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COMMUNICATION

Automated solid phase synthesis of teichoic acids†‡

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This communication describes the first automated solid phase synthesis of teichoic acids (TAs) and the preparation by this method of a number of well-defined TA structures, which were probed for their antigenicity. An opsonophagocytic killing assay revealed a clear TA-length–activity relationship and indicated a promising candidate for future vaccine development.

Teichoic acids (TAs) are polyanionic glycopolymers built up from repeating alditol (*e.g.* glycerol, ribitol or mannitol) phosphate units, which are seemingly randomly decorated with D-alanine esters and carbohydrate substituents. They are prominent constituents of the cell wall of Gram-positive bacteria,[1] and are important for cell wall integrity, nutrient uptake, cation homeostasis, neutralizing antibiotics. They mediate various extracellular interactions and since they protrude from the bacterial cell wall they interact with our immune system. In this regard they have been implied as antigenic structures and as immunostimulatory molecules that act on the innate immune system through interaction with Toll-like receptors.[2] Because of the microheterogeneity of TAs, the establishment of structure–activity relationships has been a challenge and the biological activity of TA preparations has been the subject of debate.[3] We therefore set out to develop synthetic strategies to produce well-defined structures.[4,5] The repetitive nature of the TA structures and the fact that the repeating units are connected through phosphodiester linkages make them attractive synthetic targets for an automated solid phase approach. Besides the potential to rapidly access a library of compounds, automated solid phase synthesis is also very attractive for the assembly of longer oligomers, for which solution phase synthesis is too time and labour intensive. We here describe the development of automated solid phase methodology for the synthesis of TA structures, its use in the generation of a small TA-library and the initial assessment of the antigenic properties of the synthetic TA fragments.

Our first focus was the generation of TA structures consisting of repeating glycerol phosphate residues, as present on the cell wall of Gram-positive bacteria like *Staphylococcus aureus* and the commensal bacterium *Enterococcus faecalis*.[1] Whereas the health threat of *S. aureus* is widely recognized, the latter species has been regarded to be relatively harmless. Recently, however it has become clear that *E. faecalis* can present a serious risk, especially to immunocompromized individuals, causing bacterimea, urinary tract infections, peritonitis and endocarditis.[6] The presence of multiple antibiotic resistant determinants in most clinically relevant enterococci isolates urges the development of alternative treatment and prevention strategies.[7] In this context, the determination of specific antigenic epitopes can form the basis for future vaccine development.

Based on the state-of-the-art in nucleic acid synthesis[8] we decided to employ cyanoethyl protected phosphoramidite building blocks[9] of which the alcohol function to be elongated was protected with the dimethoxytrityl (DMT) group to allow mildly acidic, yet fast and efficient cleavage and simultaneous online monitoring of coupling efficiency. The automated synthesis of the TA oligomers was executed on controlled pore glass (CPG), with a base labile succinyl linker using a commercially available ÄKTA™ oligopilot™ synthesizer. To investigate the use of C-2 functionalized glycerol phosphoramidite building blocks we explored the incorporation of α-glucosyl substituted glycerol phosphates in our synthetic scheme, because α-glucopyranose is a common substituent of *S. aureus* TA. With the aim to provide TA fragments that can be functionalized, an aminohexanol spacer was incorporated.

The following building blocks were required: glycerol phosphoramidite **1a** and α-glucosyl glycerolphosphoramidite **2a**, to build up the glycerol phosphate chain, glycerol succinate **1b** and **2b** to functionalize aminopropyl-CPG, and aminohexanol phosphoramidite **3** to terminate the TA chains. We previously reported[5] the synthesis of glycerol **1** and spacer **3** and the synthesis of the glucosyl building block **2** is depicted in Scheme 1.‡

A key step in the construction of building block **2a/b** is the stereoselective introduction of the glycosidic bond, which was

