Invited Paper

Synthetic Chemistry and Function of Bacterial Cell Surface Glycoconjugates[+]

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Typical bacterial glycoconjugates are known to stimulate immunological systems of higher animals and thereby play important roles in the primary defense of animals against bacterial infection.Lipopolysaccharide (LPS) of gram-negative bacteria is a representative of such glycoconjugates.LPS was first discovered as a potent bacterial toxin and named endotoxin but was soon found to exhibit immunostimulating activity.By the use of our synthetic pure preparations, the lipophilic partial structure of LPS, designated lipid A, proved to be the active entity responsible for both endotoxic and immunostimulating activities of LPS.This paper deals with our recent chemical synthesis and functional study of lipid A and related compounds.Synthesis is described of its various structural analogues, radio-labeled compound and Re-type LPS that contains two additional sugar moieties linked to lipid A.

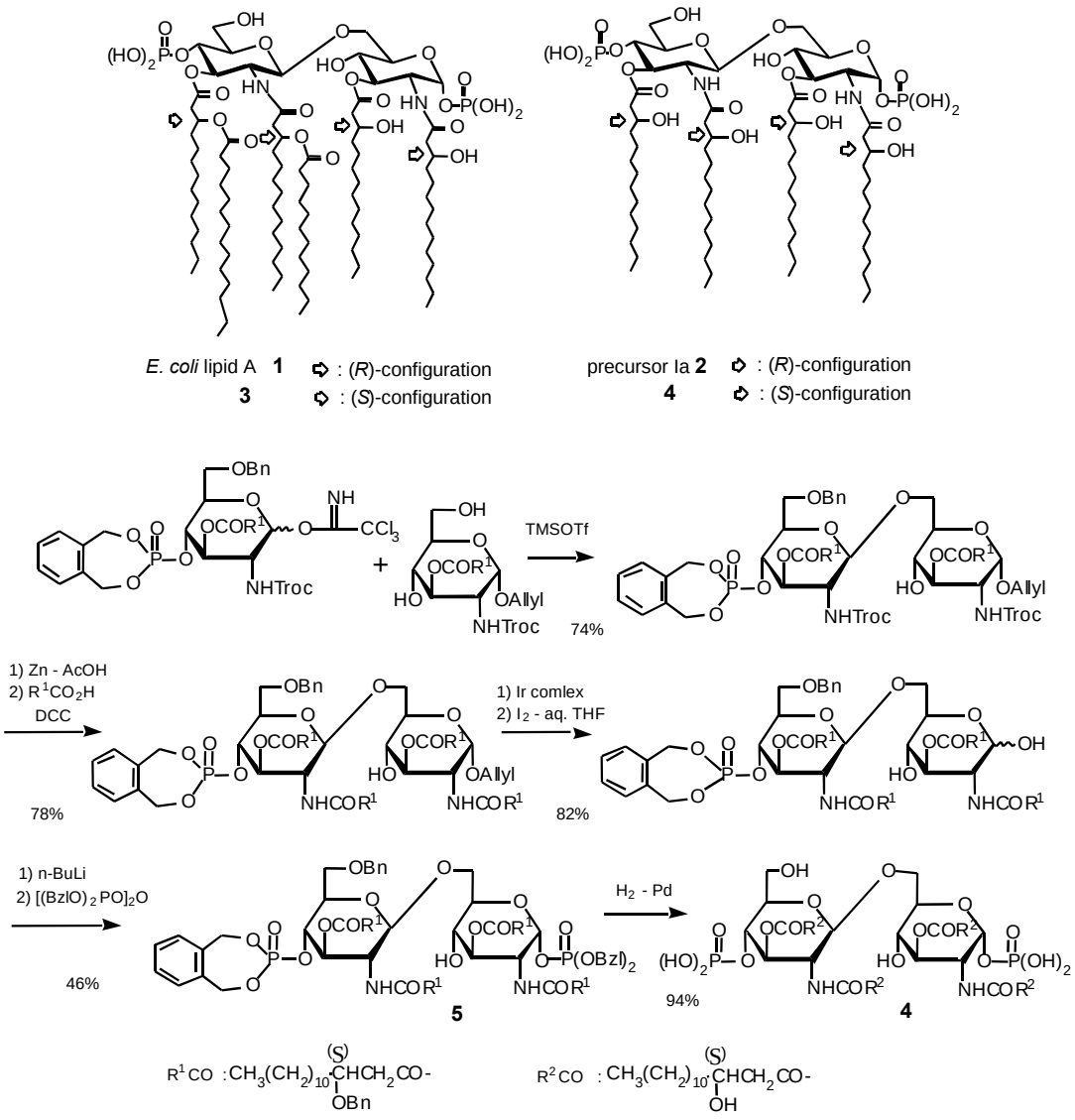
Bacterial cells are covered with a rigid cell envelope which is mainly composed of complex glycoconjugates having unique structures never found in higher animals. When infection of bacteria occurs, defensive cells of animals such as macrophages and lymphocytes start their action and produce protein mediators (cytokines) such as interleukins, interferons, and tumor necrosis factors to enhance the entire defense system. Interestingly, not whole living cells of bacteria are required for such activation of defense mechanism but certain typical cell surface glycoconjugates represent the role of the living bacteria. It was further demonstrated that definite chemical entities corresponding to particular partial structures of the glycoconjugates possess the activity.

