Synthesis of the lipoteichoic acid of the *Streptococcus* species DSM 8747

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ABSTRACT

The lipoteichoic acid (LTA) of the *Streptococcus* species DSM 8747 consists of a β -D-galactofuranosyl diacylglycerol moiety (with different acyl groups) that is linked via 6-O to a poly(glycerophosphate) backbone; about 30% of the glycerophosphate moieties carry at 2-O hydrolytically labile D-alanyl residues. As typical LTA for this array of compounds LTA **1a** was synthesized. To this end, from D-galactose the required galactofuranosyl building block **5** was obtained. The anomeric stereocontrol in the glycosylation step with 1,2-O-cyclohexylidene-*sn*-glycerol (**4**) was based on anchimeric assistance, thus finally leading to the unprotected core glycolipid **16**. Regioselective protection and deprotection procedures permitted the defined attachment of the pentameric glycerophosphate **3** to the 6-hydroxy group of the galactose residue. Introduction of four D-alanyl residues led after global deprotection and purification to target molecule **1a** possessing on average about two D-alanyl residues at 2-O of the pentameric glycerophosphate backbone, thus being in close accordance with the structure of the natural material.

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1. Introduction

A unique lipoteichoic acid (LTA) was isolated from the cell wall of the *Streptococcus* species DSM 8747 and structurally assigned by Roethlisberger et al.^{1,2} (Fig. 1, **1**). The LTA comprises a linear (1–3)-linked poly(glycerophosphate) chain which is partly substituted with 2-O-D-alanyl residues; this part is phosphodiester linked to the 6-hydroxy group of 3-O-(β -D-galactofuranosyl)-1,2-di-O-acyl-*sn*-glycerol; thus, **1** belongs to the type I LTA structures.³ Noteworthy for the structure of **1** is the average length of only about ten glycerophosphate residues with about three D-alanyl residues attached and the core of the lipid anchor is proposed to possess the rare *mono*-hexosyl-1,2-diacyl-*sn*-glycerol structure. This Gram-positive bacterium is genetically closely related to *Streptococcus pneumoniae* that encompasses in its cell wall a structurally totally different LTA, belonging to type IV LTA.^{3b} A total synthesis of this LTA has been recently accomplished,⁴ thus the previous structural assignment was confirmed, yet the biological studies with this synthetic material led to unexpected results.^{4,5} Hence, the synthesis of the *Streptococcus* species DSM 8747 was of great interest as well.

As indicated in Figure 1, LTA **1** varies in the acyl residues in the chain length of the glycerophosphate backbone, as well as in the number and location of the attached D-alanyl residues that are hydrolytically labile and cleaved rapidly even at pH 8.5.⁶ Our

previous studies of *Staphylococcus aureus* revealed that a backbone length of about five to six residues is sufficient for biological activity.⁷ Hence, we decided to synthesize LTA **1a** having five glycerophosphate moieties with up to four D-alanyl residues attached before final deprotection. Thus, the hydrolytic lability of the D-alanyl residues and their importance for biological activity has been taken into account and after workup of the target compound at least the average number of D-alanyl residues should be available (**1a** ≈ 0.3 $n = 1.5$ D-Ala residues). For the acyl chain, myristoyl (C₁₄) residues were chosen.

2. Results and discussion

The synthesis design of LTA **1a** is outlined in the retro-synthetic scheme (Scheme 1). For a convergent synthesis of the molecule, a disconnection between the glycolipid core and the oligo(glycerophosphate) moiety is proposed leading to intermediates **2** and **3**; their further disintegration furnishes building blocks **4**–**7**. Thus, for the β -linkage in intermediate **2** neighbouring group participation for the galactofuranosyl donor **5** is employed. For convenience, the totally benzoylated donor is used in the reaction with known 1,2-cyclohexylidene-*sn*-glycerol (**4**)⁸ as acceptor, thus requiring in the following steps for the galactosyl residue an exchange of protecting groups, that is, temporary protection at 6-O and benzyl protection at 2-, 3-, and 5-O. Known intermediate **3**^{7,9} with temporary 2-O-MPM (4-methoxyphenylmethyl) protection permits selective cleavage in the presence of O-benzyl protecting groups, thus allowing introduction of the D-alanyl residues as the second

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Chemical structures of various phosphonate and phosphonate-protected nucleosides:

- 1a:** R = H, D-Ala (minimum 1.5 D-Ala). A linear chain of six phosphonate groups linked by ester bonds, with a terminal D-alanine residue.
- 2:** A nucleoside derivative with a ribose sugar, a phosphate group, and a long alkyl chain (C₁₃H₂₇).
- 3:** A linear chain of six phosphonate groups linked by ester bonds, with a terminal D-alanine residue.
- 4:** A nucleoside derivative with a ribose sugar, a phosphate group, and a long alkyl chain (C₁₃H₂₇).
- 5:** A nucleoside derivative with a ribose sugar, a phosphate group, and a long alkyl chain (C₁₃H₂₇).
- 6:** A nucleoside derivative with a ribose sugar, a phosphate group, and a long alkyl chain (C₁₃H₂₇).
- 7:** A nucleoside derivative with a ribose sugar, a phosphate group, and a long alkyl chain (C₁₃H₂₇).

last step and thereafter global hydrogenolytic O-debenzylation under mild conditions. This way, as previously found by us in the synthesis of *S. aureus* LTA,⁷ another type I LTA, no or little loss of the hydrolytically labile D-alanyl residues is expected. Therefore, the previously prepared building blocks **6**^{7,9,10} and **7**^{7,8,11} will be employed for the synthesis of **3**.

group in the presence of ethylene glycol as nucleophile and *p*-toluenesulfonic acid (*p*-TsOH) as catalyst afforded glycerol derivative **14**. Treatment of this compound with myristic acid in the presence of dicyclohexylcarbodiimide (DCC) as condensing agent and 4-dimethylaminopyridine (DMAP) as catalyst provided diacylglycerol derivative **15**. Hydrogenolytic O-debenzylation of **15** with Pd/C as catalyst in the presence of trifluoroacetic acid (TFA) gave the glycolipid **16** that was of interest for comparisons in the biological studies.

