New Efficient Route for Synthesis of Lipid A by using Affinity Separation

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Abstract: New efficient synthesis of lipid A, an immunostimulating glycoconjugate of bacteria, was achieved for the construction of lipid A library by using synthesis based on affinity separation (SAS), where the compounds possessing a barbituric acid (BA)-tag are selectively and rapidly purified by interaction with an artificial receptor for BA. Glycosylation of a glycosyl acceptor possessing the BA-tag with a 4'-phosphorylated *N*-Troc glucosaminyl trichloroacetimidate gave the disaccharide 4'-phosphate, which was purified by the affinity separation. Successive removal of protective groups and introduction of acyl groups were then effected and the synthetic intermediate at each step was purified by the affinity separation. Cleavage of the tag and subsequent deprotection afforded *Escherichia coli* lipid A. SAS enabled the rapid preparation of lipid A, therefore, proved to be a promising method for synthesis of other complex glycoconjugates.

Key words: lipid A, high throughput synthesis, affinity separation, barbituric acid, library

Lipopolysaccharide (LPS), also termed endotoxin, is a cell surface amphiphile characteristic of Gram-negative bacteria. LPS induces various cytokines to exhibit potent toxic activity such as pyrogenicity or lethality as well as beneficial activities such as antitumor activity.[1] LPS is composed of a hydrophilic polysaccharide portion and a hydrophobic lipid part. The lipid part was named lipid A; its chemical structure and the full endotoxic responsibility were established by our chemical synthesis of *Escherichia coli*-type lipid A **1** (Figure 1).[2,3] Lipid A of various bacterial origin were shown to be structurally closely related and to consist of: 1) β (1→6) disaccharide of two D-glucosamines, 2) phosphono groups at the reducing end and the 4-position of the non-reducing sugar and 3) long-chain acyl groups bound at 2, 2', 3, and 3' positions. Many structural variations of lipid A, however, have been reported mainly with regard to the acyl moieties: their types, numbers, and locations on the hydrophilic sugar backbone. It has been shown that both phosphono groups at the 1- and 4'-positions and acyl groups are crucial for the biological activities of lipid A.[4–7]

Structurally diverse lipid A analogues having various types of acyl groups have been required for further investigation of the biological importance of acyl groups. We therefore established a divergent synthetic route in the previous study: a disaccharide 4'-phosphate was first constructed as a common synthetic intermediate and all acyl moieties were then introduced step by step to the respective positions.[8] Novel lipid A analogues which possess four (*R*)-3-hydroxyacyl moieties of shorter chain length were synthesized via the synthetic route and the biological tests clearly showed the critical importance of the chain length of the acyl moieties in lipid A for the activity. In the previous route, both amino groups were protected with trichloroethoxycarbonyl (Troc) group and hence the same acyl group was introduced to both amino groups. In the present paper, we wish to report a new efficient synthesis of lipid A by using a solution and solid-phase hybrid synthesis via a novel divergent synthetic route, where different acyl groups can be introduced to 2, 2', 3, and 3' positions.

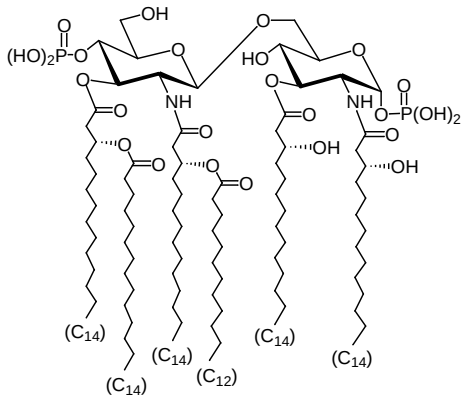

Figure 1 Lipid A of *Escherichia coli* (**1**)

The solid-phase strategy has greatly contributed to recent development in combinatorial chemistry.[9] Solid-phase synthesis of complex glycoconjugates is, however, still immature though various solid-phase oligosaccharide synthesis have been reported.[10] Recently we reported a hybrid methodology of solid-phase synthesis and traditional solution synthesis: the reactions were carried out in solutions and the desired products were isolated rapidly by the host-guest interaction between the solid-support and the desired products which possess the host and guest structures, respectively.[11] This methodology was termed synthesis based on affinity separation (SAS). We have used two interactions for SAS. One is interaction of a crown ether and polymer-supported ammonium ion[11a,b] and the other is the specific molecular recognition between a barbituric acid derivative and its artificial receptor, which form tight complex with 6 hydrogen bonds[11c] (Figure 2). Several peptides, heterocycles, and oligosaccharides were synthesized by SAS. In the present study,

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we applied the latter method to the synthesis of a complex glycoconjugate lipid A.

