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Lipid A Structures Containing Novel Lipid Moieties: Synthesis and Adjuvant Properties

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Abstract—Structurally well-defined immune stimulatory molecules are important components of new generation molecular vaccines. In this paper, the design and synthesis of two lipid A analogues containing an unnatural tri-lipid acyl group are described. In a totally synthetic liposomal vaccine system, these re-designed lipid A analogues demonstrate potent immune stimulatory properties including antigen specific T-cell activation.

With a steady growth in research towards molecular vaccines for various applications, both in therapy and prophylaxis, a significant attention is drawn towards structurally well defined immune stimulatory adjuvants.[1] However, the structural requirements for an adjuvant and the molecular and cellular mechanisms underlying their activities in vivo remain poorly understood.[2] Nevertheless, the discovery of mammalian Toll-like receptors (TLRs)[3] and their essential role in innate and adaptive immune responses[4] has led to a better understanding of adjuvant actions. Recent identification of a broad spectrum of specific TLR binding structural features[4] of pathogenic origin has expanded the repertoire of adjuvants and their structural definition far beyond the traditional secondary role of the crude adjuvant preparations based on bacterial cell wall, such as Freunds' complete adjuvant (FCA).[5]

Lipopolysaccharide (LPS) preparations from Gram-negative bacteria are effective in activating innate immunity of the host following bacterial infection. Lipid A, the lipid anchor of LPS, has been shown to be responsible for the biological activities of LPS in most in vitro and in vivo test systems. Structure–activity relationship of lipid A has been a subject of wide research interest over the last two decades.[6,7] Recently, Seydel et al.[8–10] showed that the agonistic and antagonist activity of lipid A are governed by the intrinsic conformation of lipid A, which in turn is defined mainly by the number of charges, the number of acyl chains, and the distribution and degree of un-saturation in the lipid chains of the molecule. Furthermore, lipid A has been suggested to be a ligand for TLR4,[11,12] the Toll-like receptor involved in the mediation of immune responses to LPS/lipid A. Thus, structural mimics of natural lipid A can function as potential ligands for TLR4 and display agonistic or antagonistic activity to bacterial LPS. In this paper we report the synthesis and preliminary biological evaluations of two lipid A analogues, **1** and **2** (Fig. 1), which are designed primarily as immune stimulatory adjuvants.

Recently, Johnson et al.[13] reported the syntheses of a group of 3-*O*-desacyl monophosphoryl lipid A derivatives with different acyl chain lengths. Their studies suggest that fatty acid structure may be more important than the presence or absence of the 1-phosphate group in determining the biological activity of lipid A molecules. Compounds **1** and **2** are monophosphorylated analogues, incorporating an unnatural tri-lipid group incorporating an ether linkage.[14] Although 3-(*R*)-hydroxy myristic acid **3** (Fig. 2) is the most commonly found fatty acid in natural lipid A, lipid acids such as **6**, derived from two molecules of **3**, are not known in natural lipid A structures. It is generally believed that subtle modifications to the lipid chains of lipid A may affect hydrophobic balance and induce conformational changes that may alter cellular uptake and expression of endotoxin activities of lipid A.[9,10,15] In an effort to determine whether the immune system, or perhaps TLR4 itself, is tolerant

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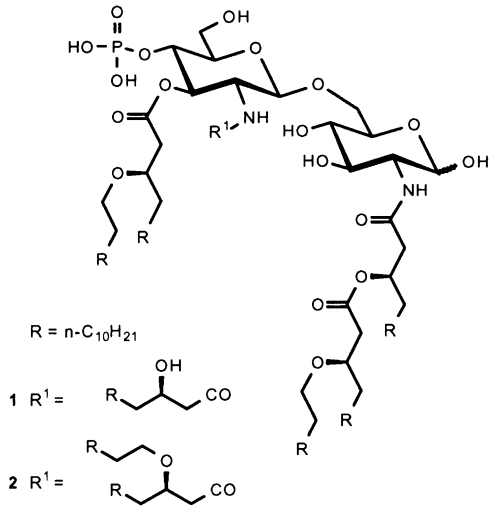


Figure 1. Designed lipid A analogue **1** and **2** containing unnatural tri-lipid moiety.

