Synthetic methods to glycerol teichoic acids

Hogendorf, W.F.J.

Chapter 7

Summary and Future Prospects

This thesis describes the development of synthetic methodologies to acquire structurally defined fragments of teichoic acids (TAs). TAs are an important class of phosphodiester containing biopolymers that occur in the cell wall of Gram-positive bacteria.[1-5] The structure of TAs mostly consists of linear polymers of alditol (glycerol, ribitol) phosphates, non-stoichiometrically substituted with D-alanyl and carbohydrate moieties. The TA structure is related to the type of bacteria and is dependent on the conditions these encounter. TAs not only take part in vital functions of the bacteria but also are immunologically active. In the introductory chapter the discovery, the structural classification, as well as physiological and immunological aspects of TAs is briefly discussed. In addition, selected examples of reported syntheses to TA fragments, with a focus on phosphor chemistry are presented. The synthetic efforts described in this thesis are mainly directed to TAs originating from *Enterococcus faecalis*. This normally harmless commensal has become a considerable threat in hospitals as growing antibiotic resistance makes this species difficult to treat.[6-10] In collaboration with the group of Huebner, specialists on the pathogenesis, treatment, and prevention of enterococcal infections, the prepared TAs are immunologically evaluated.

Chapter two describes the solution phase synthesis of a spacer containing (kojibiosyl) glycerol hexamer, a striking fragment of a TA from *Enterococcus faecalis*.[9] Benefit was taken from knowledge on oligonucleotide synthesis, indicating that phosphoramidite chemistry is the most efficient procedure for the formation of phosphodiesters and the dimethoxytrityl group is most appropriate as temporary protective group. Suitably protected glycerol and kojibiosyl glycerol phosphoramidite building blocks and an aminohexylphosphoramidite were prepared and implemented in the stepwise elongation of dibenzylglycerol to the target hexamer. Each elongation cycle comprised three reactions: 1) Phosphitylation using a phosphoramidite. 2) Oxidation of the intermediate phosphite triester. 3) Cleavage of the dimethoxytrityl group. Deprotection and purification of the target hexamer proceeded uneventful. The synthetic approach proved efficient and could be performed at a relatively large scale using only a small excess of phosphoramidite reagents.[11]

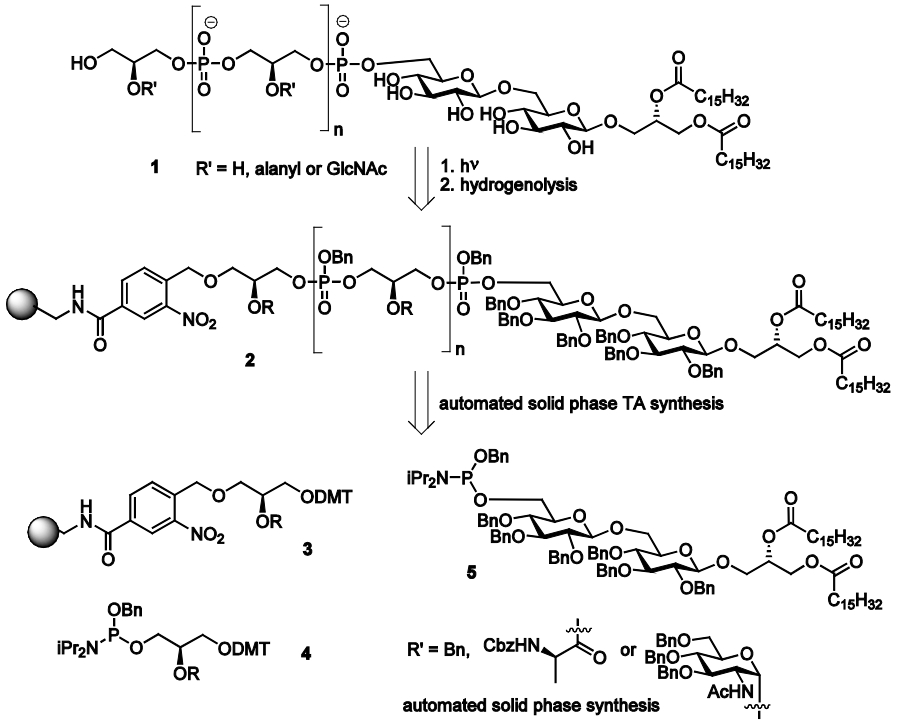
The phosphoramidite chemistry described in **chapter two** could be applied for the development of a solid phase approach, executed with the aid of a DNA synthesizer.

Chapter three deals with the construction of a small library of TA fragments using an automated solid phase synthesis approach. The library consists of linear 1,3-glycerol phosphate TAs comprising six, ten, fourteen and twenty residues and two α-glucosylated hexaglycerol phosphate TAs. The approach proved to be a fast and efficient means to obtain small quantities of a library of TA fragments.[12]

The automated solid phase synthesis protocol described in **chapter three** can potentially give access to all kinds of TAs. Intrinsically base-labile structural entities such as diacyl glycerol, which is the core structure of the glycolipid anchor of all LTAs and alanylated TAs can potentially be made using this approach. However, this excludes the 2-cyanoethyl as a phosphate protecting group and the succinyl linker to the solid-support since these require base treatment in the final stage of the synthesis. The protective group pattern should be adjusted and an example of a retro-synthesis to a fragment of the LTA of *S. aureus* using benzyl protection for the phosphate moieties and a photo-cleavable linker to the solid support is outlined in Scheme 1.

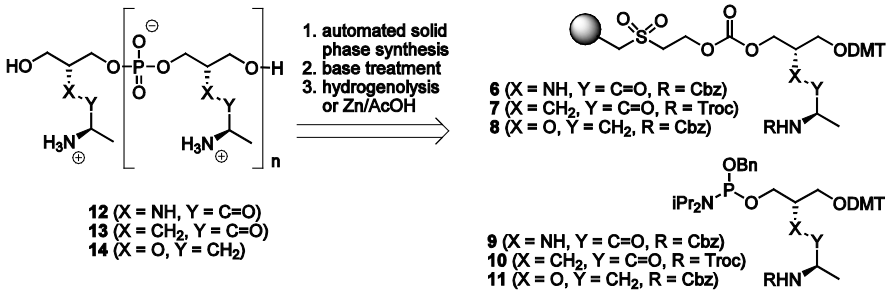
The lability of D-alanyl esters present on TAs makes it difficult to get a clear understanding of the immunomodulatory functions that these moieties possess.[13] Even synthetic TAs, the structure of which is spectroscopically ascertained, can spontaneously degrade when dissolved in the buffer belonging to *in vitro* or *in vivo*

