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CHEMICAL SYNTHESIS OF PHOSPHORYLATED TETRAACYL DISACCHARIDE CORRESPONDING TO A BIOSYNTHETIC PRECURSOR OF LIPID A

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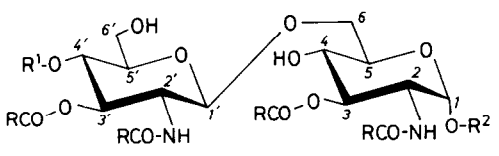
Summary: A total synthesis of 2,2'-N; 3,3'-O-tetraquis[(R)-3-hydroxytetradecanoyl]-β(1-6)-D-glucosamine disaccharide 1,4'-diphosphate is described. This is the first confirmation of the fundamental structure of lipid A since the synthetic compound exhibited most of the characteristic biological activities of natural endotoxin.

The cell-surface lipopolysaccharide (LPS) of Gram-negative bacteria is designated as endotoxin which exhibits many characteristic biological activities. Most of them have been attributed to the lipophilic portion of the molecule called lipid A which can be liberated from LPS by mild acid hydrolysis. The basic chemical structure of lipid A was postulated to be a polyacylated β(1-6)-D-glucosamine disaccharide 1,4'-diphosphate.[1] The positions of acyl groups attached to the disaccharide were unequivocally determined by our recent investigation for a purified main component of *E. coli* lipid A. We concluded that the 2,2'-amino as well as the 3,3'-hydroxyl groups of the disaccharide are acylated, revising the previously proposed distribution pattern of ester-bound acyl groups (3,4,6'-hydroxyl groups).[2] It became thus urgently important to perform a synthesis of compounds based on the revised structure.

In this paper we describe a synthesis of 2,2'-N; 3,3'-O-tetraquis[(R)-3-hydroxytetradecanoyl]-β(1-6)-D-glucosamine disaccharide 1,4'-diphosphate (1). We assumed this target as the most promising structural candidate for the so-called "acidic disaccharide precursor" in the biosynthesis of lipid A which was isolated from a certain *Salmonella* mutant.[3] Though the positions of acyl groups in the precursor were at that time not yet elucidated, it seemed to be quite reasonable to apply the above acylation pattern for it. The natural precursor containing four moles of (R)-3-hydroxytetradecanoic acid but devoid of non-hydroxylated acids[3] had been shown to exhibit most of the typical endotoxic activities, representing the simplest structural features of biological active lipid A analogs known so far. Corresponding monophosphates (2 and 3) as well as the dephospho derivative (4) were also prepared in order



RCO : $CH_3(CH_2)_{10}\underset{OH}{C}HCH_2CO$

	R^1	R^2
1	$PO(OH)_2$	$PO(OH)_2$
2	$PO(OH)_2$	H
3	H	$PO(OH)_2$
4	H	H

to elaborate the role of phosphate moieties of 1 for expression of activities. According to several biological tests the synthetic phosphorylated compounds showed endotoxic activities comparable to those of natural specimen,[4] finally proving the concept that lipid A is the active principle of endotoxin.[5] Consequently, this synthesis is the first successful chemical construction of endotoxic compounds.