The first example identified as an "active principle" of cell surface glycoconjugates was N-acetylmuramyl-L-alanyl-D-isoglutamine (so-called muramyl dipeptide, MDP) described by two independent research groups including ourselves.[1] The muramyl peptide is a common building-block of cell wall peptidoglycan. This particular molecular species with molecular weight of only around 500 exhibits the definite immunostimulating activity similar to that of the cell wall itself. This result encouraged us to continue our study on other biologically important bacterial glycoconjugates in order to identify the active structures responsible for their immunostimulation and to disclose the mechanism of their action. For synthetic organic chemists, these molecules present highly challenging targets with complex chemical structures and amphiphilic natures difficult to handle. In this presentation we will focus on our synthetic work on lipopolysaccharide, a typical bioactive glycoconjugate of gram-negative bacteria.

Synthesis of lipid A, the lipid component of lipopolysaccharide, and its analogues

Lipopolysaccharide (LPS) is the chemical entity of bacterial endotoxin which was first described as early as 1892 as a heat-stable potent toxin bound to the cells of Gram-negative organism. As the name endotoxin implies, LPS exhibits various detrimental activities such as lethal toxicity, pyrogenicity, and tissue-damaging activity, which sometimes cause serious clinical problems even today. Simultaneously, however, LPS also shows a wide range of beneficial activities related to stimulation of immunological responses. LPS is composed of a hydrophilic polysaccharide chain covalently bound to a glycolipid designated lipid A. The latter was then found to be responsible for both beneficial and toxic functions of LPS.[2] The chemical structure of lipid A from *Escherichia coli* Re mutant was deduced as **1** by us.[3] The proposed structure of lipid A (**1**) was soon confirmed by our total synthesis.[4] The synthetic lipid A showed identical biological activities to those of the natural counterpart isolated from bacterial cells.[5]

[+] Based on the lecture of Shoichi Kusumoto presented in "K.-T. Wang Bioorganic Chemistry Lectureship Symposium 2001" held on September 28, 2001 in Taipei.



Various structural analogues of lipid A (**1**) were isolated from cells of other Gram-negative bacteria. Some of them were also synthesized and tested for their activities.[6] A novel efficient synthetic route to lipid A analogues was recently established as exemplified in the scheme below.

