction

Lipopolysaccharide (LPS) is a cell surface component of Gram-negative bacteria that activates innate immunity to induce the release of inflammatory cytokines, nitric oxide, and reactive oxygen species.[1–3] LPS generally consists of a polysaccharide moiety, termed the O-antigen, a core oligosaccharide region, and the glycolipid lipid A, located at the terminal of LPS.[1–3] The lipid A moiety is widely known as the active principle for the immunostimulatory effects of LPS. Canonical *Escherichia coli* lipid A is composed of a glucosamine (GlcN) disaccharide backbone attached to two phosphate groups at the 1-position of the reducing end and the 4′-position of the non-reducing end (Figure 1). Toll-like receptor 4 (TLR4)/myeloid differentiation factor-2 (MD-2) receptor plays a central role in sensing lipid A and initiates two representative signal cascades: one is the myeloid differentiation factor 88 (MyD88)-dependent pathway, which leads to the up-regulation of inflammatory cytokines such as interleukin-6 (IL-6), and the other is the TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathway, which is associated with type I interferon production.[4] Overactivation of TLR4/MD-2 by potent LPS or lipid A, such as canonical *E. coli* LPS, can cause excessive inflammation and severe sepsis.

Recently, the immunomodulatory function of LPS in fermented foods has attracted considerable attention, and its application in health foods and pharmaceutical drugs is anticipated.[5] Black unpolished rice vinegar, known as kurozu, is a traditional Japanese fermented food that has been reported to have various effects, such as improving

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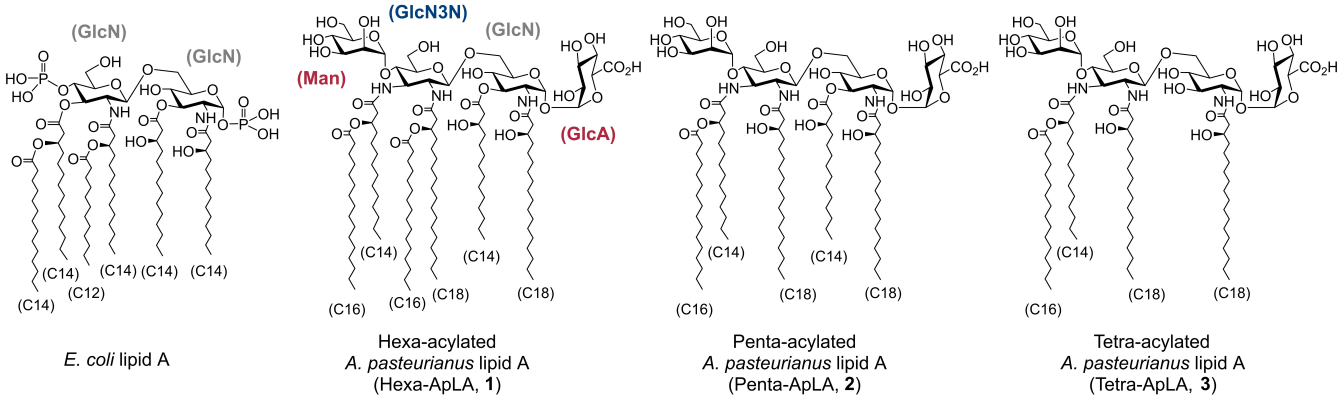
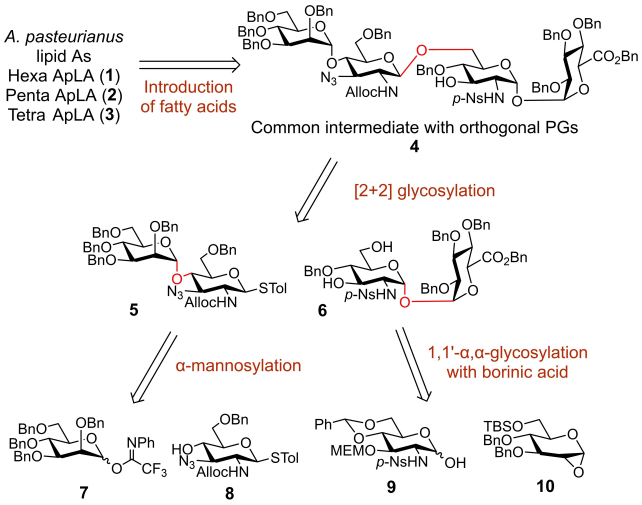


Figure 1. Chemical structures of *A. pasteurianus* lipid As **1**–**3**. GlcN = glucosamine, GlcN3 N = 2,3-diamino-2,3-dideoxyglucose, Man = mannose, GlcA = glucuronic acid.

host immune function and alleviating allergic symptoms.[6] In collaboration with Hashimoto et al., we previously found that the hydrophobic fraction of kurozu exhibited immunostimulatory activity via TLR4/MD-2.[7] Therefore, we extracted LPS from *Acetobacter pasteurianus*, an acetic acid bacterium most commonly used in kurozu fermentation, and found that *A. pasteurianus* LPS has moderate immunostimulatory activity, demonstrating its potential as an immunostimulatory component of kurozu.[8]

Furthermore, Silipo et al.[9] and Hashimoto et al.[8] characterized the chemical structure of *A. pasteurianus* lipid A and found that it has a unique tetrasaccharide backbone containing glucuronic acid (GlcA) and mannose (Man) instead of phosphate groups (Figure 1). Since the glycosyl phosphate group of lipid A is acid-labile, the GlcA modification has been suggested to be linked to the acid resistance of *A. pasteurianus* lipid A. However, the role of GlcA residues in the immunological function of lipid A is unclear, as the synthesis of *A. pasteurianus* lipid A has not been achieved. Moreover, the active principle structure of *A. pasteurianus* lipid A has not yet been identified because it was extracted as a heterogeneous mixture of three lipid As namely hexa-ApLA (**1**), penta-ApLA (**2**), and tetra-ApLA (**3**) (Figure 1).

