

Synthesis and bioactivity of fluorescence- and biotin-labeled lipid A analogues for investigation of recognition mechanism in innate immunity

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Abstract—Fluorescence- and biotin-labeled lipid A analogues were synthesized for the investigation of bacterial lipopolysaccharide (LPS)/lipid A recognition in the innate immune system. For the introduction of the labeling moiety, a hydrophilic glutaryl-glucose linker was used for maintaining the bioactivity and also for preventing self-aggregation, which causes quenching of the fluorescence. We also observed the biological activity of the labeled compounds.
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Lipid A is the active principal of bacterial lipopolysaccharide (LPS),^{1–3} which is a constituent of the outer membrane of Gram-negative bacteria. LPS has potent immunostimulating activity and is also known as endotoxin for its toxicity that sometimes causes lethal sepsis.⁴ Innate immunity is a phylogenetically ancient defense mechanism against invasion by pathogens. This defense system recognizes pathogens by the pattern recognition receptors (PRRs), which recognize common microbial components such as LPS. These molecules are called pathogen associated molecular patterns (PAMPs). Activation of immunocomponent cells by PAMPs via PRRs causes immunostimulation. Concerning the LPS and lipid A, the receptor complex consisting of Toll-like receptor 4 (TLR4)^{5,6} and MD-2 was found to be responsible for the recognition.⁷ Recent studies have revealed that TLR4/MD-2 directly recognizes LPS and lipid A. Binding assay using synthetic radio-labeled lipid A⁸ suggested that TLR4/MD-2 recognizes a single molecule of LPS and lipid A.^{9,10} On the other hand, Seydel and co-workers demonstrated that the aggregate formation

of lipid A and LPS is essential for expression of activity.¹¹ These results reflect the complex recognition mechanism of LPS, in which various PRRs such as LBP (LPS binding proteins), CD14, TLR4/MD-2, CD55 and so on are implicated.¹² However, the recognition mechanism of lipid A and LPS is still unclear.

In order to understand the immunostimulating mechanism in the innate immune system, fluorescence-labeled or biotin-labeled lipid A derivatives have been desired as probe molecules. Since natural LPS is heterogeneous and often contaminated with other PAMPs, synthetic homogeneous preparations are essential for investigation of the recognition system. However, the acid-labile and amphiphilic characteristics of lipid A made it difficult to synthesize labeled compounds possessing both the bioactivity and the desired function. In fact, we previously synthesized fluorescence-labeled lipid A with BODIPY (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diazas-indacene) group linked through a glycine linker.¹³ BODIPY-labeled lipid A, however, showed only weak fluorescence owing to self-quenching. Since lipid A forms aggregates under aqueous conditions, the hydrophobic characteristic of the BODIPY group and shorter glycine linker caused tight aggregation of the fluorescence group and quenched the fluorescence.

We thus introduced a glucose with a glutaryl group as a longer hydrophilic linker and also used more hydrophilic fluorescent groups for the lipid A labeling