The new high throughput synthetic route employed in this work is outlined in Scheme 1. The barbituric acid (BA) tag was attached to the 4-position of the glycosyl acceptor via an acylamino benzyl linker, which can be cleaved by DDQ oxidation.[12] A β (1→6) disaccharide 4'-phosphate **4** was constructed as a common key synthetic intermediate by the coupling of two monosaccharides, i.e., a glycosyl trichloroacetimidate **2** as a donor and a glycosyl acceptor **3** having the BA-tag. All acyl moieties were then introduced step by step to the respective positions. After simultaneous cleavage of the linker and the BA-tag, the glycosyl phosphate was formed by the cleavage of the allyl glycoside and subsequent phosphorylation of the 1-hydroxy group. Removal of all the benzyl-type protecting groups by catalytic hydrogenolysis afforded the desired lipid A **1**.

We first improved the synthetic procedure for the glycosyl donor **2** whose 2-amino and 3-hydroxy groups were differentially protected with Troc and allyloxycarbonyl (Alloc) groups, respectively. In our previous study, where the allyl group was used for temporary protection of the 1-position of the donor moiety,[8] we encountered difficulty in selective cleavage of the 1-*O*-allyl group in the presence of the 3-*O*-Alloc group. In the attempt to isomerize the 1-*O*-allyl group to the 1-*O*-1-propenyl group using an iridium complex ([Ir(cod)($MePh_2P$)$_2$]-PF_6), the rate of the isomerization in the presence of the Alloc group was much lower than the corresponding reaction of a substrate without the Alloc group. The reaction had to be stopped before completion in order to suppress the decomposition of the product. In the present study, the Alloc group was introduced to the 3-position (Scheme 2) after isomerization of the allyl group of **8**. The product **9** was subjected to the reductive benzylidene-ring opening as previously