Notably, the fine structure of lipid A significantly affects its capacity to activate the immune system. Previous studies have shown that the chain length, fatty acid distribution pattern, and number of phosphate groups affect its activity.[1,3,10] In this study, to investigate the immunological properties of *A. pasteurianus* lipid A, we report the divergent synthesis[11] of **1**–**3** by using the orthogonally protected tetrasaccharide intermediate **4** (Scheme 1). By evaluating the biological functions of the synthesized lipid As **1**–**3**, we identified the active principle of *A. pasteurianus* LPS. Furthermore, to clarify the molecular basis of the GlcA modification of *A. pasteurianus* lipid A, we performed a structure–activity relationship study and an acid resistance test of hexa-ApLA (**1**). Herein, by focusing on lipid A from acetic acid bacteria, we aimed to identify the immune regulatory factors in kurozu and elucidate how their unique structure is related to their functions.



Scheme 1. Retrosynthetic analysis of *A. pasteurianus* lipid As **1**–**3**. Bn = benzyl, Alloc = allyloxycarbonyl, *p*-Ns = 4-nitrobenzenesulfonyl, Tol = tolyl, Ph = phenyl, MEM = 2-methoxyethoxymethyl, TBS = *tert*-butyldimethylsilyl.

Results and Discussion

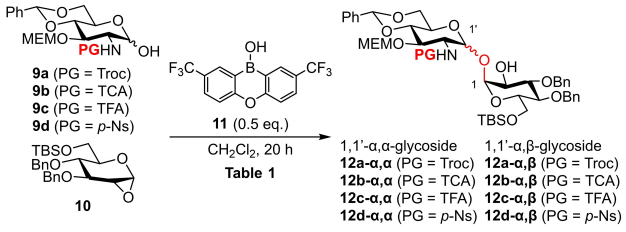
The divergent synthetic strategy for **1**–**3** is illustrated in Scheme 1. We planned to synthesize **1**–**3** by introducing fatty acids into the common tetrasaccharide intermediate **4**, which has an orthogonal protecting group pattern to enable the sequential introduction of fatty acids. Intermediate **4** was synthesized by [2 + 2] glycosylation of two disaccharide fragments, **5** and **6**. To synthesize disaccharide **5**, an α-mannosylation reaction involving mannosyl donor **7**[12] with 2,2,2-trifluoro-*N*-phenylacetoimidate[13] and 2,3-diamino-2,3-dideoxyglucose (GlcN3 N) acceptor **8** was performed. Furthermore, the construction of the 2-trehalosamine (2-amino-2-deoxy-α,α-D-trehalose) skeleton of disaccharide **6** was achieved through a borinic acid-catalyzed 1,1′-α,α-glycosylation reaction between glucosamine acceptor **9** and epoxide donor **10**.[14]

First, we constructed a 2-trehalosamine skeleton (Table 1). Notably, stereoselective 1,1′-glycosylation is challenging because four kinds of stereoisomers can be generated. Several approaches for synthesizing the 2-trehalosamine skeleton via chemical[15,16] and enzymatic[17] glycosylation have been reported. Mong et al. developed stereoselective synthesis of 2-trehalosamine in moderate yield using picolinoyl-protected trimethylsilyl glycosides as glycosyl acceptors.[15] In this study, we aimed to develop a more efficient glycosylation method for asymmetrically protected 2-trehalosamine derivatives that could be applied in the synthesis of ApLA **1**–**3** and similar useful glycosides.[18,19,20]

Recently, there have been significant advances in the development of organoboron-catalyzed 1,2-*cis*-glycosylation,[14,21–13] and Takemoto et al. reported a 1,1′-α,α-glycosylation method to construct a trehalose skeleton using a borinic acid catalyst together with a glucose acceptor and an epoxide donor[23] (Figure 2). In their study, the stereoselective reaction was supported by fixing the free hydroxy groups of the glycosyl acceptor to the 1,2-*cis*-conformation bridged by borinic acid. Inspired by this research, we hypothesized that this reaction could also be applied to the construction of a 2-trehalosamine skeleton; therefore, we attempted glycosylation between epoxide donor **10**[14] and glucosamine acceptors in the presence of borinic acid **11**[23] (Table 1).

Table 1: Investigation of 2-trehalosamine skeleton synthesis in this present study. Troc = 2,2,2-trichloroethyloxycarbonyl, TCA = trichloroacetyl, TFA = trifluoroacetyl.

Synthesis of 2-trehalosamine skeleton (This work)



Entry	Acceptor	Donor (**10**)	Temp. [°C]	Yield [%]
1	**9a**	2.0 eq.	r.t.	38 (**12a-α,α**) / 24 (**12a-α,β**)
2	**9b**	2.0 eq.	r.t.	37 (**12b-α,α**) / 5 (**12b-α,β**)
3	**9c**	2.0 eq.	r.t.	32 (**12c-α,α**) / 9 (**12c-α,β**)
4	**9d**	2.0 eq.	r.t.	58 (**12d-α,α**) / 7 (**12d-α,β**)
5	**9d**	2.0 eq.	-20	75 (**12d-α,α**) / 7 (**12d-α,β**)
6	**9d**	3.0 eq.	-20	85 (**12d-α,α**) / 9 (**12d-α,β**)

