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# Synthesis of immunoregulatory *Helicobacter pylori* lipopolysaccharide partial structures

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This Letter is dedicated to the memory of the late Professor Yoshihiko Ito

**Abstract**—The synthesis of immunoregulatory glycoconjugates, namely the active entity of lipopolysaccharide (LPS) from *Helicobacter pylori* was achieved. The results of biological activities of the LPS partial structures provide the structural basis for the immunobiological activity, especially for the immune inhibitory activity of *H. pylori* LPS.


*Helicobacter pylori* is a Gram-negative bacterium and an etiological agent of gastroduodenal diseases such as chronic gastritis, gastroduodenal ulcers and gastric cancer.[1,2] Gram-negative bacteria are distinguished by the presence of lipopolysaccharide (LPS) in their outer membrane. LPS, also called an endotoxin, is a potent immunostimulator, often causing sepsis in severe infections. However, *H. pylori* lipopolysaccharide (LPS) shows very low endotoxic activity compared to other enterobacterial preparations such as *Escherichia coli* LPS.[3–6] The *H. pylori* LPS has the characteristic lipid A component, the structure of which is distinct from those of the enterobacterial ones as in the following ways: (1) the presence of fewer, but longer fatty acid residues, (2) the absence of the 4′-phosphate group and (3) the occasional presence of an ethanolamine group linked to the glycosyl phosphate functionality.[7,8] We have previously synthesized *H. pylori* strain 206-1 lipid A, which has tri-acyl groups and ethanolamine at the anomeric phosphate (**1b**). This lipid A induced lower levels of cytokines such as IL-18 and TNF-α upon activation of the LPS receptor, a Toll-like receptor 4 (TLR4)/MD-2 complex,[9,10] as it was known that the activation leads to induction of proinflammatory cytokines such as IL-6, IL-18 and TNF-α. On the other hand, it has been recently reported that *H. pylori* LPS from other strains has inhibitory effects on TLR4 activation.[11] This discrepancy in biological activities may come from the structural heterogeneity of LPS, especially in the acyl group distribution and in the phosphate part in lipid A (Fig. 1, **1a**–**d**). Synthetic study is therefore required for elucidation of biological activity of *H. pylori* LPS, since chemical synthesis can provide homogeneous preparations.

It has been reported that the number of acyl groups is crucial for the biological activity of lipid A.[12,13] *E. coli* type lipid A having hexaacyl groups has strong endotoxic activity, whereas a biosynthetic precursor Ia having tetraacyl groups shows antagonistic activity in humans.[12] Triacyl lipid As also showed antagonistic activity though the potency was low. There have been known some bacteria, LPS of which have significant inhibitory activity against *E. coli* LPS, such as *Rhodobacter sphaeroides*,[14,15] and *Rhodobacter capsulatus*,[16] which have an unsaturated acyl group, and also *Rhizobium sin*-1[17,18] which has pentaacyl groups of relatively longer fatty acids and lactone structure at the anomeric position. However, in the case of *H. pylori*, the LPS shows weaker antagonistic activity, and at the same time, shows weak proinflammatory activity. Chain

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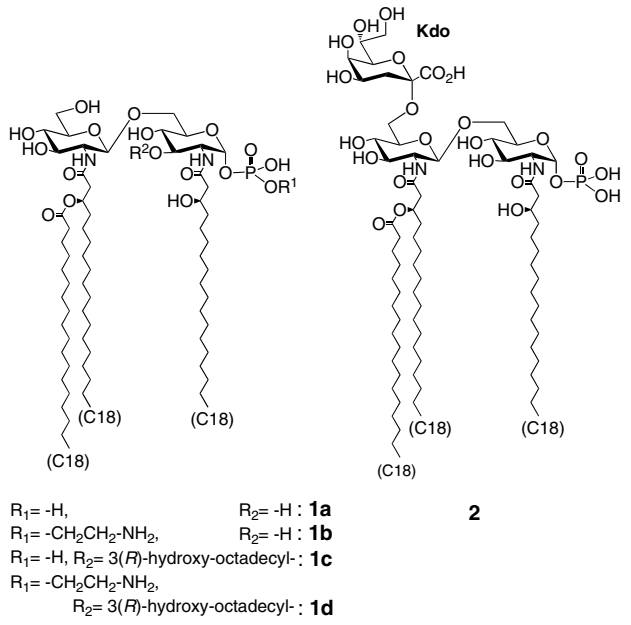


