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Synthesis and characterisation of fluorescent substrates for eukaryotic protein N-glycosylation

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ABSTRACT

Herein we report the synthesis of two fluorescently labelled analogues of C_{25} dolichol (Dol_{25}) in which the terminal isoprene unit has been replaced by a dansyl or 7-amino-4-trifluoromethylcoumarin fluorophore, a transformation enabled by the regioselective epoxidation of the terminal olefin via its bromohydrin using the van Tamelen procedure. The lipid alcohols were phosphorylated and glycosylated to obtain lipid-linked chitobiose-α-diphosphates and a lipid-linked mannosyl-β-phosphate. Biochemical assays showed that these labelled substrates are accepted by eukaryotic protein *N*-glycosylation enzymes with rates comparable to the unlabelled substrates, reconstituting a major part of the pathway up to the lipid-linked dodeca-saccharide $Glc_1Man_9GlcNAc_2$-PP-Dol_{25} and its transfer to an acceptor peptide catalysed by eukaryotic oligosaccharyltransferases (OSTs), namely the single-subunit OST STT3A from *Trypanosoma brucei* and the octameric OST complex from *Saccharomyces cerevisiae*. The fluorescent labels facilitate handling and purification of the lipid-linked glycosyl donors and acceptors and should facilitate further biochemical studies of protein glycosylation enzymes.

1. Introduction

Protein *N*-glycosylation is an important post-translational modification found in all domains of life [1,2]. In the key step, an oligosaccharyltransferase (OST) enzyme performs the *en bloc* transfer of a complex pre-assembled glycan from the lipid-pyrophosphate carrier onto an asparagine residue within a glycosylation sequon of a nascent polypeptide. Synthetic substrates have been essential for detailed investigations of the *N*-glycosylation machinery [3,4]. On one hand, they are indispensable for *in vitro* characterisation of enzymes and their activity [5]. On the other hand, structural studies with one or more substrates bound to the enzyme have revealed structural features which were not observed in the *apo* state and are key to elucidating the molecular mechanism of action [6,7].

The substrates of the enzymes involved in *N*-glycosylation fall into two categories, namely lipid-linked oligosaccharides (LLOs) and glycosyl donors. In eukaryotes, LLOs consist of a glycan linked by a pyrophosphate moiety to a dolichol lipid (*e.g.* **1**, Fig. 1). In contrast, the glycosyl donors contain a single carbohydrate linked by phosphate to a dolichol lipid (*e.g.* **2**, Fig. 1) [2]. Depending on the species, the length of dolichol lipids varies, with the most abundant lipid chains containing 75–100 carbon atoms (Dol_{75}-Dol_{100}) [8,9]. However, they all share a common structure with the α unit saturated to an (*S*) configuration and an EEω tail motif, separated by a variable number of *Z*-isoprene units (Fig. 1.) [10].

Chemical synthesis of substrates with the naturally occurring Dol_{100} lipid such as **1** and **2** has been demonstrated in the past [11,12]. However, these long lipids are hard to obtain through chemical synthesis [13,14]. The extraction of long dolichol lipids from natural sources is also problematic, which is related to their unfavourable physicochemical properties and low abundance [10,15]. In addition, LLOs and glycosyl donors containing natural dolichol are nearly insoluble in water, which poses a challenge for structural and mechanistic studies *in vitro*. Nevertheless, both bacterial [6,13,14] and eukaryotic systems [16–19] have been shown *in vitro* to accept substrates with shorter lipids, which are easier to synthesize and handle. Along these lines, we have used Dol_{25}, which can be obtained from (*S*)-citronellol and farnesol, for the synthesis of the LLO $GlcNAc_2$-PP-Dol_{25} **3** and the mannosyl

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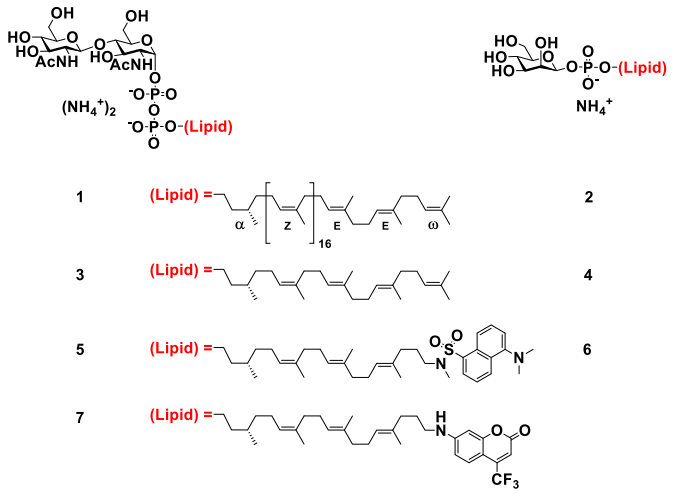


Fig. 1. Chemical structure of natural and synthetic LLOs and donors.

donor **4** (Fig. 1) [7,16,17,20]. These compounds readily dissolve into polar organic solvents or water and are well accepted as substrates by the corresponding enzymes in *in vitro* assays which use purified enzymes in detergent or reconstituted in lipid nanodiscs.