Synthesis of trehalose skeleton (Previous work, Takemoto et. al. 2020)

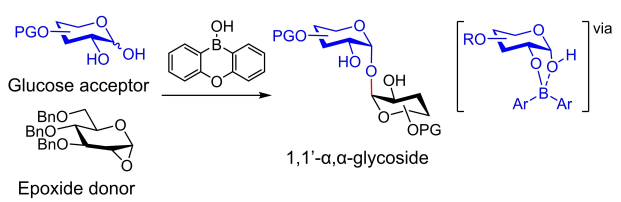
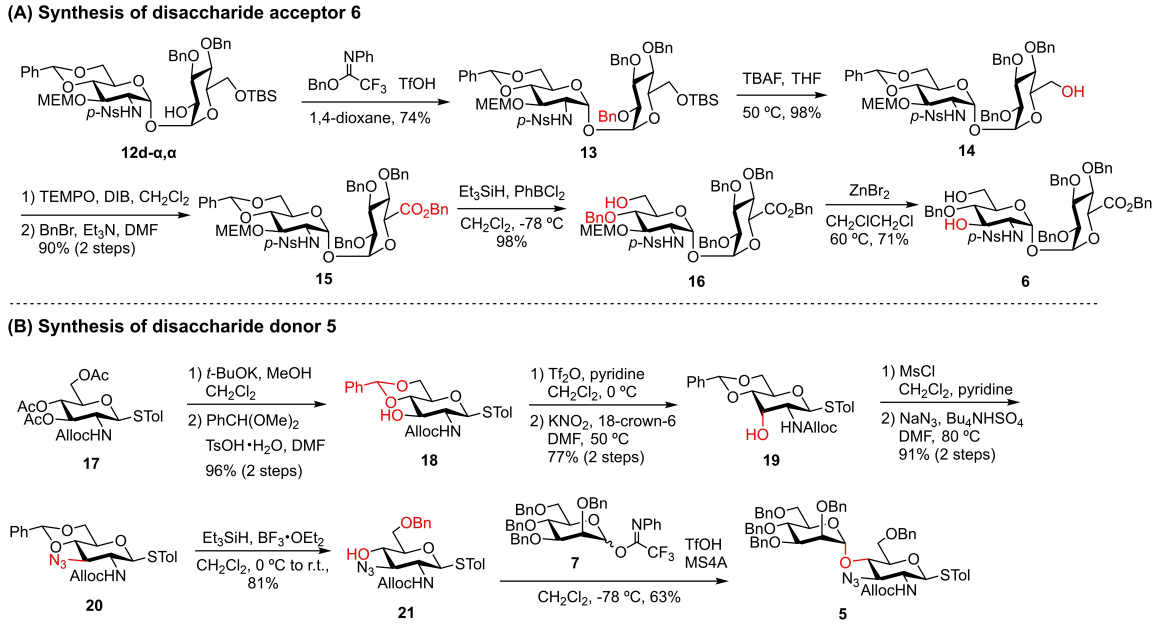


Figure 2. Synthetic method for trehalose skeleton reported in previous studies. PG = protecting groups, Ar = Aryl.

It is important to control the electron density on the nitrogen of the glucosamine acceptors in the complex with borinic acid, as suggested by Takemoto et al.[23] Accordingly, we initially investigated the effect of protecting groups (PGs) at the 2-position on borinic acid **11** catalyzed glycosylation. Considering the orthogonality with other PGs of the common tetrasaccharide intermediate **4**, we prepared **9a**–**9d**, respectively protected by 2,2,2-trichloroethyloxycarbonyl (Troc), trichloroacetyl (TCA), trifluoroacetyl (TFA), and 4-nitrobenzenesulfonyl (*p*-Ns) groups, which were easily removed in the presence of a base or thiol (Table 1, Scheme S1). Notably, only the 1,1′-α,α-glycoside **12x-α,α** and 1,1′-α,β-glycoside **12x-α,β** (where ***x*** = **a**–**d**) were observed, and neither 1,1′-β,α-glycoside nor 1,1′-β,β-glycoside were observed in all entries. The use of **9a** with a Troc group afforded the desired **12a-α,α** in 38 % yield and the undesired **12a-α,β** in 24 % yield (Table 1, entry 1). When using acceptors **9b** and **9c** with stronger electron-withdrawing PGs than the Troc group, such as the TCA and TFA groups, the selectivity improved, but the conversion decreased (Table 1, entries 2 and 3). Furthermore, when using **9d** with the *p*-Ns group, a more potent electron- withdrawing PG than the TCA and TFA groups, we successfully improved the selectivity and yield to obtain **12d-α,α** in 58 % yield and **12d-α,β** in 7 % yield (Table 1, entry 4). From these results, 1,1′-α,α selectivity tended to improve when strong electron-withdrawing PGs were used. To validate this tendency, when using acceptor **9e** with acetyl group which has weaker electron-withdrawing effect than the Troc group, the lowest selectivity was obtained in all entries, with **12e-α,α** in 34 % yield and **12e-α,β** in 23 % yield (Table S1). Furthermore, among the acceptors with electron-withdrawing PGs, the *p*-Ns-protected acceptor afforded the highest yield and selectivity. We speculated that this result might be due to the additional contribution of the pathway in which the epoxide donor **10** can be activated by a highly acidic proton in the complex with borinic acid, as suggested by Takemoto et al.[23] (detailed in Figure S2).