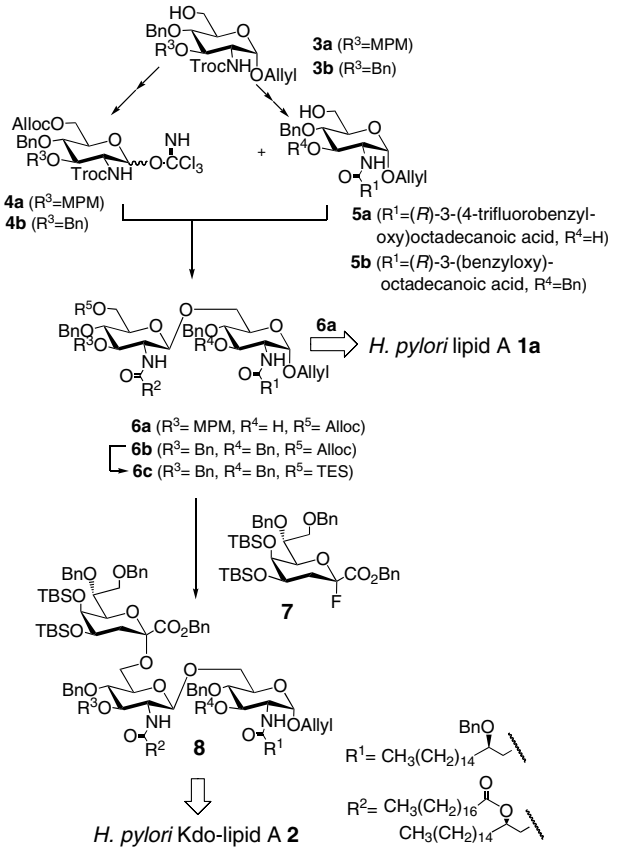

**Figure 1.** Chemical structures of *Helicobacter pylori* lipid A and Kdo-lipid A.

length of fatty acids in lipid A also influences the biological activity. A biosynthetic precursor Ia analog having shorter lipids (two C14 and two C10 fatty acids) showed much weaker antagonistic activity, whereas that having four C10 fatty acids did not show the activity.[19] On the other hand, an *E. coli* lipid A analogue having shorter lipids (two C14 and four C12 fatty acids) were ca. 100 times more active than *E. coli* lipid A.[20]

The acidic functional groups of lipid A are also crucial for the biological activity.[12,13] Monophosphoryl lipid A analogues show much weaker activity than the corresponding diphosphates and the analogues lacking phosphoryl group did not show the activity. The phosphate groups in lipid A can be replaced with carboxymethyl groups without loss of the activity in the case of *E. coli* lipid A and the tetraacyl biosynthetic precursor.[21–24] However, in our recent studies with lipid A analogues containing acidic amino acid residues, immunostimulating or antagonistic activity was observed depending on the anionic charges (phosphoric acid vs carboxylic acid).[25,26]

As for another aspect, it has been reported that *H. pylori* LPS activates TLR2.[11,27,28] Some of them also indicated that *H. pylori* LPS antagonizes TLR4.[11,28] Another parasitic bacteria, *Porphyromonas gingivalis*, which has similar acylated patterns of lipid A to the one of *H. pylori* though the acyl groups have branched terminus,[29,30] also showed TLR2 stimulatory and TLR4 antagonizing activities.[28] These biological activities are considered to be associated with atherosclerosis, which is a chronic inflammatory vascular disease and leads to cerebral stroke and cardiac affection—most major causes of death in industrial countries. The heterogeneity of the lipid A structures in *H. pylori* LPS, however, complexifies understanding the molecular basis of these biological activities. There is also a potential issue on the contamination of immunoreactive compounds in preparations from natural sources. We thus planned to synthesize the series of the lipid A and LPS partial structures to clarify the structural basis of the immunoregulatory activities.