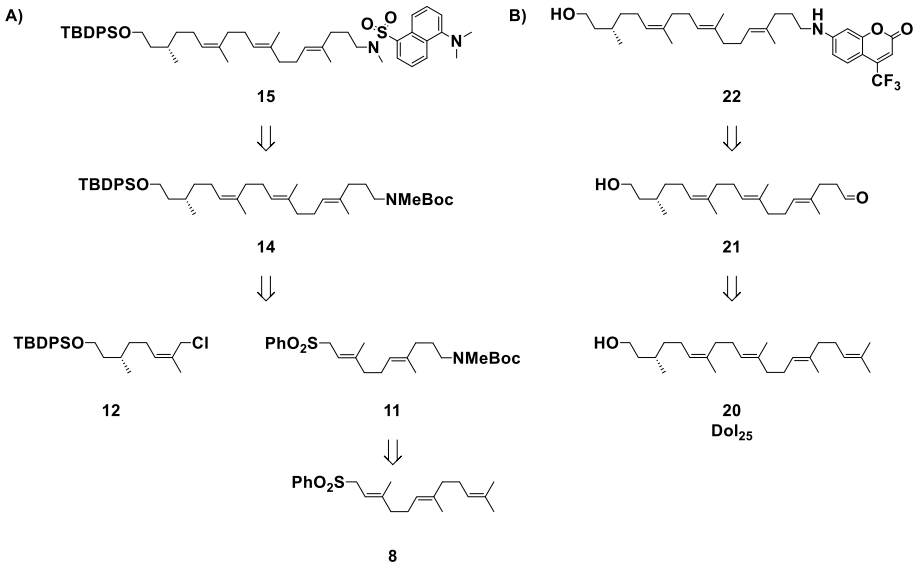
Due to the absence of strong UV absorptions, difficulties in staining on TLC, as well as instability towards raised temperatures, basic and lightly acidic conditions, handling and purification of synthetic LLOs like **3** is very challenging. With a view to obtaining probes that would be easier to manipulate, we set out to investigate whether the enzymes of the eukaryotic protein *N*-glycosylation pathway, in particular the ALG enzymes involved in LLO synthesis and OST enzymes involved in protein glycosyl transfer, might accept LLOs bearing a fluorophore. Although the synthesis of labelled LLOs would be longer than that of a plain dolichol analogue, we expected the fluorophore to facilitate detection and therefore analysis and purification considerably. Furthermore, fluorescence labelled LLOs might offer interesting opportunities to track substrate turnovers in test systems. Previous reports have shown that 2-aminopyridine and 1-aminonaphtalene labelled Dol-P are accepted by Dol-P-Man synthase [21]. The synthesis of a Man-P-Citronellol derivative bearing a nitrobenzofurazane fluorophore was also reported, designed to investigate MPD scramblase [22]. Herein we report the synthesis of the dansyl labelled LLO **5** and mannosyl donor **6**, and of the coumarin labelled LLO **7** (Fig. 1), as well as their *in vitro* characterisation showing that these fluorescence-labelled substrates are accepted with comparable efficiency to non-labelled substrate by several enzymes of the *N*-glycosylation pathway separately or in concert [23,24].

2. Results

2.1. Probe design and retrosynthetic analysis

The fact that synthetic LLOs bearing a Dol_{20} lipid with only four isoprene units were also accepted as substrates by OSTs suggested to us that the fifth isoprene unit could be substituted with a fluorophore without compromising enzyme activity [17]. Dansyl and 7-amino-4-trifluoromethylcoumarin were selected as fluorophores on account of their relatively small size and ability to enter membranes and exhibit fluorescence there [23,24]. Linking the dansyl group via a methylamine offered an interesting option to partially mimic the terminal isoprene unit while removing a polar NH group. Unfortunately, this option could not be realized in the case of the amino-coumarin fluorophore.