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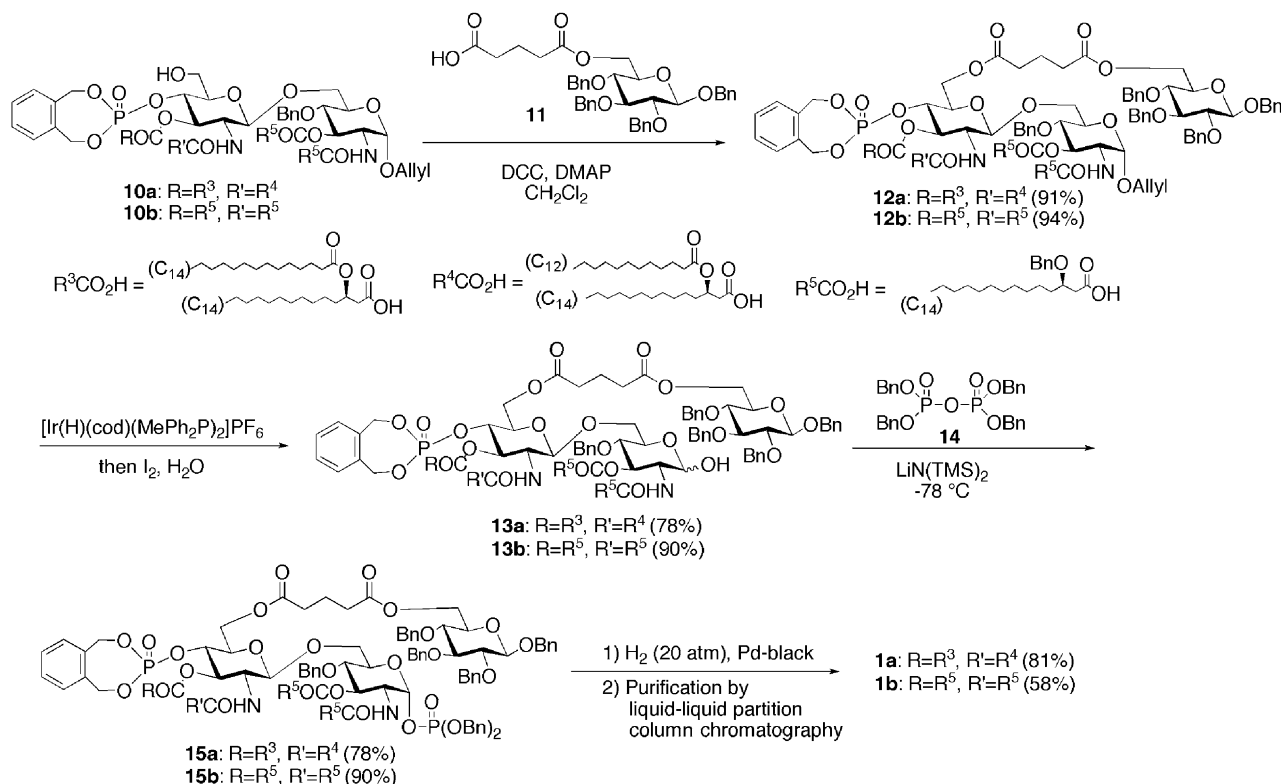
10a (for lipid A) and **10b** (for lipid IVa) as the precursor having the lipid A backbone. For the preparation of the disaccharide, we used an imidate **3a** and **3b** as the glycosyl donor, and the glycosyl acceptor **4**, both were synthesized from D-glucosamine.¹⁶ As for the synthesis of *E. coli* lipid A type intermediate **10a**, we first carried out the glycosylation of **3a** and **4** with a catalytic amount of TMSOTf to give the $\beta(1\rightarrow6)$ disaccharide **5a** in 74% yield.¹⁷ The Troc protection for the 2-amino group of **5a** was cleaved with Zn–Cu couple, and (*R*)-3-(dodecanoyl)tetradecanoic acid was subsequently introduced to the amino groups in 93% yield for two steps. The benzylidene group, which protects 4'- and 6'-hydroxyl groups, was then removed with trifluoroacetic acid to give the compound **6a** in 88% yield. After the protection of the 6'-position of **6a** with TBDMS, a phosphate group was introduced to the 4'-position of **7** using phosphhepane **8**, and succeeding oxidation with mCPBA¹⁸ gave phosphorylated compound **9a** in 83% yield for two steps. The deprotection of the TBDMS group at the 6'-position with hydrofluoric acid gave the key intermediate **10a** in quantitative yield. The intermediate for lipid IVa, **10b**, was also synthesized in a similar manner (Scheme 2).

The introduction of the glutaryl-glucose-linker to **10a** and **10b**, and the preparation for the suitable deprotected structure for the final labeling reaction are shown in Scheme 3. To the free 6'-position of compound **10a**, the glutaryl-glucose linker **11**, which was prepared from *O*-benzyl-protected glucose and glutaric anhydride, was

introduced using DCC and DMAP to give **12a** in 91% yield. The 1-*O*-allyl group was then cleaved via isomerization with $[\text{Ir}(\text{H})(\text{cod})(\text{MePh}_2\text{P})_2]\text{PF}_6$ to a vinyl group, which was subsequently removed with iodine in water, to give compound **13a** in 90% yield. The deprotected 1-position was phosphorylated with **14** and $\text{LiN}(\text{TMS})_2$ at -78°C . All the benzyl protecting groups were then cleaved by catalytic hydrogenolysis with Pd-black to give the precursor for the final labeling **1a**. The lipid IVa having a glutaryl-glucose-linker, **1b**, was also prepared (Scheme 3).

With the compounds **1a** and **1b** in hand, we synthesized fluorescence or biotin labeled compounds with labeling reagents having a hydrazide group. For the fluorescence labeling, we used Alexa Fluor[®] 568 hydrazide (Invitrogen Co.) and DCCH (7-diethylaminocoumarin-3-carboxylic acid, hydrazide) in a solution of chloroform, methanol and 1.0 M aqueous sodium acetate–acetic acid (pH 2.5).¹⁹ We first synthesized the DCCH-labeled lipid A **2a-1**. However, the bioactivity was much weaker than that of the native lipid A. We then introduced a hydrophilic fluorescence group Alexa fluor[®] 568 to synthesize compound **2a-2**. We also prepared biotin-labeled lipid A **2a-3**.²⁰ The labeled lipid IVa was also prepared in a similar way via the precursor **1b** to give DCCH-labeled lipid IVa **2b-1** and Alexa fluor[®] 568-labeled lipid IVa **2b-2**.

The bioactivities of the labeled compounds **1a**, **2a-1** and **2a-2** (Fig. 1A), and also **1b**, **2b-1** and **2b-2** (Fig. 1B) were



Scheme 3.