Lipid A generally connects to the polysaccharide domain via an acidic sugar, 3-deoxy-D-manno-2-octurosonic acid (Kdo). Because *H. pylori* lipid A lacks a 4′-phosphate group, the acidic group of Kdo might alter the anionic charge and affect recognition by the LPS receptor. In the present study, we thus synthesized the *H. pylori* LPS partial structures, lipid A **1a** and Kdo-lipid A **2**, which do not have ethanolamine at the anomeric phosphate to observe the effect of Kdo and ethanolamine in comparison with our previously synthesized ethanolamine-type lipid A (Fig. 1).[9] Scheme 1 shows the basic strategy for lipid A synthesis. Glycosyl donors **4a**/**4b** and glycosyl acceptors **5a**/**5b** were prepared from properly protected glucosamine **3a**/**3b**, respectively. The acyl group of the glycosyl acceptors **5a**/**5b** was introduced before glycosylation. Glycosyl donor, trichloroacetimidate **5a**/**5b**, which possessed a 2-*N*-Troc group, was used for β-selective glycosylation to construct the lipid A backbone. *H. pylori* lipid A **1a** was synthesized from disaccharide **6a** with 1-*O*-phosphorylation and deprotection. Kdo-lipid A **2** was synthesized by glycosylation of **6b** with Kdo donor **7** and




**Scheme 1.** Outline for the synthesis of *Helicobacter pylori* lipid A and Kdo-lipid A. Alloc = allyloxycarbonyl, Bn = benzyl, MPM = *p*-methoxybenzyl, TBS = *t*-butyldimethylsilyl, Troc = 2,2,2-trichloroethoxycarbonyl, TMS = trimethylsilyl.

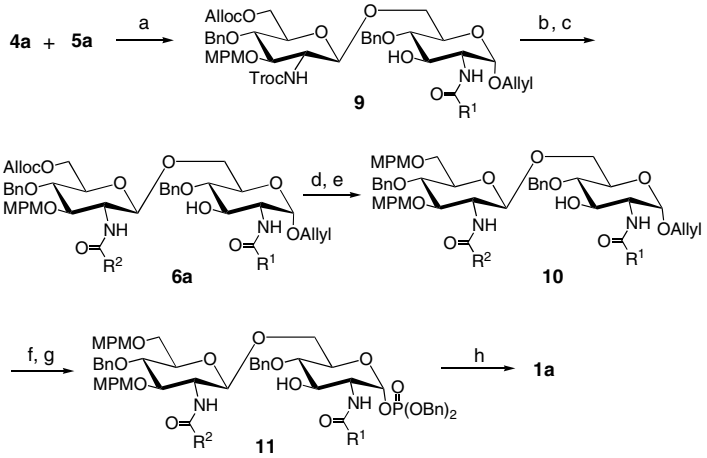
1-*O*-phosphorylation. Kdo fluoride **7** protected with TBS groups at the 4- and 5-positions was used as a donor for α-selective glycosylation, because we previously found in our *E. coli* Re-LPS synthesis that α-selectivity increased when large protective groups such as TBS were introduced at the 4- and 5-positions.[31] Benzyl-type protecting groups were used for hydroxyl, carboxy and phosphate functions, and the final deprotection of all the benzyl-type protecting groups was carried out by catalytic hydrogenolysis.