The scheme illustrates preparation of an artificial analogue **4** of a biosynthetic precursor of lipid A **2** which contains only four moles of 3-hydroxytetradecanoic acid. Compound **4** contains (*S*)-3-hydroxytetradecanoic acid instead of the corresponding (*R*)-3-hydroxy acids present in the natural counterpart **2** and lipid A (**1**).[7] The β(1-6)glycosidic linkage of **3** was formed by the imidate method with the participation of the neighboring *N*-Troc group. The use of this glycosylation method in place of the previously employed glycosyl bromide[4] enabled the protection of the distal phosphate group as an *o*-xylidene ester. This cyclic benzyl type protecting group was removed later smoothly by hydrogenolysis together with all the other benzyl groups in one step. After introduction of the glycosyl phosphate group, the protected final product **5** was carefully purified, which was essentially important to obtain a high yield of the final product by hydrogenolysis. The free lipid A was purified by centrifugal partition chromatography with a solvent system 1-butanol-THF-H_2O (9 : 7 : 20).[7] The purification procedure is applicable with some minor modifications to the synthesis of various structural analogues of lipid A.[8]

Cytokine-inducing activity as a typical biological function of LPS was tested for the synthetic compounds. Figs. 1 and 2 show the interleukin-6 (IL-6)-inducing activity as tested with human peripheral whole blood cells by the ELISA

method. The data revealed the following interesting phenomena. The number of acyl groups in the molecule is important for the nature of the activity, whereas the configurations of the 3-hydroxy acyl residues have no essential effect. Synthetic *E. coli*-type lipid A (**1**) with 3-hydroxy- and 3-acyloxyacyl residues of natural (*R*)-configurations exhibits comparable activity with that of a standard natural LPS (from *E. coli* 0111:B4 cells). An unnatural *E. coli*-type analog **3** with (*S*)-3-hydroxy acids exhibited almost the same endotoxic activity as the natural type **1**. A biosynthetic precursor of lipid A designated precursor Ia (**2**) was found to act as an antagonist to **1** and LPS, suppressing the cytokine-inducing activity of the latters.[9] The precursor-type **4** with (*S*)-acids exhibited similar but even stronger antagonistic activity than the natural-type precursor Ia (**2**).[7,8]

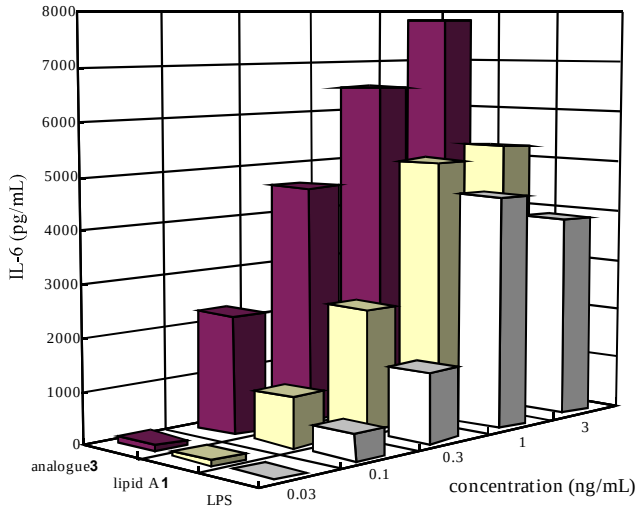


Fig. 1. IL-6 induction by synthetic *E. coli*-type lipid A **1**, its (*S*)-acyl analogue **3**, and LPS (*E. coli* 0111:B4).

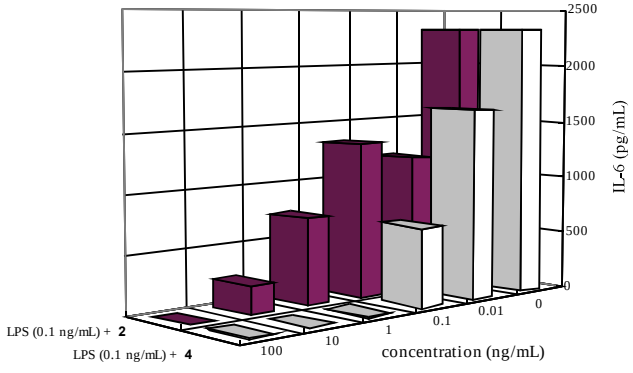
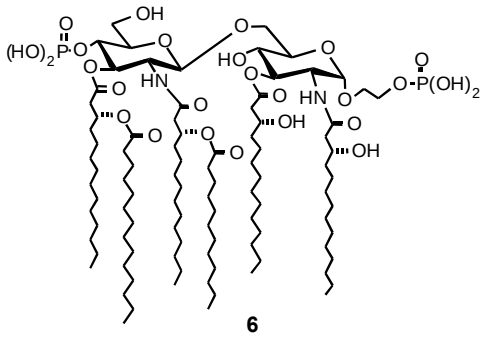


Fig. 2. Inhibitory activity of synthetic lipid A analogues **2** and **4** against IL-6 induction by LPS.