Next, we investigated employing various organoboron catalysts;[14,22,23] however, they did not improve the reaction yield (Table S1, entries 1–3). When using borinic acid **11** in entries 1–4 (Table 1), we also observed substantial hydrolysis of glycosyl donor **10**. Despite the addition of 4 A molecular sieves (MS), the yield of **12d-α,α** was not improved, and the production of the undesired trisaccharide **S10** was confirmed instead (Table S1, entry 4). We then attempted to lower the reaction temperature, which resulted in reduced decomposition of **10** and an increase in the yield up to 75 % (Table 1, entry 5). Subsequently, by increasing the equivalent of **10**, nearly all the starting material **9d** was reacted to afford **12d-α,α** in 85 % yield (Table 1, entry 6).

Following this, we synthesized the reducing-end disaccharide acceptor **6** (Scheme 2A). First, benzylation was performed at 2-position under acidic conditions using benzyl immidate and trifluoromethanesulfonic acid (TfOH) to afford **13** in 74 % yield. Next, we cleaved the *tert*-butyldimethylsilyl (TBS) group,[24] followed by oxidation[25] and benzylation at 6-position to obtain **15**. Furthermore, the regioselective reductive ring opening of the benzylidene



Scheme 2. Synthesis of the disaccharide acceptor **6** (A) and donor **5** (B). TfOH = trifluoromethanesulfonic acid, TBAF = tetrabutylammonium fluoride, THF = tetrahydrofuran, TEMPO = 2,2,6,6-tetramethylpiperidine 1-oxyl, DIB = (diacetoxyiodo)benzene, DMF = *N*,*N*-dimethylformamide. TsOH = *p*-toluenesulfonic acid, Tf_2O = trifluoromethanesulfonic anhydride, MsCl = methanesulfonyl chloride.

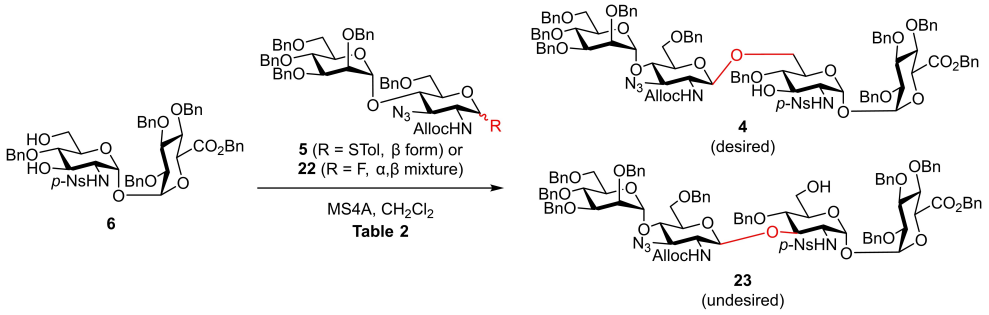
acetal was performed using triethyl silane and dichlorophenylborane[26] to afford **16** with a free hydroxy group at the 6′-position. Finally, we removed the 2-methoxyethoxymethyl (MEM) group[27] using zinc bromide to obtain disaccharide acceptor **6**.

The synthetic Scheme for non-reducing end disaccharide **5** is summarized in Scheme 2B. To construct the GlcN3 N skeleton, we employed C3 epimerization and S_N2 azidation.[28] First, methanolysis was performed to cleave the acetyl group of thioglycoside **17**[29] to obtain the triol product, followed by protection of the 4 and 6-positions using a benzylidene acetal, affording **18** in 96 % yield over two steps. The free hydroxy group at the 3-position of **18** was activated using triflate, followed by inversion of the stereochemistry through an S_N2 reaction using potassium nitrite to obtain **19**. The axial hydroxyl group at the 3-position of **19** was activated again with mesylate, and the azide group was introduced to construct the GlcN3 N skeleton in 91 % yield over two steps. Furthermore, selective reduction of the benzylidene acetal group of **20** was performed using Et_3SiH and $BF_3\cdot OEt_2$[30] to obtain **21** with a free hydroxy group at the 4-position. Finally, α-mannosylation using glycosyl donor **7**[12] was performed to yield disaccharide donor **5**. The stereochemistry of the anomeric position of **5** was determined using non-decoupling ^{13}C NMR.[31]