Scheme 2 shows the synthesis of lipid A **1a**. Glycosyl donor **4a** and the acceptor **5a** were synthesized in a manner similar to previously published methods.[31] The glycosylation was carried out in the presence of a catalytic amount of TMSOTf in THF solution at 0 °C to give disaccharide **9** in 85% yield. The Troc group at the 2′-position of **9** was cleaved with Zn–Cu couple in acetic acid, and (*R*)-3-octadecanoyloxyoctadecanoic acid was introduced with WSCD·HCl at 40 °C to give **6a** from compound **9** in 56% yield. The Alloc group at the 6′-position was cleaved with $Pd(PPh_3)_4$, $PPh_3$ and ammonium formate in THF selectively in the presence of an allyl group at 1-position, and then the MPM group was introduced at this liberated 6′-position. The allyl group at the 1-position was cleaved via isomerization to a vinyl group with an iridium complex and then with iodine and water in 87% yield, and the anomeric position was selectively phosphorylated with tetrabenzyl pyrophosphate to give **11**. Cleavage of all the benzyl-type protecting groups in **11** by hydrogenolysis gave the desired lipid A **1a**.

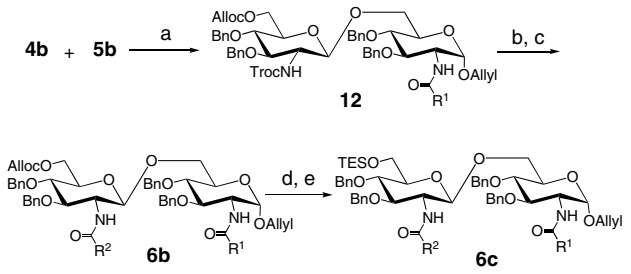
For the synthesis of Kdo-lipid A, the lipid A backbone was first prepared as shown in Scheme 3. Although we initially tried to synthesize Kdo-lipid A from **6a**, the 3′-*O*-MPM group was cleaved under the glycosylation conditions using Kdo fluoride **7** in the preliminary experiments. We, therefore, used 3-*O*-benzyl acceptor **5b** in subsequent procedures. The glycosyl donor **4b** and the acceptor **5b** were synthesized in a manner similar to compounds **4a** and **5a**, respectively.[31] The glycosylation was carried out in the presence of a catalytic amount of TMSOTf in THF solution at −78 °C to give the disaccharide **12** in 70% yield. The Troc group at the 2′-position of **12** was cleaved with Zn–Cu couple in acetic acid, and (*R*)-3-octadecanoyloctadecanoic acid was introduced with WSCD·HCl at 40 °C to give **6b** in 58% yield from compound **12**. The Alloc group at the 6′-position was cleaved with $Pd(PPh_3)_4$ in the presence of $PPh_3$ and ammonium formate in THF, and then the TES group was introduced at this position for subsequent glycosylation to give compound **6c**.

Scheme 4 shows the preparation of Kdo fluoride **7**, the glycosyl donor for the next glycosylation, from compound **13**.[31] In the previous study, we used (diethylamino)sulphur trifluoride (DAST) for fluorination. However, the reaction was difficult due to the high reactivity of DAST and a considerable amount of glycal was formed as a by-product. Hence, we used a new, mild fluorination reagent, *N*,*N*-diethyl-α,α-difluoro-(*m*-methylbenzyl)amine (DFMBA).[32] The anomeric stereoselectivity for fluorination was different for these two compounds: 3/1 (α/β) for DAST and 1/10 (α/β) for DFMBA. The formation of glycal **14** was suppressed when DFMBA was used at a lower temperature.

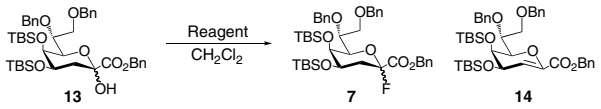
Scheme 5 shows the glycosylation between Kdo-fluoride **7** and lipid A backbone **6c**. The reaction conditions were examined using a variety of Lewis acids, solvents, and molecular sieves. The molecular sieve 5A (MS5A) was found to be effective for this reaction compared to MS4A. Because MS5A contains calcium ions, MS5A should trap fluoride anions to promote glycosylation. Thus, we successfully obtained trisaccharide **8** with the α-glycoside linkage between Kdo and lipid A, using $BF_3/Et_2O$ and MS5A in $CH_2Cl_2$ at −20 °C with a high