We designed the approach to the protected dansyl labelled dolichol **15** following the previously published route for the parent Dol_{25} [17]. Lipid **15** would be obtained by deprotection and dansylation of the Boc-protected precursor **14** resulting from the alkylation of phenyl sulfone **11** with allyl chloride **12**, derived from (*S*)-citronellol, and desulfonylation. Critically, phenyl sulfone **11** bearing the protected methylamine handle could be obtained by regioselective oxidation of the terminal double bond of the farnesol derived phenyl sulfone **8** using the van Tamelen procedure and reductive amination (Scheme 1, route A) [25]. We later discovered conditions to carry out the van Tamelen procedure on the acetyl protected form of Dol_{25} **20** to form aldehyde **21** in good yields [26], providing an alternative approach which we exemplified for the synthesis of a 7-amino-4-trifluoromethylcoumarin labelled lipid **22** (Scheme 1, route B).



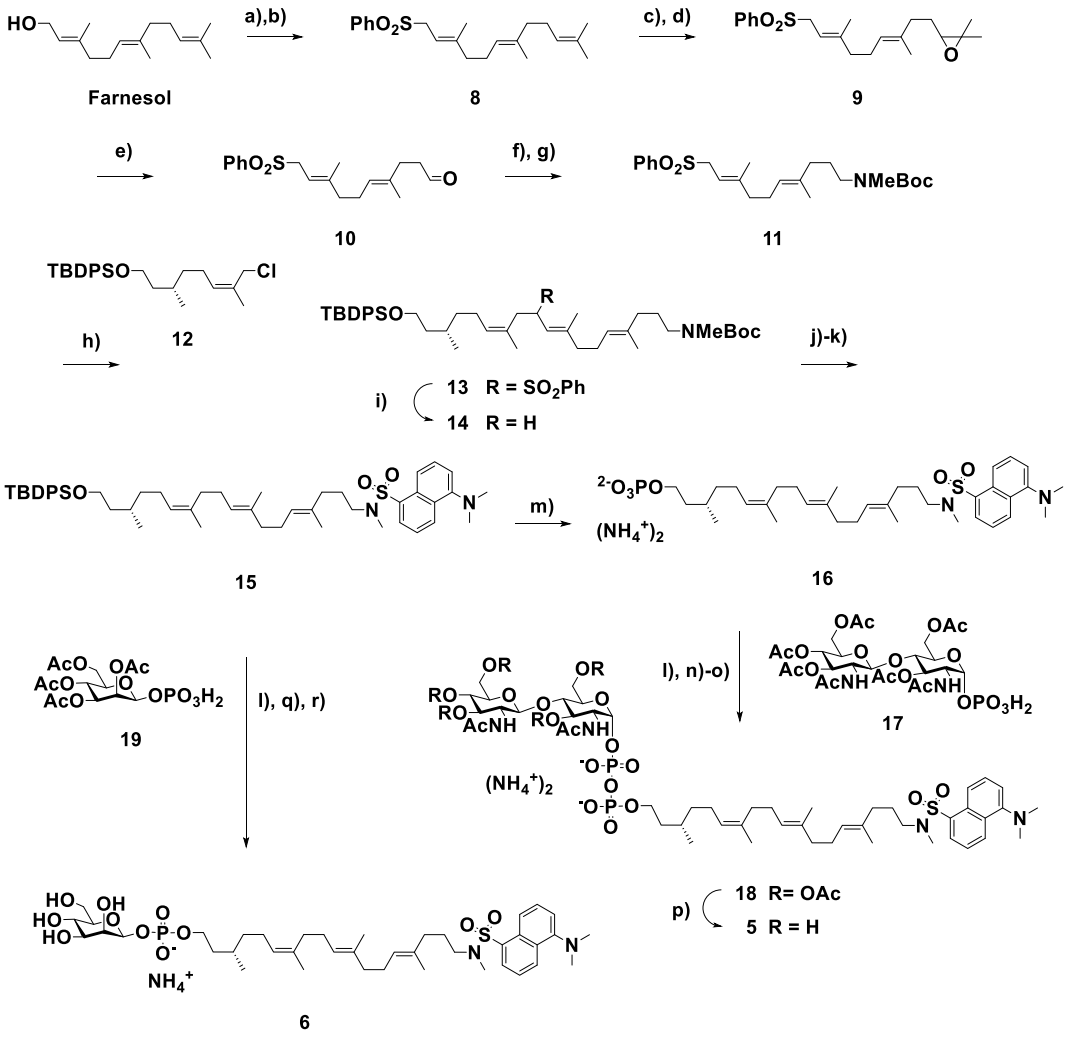
Scheme 1. Retrosynthetic analysis for fluorescence labelled dolichol lipids.

