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Synthesis and Bioactivity of a Fluorescence-Labeled Lipid A Analogue

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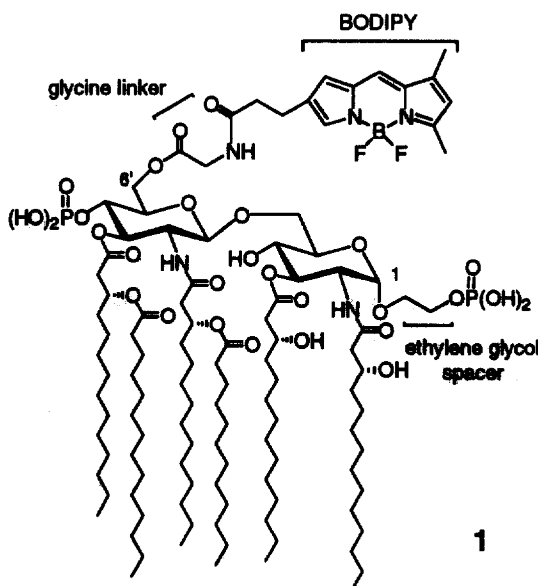
Abstract: A fluorescence-labeled phosphonooxyethyl (PE) analogue of lipid A has been successfully synthesized in 15 steps from D-glucosamine derivative **2** in total 16% yield. The analogue exhibits *Limulus* activity and cytokine-inducing activity similar to the natural lipopolysaccharide and the unlabeled PE analogue of lipid A. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: lipopolysaccharide; lipid A; fluorescence; biologically active compounds



Lipid A is the lipophilic proximal partial structure of lipopolysaccharide (LPS, endotoxin) which is a major constituent of the outermost leaflet of the outer membrane of Gram-negative bacteria [1]. The bioactivities of LPS, including pyrogenicity and enhancement of immunological responses toward higher animals, are attributed to lipid A [2]. As a tool to clarify the mode of the biological action of lipid A, a fluorescence-labeled analogue **1** of *Escherichia coli*-type lipid A has been synthesized in the present study. In the target structure **1**, the fluorescent BODIPY (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene) group was linked to the lipid A moiety through a glycine linker. To the glycosyl phosphate moiety in the lipid A structure was also inserted an ethylene glycol spacer to increase the chemical stability. The latter type of modification described as the phosphonooxyethyl (PE) analogue is known to retain the original, full biological activity of lipid A [3,4]. By virtue of these advantages, radio-labeling has been successfully effected on the PE analogue, the labeled product being used to identify the receptor on competent animal cells [5].

Since the BODIPY fluorescent group is not stable enough, especially under basic conditions, the group was introduced at the latest step before deprotection in the synthesis of **1**. To achieve selective introduction of the

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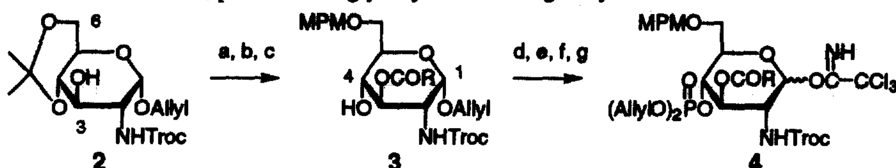
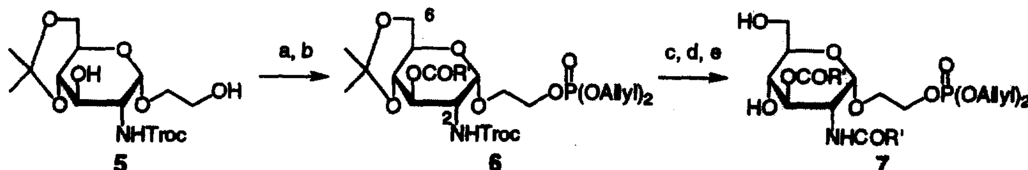
BODIPY group, the 6'-hydroxy group in synthetic precursors was protected as an MPM ether. For hydroxy and phosphoryl groups to be protected until the end of the synthesis were employed allyl-type protecting groups which were expected to be removed under nearly neutral conditions [6,7,8] without damaging the BODIPY group.

The known glucosamine derivative **2** [9] was employed as the starting material for the non-reducing-side glucosamine residue (Scheme 1). The 3-hydroxy group was acylated with (*R*)-3-(tetradecanoyloxy)tetradecanoic acid [10] under the standard conditions to afford the fully derivatized glucosamine in quantitative yield. The isopropylidene group was removed by aqueous acetic acid, and the primary 6-hydroxy group was selectively protected as the MPM ether to afford **3**. Before phosphorylation, the 1-*O*-allyl group was isomerized by the action of a cationic iridium complex [11] to achieve smooth deprotection of this protecting group later. Then the 4-hydroxy group was phosphitylated and the resultant phosphite oxidized. Successive 2-step sequence, including removal of the 1-*O*-propenyl group and the trichloroacetimidate formation, provided the glycosyl donor **4** in good yield.