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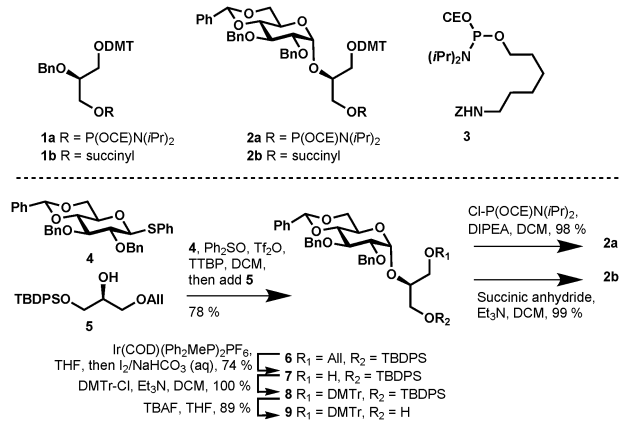
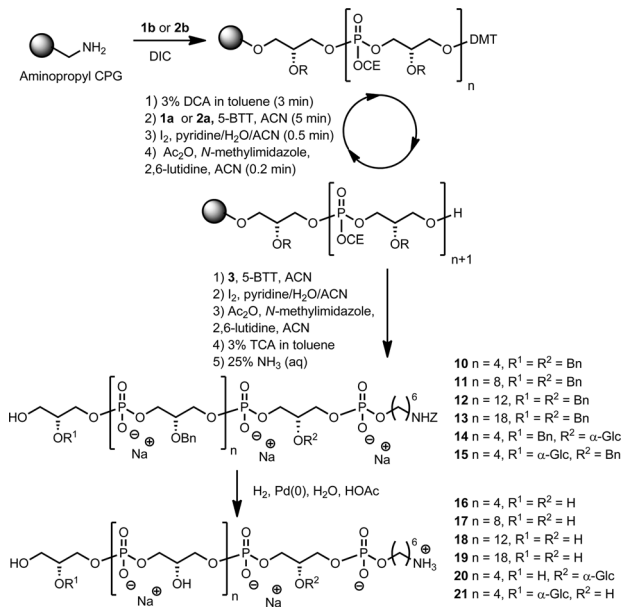


Scheme 1 Building blocks for the automated solid phase TA synthesis.

accomplished by employing benzylidene *S*-phenyl glucosyl donor **4** in a diphenylsulfoxide-triflic anhydride mediated glycosylation of glycerol alcohol **5**.[10,11] The resulting glucosyl-glycerol **6** was transformed into **2a/b** as follows: isomerization of the allyl ether and subsequent cleavage of the resulting enol ether using iodine under basic conditions liberated the primary alcohol (**7**), which was capped with a DMT group to give compound **8**. Next the silyl group was removed to provide alcohol **9**, which was either phosphitylated to provide the phosphoramidite building block **2a**, or treated with succinic anhydride to give the glucosylglycerol linker **2b**.

With the required building blocks in hand we set out to explore the automated solid phase TA oligomer synthesis (Scheme 2). As a first objective we attempted the assembly of a spacer containing TA-hexamer **10**. To this end aminopropyl CPG was functionalized with succinyl glycerol **1b**. Cleavage of the DMT group with 3% dichloroacetic acid (DCA) in toluene and concomitant determination of the loading led to a resin with a loading of 100 μmol g^{-1}. For the coupling step we treated the resin with 5 equiv. of phosphoramidite **1a** and



Scheme 2 Automated solid phase TA synthesis.

5-benzylthiotetrazole (5-BTT, 22.5 equiv.) as an activator[12] in acetonitrile for 5 minutes. Oxidation of the intermediate phosphites was achieved with I_2 in pyridine/H_2O (1 min), after which a capping step (*N*-methylimidazole/acetic anhydride (Ac_2O)/2,6-lutidine) was introduced to cap any unreacted alcohol functionalities. To complete the elongation cycle the DMT-groups were removed with 3% DCA in toluene. The coupling efficiency using this protocol was generally >98% as judged from the automatic DMT-count. In the 6th cycle of the hexamer assembly, the glycerol phosphoramidite **1a** was replaced by benzyloxycarbonyl-aminohexanol phosphoramidite **3** (2 x 5 equiv.) to terminate the sequence.§ Cleavage of the hexamer from the CPG using aqueous ammonia, and concomitant cyanoethyl removal liberated the partially protected TA-hexamer **10**. We examined two different purification protocols for the purification of the partially protected oligomers, RP-HPLC purification and anion exchange chromatography followed by desalination. In the case of hexamer **10**, the former protocol provided the desired oligomer in the highest yield (18% overall from aminopropyl CPG). At the onset of our studies we realized that the phosphodiesters in our synthetic targets could be potentially labile to base treatment, in analogy to the lability of RNA fragments.[9] To investigate the base lability of the partially protected TA oligomer, we subjected hexamer **10** to a treatment with 25% aqueous ammonia for a prolonged period of time at room temperature and at 40 °C. No degradation of the hexamer could be detected by LCMS analysis after 24 h at 40 °C indicating that transesterification of the potentially labile phosphodiesters is not a significant risk under the reaction conditions used. Using the conditions described above, the following TA-oligomers were assembled: unsubstituted 10-mer **11**, unsubstituted 14-mer **12**, unsubstituted 20-mer **13** and monoglucosyl substituted 6-mers **14** and **15**. TA-fragment **15** was assembled on the CPG resin functionalized with C-2 glycosyl glycerol succinate **2b** (loading: 100 μmol g^{-1}). The results of the syntheses are summarized in Table 1, from which it becomes clear that the syntheses of the longer and substituted fragments proceeded with even greater efficiency than the assembly of hexamer **10**. With increasing size of the oligomers, purification by ion exchange chromatography became more efficient than RP-HPLC purification. For TA 20-mer **13** and the glucosyl substituted hexamers **14** and **15** LCMS analysis of the crude reaction products showed significant peak broadening and therefore these compounds were solely purified using the ion-exchange protocol. From the excellent yields of glucosyl substituted hexamers **14** and **15** it becomes clear that the glycerol C-2 glucose substitution had no adverse effect on the coupling