2.2. Synthesis

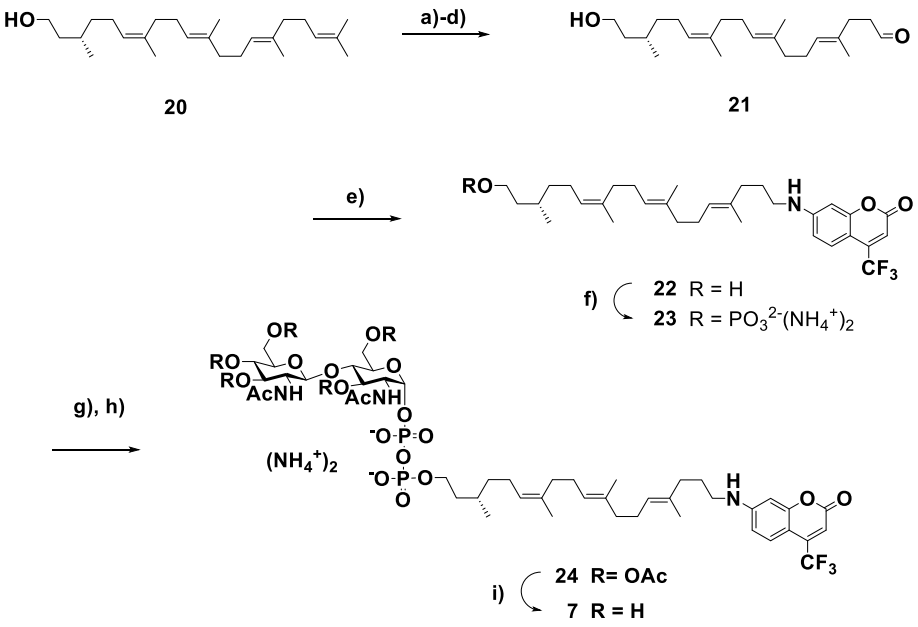
The synthesis of the fluorescent lipid **15** started by converting farnesol to the phenylsulfone **8** as described previously (Scheme 2) [17]. To oxidise the terminal olefin in **8**, we applied an optimised set of conditions for the van Tamelen procedure [25,26]. By saturating the organic solution with water, the formation of the bromohydrin from NBS and water is directed towards the terminal double bond, presumably because of the formation of lipid aggregates [27]. Subsequent treatment of the bromohydrin with K_2CO_3 in methanol yielded epoxide **9** in 51% yield. Epoxide **9** was then subjected to hydrolysis and oxidative cleavage with H_5IO_6 to afford aldehyde **10** in 77% yield [28]. Reductive amination of **10** using methylamine in ethanol and $NaBH_4$ followed by Boc protection gave access to the protected amine **11** in 69% yield over two steps. Deprotonation of the phenylsulfone in **11** with *n*-BuLi followed by alkylation with allyl chloride **12** then gave the full-length lipid **13**, which was subjected to Pd mediated desulfonylation with $LiBHEt_3$ to afford the orthogonally protected lipid **14**, isolated in 80% yield over two steps [17]. Removal of the Boc group using dry $ZnBr_2$ in CH_2Cl_2, used to avoid double bond isomerization [29], followed by treatment of the crude amine with dansyl chloride in pyridine, finally furnished the protected fluorescent lipid **15** in 95% yield over two steps (see Scheme 2).

Similar to the procedure reported earlier for the unlabelled Dol_{25} LLO **3** [17], deprotection and activation of **15** with CCl_3CN and displacement with $Bu_4NH_2PO_4$ furnished the fluorescent lipid phosphate **16** in 63% yield. Lipid **16** was then activated with carbonyldiimidazole (CDI) and coupled with chitobiose-α-phosphoric acid **17** in DMF over 4 days at ambient temperature to afford the protected, fluorescent LLO **18** in 25% yield. Ammonolysis of the *O*-acetyl groups in **18** provided the fluorescent LLO **5**, which was isolated in 48% yield after purification by reverse phase column chromatography. On the other hand, desilylation of lipid **15** and coupling of the resulting alcohol with mannose-β-phosphoric acid pentaacetate **19**, prepared as described in literature [7], followed by ammonolysis of the acetyl groups and purification by preparative reversed-phase HPLC, provided the dansyl-labelled mannosyl donor **6**. In both syntheses, the low detection threshold of the dansyl fluorophore both in solution and on TLC greatly simplified handling and purification in the late stages.