Reaction of previously synthesized **3**^{7,9} with **2** in the presence of tetrazole as catalyst afforded the phosphite triester intermediate that was oxidized with *tert*-butyl hydroperoxide to the corresponding phosphate **20** and obtained as mixture of diastereomers.

Scheme 2. Synthesis of the core glycolipid **16**. Reagents and conditions: (a) BzCl, Py (qu); (b) Ac₂O, H₂SO₄, 0 °C (95%); (c) HBr, Ac₂O, DCM, H₂O (90%); (d) CCl₃CN, DBU, DCM (qu); (e) TMSOTf (0.1 equiv), DCM (89%); (f) NaOMe, MeOH; then BnBr, NaH, DMF (96%); (g) *p*-TsOH, DCM-CH₃CN, ethylene glycol (76%); (h) DCM, DMAP, DCC (qu); (i) Pd/C, H₂, TFA (cat.), EtOAc-MeOH (90%).

Oxidative cleavage of the MPM groups with ceric ammonium nitrate (CAN) in an acetonitrile/toluene/water mixture liberated four hydroxy groups of the glycerophosphate backbone furnishing compound **21**. For the attachment of the *D*-alanyl residues, **21** was reacted with the triethylammonium salt of *N*-benzyloxycarbonyl (Cbz) protected *D*-alanine and benzotriazol-1-yl-oxytripyrrolidino-phosphonium hexafluorophosphate (PyBOP) as coupling reagent in the presence of *N*-methylimidazole, thus affording compound **22** as fully protected derivative of the target molecule. Hydrogenolytic cleavage of the six *O*-benzyl and four *Z* groups was performed with Pearlman's catalyst in a mixture of dichloromethane/methanol/water, thus providing after hydrophobic interaction chromatography (HIC) with an ammonium acetate/*n*-propanol gradient (from 85:15 to 40:60) pure target compound **1a** with about two *D*-alanyl residues attached. This material will be used for biological studies.

The charge deconvoluted ESI FT-ICR mass spectrum obtained in the negative ion mode revealed molecular mass peaks being in agreement for **1a** with zero to four alanyl residues with the highest intensity for two alanyl residues. Hence, as expected, alanyl residues have been lost in the deprotection and/or purification procedure. The MS data confirm that on average at least two alanyl residues are attached.

All attempts to record well resolved ¹H NMR spectra in deuterated THF, DMSO, water, methanol and mixtures of these solvents remained unsuccessful due to micelle and aggregate formation of the glycolipid part. The solvent for HIC (propanol/water, 1:1) was found to be the best for the NMR experiments at concentrations that were sufficient for heteronuclear correlated ¹H, ¹³C, ³¹P NMR spectroscopy. This way, a peak assignment by homo- and heteronuclear correlated NMR spectroscopy was possible (Table 1). The NMR data are in good accordance with those reported for the natural material,^{1,2} thus confirming the previous structure assignment. In the ¹H MNR spectra of **1a** three different signals for 2-H of the glycerol (Gro) residue were obtained. One signal at δ 5.38 (C-2^{Gro} = 74.9) is assigned to *O*-acylated 2-H of the diacylglycerol (DAG) residue. The signal at δ 5.30 (C-2^{Gro} = 70.1) originates from the glycerophosphate repeating units having 2-*O*-alanyl residues and the signal at δ 4.33 (C-2^{Gro} = 75.0) is due to *O*-unsubstituted glycerophosphate residues. In agreement with this also for 2-H of the alanyl residues two different signals were observed: One signal at δ 4.26 (C-2^{Ala} = 49.5) is due to ester bound 2-*O*-alanyl residues and the other signal at δ 3.73 (C-2^{Ala} = 51.2) originates from non-bound alanine, that is known to be co-eluting with LTA from the HIC column.⁶ From the signals at δ 5.38 and 5.30 an intensity ratio of about 1:2 was observed, indicating that **1a** contains about two *O*-alanyl residues. This is in agreement with the ESI-MS data. Hence, about two *O*-alanyl residues were lost in the deprotection and/or purification procedure of **1a** that seems to be more sensitive to *O*-de-alanylation than the LTA of *S. aureus*.^{7,9}

3. Summary

In conclusion, following the retro-synthetic design the LTA **1a** of the *Streptococcus* species DSM 8747 was successfully synthesized. The expected partial hydrolytic cleavage of *D*-alanyl residues during final deprotection and/or purification could be compensated by introducing beforehand a higher degree of alanylation. Thus, the ratio of the constituents of **1a** with on average two *D*-alanyl residues is in good agreement with the natural compound.