Scheme 1. Retrosynthesis of staphylococcal LTA, no significant base is used during the synthesis.



immunological evaluation assays. This problem can possibly be circumvented by the synthesis of TA fragments that are equipped with "stabilized" alanines. Schmidt and co-workers have reported on the use of alanyl amides as replacement of alanyl esters in the synthesis of an analogue of staphylococcal LTA.[14] This structural change, although adding extra potential hydrogen bond donors, minimally affects important properties of teichoic acids such as the overall charge. It is proposed to investigate the optically pure α-amino ketone as another example of a stabilized D-alanyl ester analogue. In this case the molecules lose a hydrogen bond acceptor for every replacement. A third option would be to omit the carbonyl moiety and incorporate the stereochemically pure 1-(2-amino)-propyl ether as an alanine mimetic. In this case the molecule loses an H-bond acceptor but gains more flexibility. In Scheme 2, a retrosynthetic approach to these TA mimetics is presented. In order to avoid potential epimerization of the alanyl C-2, it is recommended to use a linker to the solid support that is sufficiently base labile. Alternatively, the photo-cleavable linker, the structure of which is depicted in Scheme 1, can be amended for this purpose.

Scheme 2. Retrosynthesis of stabilized D-alanylated TA mimetics **12**-**14**.



Chapter four informs on a new light fluorous approach to the synthesis of a small library of TA fragments, having a terminal phosphate monoester. Glycerol TAs up to a length of a dodecamer and several hexamers containing (amino)glucosyl moieties, as present in TAs of several strains of bacteria, were efficiently obtained via this technique. The molecules were built up from a base labile perfuorooctylpropyl sulfonyl ethyl (F-Pse) scaffold, which concomitantly served as a phosphate protecting group. This method bears the advantages of the solution phase method (**chapter two)**, such as the scale of the reaction allowing the production of multimilligram amounts of target TAs and the small excess of expensive phosphoramidite building blocks. In addition, the highly efficient fluorous solid phase extraction (F-SPE) purification step, which is performed after each elongation cycle ensures a significantly reduced purification time.[15]

Chapter five reports on an alternative light fluorous approach to three glucosylated TA hexamers. In order to obtain TAs provided with a terminal hydroxyl function instead of a phosphate monoester, a base labile fluorous succinyl linker was developed. In addition, the synthesis towards a more acid stable tetrabenzylglucosyl glycerol phosphoramidite is described. This building block provided with acid stabile

protecting groups allowed the introduction of the (glucosyl)glycerol moiety into the TA backbone at an earlier stage of the synthesis.[16]

The chemical research described in **chapters two, three, four** and **five** can be summarized as the development of three distinctive synthetic approaches to TA fragments. These three synthetic approaches: solution phase (**chapter two**), automated solid phase (**chapter three**) and fluorous phase (**chapters four/five**) all have their advantages and disadvantages, which are summarized in Table 1. From the summary drafted in this Table one can envisage that for the development and optimization of the applied chemistry the solution phase approach suits best. For a small-scale synthesis of a library of TA fragments to screen the initial biological properties such as their antigenic potency, the automated solid phase approach is the most suitable method. If screening experiments make available a lead compound provided with an interesting immunological profile, larger quantities of this TA fragment are required for additional immunological experiments. The TA fragment can in that case be obtained on a larger scale using one of the light fluorous approaches (**chapter four/five**), which allows a more rapid and efficient synthesis compared to regular solution phase chemistry (**chapter two**).

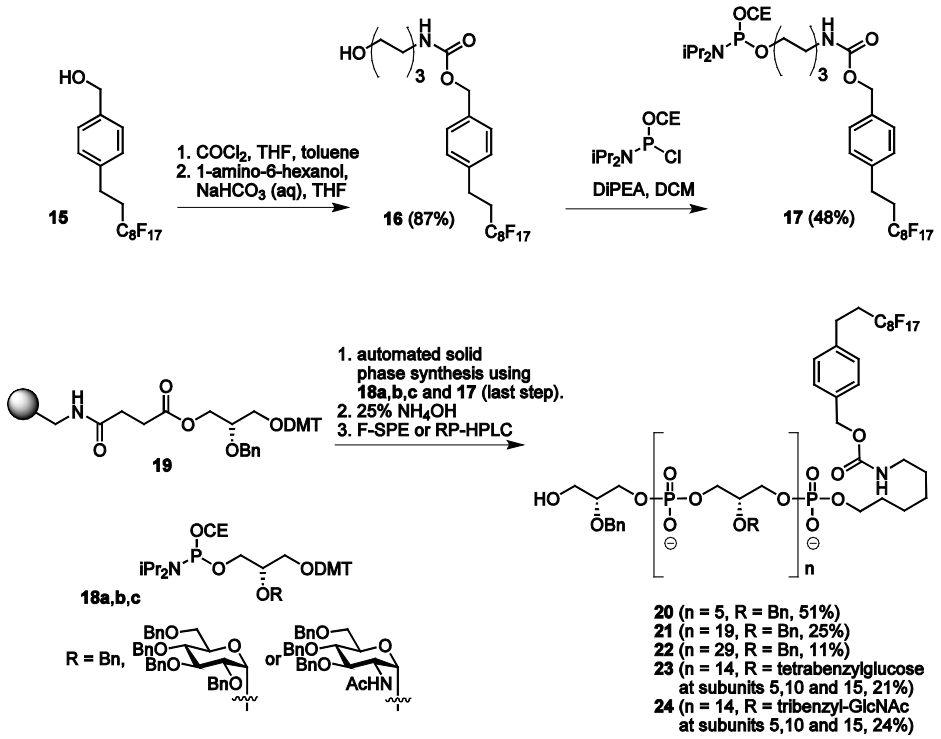
Table 1. Relative valuation of the three approaches on several practical elements. (+ = good, +/- = reasonable, - = poor)

	Solution phase	Automated solid phase	Light fluorous
Max . work scale	+ (>1 mmol)	- (10-15 μmol)	+ (>1 mmol)
Equivalent of phosphoramidite needed for full conversion	+ (1.3-3)	- (5-10)	+ 1.2-3
Maximum length (glycerol phosphate subunits)	- (6)	+ (>20)	+/- (>12)
Purification	- (silica gel)	+ (RP-HPLC or Q-sepharose)	+ (F-SPE)
Total synthesis time	- (weeks)	+ (hours)	+/- (days/weeks)