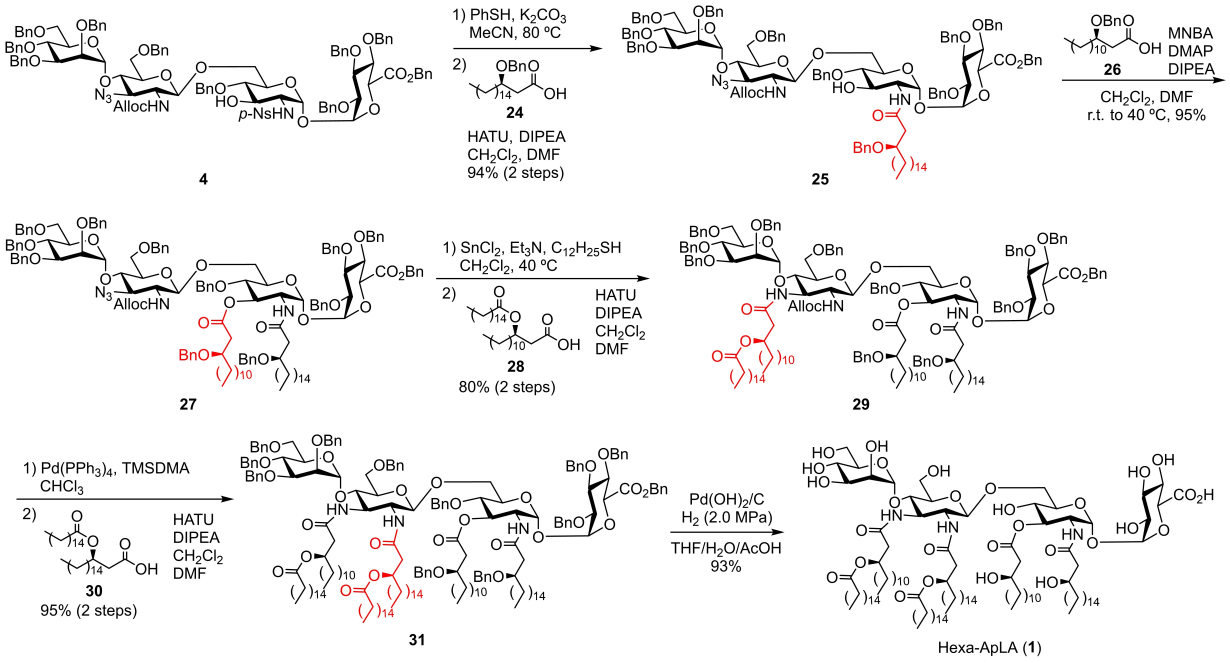
After obtaining the disaccharide donor **5** and acceptor **6**, we attempted the [2+2] glycosylation reaction (Table 2). We used thioglycosyl donor **5** with *N*-iodosuccinimide (NIS) and TfOH or *tert*-butyldimethylsilyl trifluoromethanesulfonate (TBSOTf) to afford the desired compound **4** in moderate yield; however, the undesired side-product **23**, glycosylated at the 3′-position, was also generated (Table 2, entries 1 and 2). In addition, replacing the oxidative reagent with *N*-bromosuccinimide (NBS) resulted in substantially worse selectivity than that in entry 1 (Table 2, entry 3). We switched the donor to glycosyl fluoride **22**, which was activated by $BF_3\cdot OEt_2$ to afford the desired compound **4** in excellent yield with high regioselectivity at 6′-position (Table 2, entry 4).

Next, we attempted to introduce fatty acids into the common tetrasaccharide intermediate **4** (Scheme 3). Our previous studies have shown that 2-methyl-6-nitrobenzoic anhydride (MNBA)[32] is the most effective condensation agent for the introduction of long fatty acids into this class of compounds;[33–35] however, side-products of 2-methyl-6-benzoyl amide are sometimes produced, resulting in low yields. In this study, MNBA was used for ester bond formation, and *O*-(7-azabenzotriazol-1-yl)-*N*,*N*′,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) was used for amide bond formation. Considering that *p*-Ns removal typically takes place under basic conditions, we introduced the first acyl chain into the 2′-position to avoid decomposition of the acyl group. The *p*-Ns group was removed using benzenethiol and potassium carbonate, followed by the introduction of **24**[33] using HATU and *N*,*N*-diisopropylethylamine (DIPEA) to afford **25** in 94 % yield over two steps. Subsequently, the hydroxy group at the 3′-position was acylated with MNBA, DIPEA, and 4-dimethylaminopyridine (DMAP). The azide group was reduced using tin (II) chloride[36] and odorless 1-dodecanethiol, thereby yielding the amine product. Subsequently, acyloxy fatty acid **28**[37] was introduced using HATU to obtain **29** in good yield. $Pd(PPh_3)_4$ and (dimethylamino)trimethylsilane (TMSDMA)[38] were used to remove the 2-*N*-allyloxycarbonyl (Alloc) group, and the condensation of acyloxy fatty acid **30** (Scheme S2) using HATU afforded **31** in 94 % yield.

Table 2: Investigation of [2+2] glycosylation. NIS = *N*-iodosuccinimide, NBS = *N*-bromosuccinimide, TBSOTf = *tert*-butyldimethylsilyl trifluoromethanesulfonate.



Entry	Donor (1.2 e.q.)	Reagents	Temp. [°C]	Time	Yield [%] (**4** / **23**)
1	**5**	TfOH (0.4 eq), NIS	-78 to -60	3 h	48 / 6
2	**5**	TBSOTf (0.4 eq), NIS	-78 to -40	3 h	47 / 11
3	**5**	TfOH (0.4 eq), NBS	-78	40 min	33 / 26
4	**22**	BF_3•OEt_2 (1.0 eq.)	0	40 min	83 / trace



Scheme 3. Chemical synthesis of hexa-ApLA (**1**). HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*′,*N*′,*N*′-tetramethyluronium hexafluorophosphate, DIPEA = *N*,*N*-diisopropylethylamine, DMAP = 4-dimethylaminopyridine, TMSDMA = (dimethylamino)trimethylsilane.

Finally, catalytic hydrogenolysis with $Pd(OH)_2/C$ under H_2 (2.0 MPa) was performed to achieve the first successful chemical synthesis of hexa-ApLA (**1**). The purification of hexa-ApLA (**1**) was performed by gel permeation chromatography using Sephadex™ LH-20. In addition, penta-ApLA (**2**) and tetra-ApLA (**3**) were synthesized using a similar synthetic strategy (Scheme S3).