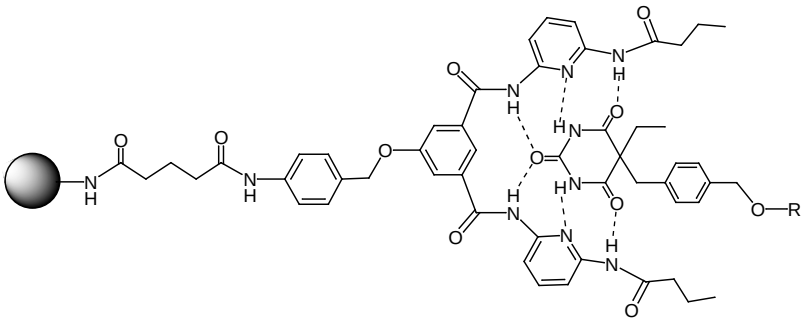
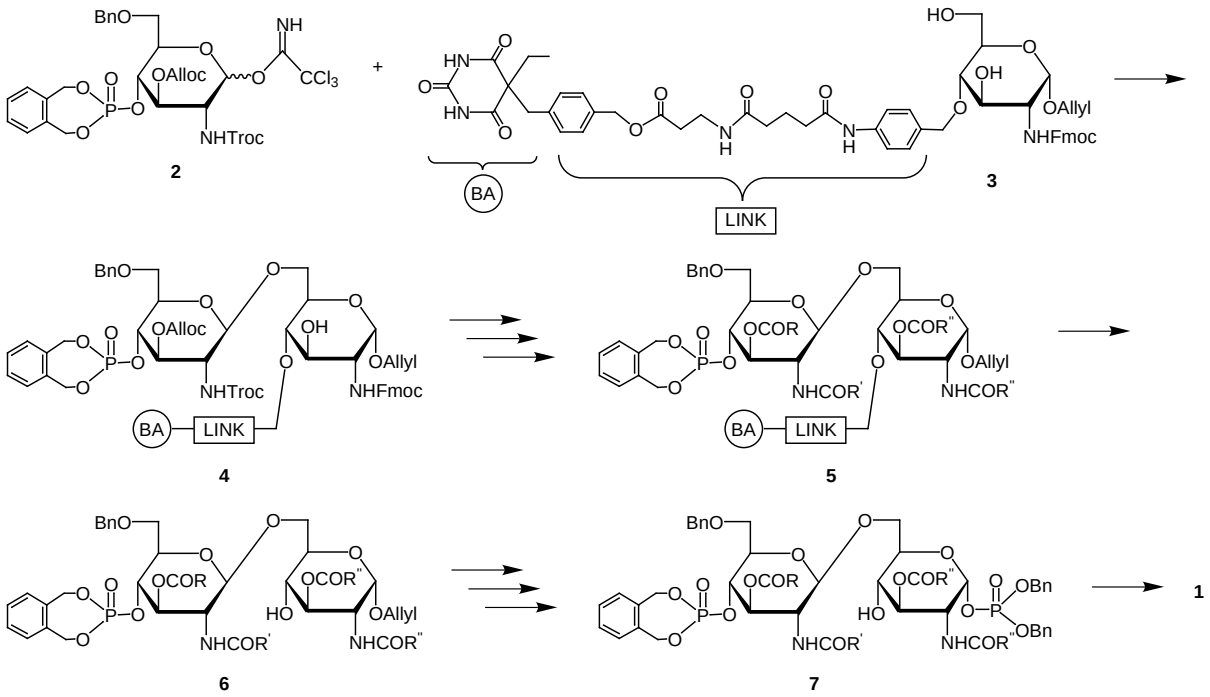
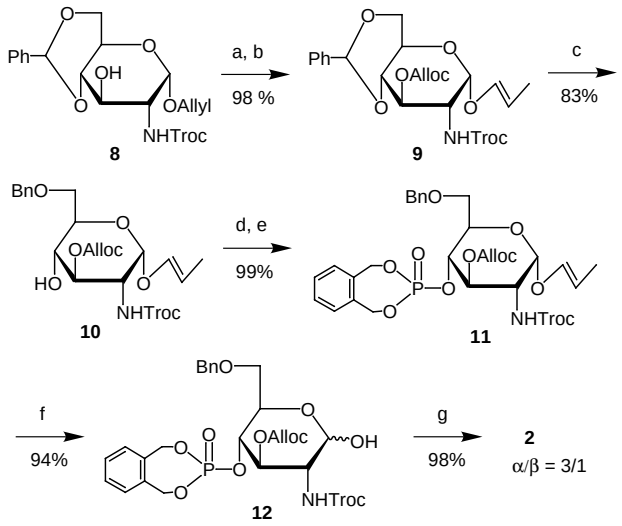


Figure 2 Host-guest interaction of a polymer-supported receptor with the barbituric acid tag