It was envisioned that the automated solid phase TA synthesis could be improved by amending the purification method with a fluorous tagging procedure.[17-19] This procedure comprises the installation of a suitable fluorous tag in the final elongation step of the automated synthesis to TAs. Because deletion sequences have been capped (acetylated) throughout the assembly process, only the target fragment will be equipped with the fluorous tag. Cleavage from the solid support and removal of the base-labile protecting groups provides a crude mixture, in which the semi-protected

target TA is the sole fluorous compound, allowing an efficient purification by F-SPE or F-HPLC. To test the viability of this purification procedure in TA synthesis, fluorous benzyl carbamate[20] protected aminohexyl phosphoramidite (**17**) was prepared from fluorous benzyl alcohol (**15**) in three steps (see Scheme 3). A small library of TA fragments, in which this fluorous tag was appended, was made. The library consisted of glycerol phosphates hexamer **20**, 20-mer **21**, 30-mer **22**, and two 15-mers, bearing three α-glucosyl or α-*N*-acetyl glucosamine residues (**23**, **24**). F-SPE proved to be an efficient purification method for hexamer **20**, although it was observed that the difference in affinity for the F-SPE column of **20** with respect to its by-products was small. Semi-protected hexamer **20** could be obtained in 51% yield. In case of 20-mer **21**, the F-SPE purification of the crude product was significantly less effective, as 20-mer **21** partially co-eluted with non-fluorous by-products. A second F-SPE purification step of the mixed fractions was required to afford the pure target **21** in 25% yield. In the case of 30-mer glycerol phosphate **22**, no separation between the fluorous and non-fluorous products was obtained with F-SPE, indicating the limit of the F-SPE purification method. To effect the purification of **22** the potency of a fluorous HPLC purification was explored. Interestingly, with this technique no significant improvement in separation of the fluorous 30-mer (**22**) from the by-products was

Scheme 3. Automated solid phase synthesis of TA fragments using a fluorous tagging strategy.



observed compared to a normal RP-HPLC (C18) purification. Apparently, the lipophilicity of the fluorous tag in these charged, semi-protected, glycerol phosphates has a larger effect on the separation than the fluorous affinity. Purification of the 30-mer with regular preparative RP-HPLC (C18) gave pure **22** in 11% yield. The glycosylated 15-mers **23** and **24** were also purified with preparative RP-HPLC. The relatively lipophilic benzylated carbohydrate residues in 15-mers **23** and **24**, led to significant peak broadening during RP-HPLC analysis using a C18 column. When the RP-HPLC purification was performed with a preparative C4 column the two semi-protected TA 15-mers were isolated in adequate yields: 21% for **23**, and 24% for **24**, respectively. These results indicate that the fluorous tagging strategy is viable for the synthesis of TA fragments. For the smaller fragments, the target compounds can be purified by exploiting the fluorous character of the TAs, where purification of the longer fragments hinges on the extra lipophilicity of the fluorous benzyl group attached to the targets, as a RP-HPLC-purification handle.

The TA fragments described in **chapters two**, **three**, **four** and **five** were tested on their antigenicity in an opsonophagocytic inhibition assay (OPIA). The antigenicity of the compounds depended on their length, and the non-substituted 20-mer inhibited the killing of *E. faecalis* more efficiently than the shorter non-substituted glycerol phosphates. Surprisingly, the fragments containing an α-glucosyl moiety, which is a substitution not found in native enterococcal LTA, showed even better inhibitory activity in the OPIA (see **chapter three**) than the 20-mer. Surprisingly, the TA fragments containing a phosphate monoester functionality (**chapter four**) all showed low activity and the bis-glucosylated hexaglycerol phosphate (**chapter five**) was less active than its monoglucosylated counterpart (structure **25**, Figure 1). The outcome of these studies indicate that the activity of the TA fragments critically depends on the substitution pattern and that more structure activity studies are warranted to arrive at a TA fragment with an optimal immunological profile.[10,12]

Chapter six describes the synthesis and immunological evaluation of a conjugate of fragment **25** with bovine serum albumin (BSA), an immunogenic carrier protein. Conjugate **26** was obtained via thiol maleimide chemistry and the conjugate contained ~20 TAs per protein (see Figure 1).[21] Serum obtained from rabbits, immunized with this conjugate, showed high opsonic activity against *E. faecalis*. Notably, two strains of different Gram-positive pathogens (*Enterococcus faecium* and *Staphylococcus aureus*) were also opsonized this rabbit anti-**26** serum. The opsonic activity against *E. faecium* was surprisingly high, as the antiserum could be diluted ~160.000 fold before the killing was reduced. In an *in vivo* endocarditis model it was shown that rats could successfully be passively immunized against *E. faecalis* using the rabbit antiserum raised against conjugate **26**.[22] A next step in the evaluation of conjugate **26** as an experimental vaccine modality would be to test the possibility of active immunization.