4. Experimental details

4.1. General

Solvents were dried according to standard procedures. NMR spectroscopic measurements were performed at 22 °C with Bruker DRX 600, Bruker Avance 600 cryo, Bruker 400 Avance, Varian Mercury 300 and Bruker AC250 spectrometers. Tetramethylsilane (TMS) or the resonances of the deuterated solvents were used as internal standards: CDCl₃, (δ = 7.24 ppm) was used as an external standard, 85% of phosphoric acid was used as an external standard for ³¹P spectra. MALDI mass spectra were recorded with a Kratos Kompact MALDI II spectrometer; 2,5-dihydroxybenzoic acid (DHB) or *p*-nitroaniline and NaI were used as matrices for positive mode measurements, and trihydroxyacetophenone (THAP) was used as the matrix for negative mode measurements. HRMS spectra were recorded with a Bruker ESI-MS mass spectrometer. High resolution Electrospray Ionization Fourier Transform Ion Cyclotron Mass Spectrometry (ESI FT-ICR MS) for **1a** was performed in the negative ion mode on a 7 Tesla APEX Qe (Bruker Daltonics). Samples preparation and instrumental settings were the same as described previously.⁴ Optical rotations were measured at 22 °C with a Büchi Polar-Monitor using the sodium D line. Thin-layer chromatography (TLC) was performed on Merck Silica Gel (60 F₂₅₄) plastic plates; compounds were visualized by treatment with a solution of (NH₄)₆Mo₇O₂₄·4H₂O (20 g) and Ce(SO₄)₂ (0.4 g) in sulfuric acid (10%, 400 mL) and then by heating to 120 °C. Flash

Scheme 3. Synthesis of the target molecule **1a**. Reagents and conditions: (a) TBDMSCl, DMAP, Et₃N, DCM (63%); (b) BnBr, NaH, DMF (90%); (c) TBAF, DCM (76%); (d) BnOP(NiPr₂)₂, diisopropylammonium tetrazolide (81%); (e) tetrazole, DCM, then *t*-BuOOH (90%); (f) CAN, CH₃CN, toluene, H₂O (85%); (g) *N*-methylimidazole, Cbz-D-alanine triethylammonium salt, PyBOP, DCM (74%); (h) Pd(OH)₂, H₂, DCM/MeOH/H₂O (7:3:2) (28%).

chromatography was performed on MN Silica Gel 60 (230–400 mesh) at a pressure of 0.2 bar. Target molecules were purified by hydrophobic interaction chromatography on octyl-sepharose as the stationary phase and the elution phase was used as a gradient of propanol (15–60%) in 0.1 mol/L ammonium acetate buffer (pH 4.8).

4.1.1. 2,3,5,6-Tetra-*O*-benzoyl- α,β -D-galactofuranosyl acetate (**10**)

To a solution of **9** (2.1 g, 3 mmol) in CH₂Cl₂ (21 mL) at 0 °C, acetic anhydride (1.13 mL) and concd H₂SO₄ (0.08 mL) were added and the mixture stirred for 30 min. The temperature was then allowed to rise to 25 °C and after 4 h the reaction mixture was quenched by adding excess satd NaHCO₃. The mixture was extracted with ethyl acetate and the organic layer was washed successively with ice water, aqueous NaHCO₃ and water and then dried over MgSO₄ and concentrated in vacuo to give crude **10**. After purification as described¹⁴ the NMR data are identical with those reported in the literature¹⁴ for **10**.

4.1.2. 2,3,5,6-Tetra-*O*-benzoyl- α,β -D-galactofuranose (**11**)

Compound **11** was obtained from **10** following the reported procedure.¹⁵

4.1.3. *O*-(2,3,5,6-Tetra-*O*-benzoyl- α,β -D-galactofuranosyl) trichloroacetimidate (**5**)

Reaction of **11** with CCl₃CN and DBU in CH₂Cl₂ at room temperature afforded known **5**¹⁶ in practically quantitative yield.

4.1.4. 3-*O*-(2,3,5,6-Tetra-*O*-benzoyl- α,β -D-galactofuranosyl)-1,2-*O*-cyclohexylidene-*sn*-glycerol (**12**)

To a solution of **5** (2.6 g, 3.5 mmol) and 1,2-*O*-cyclohexylidene-*sn*-glycerol (**4**) (750 mg, 4.3 mmol; commercial material) in CH₂Cl₂ (60 mL) was added TMSOTf (70 μ L, 0.35 mmol) under argon. The reaction was stirred for 3 h and then quenched with NEt₃ (0.2 mL). The solvent was evaporated in vacuo. Flash silica gel column chromatography (petroleum ether/ethyl acetate = 5:1) gave **12** (2.2 g, 89%) as oil. ¹H NMR (400 MHz, CDCl₃): δ 8.01–7.81 (m, 8H), 7.51–7.19 (m, 12H), 6.0–5.97 (m, 1H), 5.55 (d, 1H, *J* = 5.0 Hz), 5.41 (d, 1H, *J* = 1.0 Hz), 5.29 (s, 1H, anomeric proton), 4.71–4.63 (m, 2H), 4.58 (dd, 1H, *J* = 3.8, 5.0 Hz), 4.25 (m, 1H), 3.97 (dd, 1H, *J* = 6.4, 8.3 Hz), 3.78–3.71 (m, 2H), 3.52 (dd, 1H, *J* = 5.7, 10.4 Hz), 1.54 (m, 8H), 1.33 (m, 2H). ¹³C NMR (101 MHz, CDCl₃): δ 166.12, 165.75, 165.65, 165.40, 133.52, 133.40, 133.26, 133.12, 129.97, 129.85, 129.73, 129.56, 129.48, 129.03, 128.88, 128.46, 128.43, 128.41, 128.38, 110.08, 105.96 (*J*_{C,H} = 176.7 Hz, anomeric carbon), 81.86, 81.63, 77.51, 77.35, 77.23, 77.03, 76.71, 74.02, 70.37, 67.97, 66.27, 63.53, 36.40, 35.01, 25.13, 24.01, 23.84. ESI HRMS Calcd for C₄₃H₄₂O₁₂: [M+Na]⁺ *m/z* 773.2568. Found: [M+Na]⁺ *m/z* 773.2730.