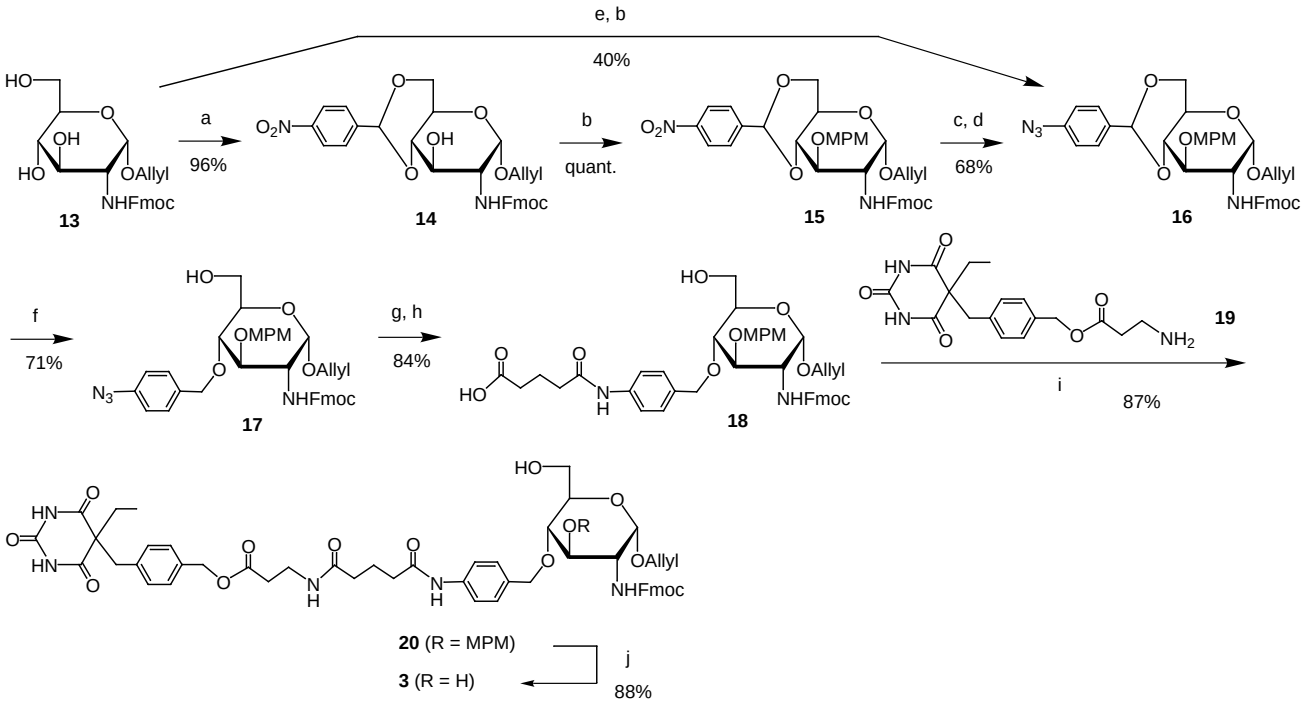
Scheme 1 Strategy for the high throughput synthesis of lipid A by using the affinity separation

reported to give the 6-O-benzylated **10** in a good yield with high regioselectivity.[13] Phosphitylation on the free 4-hydroxy group followed by oxidation[14] gave the phosphate **11** in 99% yield. The 1-propenyl group was smoothly deprotected with iodine and water to give the 1-hydroxy product **12** in 94% yield. Compound **12** was then converted as usual to the trichloroacetimidate **2** in a good yield.



Scheme 2 Synthesis of the glycosyl donor. a) $[Ir(cod)(MePh_2P)_2]PF_6$, H_2, THF; b) AllocCl, pyridine, CH_2Cl_2; c) $Me_2NH{\cdot}BH_3$, $BF_3{\cdot}Et_2O$, CH_3CN; d) *N*,*N*-Diethyl-1,5-dihydro-3*H*-2,4,3-benzodioxaphosphepin-3-amine, 1*H*-tetrazole, CH_2Cl_2; e) *m*CPBA, -20 °C; f) I_2, water, THF; g) CCl_3CN, Cs_2CO_3, CH_2Cl_2

The acceptor **20** tagged with BA moiety was prepared as shown in Scheme 3 via the 4-*O*-*p*-azidobenzyl ether **17**, which was prepared from a 4,6-*O*-*p*-nitrobenzylidene derivative **14**. We first attempted to introduce the *p*-nitrobenzyl group to the 6-position of *N*-Fmoc glucosamine allyl glycoside **13** (Fmoc = 9-fluorenylmethoxycarbonyl) by a new one-pot reductive benzylation using benzaldehydes, TMS_2O, TMSOTf, and Et_3SiH.[8] Unexpectedly, the *p*-nitrobenzylidenated product **14** was obtained in a good yield by the reaction using *p*-nitrobenzaldehyde. Compound **14** was not formed in the absence of Et_3SiH. The 3-hydroxy group in **14** was *p*-methoxybenzylated by using *p*-methoxybenzyl (MPM) trichloroacetimidate to give **15**. Since nitrobenzylidene moiety is electron-deficient, reductive opening of the nitrobenzylidene ring did not proceed under attempted several reaction conditions. We then transferred the *p*-nitrobenzylidene **15** to *p*-azidobenzylidene **16** via reduction of the nitro group to an amino group with Zn–Cu and acetylacetone followed by nucleophilic aromatic substitution of diazonium salts by an azido group. The azidobenzylidene derivative **16** was also prepared by direct *p*-azidobenzylidenation of **13** by the use of *p*-azidobenzaldehyde, TMS_2O, TMSOTf, and Et_3SiH followed by introduction of the MPM group. The *p*-azidobenzylidene **16** was then subjected to reductive opening with $BH_3{\cdot}Me_2NH$ and $BF_3{\cdot}Et_2O$ in CH_2Cl_2. The reaction proceeded smoothly to form the desired 4-*O*-*p*-azidobenzylated glucosamine derivative **17**, regioselectively. The azido group of **17** was reduced by Zn and HOAc while the resulting amino group was coupled with glutaric anhy-