LLO **7** bearing the coumarin fluorophore was prepared in a four step sequence starting from the known, previously synthesized lipid Dol_{25} (**20**) [17]. Acetylation of the primary alcohol of **20**



Scheme 2. Conditions: a) PPh_3, CBr_4, CH_2Cl_2, rt, 4 h; b) $PhSO_2Na$, DMF, rt, 16 h, 80% over 2 steps; c) NBS, tBuOH, H_2O, rt, 1 h; d) K_2CO_3, MeOH, rt, 16 h, 51%; e) H_5IO_6, THF, rt, 2 h, 77%; f) $MeNH_2$, EtOH, rt, 1 h then $NaBH_4$, 0 °C, 75 min; g) Boc_2O, CH_2Cl_2, rt, 16 h, 69% over 2 steps; h) BuLi, THF, −78 °C, 2 h, then **12**, THF, −78 °C, 2 h; i) Pd (dppp)Cl_2, $LiBHEt_3$, THF, −78 °C to rt, 16 h, 80% over 2 steps; j) $ZnBr_2$, CH_2Cl_2, rt, 16 h; k) DNSCl, Pyridine, rt, 2 h, 95% over 2 steps; l) TBAF, THF, rt, 16 h, 84%; m) CCl_3CN, $NBu_4H_2PO_4$, CH_2Cl_2, rt, 8 min, 63%; n) CDI, DMF, rt, 2 h, then MeOH; o) **17**, DMF, rt, 4 days, 25% over 2 steps; p) NH_3, MeOH, rt, 16 h, 48%; q) **15**, **19**, CCl_3CN, pyridine, 65 °C, 17 h; r) NH_3, MeOH, rt, 16 h, 16% over 2 steps.



Scheme 3. Conditions: a) Ac_2O, Pyridine, CH_2Cl_2, rt, 16 h, quant.; b) NBS, tBuOH, H_2O, rt, 90 min; c) K_2CO_3, MeOH, rt, 16 h; d) H_5IO_6, THF, 0 °C, 2 h, 32% over 4 steps; e) 7-amino-4-trifluoromethylcoumarin, $NaBH_3CN$, MeOH, rt, 16 h 34%; f) CCl_3CN, $NBu_4H_2PO_4$ CH_2Cl_2, rt, 8 min, 22%; g) CDI, DMF, rt, 2 h, then MeOH; h) **17**, DMF, rt, 7 days, 22% over 3 steps; i) NH_3, MeOH, rt, 16 h, 8%.

followed by van Tamelen epoxidation of the terminal double bond, hydrolysis and cleavage by H_5IO_6 as described above for sulfone **8**, furnished the desired aldehyde **21**, which was isolated in 32% yield along with 25% of recovered **20** [25,26,28]. Reductive amination of **21** with 7-amino-4-trifluoromethylcoumarin and $NaBH_3CN$ then afforded the fluorescent lipid **22** in 33% yield. Unfortunately, attempts to introduce an *N*-methyl group in **22** to better mimic the terminal isoprene unit as in Dol_{25} were unsuccessful (see Scheme 3).

Activation of the alcohol group in **22** by Cl_3CCN and displacement with $Bu_4NH_2PO_4$ gave the fluorescent lipid phosphate **23** in 22% yield. After activation with CDI, the lipid was stirred with **17** in DMF at ambient temperature for 7 days to give the protected LLO **24** in 22% yield. Ammonolysis of the *O*-acetyl groups then furnished the fluorescent LLO **7** in only 8% yield after manual reverse phase chromatography. This low yield reflected partial degradation of the coumarin fluorophore under the deprotection conditions, which was also observed in the phosphorylation of **22**. The fluorescence characteristics of the dansyl labelled LLO **5** (λ_{ex} = 337 nm, λ_{em} = 525 nm) and coumarin labelled LLO **7** (λ_{ex} = 394 nm, λ_{em} = 499 nm) were found to be in the expected ranges (supporting information Fig. S1).

3. Conclusion

In summary, we demonstrated two synthetic approaches to dolichol derived lipids bearing fluorescent moieties and their incorporation into LLOs by chemical synthesis and their use as enzyme substrates. Dansyl labelled LLO **5** was obtained from modified lipid building blocks which were joined and functionalised to arrive at the LLO in a 24 steps, 16 steps longest linear sequence (LLS) synthesis. The dansylated lipid **15** was also used to prepare the modified mannose-lipid donor **6**. Furthermore, we prepared an aminocoumarin labelled LLO **7** by a second route starting from the Dol_{25} lipid **20** in a synthesis totalling 21 steps, 15 steps LLS from commercial starting materials. This synthesis was unfortunately low yielding due to partial decomposition of the amino-coumarin fluorophore in the last step of the synthesis.