4.1.5. 3-*O*-(2,3,5,6-Tetra-*O*-benzoyl- β -D-galactofuranosyl)-1,2-*O*-cyclohexylidene-*sn*-glycerol (**13**)

Compound **12** (2.2 g, 3 mmol) in MeOH (20 mL) was treated with NaOMe (0.5 mL, 1 M in MeOH), then the solvent was removed after 2 h. The crude debenzoylation compound was dissolved in DMF (20 mL), BnBr (1.8 mL, 15 mmol) and NaH (800 mg, 60% in

Table 1
NMR data of **1a**^{a,b,c}

¹ H NMR data Chemical shift		¹³ C NMR data Chemical shift	
Proton	δ (ppm)	Carbon	δ (ppm)
<i>β</i> -D-Galf			
1-H	4.97	C-1	108.3
2-H	4.04	C-2	81.8
3-H	4.10	C-3	76.8
4-H	3.97	C-4	84.3
5-H	3.89	C-5	70.8
6a-H	3.9 ^c	C-6	67.0
6b-H	3.9 ^c		
<i>Ala</i> (due to heterogeneity, two-different types of <i>Ala</i> and three of <i>Gro</i> were identified) (see MS)			
2-H ^(free)	3.76	C-1	170.5
2'-H ^(bound)	4.26	C-2 ^(free)	51.2
3-H ^(free)	1.59	C-2 ^(bound)	49.5
3'-H ^(bound)	1.47	C-3 ^(free)	16.0
		C-3 ^(bound)	16.8
<i>Gro</i>			
1,1'-H	3.4–3.8 ^c	C-1	64 ^c
2-H ^{O-Acyl}	5.38	C-2 ^{O-Acyl}	74.9
2-H ^{O-Ala}	5.30	C-2 ^{O-Ala}	71.4
2-H ^{OH}	4.33	C-2 ^{OH}	70.1
3-H	3.4–3.8 ^c	C-3	65 ^c
<i>Fatty acids</i> (14:0)			
2-H	2.28	C-1	170.7
3-H	1.59	C-2	34.6
4-H	1.2	C-3	25.3
4-H-13-H	1.02–1.08	C-4–C-13	22.8–24.50 ^c
14-H	0.811	C-14	14.3
³¹ P	+0.82 ppm (br s)		

^a ¹H, ¹³C, ³¹P NMR [600 MHz/150.9 MHz/243 MHz, *n*-propanol-*d*₆/D₂O approx. 1:1 (V/V), 300 K]; ¹³C assignments based on HMQC and HSQC-DEPT.

^b Other signals: *n*-propanol (δ, ppm), δ_{1-H} 4.10, δ_{C-1} 64.04, δ_{2-H} 1.20, δ_{C-2} 30.3, δ_{3-H} 0.78, δ_{C-3} 23.5.

^c Non-resolved signals overlapping with signals of the *n*-propanol solvent.

oil, 20 mmol) were added and the reaction mixture stirred overnight, then poured into ice water (100 mL) and extracted with EtOAc. The obtained extract was washed with water and brine, dried over MgSO₄, and evaporated in vacuo. The residue was purified by silica gel column chromatography (petroleum ether/ethyl acetate = 8:1) to obtain **13** (2.5 g, 96%) as oil. ¹H NMR (400 MHz, CDCl₃): δ 7.38–7.29 (m, 20H), 5.12 (s, 1H, anomeric proton), 4.77–4.47 (m, 7H), 4.34–4.28 (m, 2H), 4.16–4.14 (m, 1H), 4.07–4.03 (m, 3H), 3.87–3.71 (m, 5H), 3.48 (dd, 1H, *J* = 6.4, 10.5 Hz), 1.65–1.58 (m, 8H), 1.43 (m, 2H). ¹³C NMR (101 MHz, CDCl₃) δ 138.41, 138.27, 137.86, 137.63, 128.43, 128.39, 128.35, 128.31, 128.29, 127.97, 127.93, 127.85, 127.76, 127.64, 127.59, 109.85, 106.34 (anomeric carbon), 88.39, 82.74, 80.97, 77.40, 77.09, 76.77, 76.24, 73.99, 73.46, 73.34, 72.10, 71.92, 70.86, 67.87, 66.49, 36.44, 35.01, 25.19, 24.05, 23.87. ESI HRMS Calcd for C₄₃H₅₀O₈: [M+Na]⁺ *m/z* 717.3398. Found: [M+Na]⁺ *m/z* 717.3415.