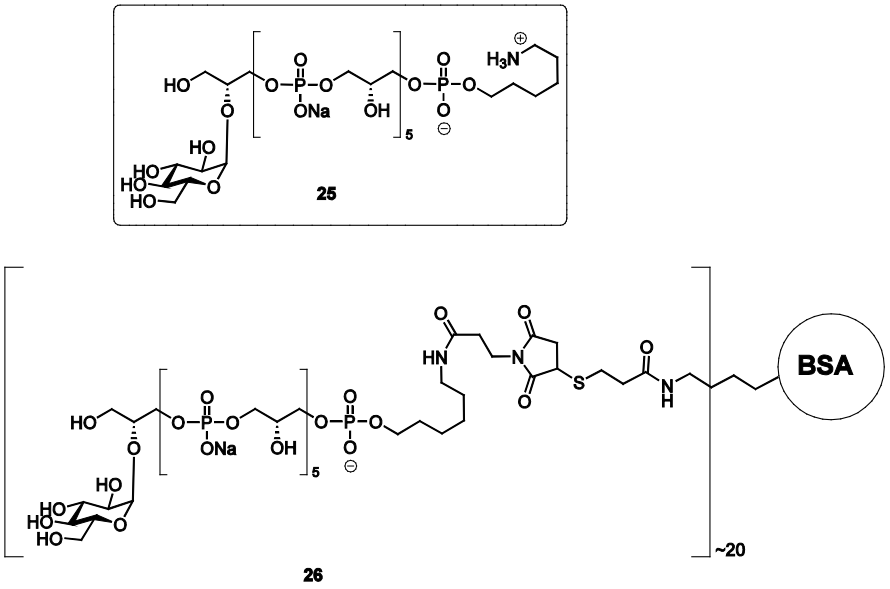
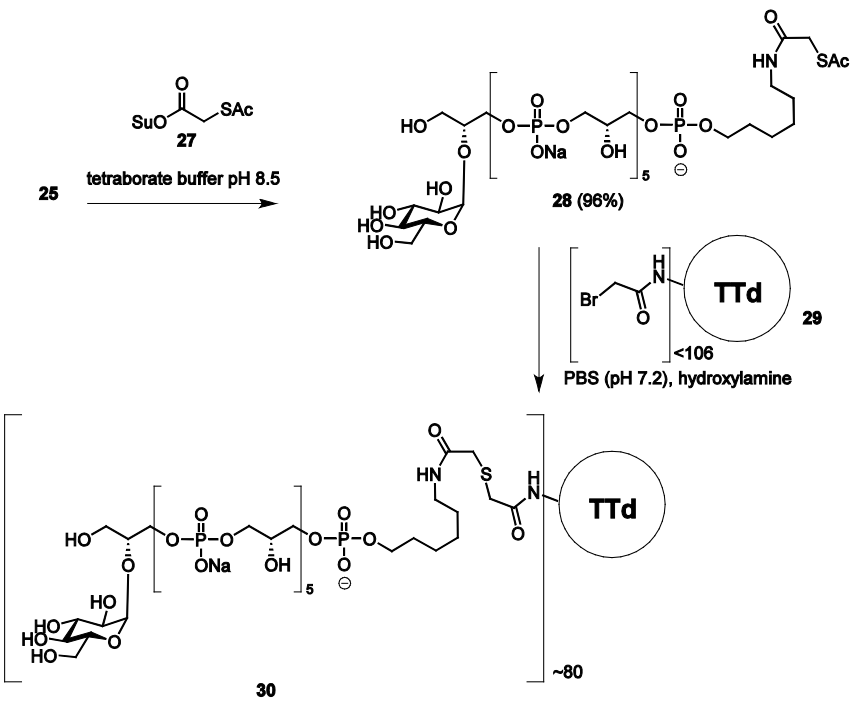


Figure 1. Hexamer **25**, the TA fragment with the highest inhibitory activity, and its BSA conjugate (**26**).

Scheme 4. Synthesis of TA-TTd conjugate **30**.



When TA-BSA conjugate **26** proves successful in the additional immunological evaluation, it is needed to replace BSA by a protein carrier that is more suitable for clinical studies. One of these carrier proteins is tetanus toxoid (TTd), which is used in several conjugate vaccines. A notable example is the Quimihib®-vaccine, a TTd-conjugate of the (synthetic) capsular polysaccharide of *Haemophilus influenza*.[21] In anticipation, TA fragment **25** was equipped with a (2-thioacetyl)acetyl moiety using the corresponding hydroxysuccinimide ester (**27**) to give modified TA **28** in 96% yield (see Scheme 4). The thiol functionality in **28** was liberated in a buffer containing hydroxylamine, and coupled *in situ* to bromoacetylated-TTd (**29**) as described earlier by Hoogerhout and co-workers.[23,24] An H_2SO_4/phenol colorimetric assay[25] of the resulting conjugate (**30**) indicated the presence of carbohydrates on the protein and a protein/sugar ratio of ~1.25/1 (~80 TAs per TTd). The immunological evaluation of this conjugate is currently in progress.

In conclusion, this thesis describes the development of three distinctive synthetic methodologies to well defined TA fragments. The solution phase, automated solid phase and light fluorous approaches all have their specific merits and can be amended for different purposes. The synthetic TA fragments have been evaluated on their antigenicity and the most potent compound has been conjugated to a protein carrier. The TA-protein conjugate shows promising results in initial *in vitro* and *in vivo* immunological assays and can be used to passively immunize rats against *E. faecalis* an increasingly important Gram-positive threat in healthcare. Further biological studies will indicate whether the conjugate is a suitable candidate for clinical trials.

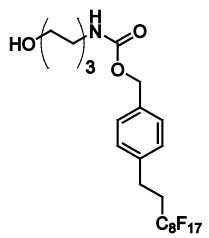
Experimental section

General Procedures and Material: All chemicals (Acros, Fluka, Merck, Schleicher & Schuell, Sigma-Aldrich, Genscript, Fluorous Technologies) were used as received and reactions were carried out dry, under an argon atmosphere, at ambient temperature, unless stated otherwise. Column chromatography was performed on Screening Devices silica gel 60 (0.040-0.063 mm). TLC analysis was conducted on HPTLC aluminium sheets (Merck, silica gel 60, F245). Compounds were visualized by UV absorption (245 nm), by spraying with 20% H_2SO_4 in ethanol or with a solution of $(NH_4)_6Mo_7O_{24}\bullet 4\ H_2O$ 25 g/l and $(NH_4)_4Ce(SO_4)_4\bullet 2\ H_2O$ 10 g/l, in 10% aqueous H_2SO_4 followed by charring at +/- 140 ^oC. ^{31}P, ^1H, and ^{13}C NMR spectra were recorded with a Bruker AV 400 (161.7, 400 and 125 MHz respectively) or a Bruker DMX 600 (600 and 150 MHz respectively). NMR spectra were recorded in $CDCl_3$ with chemical shift (δ) relative to tetramethylsilane, unless stated otherwise. When D_2O was used, ^1H-NMR spectra were recorded with chemical shift relative (δ) to HDO (4.755 ppm), ^{31}P spectra were measured with chemical shift relative to 85% H_3PO_4 (external standard) and ^{13}C-NMR spectra were recorded with chemical shift relative to TMS (external standard). High resolution mass spectra (HRMS) were recorded by direct injection (2 µl of a 2 µM solution in water/acetonitrile; 50/50; v/v and either 0.1% formic acid or 10mM ammonium formate for the oligomers) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 275 ^oC) with resolution R = 60000 at m/z 400 (mass range m/z = 150-2000) and dioctylphthalate (m/z = 391.28428) as a lock mass. The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan).