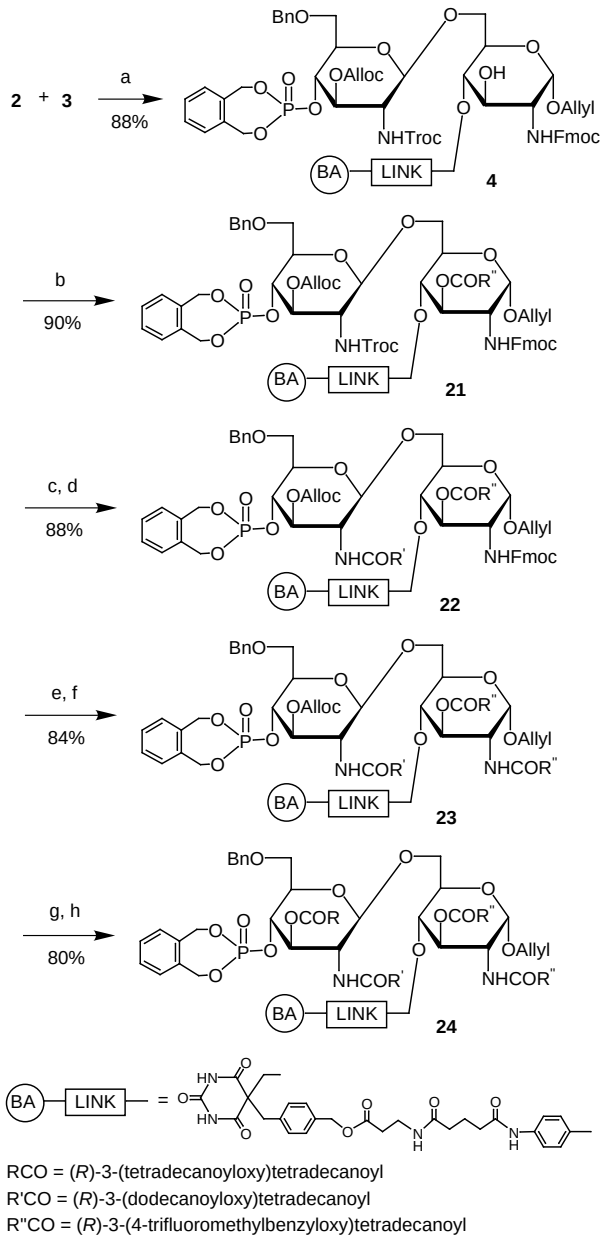
Scheme 3 Synthesis of the glycosyl acceptor having the BA-tag. a) 4-Nitrobenzaldehyde, TMS_2O, TMSOTf, Et_3SiH, THF; b) 4-Methoxyphenylmethyl trichloroacetimidate, $Sn(OTf)_2$, THF; c) Zn–Cu, acetylacetone, THF; d) $NaNO_2$, HCl, NaN_3, water, THF; e) 4-Azidobenzaldehyde, TMS_2O, TMSOTf, Et_3SiH, THF; f) $Me_2NH{\cdot}BH_3$, $BF_3{\cdot}Et_2O$, CH_2Cl_2; g) Zn, HOAc; h) glutaric anhydride, CH_2Cl_2; i) DIC, CH_2Cl_2, affinity separation; j) $BF_3{\cdot}Et_2O$, CH_2Cl_2, affinity separation.

dride. The product **18** was then coupled with BA-tag **19** by using diisopropylcarbodiimide (DIC). The desired BA-tagged product **20** was purified by the affinity separation as follows. The reaction mixture in CH_2Cl_2 was directly applied to a resin column filled with CH_2Cl_2. The tagged **20** was retained in the column, whereas all untagged compounds such as excess **18**, DIC, and *N*,*N*'-diisopropylurea were washed out from the column simply by elution with CH_2Cl_2. Elution of **20** with CH_2Cl_2–methanol (1:1) and concentration of the eluate gave purified **20**, whose MPM group was then removed with $BF_3{\cdot}Et_2O$ in CH_2Cl_2 to give the glycosyl acceptor **3**.[15]