4.1.6. 3-O-(2,3,5,6-Tetra-O-benzyl-β-D-galactofuranosyl)-sn-glycerol (**14**)

Galactofuranoside **13** (2.4 g, 3.4 mmol) was dissolved in DCM-CH₃CN (60 mL, 1:1) and reacted with ethanediol (2.4 mL) under acidic condition (pH 1, 800 mg of *p*-TsOH); after 5 h at room temperature, the reaction was quenched by adding sat. NaHCO₃ and worked up as usual. Flash silica gel column chromatography (petroleum ether/ethyl acetate = 2:1) gave **14** (1.5 g, 76%) as oil. ¹H NMR (400 MHz, CDCl₃): δ 7.37–7.32 (m, 20H), 5.10 (s, 1H, anomeric proton), 4.75–4.34 (m, 8H), 4.22 (dd, 1H, *J* = 3.8, 6.2 Hz), 4.05 (dd, 1H, *J* = 2.6, 6.2 Hz), 4.01 (m, 1H), 3.85–3.78 (m, 2H), 3.75–3.57 (m, 6H), 2.56 (br s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 138.29,

138.18, 137.58, 137.50, 128.49, 128.44, 128.41, 128.33, 128.30, 128.06, 127.94, 127.93, 127.89, 127.72, 127.66, 106.20 (anomeric carbon), 87.65, 82.40, 81.99, 77.41, 77.29, 77.09, 76.77, 76.44, 73.47, 73.31, 72.17, 71.91, 70.49, 70.43, 70.03, 63.85. ESI HRMS Calcd for C₃₇H₄₂O₈: [M+Na]⁺ *m/z* 637.7138. Found: [M+Na]⁺ *m/z* 637.7444.

4.1.7. 3-O-(2, 3, 5, 6-Tetra-O-benzyl-β-D-galactofuranosyl)-1,2-di-O-myristoyl-sn-glycerol (**15**)

Compound **14** (1.5 g, 2.4 mmol) was dissolved in DCM (30 mL) and DCC (1.5 g, 7 mmol), DMAP (30 mg, 0.24 mmol) and myristic acid (1.6 g, 7.2 mmol) were added; after stirring overnight the reaction was quenched by adding 0.2 mL MeOH and 0.2 mL HOAc, then concentrated under reduced pressure and filtered through Celite (washing with petroleum ether/EtOAc = 8:1). Flash silica gel column chromatography (petroleum ether/ethyl acetate = 10:1) gave **15** (2.4 g, 98%) as oil. ¹H NMR (400 MHz, CDCl₃): δ 7.27–7.18 (m, 20H), 5.16 (m, 1H), 4.99 (s, 1H, anomeric proton), 4.65–4.34 (m, 7H), 4.24–4.28 (m, 2H), 4.10 (dd, 1H, *J* = 6.4, 12.0 Hz), 4.03–4.01 (m, 1H), 3.95–3.89 (m, 2H), 3.70–3.66 (m, 2H), 3.61–3.58 (m, 2H), 3.49 (dd, 1H, *J* = 5.0, 11.0 Hz), 2.23–2.18 (m, 4H), 1.55–1.50 (m, 4H), 1.18 (m, 40H), 0.81 (m, 6H). ¹³C NMR (101 MHz, CDCl₃) δ 173.40, 173.01, 138.39, 138.29, 137.82, 137.58, 128.43, 128.37, 128.34, 128.28, 128.27, 127.95, 127.86, 127.74, 127.63, 127.59, 106.02 (anomeric carbon), 88.20, 82.67, 81.18, 77.38, 77.06, 76.74, 76.25, 73.45, 73.33, 72.06, 71.87, 70.85, 69.72, 65.30, 62.62, 34.34, 34.14, 31.95, 29.72, 29.68, 29.53, 29.39, 29.33, 29.17, 29.14, 24.97, 24.92, 22.72, 14.15. ESI HRMS Calcd for C₆₅H₉₄O₁₀: [M+Na]⁺ *m/z* 1057.6739. Found: [M+Na]⁺ *m/z* 1057.6745.

4.1.8. 3-O-(β-D-Galactofuranosyl)-1,2-di-O-myristoyl-sn-glycerol (**16**)

Pd/C (100 mg) was added to **15** (2 g, 1.9 mmol), TFA (0.1 mL) in EtOAc–MeOH (84 mL, 20:1 = V:V), and the reaction mixture was stirred overnight under hydrogen atmosphere. The reaction was filtrated through Celite and washed with MeOH. After removal of the solvent under reduced pressure, **16** was obtained as solid (1.1 g, 90%). [α]_D²⁰ –19.3 (c 0.9, CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ 5.24–5.22 (m, 1H), 5.0 (s, 1H, anomeric proton), 4.37 (dd, 1H, *J* = 3.7, 12.0 Hz), 4.16–4.05 (m, 4H), 3.94 (m, 1H), 3.82–3.75 (m, 3H), 3.65 (dd, 1H, *J* = 5.3, 11.0 Hz), 3.41 (br s, 4H), 2.35–2.30 (m, 4H), 1.62–1.60 (m, 4H), 1.27 (m, 40H), 0.89 (m, 6H). ¹³C NMR (101 MHz, CDCl₃) δ 173.70, 173.40, 108.57 (anomeric carbon), 86.82, 79.68, 78.39, 77.34, 77.03, 76.71, 70.99, 69.80, 66.07, 64.05, 62.52, 34.30, 34.12, 31.93, 29.71, 29.69, 29.67, 29.52, 29.37, 29.31, 29.16, 29.12, 24.92, 24.88, 22.70, 14.12. ESI HRMS Calcd for C₃₇H₇₀O₁₀: [M+Na]⁺ *m/z* 697.4861. Found: [M+Na]⁺ *m/z* 697.4900.