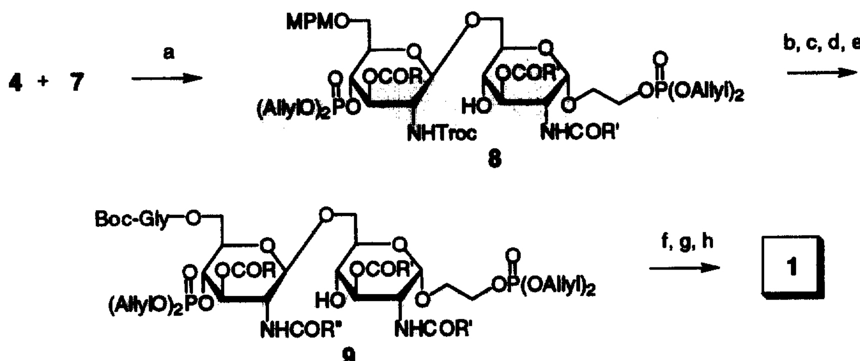
Scheme 1. a) (*R*)-3-(tetradecanoyloxy)tetradecanoic acid, DCC, DMAP, CH_2Cl_2; b) 90% AcOH, reflux, 87% for 2 steps; c) MPMOC(=NH)CCl_3, CSA, CH_2Cl_2, 89%; d) H_2-activated $[Ir(cod)(MePh_2P)_2]PF_6$, THF, 100%; e) $(AllylO)_2PN^iPr_2$, 1*H*-tetrazole, CH_2Cl_2, then ^tBuOOH, 92%; f) I_2, pyridine, H_2O, THF, 86%; g) CCl_3CN, Cs_2CO_3, CH_2Cl_2, 100%.

The synthesis of the reducing-side unit **7** started from **5** which was prepared from D-glucosamine in 3 steps ((i) 2,2,2-trichloroethyl chloroformate, $NaHCO_3$; (ii) ethylene glycol, Dowex® 50W×8; (iii) 2,2-dimethoxypropane, CSA) in 50% yield (Scheme 2). Phosphorylation of the distal primary hydroxy group in **5** by the phosphorochloridate method was first attempted using DMAP/pyridine or pyridine alone as a base. In these cases the yield of the desired monophosphate was not satisfactory, being in the range of 55–68%, because 13–16% of the bisphosphate was formed. The formation of the latter over-phosphorylated by-product was found to be suppressed completely when the more bulky 2,6-lutidine was used for the reaction. 2,6-Lutidine also worked well in the next acylation at the remaining 3-hydroxy group with (*R*)-3-(allyloxycarbonyloxy)tetradecanoic acid [8] to afford **6** in high yield. The alternative use of DMAP or DMAP·HCl for this acylation caused elimination at the base-sensitive allyloxycarbonyloxy moiety. The Troc group for the 2-amino protection of **6** was then removed by the action of zinc-copper couple, and (*R*)-3-(allyloxycarbonyloxy)tetradecanoic acid [8] was introduced again to the amino group. The isopropylidene group was removed to give **7** to be used for the disaccharide formation.



Scheme 2. a) $(AllylO)_2POCl$, pyridine, 2,6-lutidine, CH_2Cl_2, 95%; b) (*R*)-3-(allyloxycarbonyloxy)tetradecanoic acid, DCC, 2,6-lutidine, CH_2Cl_2, 98%; c) Zn-Cu, AcOH; d) (*R*)-3-(allyloxycarbonyloxy)tetradecanoic acid, WSCD·HCl, HOAt, CH_2Cl_2, 90% for 2 steps; e) 90% AcOH, reflux, 90%.

The glycoside coupling of **4** and **7** proceeded smoothly with a catalytic amount of TMSOTf to afford the β(1→6) disaccharide **8** in 87% yield (Scheme 3). Subsequent removal of the *N*-Troc group followed by acylation with (*R*)-3-(dodecanoyloxy)tetradecanoic acid [10] gave the fully acylated compound. After the 6'-*O*-MPM group was removed, the introduction of the glycine linker to the 6'-position was carried out by the coupling with *N*-Boc-glycine in the presence of DCC and bulky 2,4,6-collidine to afford **9** selectively. The reaction was quenched before completion to obtain a satisfactory regioselectivity, recovering 35% of the unreacted 4,6'-dihydroxy compound.[1] The Boc group in **9** was removed under acidic conditions, and the further introduction of the fluorescent group[2] was effected with the aid of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (WSCD) hydrochloride in 85% yield. Finally, all the allyl-type protecting groups for phosphoryl and hydroxy functions were removed by treatment with a palladium(0) catalyst [6,7] to afford the target compound **1** in a pure state as judged from TLC, NMR, and MS. The total yield was 16% for 15 steps from **2**.