Synthesis of radio-labeled lipid A analogues

Chemical synthesis has another obvious advantage that one can prepare suitably labeled derivatives of lipid A which are expected to be important tools for the investigation of binding proteins and the mode of biological action. We next attempted to incorporate radioactivity to the molecule of phosphonooxyethyl analogues **6** of lipid A.[10] In this artificial compound one of the phosphate groups of lipid A is bound not directly to the glycosidic hydroxy group but through an α-glycosidically linked ethylene glycol moiety. The phosphonooxyethyl analogue is stable enough so that its preparation was much easier than the corresponding natural lipid A. In fact, **6** was obtained through short-step transformations and purified readily by simple ion-exchange chromatography.[10] In spite of such modification, the phosphonooxyethyl analogue **6** showed indistinguishable endotoxic activity from that of **1**.



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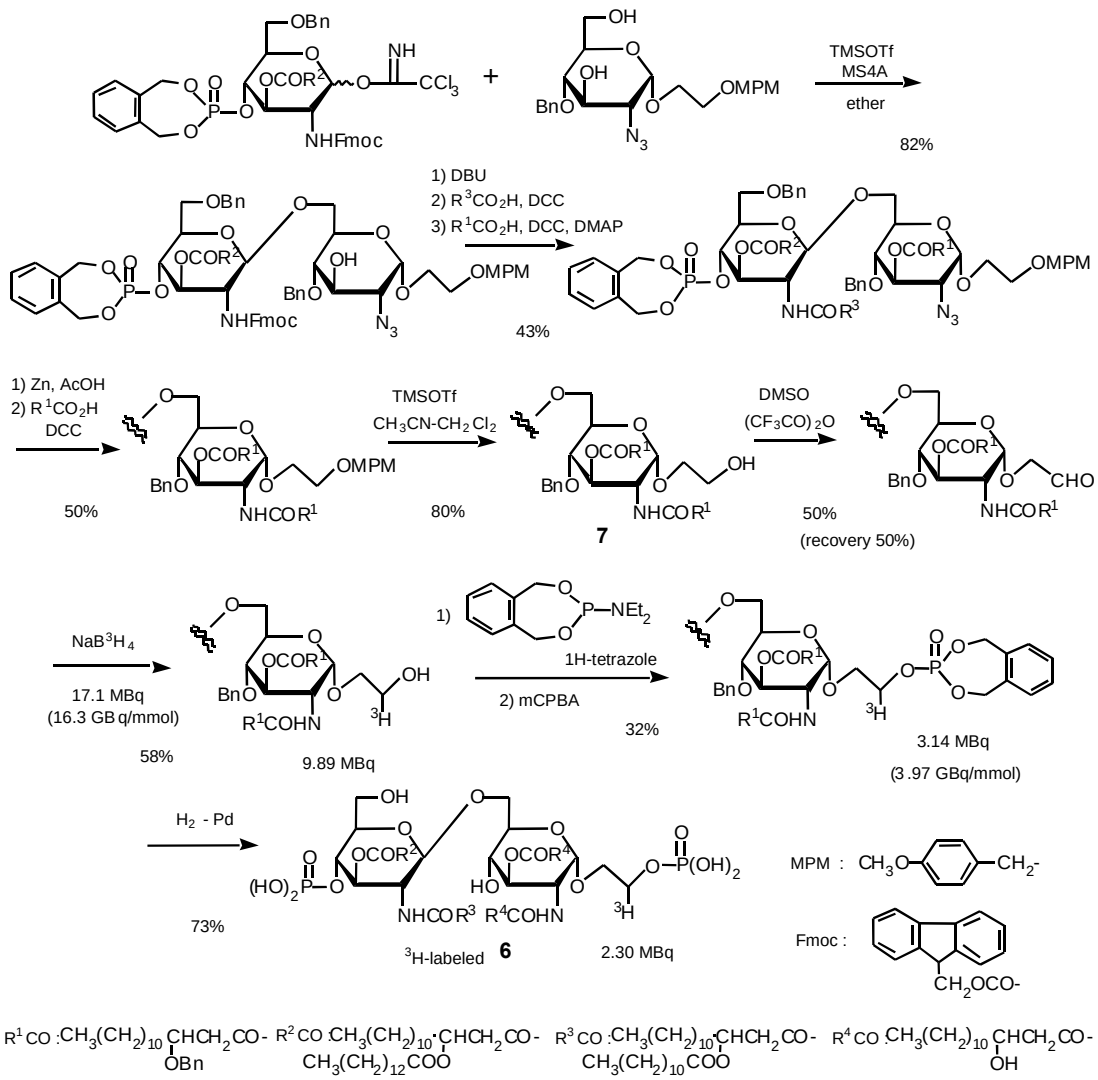
The phosphonooxyethyl analogue, therefore, seemed to be an ideal target of the synthesis of radio labeled derivative. We attempted to introduce radioactivity at the ethylene glycol unit of these molecules. An entirely new route was exploited for a tritium-labeled **6** as illustrated in the scheme below by employing a similar strategy to that for the synthesis of **4** above.