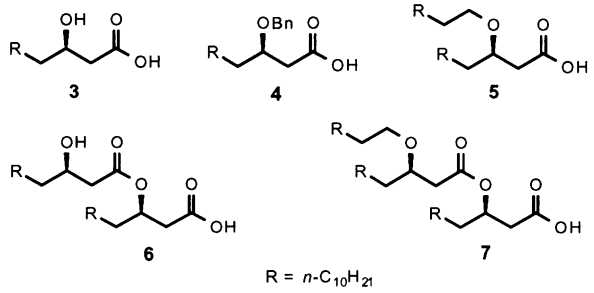
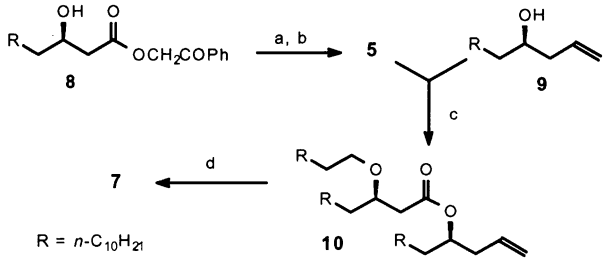


Figure 2. Structures of natural (**3**) and unnatural (**5**–**7**) lipid acids.

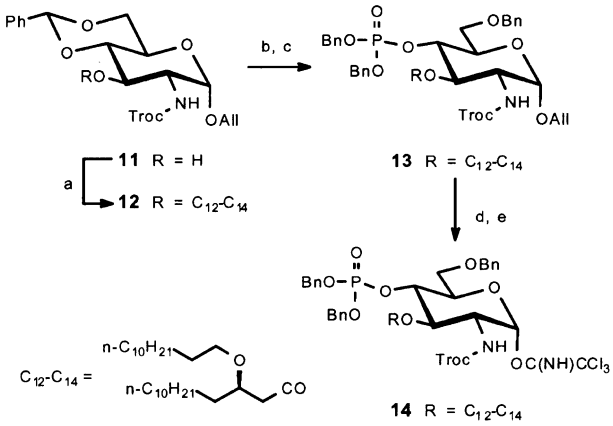
towards substantial structural modifications in the lipid chains of lipid A, we designed an unnatural tri-lipid acid **7** (Fig. 2) as one lipid component of lipid A structures.

The synthesis of both lipid acids **5** and **7** is described in Scheme 1. Reaction of the readily available **8**[16] with dodecyl triflate, followed by the removal of phenacyl protecting group afforded **5**. Further, **5** was coupled with **9**[17] to give the tri-lipid **10**, which upon $KMnO_4$ oxidation furnished the tri-lipid acid **7** in good overall yield.

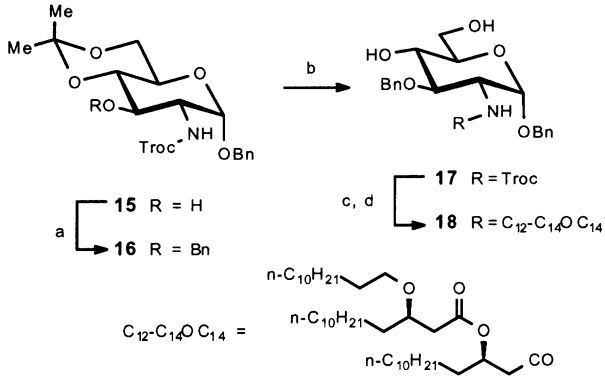
Well-established synthetic strategies for lipid A molecules[13,18–21] mainly differ in the choice of protecting group(s) and glycosylation donors in constructing the β-1,6-di-glucosamine unit. Here, we choose benzyl group as the global protecting group and the trichloroacetimidate as the glycosylation donor. Scheme 2 describes the preparation of glycosyl imidate **14**. The introduction of lipid **5** to the 3-*O*-position of monosaccharide **11**[22] gave **12**. Regio-selective reductive ring opening of the benzylidene group in **12**, followed by dibenzylphosphite introduction and oxidation gave **13**, which was then converted to the imidate **14** after allyl group removal. The glycosyl acceptor **18** was prepared starting from the readily accessible **15**[23] (Scheme 3), through the following sequence of reactions: benzylation (→**16**), removal of isopropylidene group (→**17**),



Scheme 1. (a) Dodecyl triflate, K_2CO_3, $ClCH_2CH_2Cl$, 68%; (b) Zn, HOAc, 86%; (c) DCC, DMAP, CH_2Cl_2, 81%; (d) $KMnO_4$, hexane–HOAc, 0 °C, 61%.