**Scheme 2.** Synthesis of *Helicobacter pylori* lipid A. (a) TMSOTf (0.1 equiv), MS4A, THF, 0 °C, 1 h, 85%; (b) Zn–Cu, AcOH, rt; (c) (*R*)-3-octadecanoyloxyoctadecanoic acid, WSCD·HCl, HOAt, $CHCl_3$, 40 °C, 56% (two steps); (d) $Pd(PPh_3)_4$ (1.2 equiv), $PPh_3$ (3 equiv), $HCOONH_4$ (5 equiv), THF, rt, 2 h, 94%; (e) MPM imidate Sn(OTf), MS4A, THF, 88%; (f) $[Ir(H)(cod)(MePh_2P)_2]PF_6$, then $I_2$, $H_2O$, 87%; (g) tetrabenzyl pytrophosphate, $LiN(TMS)_2$, THF, −78 °C, 47%; (h) $H_2$ (15 kg cm$^{-2}$), Pd (black), THF. $R^1$: (*R*)-3-(4-trifluorobenzyloxy)octadecanoyl, $R^2$: (*R*)-3-octadecanoyloxyoctadecanoyl, HOAt = 1-hydroxy-7-azabenzotriazole, TMSOTf = trimethylsilyl trifluoromethanesulfonate, WSCD = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride.



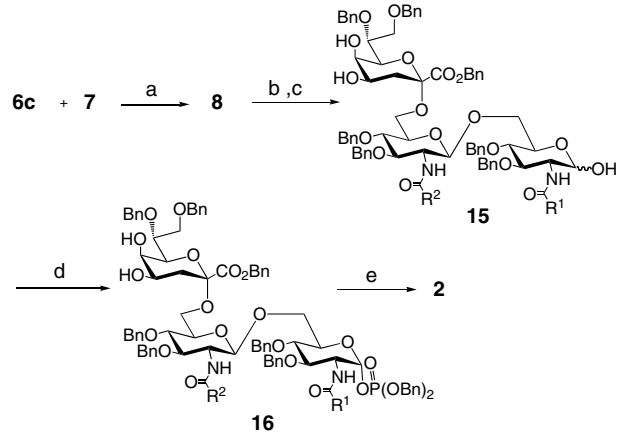
**Scheme 3.** Synthesis of lipid A backbone disaccharide for *Helicobacter pylori* Kdo-lipid A. (a) TMSOTf (0.2 equiv), MS4A, THF, −78 °C, 1 h, 80%; (b) Zn–Cu, AcOH, rt; (c) (*R*)-3-octadecanoyloxyoctadecanoic acid, WSCD·HCl, HOAt, $CHCl_3$, 40 °C, 58% (two steps); (d) $Pd(PPh_3)_4$, $PPh_3$, $HCOONH_4$, THF, 94%; (e) TESCl, imidazole, $CHCl_3$, 88%. $R^1$: (*R*)-3-benzyloxyoctadecanoyl, $R^2$: (*R*)-3-octadecanoyloxyoctadecanoyl, TES = triethyl.



| Entry | Reagent | Conditions | Time | Yield[a] |
|---|---|---|---|---|
| 1 | DAST (3 equiv) | -78 °C | 3 h | 88% (α:β= 3:1), **14** (7%) |
| 2 | DFMBA (2 equiv) | 60 °C (microwave 50 W) | 10 min | 68% (α:β= 1:10), **14** (12%) |
| 3 | DFMBA (4 equiv) | -14 °C | 1.5 d | 88% (α:β= 1:10), **14** (ND) |

[a] The ratio of the αβglycoside and glycal **14** was determined by the area ratio of the $PhCH_2OCO$ signal in $^1$H-NMR.