Table 1 Results from the automated solid phase synthesis and deprotection

Entry	Compound	Yield (RP-HPLC)	Yield (ion exchange)	Deprotection (yield)
1	**10**	18%	10%	**16** (65%)
2	**11**	29%	16%	**17** (78%)
3	**12**	8%	21%	**18** (84%)
4	**13**		24%	**19** (95%)
5	**14**		32%	**20** (68%)
6	**15**		36%	**21** (86%)

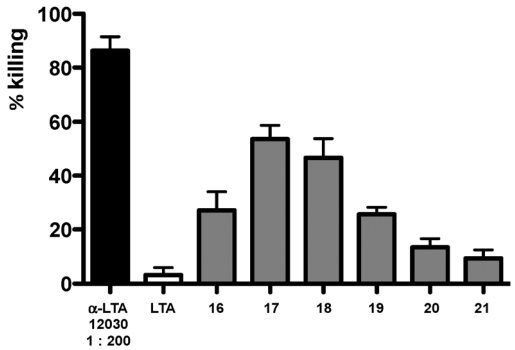


Fig. 1 Opsonophagocytic inhibition assay. α-LTA 12030 represents the killing of the sera at 1 : 200 dilution. LTA is used as a positive control and is used at a concentration of 100 mg mL^{-1}.

efficiency of the building blocks used. Notably, the benzylidene functionality on the glucosyl moiety in **15** proved to be stable to the repeated detritylation steps. Global deprotection of the partially protected TA-fragments was achieved by hydrogenolysis of the benzyl ethers, CBz-group and benzylidene functionality over palladium black followed by desalination (Scheme 2), uneventfully leading to TA-target structures **16–21**, as summarized in Table 1. The structure of the final products was confirmed by NMR spectroscopy and high resolution mass spectrometry. In the NMR spectra the relative ratio of the integrals of the peaks belonging to the spacer CH_2 groups, the spacer CH_2–N group and the terminal glycerol CH_2–O moiety with respect to the bulk signal originating from all of the other glycerol protons provided proof for the integrity of the structures.

The prepared TA fragments were tested in an opsonophagocytic inhibition assay to establish their antigenic activity.[13] In this assay, rabbit sera raised against purified *E. faecalis* LTA are used to kill *E. faecalis bacteria*. Blocking the opsonic antibodies in the sera with an inhibitor will lead to reduced killing.‡ The inhibitory potential of the synthesized TA fragments at a concentration of 100 μg mL^{-1} is displayed in Fig. 1, which shows that the smallest fragment tested, hexamer **16**, is capable of inhibiting the opsonophagocytic killing. For the 10-, 14- and 20-mer a length-dependence is observed for the inhibitory potential. The longer the fragments are the better the binding is to the opsonic antibodies, resulting in reduced killing. Interestingly, the glucose substituted TA fragments **20** and **21** were found to be very potent inhibitors. This is striking since the α-glucosyl substituent is found in *S. aureus* TA, but has not been found in *E. faecalis* TA. Nonetheless, compound **21** proved to be the most active compound of the series, making this a promising candidate for the future development of a vaccine comprising **21** or a close analogue as a synthetic TA-antigen.

We have described the development of automated solid phase methodology to synthesize glycerol phosphate teichoic acid fragments. Tailor made glycerol phosphoramidite building blocks were used in combination with a commercially available synthesizer, to produce partially protected TA fragments. With a full coupling cycle, taking approximately 15 minutes, a TA 20-mer was produced in 5 hours. Functionalized glycerolphosphate building blocks could also be used in the synthesis to allow the assembly of substituted TA fragments. Employing the solid phase methodology, a small library of TA fragments was generated which was tested for activity in an opsonophagocytic inhibition assay, revealing a clear TA-length–activity relationship. The assay also revealed glucosyl substituted TA-hexamers **20** and **21** as promising lead candidates for future vaccine development. We are currently exploring the full scope of the method in the generation of a larger library of TAs, bearing diverse substitution patterns to unravel more detailed structure–activity relationships.

Notes and references

§ The use of the commercially available *N*-MMT-6-aminohexanol phosphoramidite led to the formation of *N*-cyanoethyl side products in the cleavage/deprotection step. Although this could be prevented by the use of 1,4-dithiothreitol (DTT) in the cleavage cocktail, we prefer the use of the stable benzyl carbamate **3**.

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