4.1.9. 3-O-(6-O-tert-Butyldimethylsilyl-β-D-galactofuranosyl)-1,2-di-O-myristoyl-sn-glycerol (**17**)

Triethylamine (0.2 mL, 1.2 mmol), 4-dimethylaminopyridine (95 mg, 0.8 mmol) and *tert*-butyldimethylsilyl chloride (142 mg, 0.9 mmol) were added to a solution of **16** (530 mg, 0.78 mmol) in dry dichloromethane (4 mL). After stirring for 6 h at room temperature, the solution was diluted with EtOAc and washed with sat. aqueous NaHCO₃ and water and then dried over MgSO₄. After concentration in vacuo, the residue was separated by flash silica gel column chromatography (petroleum ether/ethyl acetate = 4:1–1:1) to give **17** (390 mg, 63%) as a waxy material. [α]_D²⁰ –17.3 (c 1.3, CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ 5.09 (m, 1H), 4.90 (s, 1H, anomeric proton), 4.25 (dd, 1H, *J* = 4.3, 11.8 Hz), 4.01–3.99 (m, 2H), 3.94 (m, 1H), 3.89 (s, 1H), 3.82 (m, 1H), 3.72 (dd, 1H, *J* = 5.9, 11.0 Hz), 3.61 (d, 1H, *J* = 2.3 Hz), 3.60 (s, 1H), 3.50 (dd, 1H, *J* = 4.6, 11.0 Hz), 2.22–2.19 (m, 4H), 1.52–1.49 (m, 4H), 1.18–1.15

(m, 40H), 0.81–0.76 (m, 15H), –0.02 (s, 6H). ^{13}C NMR (100 MHz, CDCl_3): δ 173.40, 173.13, 108.75 (anomeric carbon), 86.72, 78.86, 78.64, 77.52, 77.48, 77.36, 77.25, 77.21, 77.05, 76.73, 71.74, 69.77, 65.86, 63.93, 62.25, 34.29, 34.12, 31.96, 29.72, 29.69, 29.67, 29.52, 29.39, 29.32, 29.17, 29.13, 25.86, 24.91, 22.72, 18.27, 14.15, –5.33. HRMS Calcd for $\text{C}_{43}\text{H}_{84}\text{O}_{10}\text{Si}$: $[\text{M}+\text{Na}]^+$ m/z 811.5834. Found: $[\text{M}+\text{Na}]^+$ m/z 811.6856.

4.1.10. 3-O-(6-O-tert-Butyldimethylsilyl-2,3,5-tri-O-benzyl- β -D-glactofuranosyl)-1,2-di-O-myristoyl-sn-glycerol (**18**)

To a solution of **17** (560 mg, 0.7 mmol) and benzyl bromide (0.5 mL, 4 mmol) in DMF (3 mL) was added 60% NaH (120 mg, 3 mmol). The resulting suspension was stirred for 2 h at room temperature, poured into ice water (10 mL), and extracted with EtOAc. The obtained extract was washed with water and brine, dried over MgSO_4 , and evaporated in vacuo. The residue was purified by silica gel column chromatography (petroleum ether/ethyl acetate = 15:1) to give **18** (700 mg, 90%) as a colourless syrup. $[\alpha]_D^{25}$ –11.6 (c 1.3, CHCl_3); ^1H NMR (400 MHz, CDCl_3): δ 7.30–7.21 (m, 15H), 5.20 (m, 1H), 5.02 (s, 1H, anomeric proton), 4.65–4.37 (m, 6H), 4.24–4.20 (m, 2H), 4.16–4.12 (m, 1H), 4.05 (dd, 1H, J = 2.6, 7.2 Hz), 3.98–3.93 (m, 2H), 3.75–3.71 (m, 3H), 3.54–3.50 (m, 2H), 2.24–2.19 (m, 4H), 1.57–1.54 (m, 4H), 1.20 (m, 40H), 0.87–0.74 (m, 15H), –0.02 (s, 6H). ^{13}C NMR (100 MHz, CDCl_3): δ 173.40, 172.98, 138.51, 137.93, 137.68, 128.45, 128.43, 128.37, 128.34, 128.30, 127.98, 127.96, 127.92, 127.88, 127.85, 127.82, 127.80, 127.76, 127.69, 127.66, 127.63, 127.60, 106.17 (anomeric carbon), 88.34, 82.58, 80.50, 77.42, 77.10, 76.78, 73.67, 72.10, 71.89, 69.75, 63.28, 62.68, 34.38, 34.18, 31.99, 29.75, 29.72, 29.56, 29.42, 29.36, 29.21, 29.17, 25.98, 25.01, 24.96, 22.75, 18.31, 14.18, –5.33, –5.39. HRMS Calcd for $\text{C}_{64}\text{H}_{102}\text{O}_{10}\text{Si}$: $[\text{M}+\text{Na}]^+$ m/z 1081.6242. Found: $[\text{M}+\text{Na}]^+$ m/z 1081.5713.