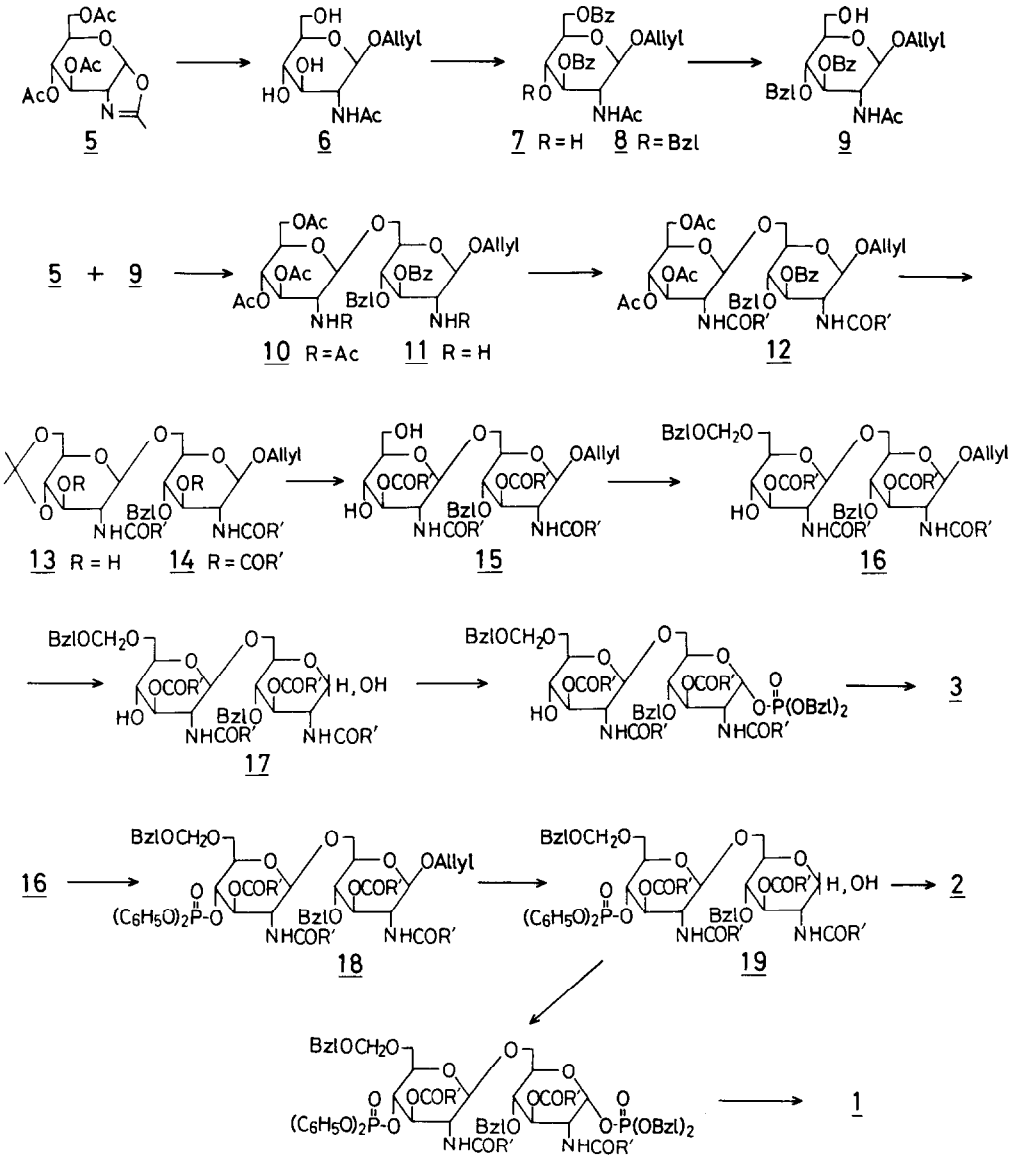
The synthesis was performed in following steps by utilizing the knowledges and experiences accumulated in our previous synthetic works since several years:[6] i) preparation of β(1-6)disaccharide, ii) N- and O-acylation with 3-benzyloxy fatty acid, iii) phophorylation and iv) hydrogenolytic removal of the protecting groups.[7]

The component for the reducing glucosamine of disaccharide was prepared from peracetyl oxazoline (5) via a β-allyl glycoside (6). Benzoylation of 6 (benzoyl chloride in pyridine - THF at -50°C for 4 hr) afforded 3,6-di-O-benzoate (7) (62%, mp 186-187°C). The position of the benzoyl groups of 7 were confirmed by NMR. Acid catalyzed benzylation[8] of 7 (benzyl trichloroacetimidate - trifluoromethanesulfonic acid in CH_2Cl_2 at 0°C for 3 hr) gave 4-O-benzyl-dibenzoate (8) (34%, mp 157-158°C).[9] Though the yield of 8 was not high enough, this approach seems to be the most practical one to prepare a 4-O-monobenzylated derivative in relatively short reaction steps. On transesterification (0.02N NaOMe at room temperature for 2.5 hr), one of the benzoyl groups was preferentially splitted off to give 9 (68%, mp 165-167°C).