**Scheme 4.** Fluorination of Kdo.



**Scheme 5.** Synthesis of *Helicobacter pylori* Kdo-lipid A. (a) $BF_3{\cdot}Et_2O$ (10 equiv), MS5A, $CHCl_3$, −20 °C, 1.5 h, 85%; (b) 47% $HF{:}CH_2Cl_2{:}CH_3CN$ = 1:5:5, 81%; (c) $[Ir(H)(cod)(MePh_2P)_2]PF_6$, then $I_2$, $H_2O$, 78%; (d) tetrabenzyl pyrophosphate, $LiN(TMS)_2$, THF, −78 °C, 47%; (e) $H_2$ (20 kg $cm^{-2}$), Pd (black), THF, 91%. $R^1$: (*R*)-3-benzyloxyoctadecanoyl, $R^2$: (*R*)-3-octadecanoyloxyoctadecanoyl.

yield. The stereochemistry at the anomeric position was determined with the chemical shift of the protons at the 3′-position from $^1$H NMR in comparison with the previously reported data.[31] Then, the TBS groups were removed with HF, and the allyl group at the 1-position was cleaved via isomerization to a vinyl group with iridium complex to give **15** in 78% yield. After selective phosphorylation at the anomeric position with tetrabenzyl pyrophosphate, all the benzyl-type protecting groups were removed by hydrogenolysis to give the desired compound **2**.

The immunostimulating activities of the synthetic *H. pylori* lipid A **1a** and Kdo-lipid A **2** were examined by measuring IL-6 production from human whole blood cells (HWBC)[33] (Fig. 2). Both **1a** and **2** did not induce IL-6, but showed competitive inhibition of IL-6 induction by *E. coli* LPS (0.5 ng/mL, O111:B4). Kdo-lipid A **2** showed more potent inhibitory activity than lipid A **1a**.

These results demonstrated that the H-form tri-acylated lipid A **1a** and Kdo-lipid A **2** of *H. pylori* competitively inhibit *E. coli* LPS immunostimulation. These results indicated that **1a** and **2** are TLR4 antagonist but not TLR2 or TLR4 agonist. We also observed that introducing an acidic Kdo residue to lipid A enhanced this inhibitory activity by 10-fold. The present study provides the molecular basis for antagonistic activity of *H. pylori* LPS from certain strains.

It should be noted that the ethanolamine-form of lipid A **1b** showed weak immunostimulating activity in our previous study.[10] These results indicate that the number of anionic charges influences the biological activity of lipid A and LPS. Similar results were obtained from our studies with lipid A analogues containing acidic amino acid residues as mentioned above. Depending on the anionic charges (phosphoric acid vs carboxylic acid) in the lipid A analogues, immunostimulating or antagonistic activity was observed.[26] The present study also explains why some strains of *H. pylori* LPS show weak immunostimulating activity while others show antagonistic activity.

In conclusion, we synthesized tri-acylated lipid A **1a** and LPS partial structure **2** of *H. pylori* and found that both compounds antagonize *E. coli* LPS immunostimulation.

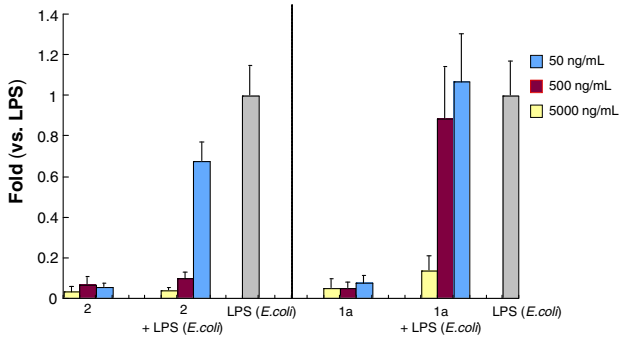



**Figure 2.** *Helicobacter pylori* Kdo-lipid A and lipid A competitively inhibit IL-6 induction by *E. coli* LPS. Noted concentrations of Kdo-lipid A **2a** and lipid A **1a** and 0.5 ng/mL of *E. coli* (O111:B4) LPS were added to human whole blood cells (HWBC). Amount of IL-6 produced by HWBC was determined by ELISA. Data represent averages of three repeated assays with standard deviations from individual experiments.

In future studies, we will use these *H. pylori* LPS compounds to investigate the biological activities of LPS and the factors that mediate host receptor LPS recognition.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2007.07.036.

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