4.1.11. 3-O-(2,3,5-Tri-O-benzyl- β -D-glactofuranosyl)-1,2-di-O-myristoyl-sn-glycerol (**19**)

The solution of **18** (900 mg, 0.85 mmol) in DCM (5 mL) was treated with TBAF (1 M solution in THF, 4 mL). The reaction mixture was stirred overnight at room temperature, then diluted with EtOAc and washed with saturated aqueous NH_4Cl and water, the organic phase was dried over MgSO_4 and the solvent was evaporated in vacuo. Purification by silica gel column chromatography (petroleum ether/ethyl acetate = 10:1) yielded **19** (600 mg, 76%) as a colourless syrup. $[\alpha]_D^{25}$ –15.1 (c 1.0, CHCl_3); ^1H NMR (400 MHz, CDCl_3): δ 7.31–7.22 (m, 15H), 5.20 (m, 1H), 5.02 (s, 1H, anomeric proton), 4.57–4.42 (m, 6H), 4.34–4.27 (m, 2H), 4.16–4.12 (m, 2H), 3.98–3.96 (m, 2H), 3.71–3.56 (m, 5H), 2.28–2.23 (m, 4H), 1.55 (m, 4H), 1.20 (m, 40H), 0.84–0.82 (m, 6H). ^{13}C NMR (101 MHz, CDCl_3): δ 173.55, 173.12, 138.22, 137.46, 137.30, 128.53, 128.45, 128.12, 128.08, 127.93, 127.84, 106.10 (anomeric carbon), 87.92, 81.77, 77.37, 77.25, 77.05, 76.73, 72.12, 69.78, 65.41, 62.66, 61.83, 34.16, 31.96, 29.73, 29.69, 29.54, 29.40, 29.34, 29.16, 24.98, 24.93, 22.73, 14.16. HRMS Calcd for $\text{C}_{58}\text{H}_{88}\text{O}_{10}$: $[\text{M}+\text{Na}]^+$ m/z 967.6270. Found: $[\text{M}+\text{Na}]^+$ m/z 967.6251.

4.1.12. 3-O-[6-O-(Benzyloxy-diisopropylaminophosphinyl)-3,5,6-tri-O-benzyl- β -D-galactofuranosyl]-1,2-di-O-myristoyl-sn-glycerol (**2**)

Bis(diisopropylamino)benzyloxyphosphine (165 mg, 0.5 mmol) and diisopropylammonium tetrazolide (83 mg, 0.5 mmol) were added to the solution of **19** (230 mg, 0.24 mmol) in DCM (5 mL); the reaction was kept at room temperature under Ar for 1 h and then diluted with DCM, washed with saturated aqueous NaHCO_3 and water. The organic phase was dried over MgSO_4 and the solvent was evaporated in vacuo below 30 °C. Purification by flash silica gel column chromatography (petroleum ether/ NEt_3 = 8:1)

yielded **2** as oil (230 mg, 81%). This mixture of diastereomers was immediately used in the reaction with **3** to afford **20**.

4.1.13. Compound 20

To a solution of glycerol moiety **3** (48 mg, 0.03 mmol) and phosphite **2** (50 mg, 0.04 mmol) in DCM (2 mL), tetrazole (0.2 mL, 0.45 M in CH_3CN) was added; the reaction mixture was stirred for 6 h under Ar and then $^t\text{BuOOH}$ (20 μL , 5.4 M in decane) was added. After 2 h, the mixture was diluted with DCM and washed with saturated aqueous NaHCO_3 and water. The organic phase was dried over MgSO_4 and the solvent was evaporated in vacuo at less than 30 °C. Purification by flash silica gel column chromatography (toluene/acetone = 3:1) yielded the phosphate **20** as oil (70 mg, 90%, mixture of diastereomers). ^1H NMR (400 MHz, CDCl_3): δ 7.37–7.21 (m, 70H), 6.82 (m, 8H), 5.27 (m, 1H), 5.04–5.01 (m, 10H, include anomeric proton), 4.71–4.48 (m, 17H), 4.33–3.99 (m, 24H), 3.76–3.56 (m, 21H, include $\text{CH}_3\text{-O-Ph}$), 2.34–2.31 (m, 4H), 1.63 (m, 4H), 1.30 (m, 40H), 0.94–0.92 (m, 6H). ^{13}C NMR (101 MHz, CDCl_3): δ 173.38, 172.98, 159.36, 138.16, 138.03, 137.91, 137.64, 137.45, 135.72, 129.65, 129.54, 128.62, 128.48, 128.42, 128.39, 128.35, 128.21, 127.98, 127.94, 127.79, 127.70, 127.64, 113.83, 106.17 (anomeric carbon), 87.77, 77.40, 77.29, 77.08, 76.77, 75.45, 73.45, 72.24, 72.08, 71.93, 69.65, 69.47, 69.13, 65.89, 62.61, 55.22, 34.34, 34.14, 31.96, 29.73, 29.69, 29.55, 29.40, 29.35, 29.19, 29.16, 24.98, 24.93, 22.73, 14.16. ESI HRMS (neg. ion mode) Calcd for $\text{C}_{154}\text{H}_{197}\text{O}_{39}\text{P}_5$: $[\text{M-Bn}]^-$ m/z 2734.1578. Found: m/z 2734.1578.