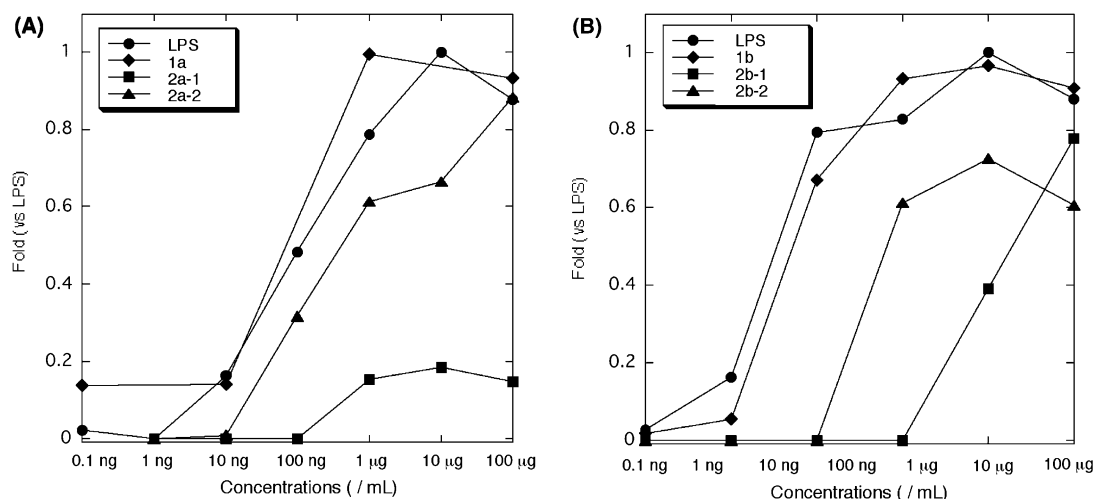


Figure 1. (A) The *Limulus* activities of compounds **1a**, **2a-1** and **2a-2**, and (B) **1b**, **2b-1** and **2b-2**, comparing with LPS from *E. coli* 0111:B4, assayed with Endospecy Test® (Seikagaku Co., Tokyo).

examined with the *Limulus* test, which is the method for detection of LPS and lipid A with limulus amebocyte lysate (lal).²¹ The activities were compared with lipopolysaccharide (LPS) from *E. coli* 0111:B4. Compound **1a** had almost the same activity with the LPS, and Alexa fluor® 568-labeled compound **2a-1** showed a little lower but satisfactory activity, while the DCCCH-labeled lipid A **2a-2** had only much lower activity. The hydrophobicity of DCCCH might cause the self-aggregation to distort the active conformation and lead to the lower bioactivity. The lipid IVa derivatives, **1b**, **2b-1** and **2b-2**, also showed a similar tendency of the activity (Fig. 1B).

We next measured the fluorescence of Alexa fluor® 568-labeled compounds **2a-2** and **2b-2** in comparison with the fluorescence-labeling reagent. In the measurement, slight reduction of intensity of fluorescence at **2a-2** and **2b-2** was observed, presumably because of self-aggregation. However, the detection limit was almost the same with the fluorescence-labeling reagent, about 1.8 pmol/mL in aqueous solution (ex. 577 nm, em. 595 nm), which was much lower than the lower limit of the bioactive concentration. These results showed that Alexa fluor® 568-labeled compounds **2a-2** and **2b-2** are applicable for the investigation of the recognition and signal transduction mechanism.

In conclusion, we have obtained a practical fluorescence-labeled *E. coli* lipid A and antagonistic lipid IVa, and also the biotin-labeled analogues, by introducing a new hydrophilic glutaryl-glucose linker, which has a reactive aldehyde group. We are now investigating the recognition mechanism of lipid A in the innate immune system, by using these labeled compounds.

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19. Typical procedure for the introduction of labeling group (synthesis of **2a-2**): To a solution of **1a** (1.4 mg, 0.67 mmol) with $\text{CHCl}_3/\text{MeOH}/0.1 \text{ M}$ aqueous sodium acetate–acetic acid (pH 2.5) = 25:25:7 (0.2 mL) was added Alexa Fluor[®] 568, hydrazide (1.0 mg, 1.3 mmol). The reaction mixture was stirred at room temperature for 2 d. The reaction mixture was diluted with *tert*-butyl alcohol and sterilized water, and lyophilized. The crude product was purified by gel permeation column chromatography (Sephadex[®] LH-20, $0.6 \times 30 \text{ cm}$, $\text{CHCl}_3/\text{MeOH}/\text{TEA} = 1:1:1.6 \times 10^{-5}$) and then purified by liquid–liquid partition column chromatography (10 g of Sephadex[®] LH-20, $\text{CHCl}_3/\text{MeOH}/\text{water}/2\text{-propanol} = 8:8:1:12$; The organic layer was the stationary phase, and the aqueous layer was the mobile phase). After removal of the solvent under reduced pressure, the residue was lyophilized from sterilized water to afford **2a-2** as a blue solid (1.25 mg, 60%). ESI-TOF MS (negative) *m/z* 920.6 $[\text{M}-\text{Na}-2\text{H}]^{3-}$, 690.5 $[\text{M}-\text{Na}-3\text{H}]^{4-}$.
20. As for the biotin labeled **2a-3**, the *Limulus* activity was tested and the result suggested the compound has similar activity to the non-labeled compound. However, the sample included small amount of non-labeled **1a** because of the difficulty of the purification, and further purification and the detailed examination of the biological activities are now under investigation.
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