A disaccharide intermediate **7** was prepared which contains all the acyl groups and 4′-phosphate as well as the glycosidically bound ethylene glycol. A tritium was incorporated to the distal hydroxylmethylene group by NaB^3H_4-reduction of the corresponding aldehyde derivative. Phosphorylation followed by HPLC purification and hydrogenolytic deprotection afforded highly pure tritium-labeled **6** with high specific radioactivity.[11,12] The labeled compound was used to detect lipid A-binding proteins on the cell surface of macrophages.[12]

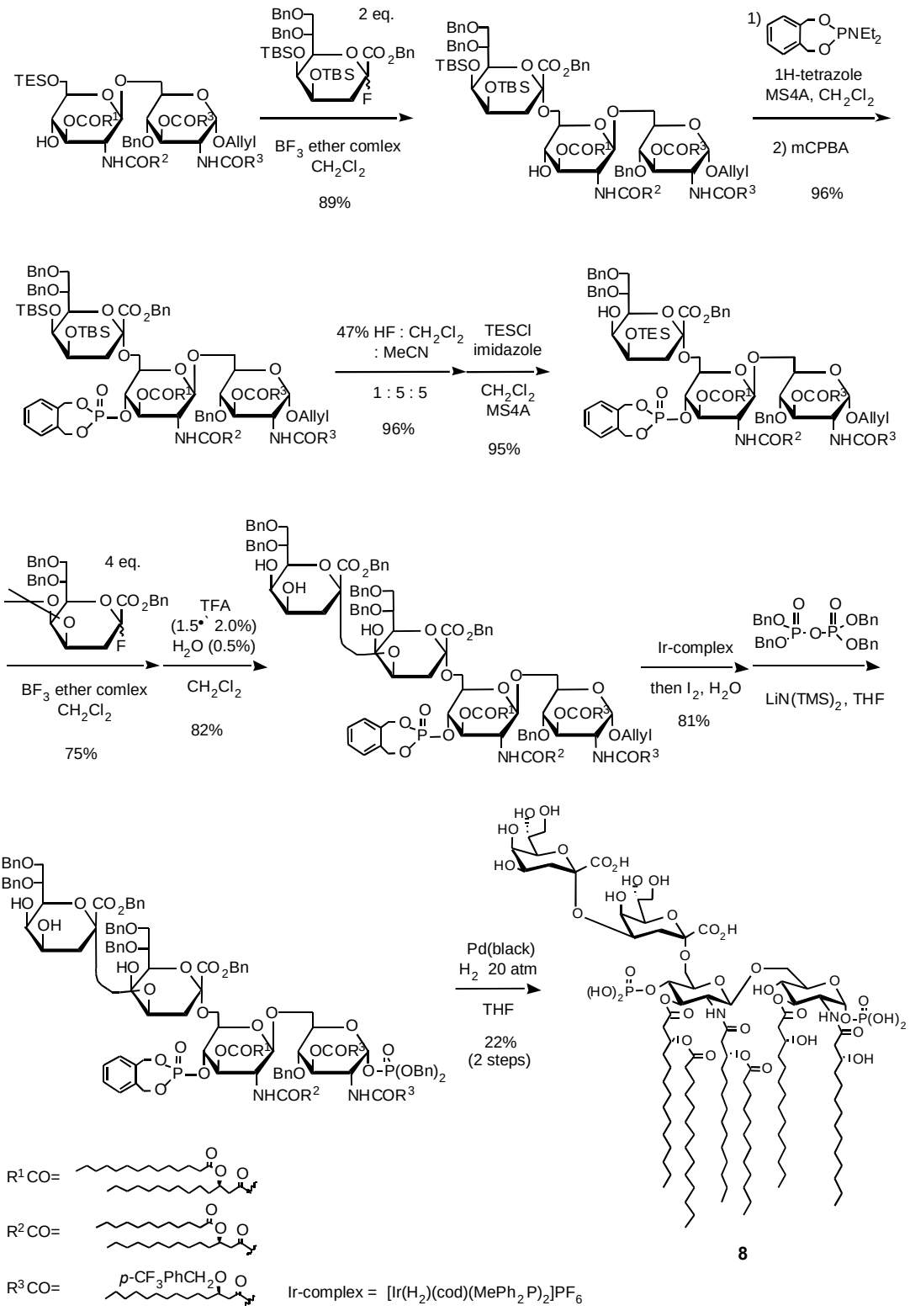


Synthesis of Re-type lipopolysaccharide (Re LPS)

As described, lipid A has proved to be the endotoxic principle of bacterial LPS. However, lipid A itself is an artificial molecule never exists in nature in the free form: it is obtained only after mild acid hydrolysis of LPS. The most simple natural LPS ever found on living bacterial cells is one called Re-type LPS produced by *E. coli* Re mutant. Re LPS (**8**) contains only two additional acidic sugar moieties called 3-deoxy-D-manno-2-octulosonic acid (Kdo) linked to the 6′-position of lipid A. We also started a synthetic approach to Re LPS which is a highly challenging target containing both base-labile ester functionalities and the acid-labile glycosyl phosphate and ketosidic linkages. After solving several basic problems, such as stereoselective formation of α-Kdo linkages, the synthesis was started with an acylated glucosamine disaccharide devoid of the phosphate as shown in the scheme.