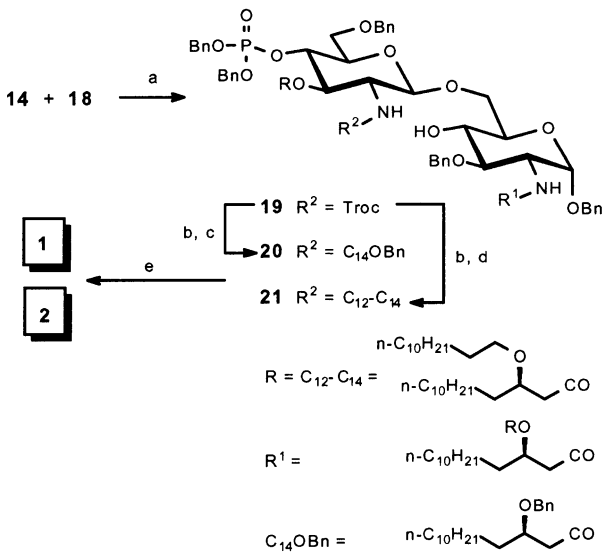
Scheme 2. (a) **5**, EDCI, DMAP, CH_2Cl_2, 92%; (b) $BH_3{\cdot}Me_2NH$, $BF_3{\cdot}OEt_2$, CH_3CN, 72%; (c) (i) $(BnO)_2PN(iPr)_2$, tetrazole, CH_2Cl_2; (ii) *m*-CPBA, CH_2Cl_2, 0 °C, 72% (two steps); (d) (i) [bis(methyldiphenylphosphine)](1,5-cyclooctadiene) iridium(I) hexafluorophosphate, THF; (ii) NBS, THF–H_2O, 79% (two steps); (e) CCl_3CN, DBU, CH_2Cl_2, 61%.



Scheme 3. (a) $BnOC(NH)CCl_3$, CF_3SO_3H, hexane–CH_2Cl_2, 0 °C, 56%; (b) HOAc–H_2O, 45 °C, 95%; (c) Zn, HOAc, 95%; (d) **7**, DCC, CH_2Cl_2, 38%.

removal of the Troc-group and introduction of the tri-lipid acid (→**18**).

Glycosylation reaction between **14** and **18** afforded the disaccharide **19**[24] in moderate yield (Scheme 4). After the removal of the Troc-group, two different lipid acyl groups (**4** and **5**, Fig. 2) were introduced at 2′-amine to give **20** and **21**, which upon catalytic hydrogenolytic



Scheme 4. (a) $BF_3{\cdot}OEt_2$, CH_2Cl_2, 56%; (b) Zn, HOAc, 97%; (c) **4**, DCC, CH_2Cl_2, 76%; (d) **5**, DCC, CH_2Cl_2, 75%; (e) Pd/C, H_2, THF–HOAc, 87% for **1** and **2**.

de-benzylation afforded the designed lipid A analogue **1** and **2**,[25] respectively.

Compounds **1** and **2** were tested for immune stimulatory (adjuvant) properties in terms of their ability to induce antigen specific T-cell proliferation (blastogenesis) and secretion of cytokine interferon-gamma (IFN-γ) (Fig. 3). Liposomal vaccine formulation containing MUC1-derived peptide BLP25[26] as an antigen and lipid A as an adjuvant was used to immunize mice.[27] Preliminary test results show that both **1** and **2** induce the same magnitude of IFN-γ level (ρg/mL) and T-cell proliferation (CPM) as the natural **Lipid A**[28] preparation, indicating that **1** and **2** are potent immune stimulatory agents for antigen specific T-cell activation. These data show that murine immune system, probably mediated by TLR4, exhibits greater tolerance to structural modifications of lipid chains in lipid A than previously thought.

The fact that **1** is strongly active as an immunostimulant is somewhat surprising in view of the observation by Seydel et al.[8–10] that biological activities of lipid A are determined by the shape of the molecule. Analogue **1**, containing one phosphoryl group and six acyl chains with a symmetric distribution (3/3), is expected to have a small tilt angle and a cylindrical shape, the molecular conformation that tends to exhibit low or no agonistic activity, but probable antagonistic activity. Unexpectedly, our result shows that **1** has strong agonistic activity of lipid A. Whether the tri-lipid moiety in **1** differs structurally from conventional three lipid chains (2+1) and has different impact on the whole molecular shape of lipid A need further investigation.