To investigate the immune properties of the synthesized ApLA **1**–**3**, nuclear factor-kappa B (NF-κB) activation was evaluated using HEK-Blue™ murine TLR4 cells (Figure 3A) transfected with genes for factors necessary for activating the murine TLR4 system, such as murine TLR4, MD-2, and CD14. *E. coli* LPS and lipid A were used as positive controls. Among the synthesized lipid As **1**–**3**, the order of NF-κB activation ability was hexa-ApLA (**1**) > penta-ApLA (**2**) > tetra-ApLA (**3**). ApLA **1**–**3** all exhibited weaker activity than the canonical *E. coli* lipid A. Furthermore, we performed a competition assay against *E. coli* LPS and found that only tetra-ApLA (**3**) exhibited antagonistic activity (Figure 3B), demonstrating that **3** is a partial agonist of mTLR4/MD-2. Next, we evaluated the immunological activities of **1**–**3** in HEK-Blue™ human TLR4 cells (Figure 3C). Lipid A **1**–**3** exhibited significantly weaker activity in HEK-Blue™ human TLR4 cells compared to that in

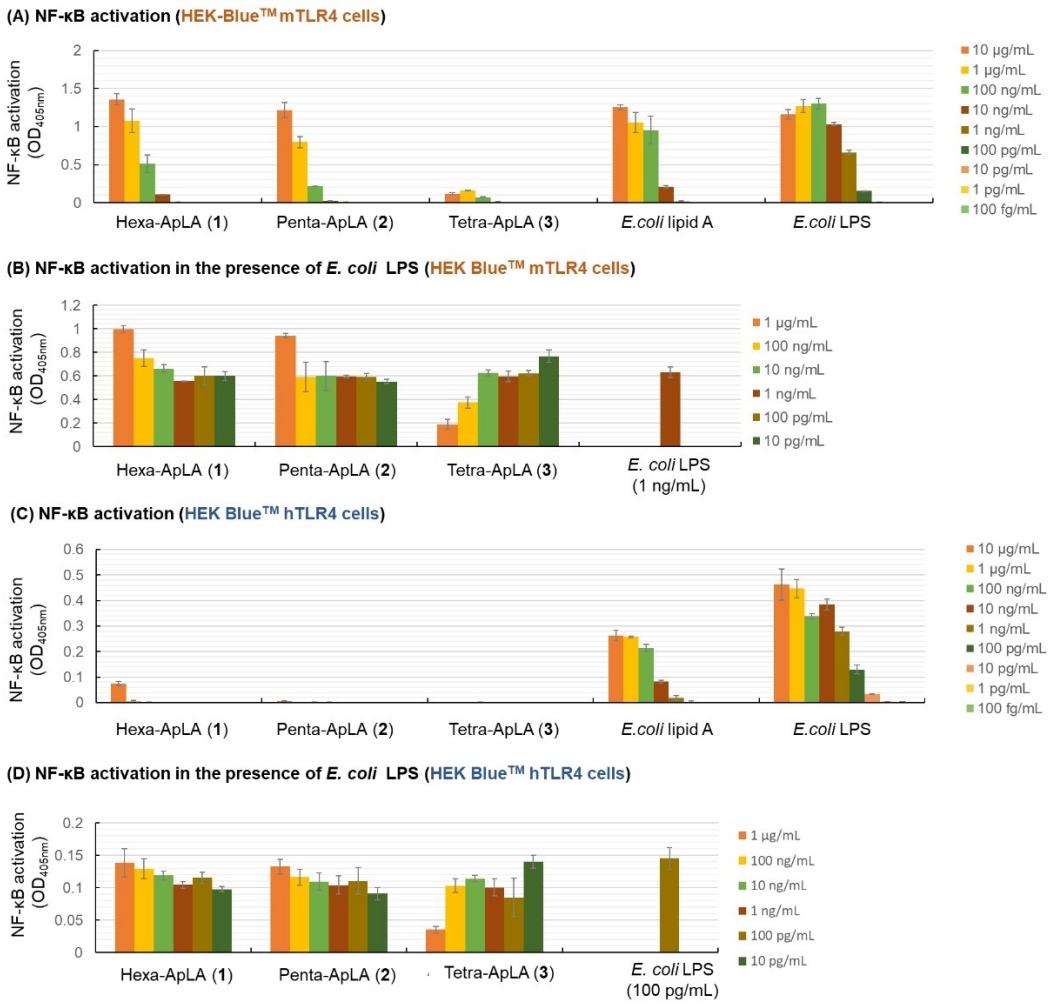


Figure 3. Immunological function of synthesized *A. pasteurianus* lipid A **1**–**3**. (A) and (C) *A. pasteurianus* lipid A **1**–**3**-mediated NF-κB activation in HEK-Blue™ mTLR4 (A) and HEK-Blue™ hTLR4 cells (C) was evaluated using secreted alkaline phosphatase (SEAP) reporter assay. (B) and (D) *A. pasteurianus* lipid A **1**–**3**-mediated NF-κB activation in the presence of *E. coli* LPS in HEK-Blue™ mTLR4 (B) and HEK-Blue™ hTLR4 cells (D) was evaluated using SEAP reporter assay. Results represent the mean ± standard deviation (SD) of three independent experiments.

HEK-Blue™ murine TLR4 cells. Hexa-ApLA (**1**) exhibited the most potent activity in HEK-Blue™ TLR4 cells among compounds **1**–**3**. In addition, a competition assay showed that **3** inhibited the activity of *E. coli* LPS in HEK-Blue™ human TLR4 cells (Figure 3D). From these results, we conclude that hexa-ApLA (**1**) and penta-ApLA (**2**) are active principles of *A. pasteurianus* LPS, which is consistent with previous studies on canonical diphosphate-type lipid A, showing that hexa-acylated lipid A acts as a strong agonist, whereas hypo-acylated lipid A acts as a weaker agonist or an antagonist.[3,33,35]

Following this, to investigate the ability of ApLA **1**–**3** to activate both the MyD88-dependent and TRIF-dependent pathways, we evaluated the induction of MyD88-dependent cytokines, such as IL-6 and tumor necrosis factor (TNF)-α, and TRIF-dependent chemokines, such as monocyte chemoattractant protein-1 (MCP-1), in murine bone marrow-derived dendritic cells (BMDCs) (Figure S3). We found that hexa-ApLA (**1**) and penta-ApLA (**2**) induced IL-6, TNF-α, and MCP-1, indicating that they can activate both MyD88-dependent and TRIF-dependent pathways.