The synthesis was completed by stepwise introduction of two Kdo moieties and both phosphate followed by catalytic hydrogenolysis. Purification was also effected by partition chromatogry to afford the highly pure first synthetic LPS **8**.[13] The intrinsic biological activities such as cytokine induction of Re LPS and its partial structure lacking one Kdo were determined for the first time, the effects of contaminating bacterial components being thereby completely excluded.

Recently, the basic framework of a new defense system called “innate immunity” has been disclosed mainly by molecular biological approaches. This is a very basic mechanism which animals are given by born to protect themselves against a wide range of invading microorganisms. A family of toll-like receptors (TLRs) were characterized and shown to play thereby important roles to recognize various bacterial glycoconjugates. By the use of our homogeneous synthetic



lipid A preparations, participation of TLR4 was unequivocally proved in the process of cell activation by lipid A.[14]

Synthetic organic chemistry is a powerful tool particularly in functional study of biological active complex glycoconjugates as exemplified here. The major merit of chemical synthesis in this field is its ability to produce homo-

geneous and definite compounds including specifically labeled ones. Highly pure such preparations which are often never available from natural sources are of crucial importance for determination of the active entities and also for the study of action mechanisms. Synthetic chemists can thereby take great pleasure in solving various problems and of course in having the final success. In that way the frontier of chemistry may be ever expanding.

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Key Words

Synthesis; Glycoconjugates; Lipid A; Lipopolysaccharide; Biological activity; Cytokines; Immunostimulation; Innate immunity.

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