Coupling of 9 with the oxazoline 5 (in $CHCl_3$ in the presence of p-TsOH under reflux for 7 hr) afforded a β(1-6)disaccharide (10) (64%, mp 222-224°C, $[\alpha]_D^{17}$ -38.1° (c 0.43, $CHCl_3$)). Removal of both N-acetyl groups was effected with Meerwein's reagent as described previously[3,10] [i) Et_3OBF_4 in CH_2Cl_2 in the presence of anhydrous K_2CO_3, ii) dil. HCl in THF] and the free amino groups were acylated with an optically pure protected hydroxy acid, i.e., (R)-3-benzyloxytetradecanoic acid[6] (dicyclohexylcarbodiimide in THF) to yield N,N'-diacylated disaccharide (12) (41% from 10, mp 165-169°C). After removal of all ester-type protecting groups from 12 (0.05N NaOMe - THF 4:1 at room temperature for 20 hr), the two hydroxyl groups on positions 4' and 6' were reprotected by conversion into the isopropylidene derivative 13 (2,2-dimethoxypropane - p-TsOH in $CHCl_3$- acetone 1:1 at room temperature for 1.5 hr; 88%, mp 106-110°C). The remaining two free hydroxyl groups in turn were acylated with (R)-3-benzyloxytetradecanoic acid (dicyclohexylcarbodiimide and dimethylaminopyridine in CH_2Cl_2 at room temperature for 4 hr) to afford a disaccharide (14) with four acyl groups at desired positions. The isopropylidene group of 14 was then removed (90% acetic acid at 90°C for 2 hr) to give 15 (82% from 13, mp 117-119°C, $[\alpha]_D^{17}$ -14.5° (c 0.40, $CHCl_3$)) whose primary hydroxyl group was then selectively protected (benzyloxymethyl chloride - ethyldiisopropylamine in CH_2Cl_2 at room temperature). 6-Monobenzyloxymethyl derivative (16) thus obtained (96%, mp 137-139°C, $[\alpha]_D^{19}$ -12.3° (c 1.12, $CHCl_3$)) was used as a common intermediate for the synthesis of three phosphorylated derivatives as described below.

The next steps in the synthesis are the introduction of phosphate moieties on 1- and/or 4-positions. The glycosidic allyl group of 16 was removed by isomerization with an iridium complex ($[Ir(COD)(PCH_3(C_6H_5)_2)_2]PF_6$ in THF at 50°C for 1 hr)[11] followed by cleavage (I_2 in aqueous THF)[12] to give 17 (66% from 15, mp 116-121°C, $[\alpha]_D^{15}$ +3.1° (c 1.11, $CHCl_3$- MeOH 3:1)). The glycosidic position was then phosphorylated with the dibenzyl phosphorochloridate - butyllithium procedure in THF.[13] Glycosyl phosphates with α-configurations of N-acyl

glucosamine derivatives could be selectively obtained by this procedure. Although the primary 6'-hydroxyl group had to be protected, no protection of other secondary hydroxyl groups was necessary. In order to avoid a decomposition of the instable dibenzyl ester of the glycosyl phosphate, the reaction mixture was directly subjected to hydrogenolysis (H_2 - Pd-black, at 6 kg/cm^2). The 1-monophosphate (3) (33% from 17, $[\alpha]_D^{19}$ -2.4° (c 1.02, $CHCl_3$- MeOH 3:1)) was isolated with a silica gel column ($CHCl_3$- MeOH - H_2O - Et_3N 50:20:3:0.2) and converted into its free acid form by a combination of electrodialysis[14] and acidic precipitation (0.1N HCl at 0°C).[15,16]



Ac : CH_3CO , Bzl : $C_6H_5CH_2$, Bz : C_6H_5CO , Allyl : CH_2=$CHCH_2$, R'CO : $CH_3(CH_2)_{10}CHCH_2CO$ (OBzl)

For phosphorylation of the secondary 4'-hydroxyl group, the procedure of Szabó et al. was employed (diphenyl phosphorochloridate - pyridine - dimethylaminopyridine in CH_2Cl_2 at room temperature for 7 hr).[17] The protected 4'-monophosphate (18) (76%, mp 71-72°C) thus obtained was converted into the free 4'-monophosphate (2) via the following series of deprotection reactions. Removal of the allyl group as above [i) Ir complex, ii) I_2] afforded 19. It was hydrogenolyzed [i) H_2 - Pd-black, ii) H_2 - PtO_2] to give 2, which was purified by acidic precipitation (32% from 18, $[\alpha]_D^{15}$ -13.6° (c 0.84, $CHCl_3$- MeOH 3:1)).[15,16]

The 1,4'-diphosphate (1) was prepared from 19 as follows. The hydroxyl group at the glycosidic position of 19 was phosphorylated with butyllithium and phosphorochloridate as described above and the product was immediately hydrogenolyzed [i) Pd-black, ii) PtO_2]. The final product 1 was isolated as free acid by the procedure described for 3 (20% from 19, $[\alpha]_D^{19}$ +2.1° (c 1.05, $CHCl_3$- MeOH 3:1)).[16] The tetraacyl disaccharide without phosphate (4) (mp 210-212°C dec), which may be used as a reference compound in biological tests, was also prepared by conventional deprotection from 15.

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