All fluorescent compounds were accepted either as acceptor or donor substrates of key enzymes of the *N*-glycosylation pathway *in vitro*, showing that the increased steric demand and difference in polar surface introduced by the fluorescent moieties do not compromise enzyme activity within a factor of two. The case of the combination of **3** and ScOST is notable, as the fluorescent LLO matched the non-fluorescent reference in turnover rate and number. These experiments show that the enzymes of the eukaryotic *N*-glycosylation pathway tolerate the addition of small fluorophores to the lipid tail of their substrates without preventing their activity *in vitro*. While the present work primarily focused on obtaining synthetic LLOs and glycosyl donors that would be easier to handle and purify and checking their compatibility with enzymes of the protein glycosylation pathway, the fluorescent labels might also be used as markers for biochemical studies. In this context, the aminocoumarin fluorophore displays a more favourable excitation wavelength (λ_{ex} = 394 nm) than the dansyl group (λ_{ex} = 337 nm) and might be more favourable for such investigations.

3.1. Biochemical activity testing

With these three fluorescent compounds in hand, we set out to characterise their properties and applicability in *in vitro* enzymatic assays. For the fluorescent LLOs **5** and **7**, we evaluated their ability to serve (1) as glycan donors for eukaryotic OST enzymes, and (2) as acceptor substrates of glycosyltransferases involved in LLO glycan elongation. To assess their ability as glycan donors, we performed *in vitro* glycosylation assays using the octameric OST (ScOST) from *S. cerevisiae* or the single subunit OST STT3A from *T. brucei*. We observed that both OST enzymes accepted **5** and **7** as glycan donor substrates with turnover rates comparable to those observed when using the non-fluorescent LLO $GlcNAc_2$-PP-Dol_{25}. (Table 1 and Fig. 2A) [17,30].

To assess the ability of **5** and **7** to act as acceptor substrates for glycosyltransferases, we tested them with purified membrane-associated and integral membrane glycosyltransferases involved

Table 1
Turnover rates for TbSTT3A and ScOST with the different LLO analogues.[a]

	LLO analogue	Turnover rate for (pep/min)[a]	
		*Tb*STT3A	*Sc*OST
3	$GlcNAc_2$-PP-Dol_{25}	28.5 ± 2.5	12.0 ± 0.5
5	$GlcNAc_2$-PP-Dol_{22}-Dansyl	13.9 ± 0.9	12.2 ± 1.2
7	$GlcNAc_2$-PP-Dol_{22}-Coumarin	20.9 ± 0.9	6.6 ± 0.5

[a] See the footnote to Fig. 2A for assay details and conditions. Turnover rates in numbers of peptides glycosylated per OST per minute.

in the LLO biosynthesis pathway [7,16] (Fig. 2B). We first elongated the glycan moiety of **5** and **7** using the purified ALG1, ALG2 and ALG11 mannosyltransferases. In eukaryotic cells, these enzymes act on the cytoplasmic side of the endoplasmic reticulum and use GDP-Man as donor substrate, yielding $Man_5GlcNAc_2$-PP-Dol_n. Subsequently, we elongated the LLO glycan using purified integral membrane enzymes that utilize mannosyl-β-dolichylphosphate **4** and its β-glucosyl analogue Glc-β-P-Dol_{25} as donor substrates [7]. First, we added four mannose units using the mannosyltransferases ALG3, ALG9 and ALG12, and **4** (Man-β-P-Dol_{25}) as the glycosyl donor. Finally, we added a terminal α-1,3 glucose using the glucosyltransferase ALG6 and Glc-β-P-Dol_{25}. We observed glycan elongation of both fluorescent LLOs **5** and **7**, to the dodecasaccharide $Glc_1Man_9GlcNAc_2$, indicating that the fluorophore group bound to the lipid carrier does not prevent the activity of the tested glycosyltransferase enzymes (Fig. 2C).

We also evaluated whether the fluorescent mannose donor **6** could be used as donor substrate by the glycosyltransferases ALG3, ALG9 and ALG12 to synthesize the undecasaccharide LLO $Man_9GlcNAc_2$-PP-Dol_{25} from $Man_5GlcNAc_2$-PP-Dol_{25} (Fig. 2B and D). There we observed complete elongation of the LLO, demonstrating that **6** can be used as a mannose donor by the three ER luminal mannosyltransferases (Fig. 2D).