Procedure for automated solid-phase synthesis, purification and global deprotection of TA oligomers: Modified polystyrene support (amino PS, Fluka) was loaded with glycerol succinates and the loading was determined (loading: 100 μmol/g CPG) using the method described by Pon.[ref] The automated syntheses were performed on a synthesizer (ÄKTA™ oligopilot plus™, GE Healthcare) on a scale of 100-150 mg immobilized **19** (10-15 μmol glycerol derivative) and started off with acidolysis of the dimethoxytrityl ether using 3% dichloroacetic acid in toluene (15 ml, 3 min). After flushing with acetonitrile (5ml, 1 min), the resulting alcohol was reacted with phosphoramidites **18a** (0.2 M in MeCN, 5 eq) and 5-benzylthiotetrazole (BTT, 0.3M in MeCN, 22.5 eq) for 5 min using a cycled flow. After flushing with MeCN (5ml, 1 min), oxidation of the intermediate phosphite was performed using I_2 (0.05 M in pyridine/H_2O 9/1, 2ml, 1 min). A flushing step with MeCN (5ml, 1 min) was followed by a capping step (1 ml of a 1/1 mixture of capping solution A (20 v/v% *N*-methylimidazole in MeCN) and capping solution B (20 v/v% Ac_2O, 15 v/v% 2,6-lutidine in MeCN for 12s). After flushing with MeCN (5ml, 1 min), a detritylation step was performed using the before mentioned cocktail and the molecule was elongated using phosphoramidites **18a,b** or **c** using the same set of reactions (coupling, oxidation, capping, detritylation). The average coupling efficiency was measured by quantitative UV-detection (400 nm) of the dimethoxytrityl cation during each detritylation step. When the desired length was obtained, spacer phosphoramidite **17** (0.2 M in ACN, 2 x 5 eq, 2 x 5 min) was coupled to the PS-TA-oligomer using BTT (0.3M in MeCN, 2 x 22.5 eq) and, subsequently treated with I_2 (0.05 M in pyridine/H_2O 9/1, 2ml, 1 min), before it was released from the solid support using 25% NH_4OH (10ml, 1h, the cyanoethyl groups are concomitantly released at this stage). The solvents were then removed *in vacuo* before the crude oligomer was purified using F-SPE or RP-HPLC.

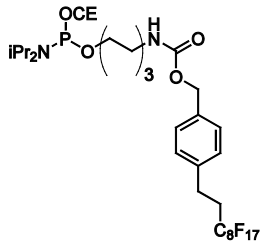
F-SPE: The crude product was taken up in 0.5 ml H_2O and applied to a small column containing fluoroflash™ fluorous silica (4g) which was preeluted with H_2O. The column was eluted with H_2O and 10% CH_3CN in H_2O until all the non-fluorous by-products were removed. The fluorous product was then eluted from the column with a 2/2/1 mixture of acetony/CH_3CN/H_2O. Lyophilization gave the partially protected oligomer of which the integrity and purity was confirmed by LC-MS, HRMS and NMR (^1H, ^{13}C, ^{31}P) analysis.

RP-HPLC: Gilson preparative HPLC system; column: Alltima C18 or C4, particle size: 5 μm, dimensions: 10/250 mm; eluent: (10 mM NH_4OAc in H_2O for C18 or 25mM TEAA for in H_2O for C4)/MeCN, 9/1 → 1/9, detection: UV (215 and 254 nm), the fractions containing product were collected and the solvents were removed under reduced pressure. Repeated lyophilization (trice) of the residue gave the partially protected oligomer of which the integrity and purity was confirmed by LC-MS, HRMS and NMR (^1H, ^{13}C, ^{31}P) analysis.



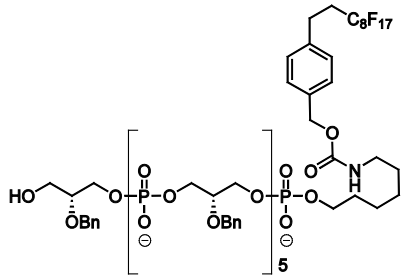
4-(2-perfluorooctylethyl)benzyl *N*-(6-hydroxyhexyl)carbamate (16)
To a cooled (0 °C) solution of fluorous benzyl alcohol **15** (1.11 g, 2.00 mmol) in THF (20 ml) was added phosgene (2M in toluene, 2.50 ml, 5 mmol). After stirring for 90 minutes the volatiles were removed under reduced pressure and the residue coevaporated with THF (10 ml). The crude chloroformate was dissolved in a mixture of THF (30 ml) and sat. aq. $NaHCO_3$ (12 ml) after which 1-amino-6-hexanol (2.34 g, 20 mmol) was added. After stirring overnight the mixture was diluted with Et_2O (300 ml) and washed with brine (2 x 150 ml). The organic layer was dried (Na_2SO_4) and concentrated *in vacuo*. Crystallization (EtOAc/PE) of the residue afforded the title carbamate (**16**, 1.21 g, 1.74 mmol, 87%) as an amorphous off-white solid. ^1H NMR (300 MHz): δ = 1.30 - 1.41 (m, 4H, 2 x CH_2 hexylspacer), 1.47 - 1.60 (m, 4H, 2 x CH_2 hexylspacer), 2.27

- 2.48 (m, 2H, $F_{17}C_8CH_2CH_2$-), 2.86 - 2.98 (m, 2H, $F_{17}C_8CH_2CH_2$-), 3.11 - 3.21 (m, 2H, CH_2-N hexylspacer), 3.58 (t, 2H, *J* = 6.5 Hz, CH_2-O hexylspacer), 5.06 (s, 2H, CH_2 F-Bn), 5.41 (bs, 1H, NH), 7.22 (d, 2H, *J* = 7.8 Hz, H_{arom}), 7.32 (d, 2H, *J* = 7.9 Hz, H_{arom}); ^{13}C NMR (75 MHz): δ = 25.1, 25.9, 26.2 (2 x CH_2 hexylspacer, $F_{17}C_8CH_2CH_2$-), 29.6, 32.1 (2 x CH_2 hexylspacer), 32.7 (t, *J* = 22 Hz, $F_{17}C_8CH_2CH_2$-), 40.7 (CH_2-N hexylspacer), 61.9 (CH_2-O hexylspacer), 66.1 (CH_2 F-Bn), 128.3 - 128.4 (CH_{arom}), 134.9 (C_q F-Bn), 138.9 (C_q F-Bn).