Glycosylation reaction of the acceptor **3** with the imidate **2** was carried out in CH_2Cl_2 at –20 °C by the use of TMSOTf as a catalyst to give the desired β-disaccharide in 88% yield after affinity separation (Scheme 4). Acyl groups were then introduced to the disaccharide step by step. Acylation of the 3-position of **4** with (*R*)-3-(4-trifluoromethylbenzyloxy)tetradecanoic acid[16,17] proceeded smoothly by using DIC and DMAP to give **21**. Cleavage of 2'-*N*-Troc group with Zn–Cu couple was followed by *N*-acylation with (*R*)-3-(dodecanoyloxy)tetradecanoic acid[3b,16] and DIC. The third acyl group was introduced to the 2-amino group after cleavage of the Fmoc group followed by *N*-acylation using DIC. The 3'-*O*-Alloc group was then removed with $Pd(PPh_3)_4$, HCO_2H, and butylamine. Final acylation to the 3'-position of **23** with (*R*)-3-(tetradecanoyloxy)tetradecanoic acid[3b,16] was carried out by using DIC and DMAP to give fully acylated disaccharide **24**. After each reaction step, the product was rapidly separated from the reaction mixture by the affinity separation. Though with less affinity than the BA-tag, DMAP was unexpectedly also retained to the column of BA-receptor so that the fractions of BA-tagged products **21** and **24** were inevitably contaminated with DMAP even after affinity separation. Successive silica-gel short column chromatography was, therefore, used for the complete removal of DMAP.

The *p*-acylaminobenzyl linker was not smoothly cleaved by conventional catalytic hydrogenolysis. The linker moiety should hence be removed before 1-*O*-phosphorylation, since the glycosyl phosphate moiety is chemically unstable and might be damaged during the cleavage of the linker. We previously reported that *p*-acylaminobenzyl ethers were selectively cleaved by DDQ oxidation in a rate comparable to the cleavage of a MPM group.[12] Treatment of **4** with DDQ (1.2 equivalents) was thus examined as a model reaction in CH_2Cl_2–H_2O (10:1) at room temperature for 33 hours. The desired 4-*O*-free product **25** was obtained only in 19% yield, whereas undesired 6'-*O*-debenzylated compound **26** was formed in 26% yield by over-oxidation of **25** (Table 1, entry 1). By contrast, treatment of **4** with 10% TFA in CH_2Cl_2 afforded the desired compound **25** in 59% yield after purification by preparative silica-gel TLC (entry 2).

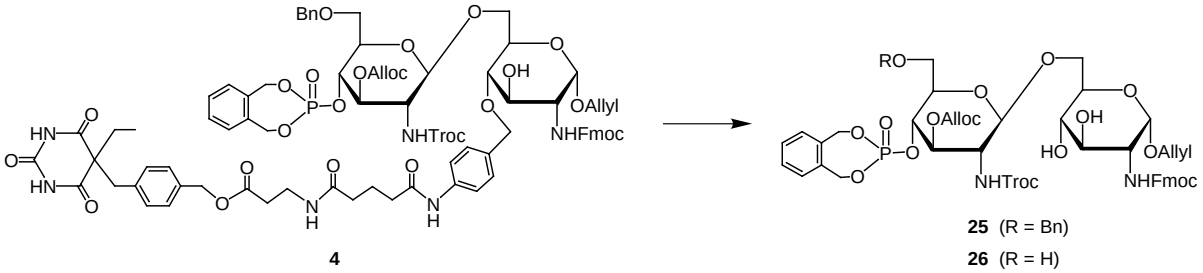
Cleavage of the BA tag moiety in **24** was thus carried out in 20% TFA in $CHCl_3$ to give the desired **6** in 15% yield (Scheme 5). The low yield of this step can be explained as



Scheme 4 Glycosylation and acylation to form the basic structure of lipid A. a) TMSOTf, MS4A, CH_2Cl_2; b) (*R*)-3-(4-trifluoromethylbenzyloxy)tetradecanoic acid, DIC, DMAP, CH_2Cl_2, affinity separation then silica-gel chromatography; c) Zn–Cu, HOAc; d) (*R*)-3-(dodecanoyloxy)tetradecanoic acid, DIC, CH_2Cl_2, affinity separation; e) DBU, CH_2Cl_2; f) (*R*)-3-(4-trifluoromethylbenzyloxy)tetradecanoic acid, DIC, CH_2Cl_2, affinity separation; g) $Pd(PPh_3)_4$, HCO_2H, *n*-$BuNH_2$, THF; h) (*R*)-3-(tetradecanoyloxy)tetradecanoic acid, DIC, DMAP, affinity separation then silica-gel chromatography.