To elucidate the role of the unique hexa-ApLA (**1**) structure, we performed a structure–activity relationship study. Previous X-ray crystallographic analysis[39] and structure–activity relationship studies[40] have clearly demonstrated that the two phosphate groups of *E. coli* lipid A are crucial for its biological activity and interaction with TLR4/MD-2. Furthermore, previous reports have shown that replacing the phosphate group with either carboxylate[41] or methyl carboxylate[42] groups at the reducing end does not significantly affect lipid A activity. Thus, we hypothesized that the carboxylate of GlcA in hexa-ApLA (**1**) plays a role similar to that of the phosphate groups of *E. coli* lipid A in its interaction with TLR4/MD-2. In this study, we synthesized analog **32** via methyl esterification of the carboxylate of hexa-ApLA (**1**) under acidic condition (Figure 4A). We evaluated the immunostimulatory activity of **32** in HEK-Blue™ murine TLR4 cells and HEK-Blue™ human TLR4 cells and found that its activity was significantly lower than

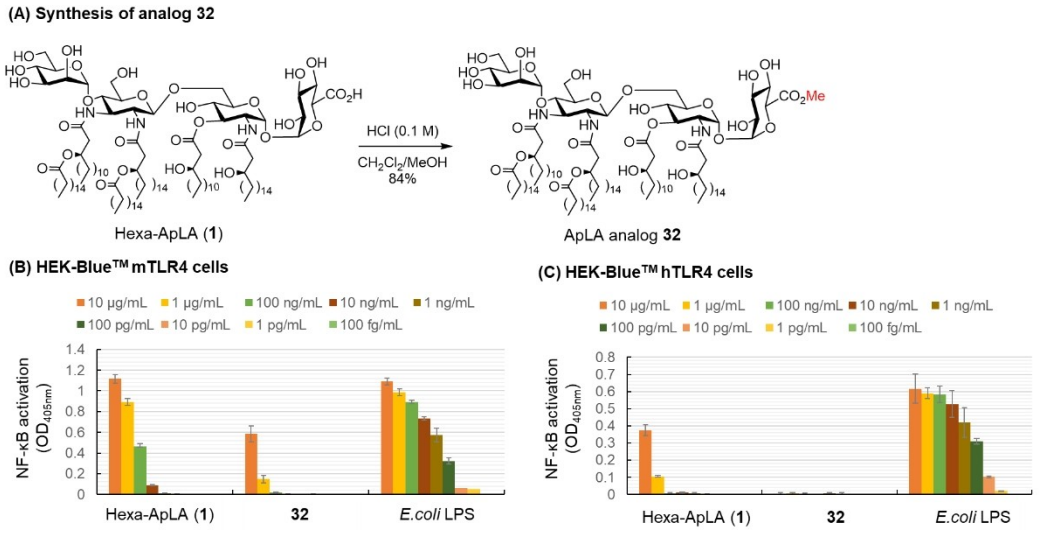


Figure 4. The effect of the GlcA group in hexa-ApLA (**1**) on its immunological function. (A) Synthesis of the ApLA analog **32**. NF-κB activation in (B) HEK-Blue™ mTLR4 cells and (C) HEK-Blue™ hTLR4 cells was evaluated using an SEAP reporter assay. Results represent the mean ± standard deviation (SD) of three independent experiments.

that of hexa-ApLA (**1**) (Figure 4B, 4C). These results revealed that the carboxylate of GlcA is a key structure for the biological activity of hexa-ApLA (**1**), and this structure was proposed to play an alternative role to the phosphate group of *E. coli* lipid A.

Finally, we performed an acid resistance test using hexa-ApLA (**1**). As mentioned above, the GlcA modification of *A. pasteurianus* lipid A has been suggested to be related to lipid A stability under acidic conditions. In this study, to confirm acid resistance, hexa-ApLA (**1**) and *E. coli* lipid A were incubated at 37 °C in solutions of CH_2Cl_2/H_2O with TFA. After 4 h, the removal of anomeric phosphate from *E. coli* lipid A was confirmed using thin-layer chromatography (TLC) and mass spectrometry (MS). However, in the case of hexa-ApLA (**1**), such decomposition was not observed (Figure S4A). Furthermore, we quantified the amount of lipid A using high-performance liquid chromatography (HPLC) and found that the amount of *E. coli* lipid A decreased in a TFA concentration-dependent manner, whereas the amount of hexa-ApLA (**1**) remained unchanged in TFA solutions (Figure S4B). These results revealed that hexa-ApLA (**1**) is more stable under acidic condition than *E. coli* lipid A.

Recently, we have demonstrated that symbiotic bacterial lipid A contributes to host immunomodulation. We previously performed structural characterization and chemical synthesis of lipid A derived from *Alcaligenes faecalis*, which inhabits gut-associated lymphoid tissues, Peyer's patches. We revealed that *A. faecalis* lipid A enhances the production of IgA antibodies without causing significant toxicity and contributes to the homeostasis of gut mucosal immunity.[35,43] Furthermore, we found that the function of parasitic bacterial lipid A is related to immune evasion and the development of chronic inflammatory diseases.[33,34] These studies strongly suggest that bacterial characteristics are closely related to the function of their lipid A. In this study, our results clearly demonstrate that hexa-ApLA (**1**) possesses stronger acid resistance than *E. coli* lipid A. This high chemical stability may be due to an evolutionary adaptation for surviving in the acidic environments produced by acetic acid bacteria. Symbiotic *A. faecalis*, as well as parasitic *Helicobacter pylori* and *Porphyromonas gingivalis*, also acquired distinctive lipid A structures to survive within their hosts. This study offers a new example of the structures and functions of lipid As, which are closely related to the habitats of bacteria.