4. Experimental

4.1. General methods

Dry solvents were obtained by filtering over columns of dried aluminium oxide under positive argon pressure. Dry pyridine, DMF, NEt_3, DMPU and toluene were obtained as dry solvents from commercial suppliers and used without fur-ther purification. Other reagents were obtained from commercial suppliers and used as

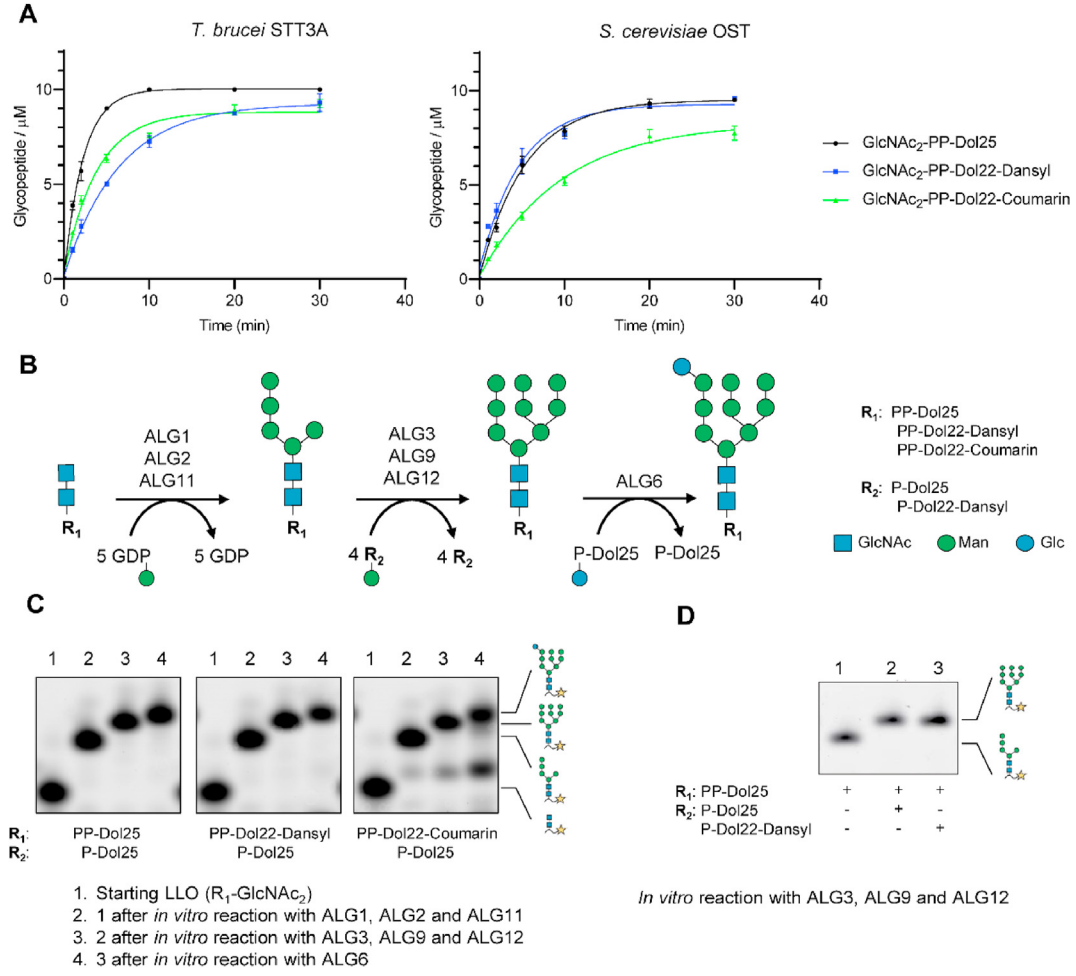


Fig. 2. A. In vitro glycosylation assay, using purified single-subunit OST from Trypanosoma brucei, TbSTT3A (left) or octameric OST complex from *Saccharomyces cerevisiae* (right) to transfer the glycan of **3**, **5** and **7** onto fluorescently labelled peptides. n = 3 replicates, error bars indicate SD. **B.** Schematic of the enzymatic elongation of synthetic LLO analogues **3**, **5** and **7** using purified ALG enzymes. Glycans are represented using the official symbol nomenclature for glycans (SNFG) **C.** Enzymatic elongation of fluorescently labelled LLO analogues. Elongated glycans were transferred to a fluorescently labelled peptide using purified TbSTT3A prior to separation on an SDS-tricine gel. **D.** Enzymatic elongation of $Man_5GlcNAc_2$-PP-Dol_{25} using fluorescently labelled donor substrate **6**. Elongated glycans were transferred to a fluorescently labelled peptide using purified TbSTT3A prior to separation on an SDS-tricine gel.

received unless stated otherwise. Where the use of degassed solvents is stated, the solvents were repeatedly frozen in liquid N_2, the headspace evacuated, and the solvent allowed to thaw under closed vacuum.