Scheme 3. a) TMSOTf, MS4A, $(CH_2Cl)_2$, -20 °C, 87%; b) Zn-Cu, AcOH; c) (*R*)-3-(dodecanoyloxy)tetradecanoic acid, WSCD·HCl, CH_2Cl_2, 90% for 2 steps; d) DDQ, H_2O, CH_2Cl_2, 89%; e) *N*-Boc-glycine, DCC, 2,4,6-collidine; f) TFA, CH_2Cl_2, 51% for 2 steps; g) BODIPY FL C3-OH®, WSCD·HCl, CH_2Cl_2, 85%; h) $Pd(PPh_3)_4$, PPh_3, $HCOONH_4$, THF, 87%.

The bioactivities of **1** were evaluated in comparison to those of LPS (*E. coli* 0111:B4) and the unlabeled PE analogue of lipid A [3,4]. The hemolymph coagulation activity on horseshoe crab, the so-called *Limulus* activity,[3] was examined first. The labeled compound **1** exhibits potent *Limulus* activity: the minimal concentrations for exhibition of 50% of full activity (ED_{50}) were estimated to be 5.0×10^{-9} g/L for LPS, 1.6×10^{-7} g/L for the unlabeled PE analogue, and 3.2×10^{-7} g/L for **1**. The interleukin-6 (IL-6) induction was examined next by means of enzyme-linked immunosorbent assay in heparinized human peripheral whole-blood cells (Figure 1) [12]. Both **1** and the unlabeled PE analogue exhibit positive activity as natural LPS does, and the amount of IL-6 induced was found to be 0.45×10^{-6} g/L when 2×10^{-6} g/L of **1** was added to the blood whereas 1.1×10^{-6} g/L of IL-6 was induced by the same amount of the unlabeled PE analogue. From these results **1** was concluded to retain the endotoxic activity of lipid A but is slightly weaker than the unlabeled PE analogue, which already proved to exhibit the same level of activity as lipid A [3,4]. This fluorescence-labeled bioactive specimen **1**, thus prepared in the present study, will allow us to analyze not only its distribution on the

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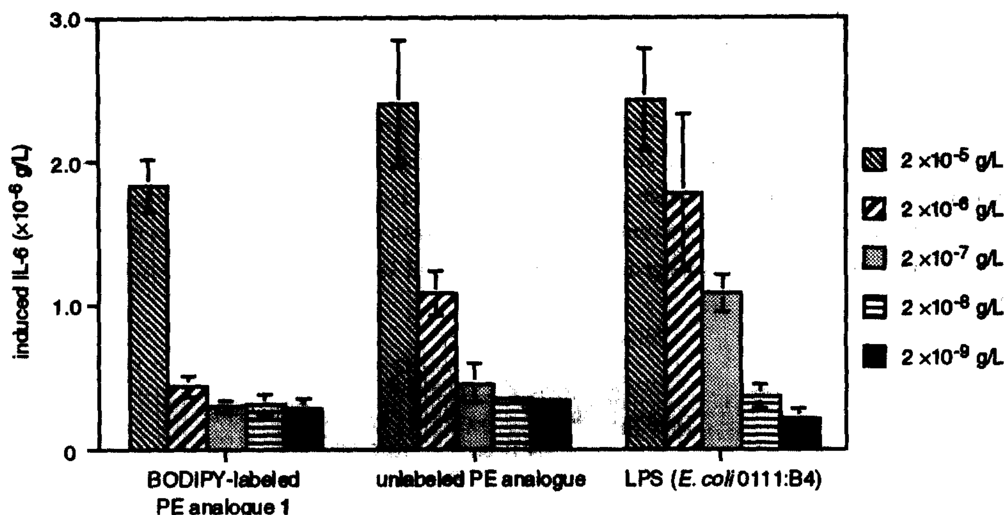
1 The longer reaction time or use of less bulky pyridine instead of 2,4,6-collidine resulted in the undesired introduction of the second *N*-Boc-glycine at the 4-hydroxy group.

2 3-BODIPY-propionic acid (BODIPY FL C3-OH®) was purchased from Molecular Probes.

3 Evaluated by Endospecy Test® (Seikagaku Corporation, Tokyo).

competent animal cells but also its mode of self-assembly; knowledge on this matter is considered to be essential to understand the biological events caused by bacterial lipopolysaccharide [13,14].

Figure 1. The IL-6 inducing activity in human peripheral whole-blood cells. The blood donor was H.F.



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