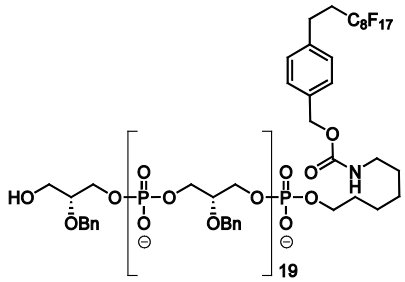
4-(2-perfluorooctylethyl)benzyl *N*-(6-*O*-[{*N*,*N*-diisopropyl}-2-cyanoethyl phosphoramidite]-hexyl)-carbamate (17)

To a solution of fluorous alcohol **16** (1.13 g, 1.62 mmol) and DiPEA (0.43 ml, 2.47 mmol) in DCM (32 ml) was added (*N*,*N*-diisopropyl)-2-cyanoethyl-chlorophosphoramidite (0.452 ml, 2.03 mmol). After stirring 90 min H_2O (10 ml) was added and the layers separated. The organic layer was washed with H_2O (10 ml) and brine (10 ml) after which it was dried (Na_2SO_4) and concentrated *in vacuo*. Purification of the residue by column chromatography (EtOAc/PE/Et_3N) gave phosphoramidite **17** (692 mg, 771 μmol, 48%) as an oily substance. ^{31}P NMR (161.7 MHz, CD_3CN): δ = 148.2; ^{1}H NMR (400 MHz, CD_3CN): δ = 1.16 - 1.20 (m, 12H, 4 x CH_3 isopropylamino), 1.30 - 1.41 (m, 4H, 2 x CH_2 hexylspacer), 1.44 - 1.51 (m, 2H, CH_2 hexylspacer), 1.56 - 1.62 (m, 2H, CH_2 hexylspacer), 2.35 - 2.49 (m, 2H, $F_{17}C_8CH_2CH_2$-), 2.63 (t, 2H, *J* = 6.0 Hz, CH_2 cyanoethyl), 2.84 - 2.90 (m, 2H, $F_{17}C_8CH_2CH_2$-), 3.09 (dd, 2H, *J* = 6.6 Hz, 13.0 Hz, CH_2-N hexylspacer), 3.55 - 3.68 (m, 4H, CH_2-O hexylspacer, 2 x CH isopropylamino), 3.71 - 3.82 (m, 2H, CH_2 cyanoethyl), 5.00 (s, 2H, CH_2 F-Bn), 5.74 (t, 1H, *J* = 5.5 Hz, NH), 7.20 (d, 2H, *J* = 7.9 Hz, H_{arom}), 7.27 (d, 2H, *J* = 7.9 Hz, H_{arom}); ^{13}C NMR (100 MHz, CD_3CN): δ = 21.1 (d, *J* = 7 Hz, CH_2 cyanoethyl), 25.0 (d, *J* = 7 Hz, 4 x CH_3 isopropylamino), 26.5 (CH_2 hexylspacer), 26.7 ($F_{17}C_8CH_2CH_2$-), 27.2 (CH_2 hexylspacer), 30.7 (CH_2 hexylspacer), 31.9 (d, *J* = 7 Hz, CH_2 hexylspacer), 33.1 (t, *J* = 22 Hz, $F_{17}C_8CH_2CH_2$-), 41.6 (CH_2-N hexylspacer), 43.8 (d, *J* = 12 Hz, 2 x CH isopropylamino), 59.3 (d, *J* = 19 Hz, CH_2 cyanoethyl), 64.3 (d, *J* = 17 Hz, CH_2-O hexylspacer), 66.4 (CH_2 F-Bn), 119.5 (C_q cyanoethyl), 129.1, 129.4 (CH_{arom}), 136.9 (C_q F-Bn), 139.9 (C_q F-Bn), 157.4 (C=O carbamate).



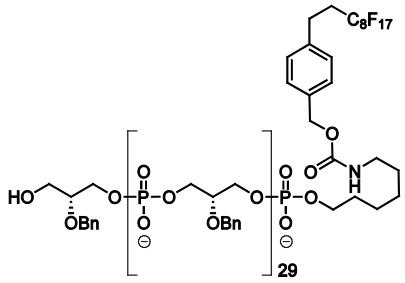
Fluorous hexa(2-benzyl)glycerol phosphate (20)

Automated solid phase TA synthesis was performed as described above using 15 μmol of immobilized glycerol (**19**). After five consecutive elongation cycles using phosphoramidite **18a** a capping step with fluorous phosphoramidite **17** was performed. After ammonia treatment the crude product was purified with F-SPE affording hexamer **20** (17.2 mg, 7.6 μmol, 51%) as an amorphous white solid. ^{31}P NMR (161.7 MHz, 3/2 D_2O/CD_3OD): δ = -0.2 (4P), 0.0 (2P); ^{1}H NMR (600 MHz, 3/2 D_2O/CD_3OD, T = 328K): δ = 1.16 - 1.29 (m, 4H, 2 x CH_2 hexylspacer), 1.34 - 1.39 (m, 2H, CH_2 hexylspacer), 1.45 - 1.51 (m, 2H, CH_2 hexylspacer), 2.29 - 2.39 (m, 2H, $F_{17}C_8CH_2CH_2$-), 2.82 (t, 2H, *J* = 7.9 Hz, $F_{17}C_8CH_2CH_2$-), 2.99 - 3.05 (m, 2H, CH_2-N hexylspacer), 3.57 - 3.68 (m, 3H, CH glycerol, CH_2 glycerol), 3.72 - 3.99 (m, 29H, 5 x CH glycerol, 11 x CH_2 glycerol, CH_2-O hexylspacer), 4.51 - 4.63 (m, 12H, 6 x CH_2 Bn), 5.01 (s, 2H, CH_2 F-Bn), 7.11 - 7.33 (m, 34H, H_{arom}); HRMS: $C_{84}H_{102}F_{17}NO_{33}P_6 + NH_4^+$ requires 2179.4827, found 2179.4840.



Fluorous (2-benzyl)glycerol phosphate 20-mer (21)