4.1.14. Compound 21

At –10 °C, $\text{Ce}(\text{NH}_4)_2(\text{NO}_3)_6$ (124 mg, 0.23 mmol) was added portion wise to a solution of **20** (80 mg, 0.028 mmol) in CH_3CN /toluene/ H_2O (60:3:4, 10 mL), the reaction was stirred for 30 min (TLC-monitoring) and then diluted with EtOAc and washed with saturated NaHCO_3 solution. The organic phase was dried over MgSO_4 and the solvent was evaporated in vacuo at less than 30 °C. Purification by flash silica gel column chromatography (toluene/acetone = 1:1) yielded **21** as oil (60 mg, 85%, mixture of diastereomers). ^1H NMR (400 MHz, CDCl_3): δ 7.37–7.21 (m, 50H), 5.26 (m, 1H), 5.08 (m, 12H), 4.64–4.61 (m, 12H), 4.33–3.99 (m, 30H), 3.78–3.58 (m, 12H), 2.34–2.30 (m, 4H), 1.62 (m, 4H), 1.30 (m, 40H), 0.94–0.92 (m, 6H). ESI HRMS (neg. ion mode) Calcd for $\text{C}_{122}\text{H}_{165}\text{O}_{35}\text{P}_5$: $[\text{M-Bn}]^-$ m/z 2253.9177. Found: m/z 2253.9277.

4.1.15. Compound 22

N -Cbz-D-Alanine triethylammonium salt (82 mg, 0.26 mmol) and PyBOP (131 mg, 0.26 mmol) were added to a solution of **21** (40 mg, 0.013 mmol) in dry DCM (4 mL) under Ar, and then N -methylimidazole (40 μL , 0.52 mmol) was added drop wise. After 5 h, the reaction mixture was diluted with DCM and washed with saturated NH_4Cl solution. The organic phase was dried over MgSO_4 and the solvent was evaporated in vacuo at less than 30 °C. Purification by flash silica gel column chromatography (toluene/acetone = 3:1–1:1) yielded a diastereomeric mixture of **22** as oil (40 mg, 74%, mixture of diastereomers). ^1H NMR (400 MHz, CDCl_3): δ 7.34–7.21 (m, 70H), 5.26 (m, 1H), 5.07–5.03 (m, 20H), 4.63–4.61 (m, 4H), 4.58–3.98 (m, 35H), 3.75–3.56 (m, 10H), 2.32–2.30 (m, 4H), 1.62 (m, 4H), 1.36–1.28 (m, 52H), 0.94–0.90 (m, 6H). ESI HRMS (neg. ion mode) Calcd for $\text{C}_{166}\text{H}_{209}\text{N}_4\text{O}_{47}\text{P}_5$: $[\text{M-Bn}]^-$ m/z 3074.2233. Found: m/z 3074.1780.

4.1.16. Compound 1a

The diastereomers of **22** (40 mg, 0.013 mmol) were dissolved in DCM/MeOH/ H_2O (7:3:2, 24 mL) and then treated with Pearlman's catalyst (5 mg) under a hydrogen atmosphere. After stirring overnight at room temperature, the reaction mixture was filtrated through Celite, washed with DCM/MeOH/ H_2O (7:3:2), and the

filtrate was concentrated under reduced pressure below 30 °C. The crude residue (**1a**) was purified with hydrophobic interaction chromatography (HIC) based on the protocol of W. Fischer¹⁹ with the following modifications. Briefly, the compound was dispersed in 0.5 mL 50 mM NH₄OAc pH 4.7/*n*-propanol 85:15 (v/v) (solvent A for HIC) under ultrasonication (5 min). The procedure was repeated twice and the resulting 1.5 mL suspension was centrifuged (Biofuge Heraeus, 13,000 rpm, 5 min, room temperature). The sediment was dissolved three times in 0.5 mL solvent A and again centrifuged. The resulting clear 2.5 mL supernatant was divided into two aliquots (1.25 mL each) and injected for two hydrophobic interaction chromatography (HIC) runs. The HIC system (Gilson) was equipped with a HiPrep 16/10 octyl agarose column (16 × 100 mm, Amersham Pharmacia Biotech). The HIC operated at a flow of 0.5 mL/min (4–7 bar) solvent A isocratic (0% B) for 75 min, then raised in 250 min linear to 100% solvent B [50 mM NH₄OAc pH 4.7/*n*-propanol 40:60 (v/v)] staying isocratic for additional 75 min at 100% B. The synthetic LTA was monitored by its UV absorbance (254 nm) and fractions (3 mL) were collected. Aliquots of 30 µL were tested for organic phosphate by a photometric test.²⁰ The phosphate positive fractions were combined that gave 6.3 mg (28%) of pure **1a**. For the NMR data see Table 1.—ESI HRMS (neg. ion mode) Calcd for C₆₄H₁₂₄N₄O₃₉P₅ [M–H][–] *m/z* 1727.5607. Found: *m/z* 1727.6639. Besides this compound in the ESI-FT-ICR mass spectrum (see Supplementary data) further species of LTA with different degree in alanylation are present: completely de-alanylated *m/z* 1443.4973 (rel. intensity 10%), (ii), mono-alanylated *m/z* 1514.5413 (13%), di-alanylated *m/z* 1585.5783 (40%), tri-alanylated *m/z* 1656.6066 (30%). These data reflect the degree in de-alanylation since they are also in agreement with the NMR analysis, in which also this kind and degree of heterogeneity in the *D*-alanylation was observed.

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