Previous studies have shown that the chain length and distribution pattern of fatty acids and the number of phosphate groups affect their activities.[1,3,10] Compared to the structure of *E. coli* lipid A, hexa-ApLA (**1**) possesses i) a GlcA residue in place of the reducing end phosphate group, ii) a Man residue instead of the non-reducing end phosphate group, and iii) a relatively longer fatty acid chain. Our results suggest that the carboxylate in the GlcA residue of hexa-ApLA (**1**) can play an alternative role to the reducing end-phosphate group. The effects of the Man residue and the relatively longer fatty acids in *A. pasteurianus* lipid A are not yet fully understood. However, these structural features may be associated with the low immunopotency of hexa-ApLA (**1**).

Our synthetic strategy for ApLA **1**–**3** employed a tetrasaccharide scaffold decorated with a set of common orthogonal protecting groups for regioselective acylation in a divergent route. Notably, this strategy can also be useful for the synthesis of similar lipid As containing the same tetrasaccharide backbone, such as *Granulibacter bethesdensis* lipid A,[44] a pathogen associated with chronic granulomatous disease (CGD). Furthermore, we developed a new strategy for the construction of a 2-trehalosamine skeleton using stereoselective 1,1′-α,α-glycosylation with a borinic acid catalyst, achieving an excellent yield. To the best of our knowledge, this is the best yield achieved through chemical

glycosylation for the synthesis of a 2-trehalosamine skeleton.[15,16] 2-Trehalosamine, isolated from *Streptomyces*, is a potent growth inhibitor of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*.[19] Additionally, its derivatives have been reported to have antimicrobial activity against *M. tuberculosis*[18] and can be used as imaging probes for mycobacteria.[20] This reaction provides an efficient approach to the synthesis of these compounds.

In this study, we focused on the acetic acid bacteria *A. pasteurianus* LPS, a potential immunostimulatory component of kurozu. Recently, LPS from fermentative bacteria have received considerable attention. Several studies have reported the immunostimulatory and allergy-suppressive effects of LPS from various fermentative bacteria, such as *Pantoea agglomerans*, *Acetobacter aceti*, and *Gluconacetobacter hansenii*.[5] In addition, because these LPSs are already present in fermented food, they are expected to be used as safe immunomodulators owing to familiarity in food consumption. This report is the first example of the synthesis and functional analysis of active lipid A from fermentative bacterial LPS. Further biological evaluation of hexa-ApLA (**1**) is currently underway.

Conclusion

In summary, we achieved the first chemical synthesis of *A. pasteurianus* lipid A to identify the active principles of *A. pasteurianus* LPS, an immune component in kurozu. The synthesis features the formation of 2-trehalosamine skeleton utilizing borinic acid-catalyzed glycosylation in high yield and stereoselectivity. Furthermore, we also revealed that the GlcA residue of *A. pasteurianus* lipid A contributes to the immunological function and the acid-resistance.

Acknowledgements

This work was supported in part by JSPS KAKENHI Grant Number JP15H05836 (KF) in Middle Molecular Strategy, JP20H04776 (AS), JP18H04620 (AS) in Frontier Research on Chemical Communications, JP16K01914 (AS), JP20K05749 (AS), JP19KK0145 (KF), JP16H01885 (KF), JP20H00404 (KF), JP20H05675 (KF), JP22J12595 (HY), AMED Grant Number JP223fa727001s0301 (AS), JP223fa627002 (KF), JP233fa727001h0002 (JK), Japan Science and Technology Agency CREST (grant number JPMJCR20R3 (KF), Osaka University Foundation for the Future (COVID-19 response fund) (KF). This research was carried out also in the frame of Multidisciplinary PhD program for Pioneering Quantum Beam Application and Quantum LEAder Resource, financially supported by Osaka university. The authors would also like to acknowledge Dr. Naoya Inazumi and Dr. Yasuto Todokoro (Osaka University) for their support in NMR measurements.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Glycolipids · lipid A · *Acetobacter pasteurianus* · acetic acid bacteria · 1,1-glycosylation

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Manuscript received: February 8, 2024
Accepted manuscript online: April 6, 2024
Version of record online: ■■, ■■

Research Articles

Glycolipids

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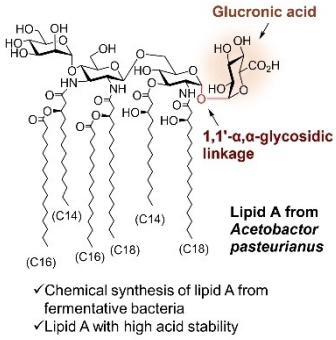
Chemical Synthesis of *Acetobacter pasteurianus* Lipid A with a Unique Tetrasaccharide Backbone and Evaluation of Its Immunological Functions



Lipid A contained in fermented foods is expected to be a safe immunostimulant. The first chemical synthesis of fermentative bacteria *Acetobacter pasteurianus* lipid A, an immune component in black rice vinegar, was achieved via borinic acid catalyzed 1,1'-α,α-glycosylation. Furthermore, glucuronic acid residue, a unique structure of *A. pasteurianus* lipid A, was found to largely contribute to its immunological and acid-stable properties.