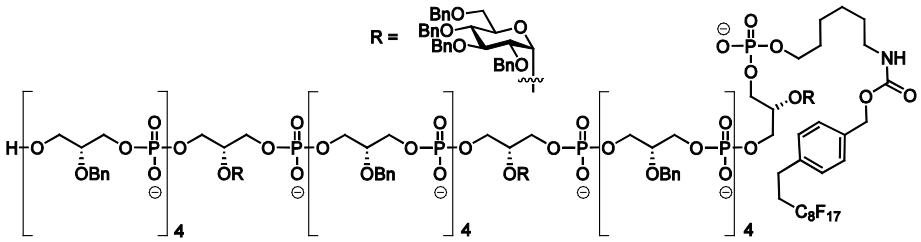
Automated solid phase TA synthesis was performed as described above using 15 μmol of immobilized glycerol (**19**). After nineteen consecutive elongation cycles using phosphoramidite **18a** a capping step with fluorous phosphoramidite **17** was performed. After ammonia treatment the crude product was purified with F-SPE. In addition, the purified oligomer was eluted through a small column containing Dowex Na^+ cation-exchange resin (type: 50WX4-200, stored on 0.5 M NaOH in H_2O, flushed with H_2O and MeOH before use). Lyophilization afforded 20-mer **21** (22.2 mg, 3.69 μmol, 25%) as an amorphous white solid. ^{31}P NMR (161.7 MHz, 3/2 D_2O/CD_3OD): δ = 1.2 (18P), 1.3 (2P); ^1H NMR (400 MHz, 3/2 D_2O/CD_3OD, T = 328K): δ = 1.10 - 1.34 (m, 6H, 3 x CH_2 hexylspacer), 1.39 - 1.49 (m, 2H, CH_2 hexylspacer), 2.11 - 2.26 (m, 2H, $F_{17}C_8CH_2CH_2$-), 2.65 - 2.71 (m 2H, $F_{17}C_8CH_2CH_2$-), 2.92 – 2.98 (m, 2H, CH_2-N hexylspacer), 3.53 - 3.99 (m, 102H, 20 x CH glycerol, 40 x CH_2 glycerol, CH_2-O hexylspacer), 4.47 - 4.59 (m, 40H, 20 x CH_2 Bn), 4.94 (bs, 2H, CH_2 F-Bn), 7.00 - 7.30 (m, 104H, H_{arom}); HRMS: $[C_{224}H_{284}F_{17}NO_{103}P_{20} + 2NH_4]^{2+}$ requires 2808.1120, found 2808.1094.



Fluorous (2-benzyl)glycerol phosphate 30-mer (22)

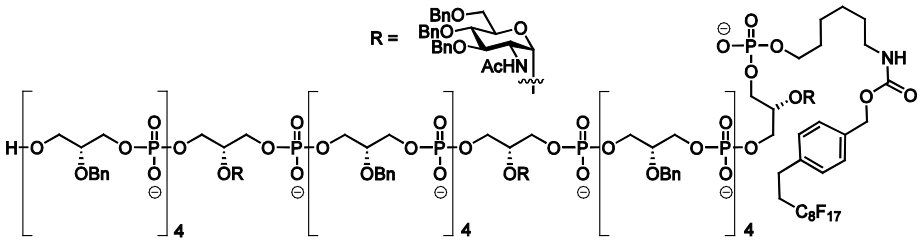
Automated solid phase TA synthesis was performed as described above using 15 μmol of immobilized glycerol (**19**). After 29 consecutive elongation cycles using phosphoramidite **18a** a capping step with fluorous phosphoramidite **17** was performed. After ammonia treatment the crude product was purified with RP-HPLC (C18). In addition, the purified oligomer was eluted through a small column containing Dowex Na^+ cation-exchange resin (type: 50WX4-200, stored on 0.5 M NaOH in H_2O, flushed with H_2O and MeOH before use). Lyophilization afforded 30-mer **22** (14.6 mg, 1.68 μmol, 11%) as an amorphous white solid. ^{31}P NMR (161.7 MHz, 7/3 D_2O/CD_3OD): δ = 1.0 (28P), 1.1 (2P); ^1H NMR (400 MHz, 7/3 D_2O/CD_3OD, T = 328K): δ = 1.01 - 1.29 (m, 6H, 3 x CH_2 hexylspacer), 1.34 - 1.41 (m, 2H, CH_2 hexylspacer), 1.85 - 2.01 (m, 2H, $F_{17}C_8CH_2CH_2$-), 2.43 - 2.51 (m 2H, $F_{17}C_8CH_2CH_2$-), 2.86 - 2.95 (m, 2H, CH_2-N hexylspacer), 3.52 - 4.03 (m, 152H, 30 x CH glycerol, 60 x CH_2 glycerol, CH_2-O hexylspacer), 4.47 - 4.59 (m, 60H, 30 x CH_2 Bn), 4.90 (bs, 2H, CH_2 F-Bn), 6.92 - 7.29 (m, 154H, H_{arom}); HRMS: $[C_{324}H_{414}F_{17}NO_{153}P_{30} + 3NH_4]^{3+}$ requires 2691.9206, found 2691.9191.

Fluorous tri(2,3,4,6-tetra-*O*-benzyl-α-D-glucosyl)glycerol phosphate 15-mer (23)



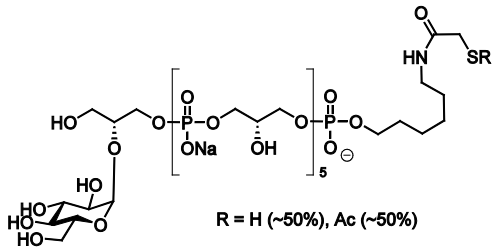
Automated solid phase TA synthesis was performed as described above using 9 μmol of immobilized glycerol (**19**). After 3 consecutive elongation cycles using phosphoramidite **18a** one elongation cycle was performed using glucosylglycerol phosphoramidite **18b**. This was followed by 4 elongations using glycerol amidite **18a** and one using glucosylglycerol amidite **18b**. The latter five-step elongation cycle was repeated once, which was followed by capping with fluorous phosphoramidite **17.** After ammonia treatment the crude product was purified with RP-HPLC (C4). Lyophilization afforded 15-mer **23** (13.5 mg, 1.88 μmol, 21%, Et_3NH^+-form) as an amorphous white solid. ^{31}P NMR (161.7 MHz, 1/1 D_2O/CD_3OD, T = 328K): δ = 1.8 - 2.4 (15P); ^1H NMR (400 MHz, 1/1 D_2O/CD_3OD, T = 328K): δ = 1.05 - 1.31 (m, 141H, 3 x CH_2 hexylspacer, 45 x CH_3 Et_3NH^+), 1.40 - 1.47 (m, 2H, CH_2 hexylspacer), 1.83 - 1.95 (m, 2H, $F_{17}C_8CH_2CH_2$-), 2.38 - 2.47 (m 2H, $F_{17}C_8CH_2CH_2$-), 2.91 - 3.00 (m, 2H, CH_2-N hexylspacer), 3.09 (q, 90H, *J* = 7.3 Hz, 45 x CH_2 Et_3NH^+), 3.34 - 4.25 (m, 95H, 15 x CH glycerol, 30 x CH_2 glycerol, CH_2-O hexylspacer, 3 x H-2, 3 x H-3, 3 x H-4, 3 x H-5, 3 x H-6, 3 x H-6'), 4.51 - 4.67 (m, 48H, 24 x CH_2 Bn), 4.81 (bs, 2H, CH_2 F-Bn), 5.09 - 5.17 (m, 3H, 3 x H-1), 6.79 - 7.26 (m, 124H, H_{arom}); LC-MS: $[C_{255}H_{303}F_{17}NO_{93}P_{15} + 2H]^{2+}$ requires 2830.8, found 2830.6.