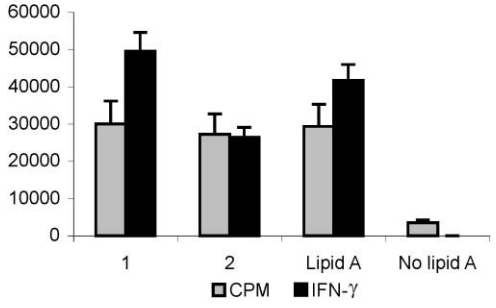


Figure 3. Adjuvant properties of synthetic lipid A analogue **1** and **2**: antigen specific T-cell proliferation (blastogenesis) (CPM, counts per min) and interferon-gamma (IFN-γ, pg/mL) production in mice immunized with MUC1-based liposomal vaccine adjuvanted with lipid A analogue. **Lipid A**: natural lipid A product obtained from *Salmonella minnesota*, R595.

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24. The regio-selectivity of the glycosylation at 6-*O*-position and its β-linkage are asserted based on the result of other similar glycosylation reaction carried out in this laboratory. The anomeric protons of the disaccharide are recognizable and its acetylated derivative confirms the 6-*O*-linkage. **19**: R_f 0.33 (hexane/ethyl acetate, 2:1). $[\alpha]_D^{20} = +24.0$ (*c* 0.3, chloroform). ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, $J = 6.5$ Hz, 15H, 5 CH_3), 1.25 (m, 90H), 1.40–1.58 (m, 10H), 2.25–2.45 (m, 6H), 2.95 (br s, 1H, OH), 3.21 (m, 1H), 3.36 (m, 3H), 3.47–3.53 (m, 2H), 3.57–3.67 (m, 4H), 3.72 (m, 2H), 3.80 (m, 2H), 4.03 (br d, $J = 11.0$ Hz, 1H), 4.25 (ddd, $J = 10.0$, 9.0, 3 5 Hz, 1H, H-2), 4.44 (d, $J = 11.5$ Hz, 1H), 4.48 (m, 3H), 4.59 (m, 2H), 4.67 (d, $J = 11.5$ Hz, 1H), 4.69 (d, $J = 11.5$ Hz, 1H), 4.75 (d, $J = 11.5$ Hz, 1H), 4.85–4.92 (m, 6H), 5.04 (m, 1H), 5.36 (m, 2H), 5.80 (d, $J = 9.0$ Hz, 1H, NH), 7.30 (m, 25H, Ar–H).
25. The ^{1}H NMR spectra of fully de-protected lipid A analogue **1** and **2** are poorly resolved in various solvents tested, indicating the aggregation of these molecules in solution. The purity of **1** and **2** were checked by HP-TLC and their identity confirmed by MS. **1**: R_f 0.20 (chloroform/methanol/water/ammonium hydroxide, 7:3:0.4:0.2). $[\alpha]_D^{20} = -3.5$ (*c* 0.2, chloroform/methanol, 4:1). ESI-MS calcd for $C_{92}H_{177}N_2O_{20}P$: 1661.3; found (negative mode): 1660.3 (87, M−H), 1661.3 (100, M−H, ^{13}C isotope peak). **2**: R_f 0.38 (chloroform/methanol/water/ammonium hydroxide, 7:3:0.4:0.2). $[\alpha]_D^{20} = +2.0$ (*c* 0.2, chloroform/methanol, 4:1). ESI-MS calcd for $C_{104}H_{201}N_2O_{20}P$: 1829.4; found (negative mode): 1828.5 (80, M−H), 1829.5 (100, M−H, ^{13}C isotope peak).
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27. Mice were immunized subcutaneously with a single injection of liposomal vaccine formulation containing MUC1-derivatived lipopeptide antigen BLP25 (40 μg per dose), H_2N–STAPPAHGVTSAPDTRPAPGSTAPPK(palmitoyl)G–OH, and a lipid A analogue (20 μg per dose), either synthetic or natural, as an adjuvant. After 9 days, the lymphocytes were taken from the draining lymph nodes of immunized mice and incubated in in vitro culture in the presence of the same boosting antigen BLP25 and antigen presenting cells (APC). T-cell proliferation was evaluated using a standard ^{3}H thymidine incorporation assay and IFN-γ level was determined in the cell culture supernatants using enzyme-linked immunoabsorbent assay (ELISA). Both CPM and IFN-γ data reported in Figure 3 were averaged from sextet experimental measurements.
28. The natural **Lipid A** product isolated from *Salmonella minnesota*, R595, was purchased from Avanti Polar Lipids, Inc. For structures of *S. minnesota* R595 lipid A, please see: Qureshi, N.; Mascagni, P.; Ribi, E.; Takayama, K. *J. Biol. Chem.* **1985**, *260*, 5271.