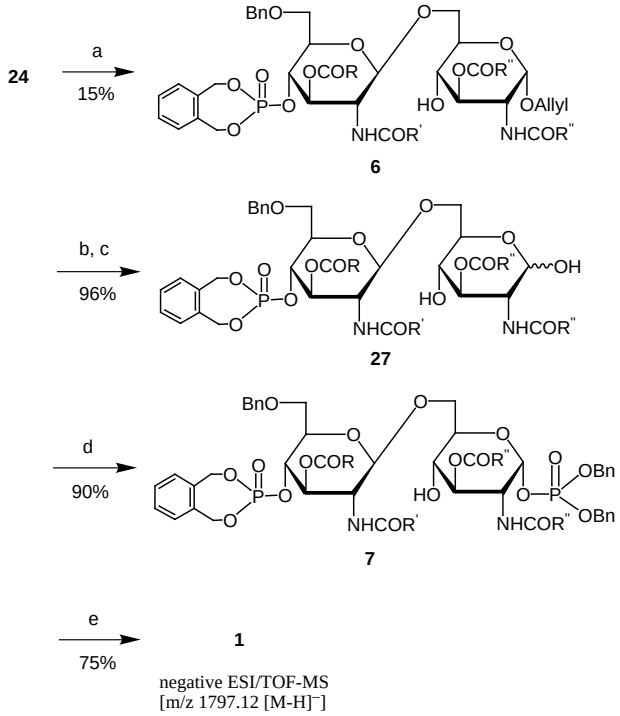
follows. Byproducts possessing the BA moiety cannot be removed by the affinity separation. All byproducts accumulated during multistep of SAS were removed after cleavage of the tag. In particular, a considerable amount of the undesired *N*-2,2-dichloroethoxycarbonylated compound was formed at the cleavage step of *N*-Troc group. Compound **6** was purified thoroughly by silica-gel column chromatography and by gel-permeation chromatography using recycling HPLC apparatus. The 1-*O*-allyl

Table 1 Cleavage of the *p*-Acylaminobenzyl Linker



Entry	Reagents	Time	Yield (%)	
			25	**26**
1	DDQ (1.2 equiv) in CH_2Cl_2–MeOH (10:1)	33 h	19	26
2	10% TFA in CH_2Cl_2	33 h	59	N.D.

group of **6** was then removed as usual to yield 1-hydroxy disaccharide **27**. Finally, the 1-*O*-α-phosphorylation was carried out via 1-O-lithiation and subsequent treatment with tetrabenzyl diphosphate in THF at –78 °C.[8,18] The protected 1,4'-bisphosphate **7** thus obtained was purified by silica-gel column chromatography. Subsequently, all the protecting groups of **7** were removed by hydrogenolysis (10 $kgcm^{-2}$ of H_2) with Pd–black in one-step. The product obtained in 75% yield was identical with *E. coli* lipid A **1** that we synthesized previously.[3,7c]



Scheme 5 Removal of the BA-tag and completion of the synthesis. a) 20% TFA in $CHCl_3$; b) $[Ir(cod)(MePh_2P)_2]PF_6$, H_2, THF; c) I_2, water, THF; d) tetrabenzyl diphosphate, $LiN(TMS)_2$, THF; e) H_2 (10 $kgcm^{-2}$), Pd–black, THF.

In summary, we established a new efficient synthetic route to lipid A by using the affinity separation protocol. Lipid A analogues can be synthesized within a few weeks by the SAS method. In our previous works, several months have been required to obtain the same lipid A by the conventional solution phase synthetic route. The present study enabled us rapid and efficient construction of lipid A libraries possessing various kinds of acyl moieties for elucidation of their structure-activity relationships.

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