Fluorous tri(2-acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-α-D-glucosyl)glycerol phosphate 15-mer (24)



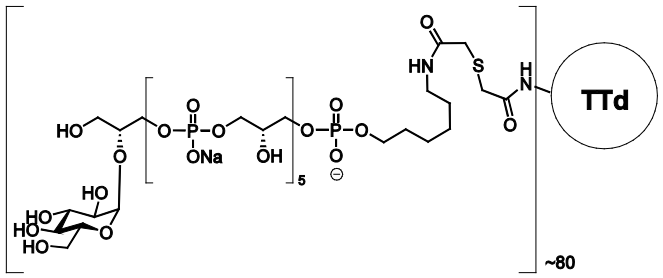
Automated solid phase TA synthesis was performed as described above using 9 μmol of immobilized glycerol (**19**). After 3 consecutive elongation cycles using phosphoramidite **18a** one elongation cycle was performed using *N*-acetylglucosaminylglycerol phosphoramidite **18c**. This was followed by 4 elongations using glycerol amidite **18a** and one using *N*-acetylglucosaminylglycerol amidite **18c**. The latter five-step elongation cycle was repeated once, which was followed by capping with fluorous phosphoramidite **17.** After ammonia treatment the crude product was purified with RP-HPLC (C4). Lyophilization afforded 15-mer **24** (15.0 mg, 2.13 μmol, 24%, Et_3NH^+-form) as an amorphous white solid. ^{31}P NMR (161.7 MHz, 1/1 D_2O/CD_3OD, T = 328K): δ = 0.3 - 0.7 (13P), 0.8 (1P), 1.0 (1P); ^1H NMR (400 MHz, 1/1 D_2O/CD_3OD, T = 328K): δ = 1.17 - 1.41 (m, 141H, 3 x CH_2 hexylspacer, 45 x CH_3 Et_3NH^+), 1.48 - 1.56 (m, 2H, CH_2 hexylspacer), 1.92 - 2.01 (3 x s, 9H, 3 x NHAc), 2.11 - 2.26 (m, 2H, $F_{17}C_8CH_2CH_2$-), 2.67 - 2.73 (m 2H, $F_{17}C_8CH_2CH_2$-), 2.94 - 3.02 (m, 2H, CH_2-N hexylspacer), 3.09 (q, 90H, *J* = 7.3 Hz, 45 x CH_2 Et_3NH^+), 3.50 - 4.13 (m, 95H, 15 x CH glycerol, 30 x CH_2 glycerol, CH_2-O hexylspacer, 3 x H-2, 3 x H-3, 3 x H-4, 3 x H-5, 3 x H-6, 3 x H-6'), 4.20 - 4.58 (m, 42H, 21 x CH_2

Bn), 4.92 (bs, 2H, CH_2 F-Bn), 4.94 - 4.97 (m, 3H, 3 x H-1), 6.92 - 7.30 (m, 109H, H_{arom}); LC-MS: $[C_{240}H_{294}F_{17}N_4O_{93}P_{15} + 3H]^{3+}$ requires 1838.5, found 1838.4.



(2-thioacetyl)acetyl glucosyl hexaglycerol phosphate (28)

To a solution of **25** (8.7 mg, 6.6 μmol) in 0.5 ml freshly prepared tetraborate buffer (0.1 M, pH 8.5) was added SATA (**27**, 30 mg, 0.13 mmol, dissolved in 300 μl DMF). The mixture was allowed to stir overnight after which it was filtered and the target compound isolated by size exclusion chromatography (HW40, 50 mM Et_3NHOAc). The purified product was lyophilized trice before it was dissolved in H_2O (~0.5 ml) and eluted to a small column containing Dowex Na^+ cation-exchange resin (type: 50WX4-200, stored on 0.5 M NaOH in H_2O, flushed with H_2O and MeOH before use). Lyophilization afforded the target oligomer (**28**, 9.2 mg, ~6.4 mmol, 96%) as a 1:1 mixture of acetate and free thiol. ^{31}P NMR (161.7 MHz, D_2O): δ = 1.2 - 1.3 (5P), 1.3 - 1.4 (1P); ^1H NMR (400 MHz, D_2O): δ = 1.23 - 1.34 (m, 4H, 2 x CH_2 hexylspacer), 1.40 - 1.51 (m, 2H, CH_2 hexylspacer), 1.52 - 1.60 (m, 2H, CH_2 hexylspacer), 2.33 (s, ~1.5H, CH_3 SAc, ~50% on), 3.08 - 3.19 (m, 2H, CH_2-N hexylspacer), 3.34 (t, 1H, *J* = 9.4 Hz, H-4), 3.37 (s, ~1H, C*H*$_2$SH), 3.46 (dd, 1H, *J* = 3.6 Hz, 9.8 Hz, H-2), 3.56 (s, ~1H, C*H*$_2$SAc), 3.64 - 4.01 (m, 36H, 6 x CH glycerol, 12 x CH_2 glycerol, CH_2-O hexylspacer, H-3, H-5, H-6, H-6'), 5.10 (d, 1H, *J* = 3.5 Hz, H-1).



TA-TTd conjugate (30)

To a solution of modified TA **28** (9.0 mg, 6.2 μmol) in H_2O (250 μl) were added bromoacetylated TTd (**29**, 7.0 mg, 47 nmol, in 2.0 ml PBS buffer pH 6.0, containing 5 MM EDTA, freshly prepared as described by van der Ley *et al.*)[ref hoogerhout] and hydroxylamine hydrochloride (2.0 M in PBS, 25 μl, 50 μmol). The mixture was mixed gently and allowed to stand overnight at room temperature after which 2-aminoethanethiol hydrochloride (18 mM in PBS pH 6.0, 225 μl, 4.0 μmol) was added. After stirring overnight, the crude modified TTd (**30**, in a total volume of 2.5 ml) was equally divided over two PD-10 columns, which were pre-equilibrated with PBS buffer (pH 7.2). The columns were eluted with PBS (pH 7.2, 3.5 ml) and the fractions containing the modified protein combined. The purified conjugate **30** was analyzed on sugar content by a phenol/sulfuric acid assay[ref] (using TA **25** as external standard, protein/sugar ratio: ~1.2/1) and stored in solution (PBS, 7 ml, ~1 mg protein/ml) at 4 °C.[ref hoogerhout]

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