TLC plates were obtained from Marcherey-Nagel (ALUGRAM Xtra Sil G/UV254) and SiO_2 for column chromatography was obtained from Sigma- Aldrich (230–400 mesh). For purification over a C18 cartridge, a RediSep Gold HPC18 (5.5 g) cartridge was used.

NMR spectra were recorded on Bruker Avance III HD 300 and 400 or Bruker Avance II 400 spectrometers. Chemical shifts (δ in ppm, J in Hz) were referenced to residual solvent resonances and are reported downfield from $SiMe_4$ [31]. ESI HRMS spectra were obtained in positive or negative ion mode on a ThermoScientific LTQ Orbitrap XL, fitted with an NSI ion source. UV/VIS spectra were recorded on an Agilent Technologies Cray 60 spectrometer.

Removal of solvents under reduced pressure generally denotes the use of a rotary evaporated with a bath temperature of 40 °C, while removal of solvents *in vacuo* was performed at ambient temperature using Schlenk equipment.

Syringe filterers were purchased from Marcherey-Nagel. Unless stated oth-erwise, a 25 mm combination filter of 1.0 μm glass fibre and 0.20 μm PTFE was used (CHROMAFIL Xtra GF/PTFE-20/25).

4.2. Compounds

4.2.1. Phenylsulfonylfarnesol **8**

A solution of Farnesol (53.44 g, 240 mmol) in dry DCM (480 mL, 2 mL per mmol) was cooled at −5 °C, then CBr_4 (85 g, 256 mmol, 1.05 eq.) was added. Subse-quently, a solution of PPh_3 (67.3 g, 256 mmol, 1.06 eq.) in dry DCM (250 mL, ca 1 mLper mmol of PPh_3) was added by dropping funnel over 90 min. The reaction was stirred in the cooling bath for 2 h, then at ambient temperature for an additional hour. Then the solvent was removed under reduced pressure and the residue was added to heptane (2 L), the resulting precipitate filtered off and the solvent removed. This procedure was repeated one more time with heptane (200 mL). The so obtained crude bromide was then dissolved in dry DMF (500 mL, ca 2 mL per mmol) and $PhSO_2Na$ (66 g, 400 mmol, 1.67 eq.) added. The resulting suspension was stirred at ambient temperature for 16 h, then diluted with heptane (2 L). This organic phase was then washed with HCl 1 M (2 × 1 L), saturated aqueous $NaHCO_3$ (1 L), saturated aqueous NaCl (1 L) and dried over Na_2SO_4, filtered over celite and the solvents removed un-der reduced pressure. Flash column chromatography (SiO_2, 15%–40% EtOAc in heptane) afforded the title compound as golden oil (71.3 g, 206 mmol, 86%).

TLC (SiO_2, 25% EtOAc in Heptane) $R_f = 0.48$ ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.89 to 7.84 (m, 2 H, H-*o*-Ph), 7.63 (tt, 1 H, $J = 7.4$, 1.2 Hz, H-*p*-Ph), 7.56 to 7.48 (m, 2 H, H-*m*-Ph), 5.19 (td, 1 H, $J = 7.9$, 0.9 Hz, H-2), 5.11 to 5.00, (m, 2 H, H-6,10), 3.80 (d, 2 H, $J = 7.9$ Hz, H-1), 1.99 (m, 8 H, H-4,5,8,9), 1.67 (s, 3 H, H–Me), 1.59 (s, 3 H, H–Me), 1.57 (s, 3 H, H–Me), 1.30 (s, 3 H, H–Me).

^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 146.5 (s, C-3), 138.8 (s, C-*q* -Ph), 135.8 (s, C-7), 133.6 (s, C-*p*-Ph), 131.4 (s, C-11), 129.1, 128.7 (s, C-*o*-Ph), 124.3 (s, C-10), 123.4 (s, C-6), 110.4 (s, C-3), 56.2 (s, C-1), 39.8 (s, C-4), 26.8 (s, C-8), 26.3 (s, C-5), 25.8 (s, C-9), 17.8 (s, C-11-Me), 16.3 (s, C-12), 16.1 (s, C-7-Me, 3-Me).

4.2.2. (E,E)-3,7,11,11-tetramethyl-1-phenylsulfonyl-10,11-epoxy-undeca- 2,6-dien **9**

To a solution of phenylsulfonylfarnesol **8** (10.42 g, 30.0 mmol, 1 eq.) in ^{t}BuOH (300 mL, 10 mL per mmol) and water (260 mL) was cooled over ice. Then water was added dropwise until slight turbidity was achieved. *Note: this should be approached like a titration, with ^{t}BuOH being added if too much water was added. The mixture should become a clear homogeneous solution upon warming to ambient temperature.* Then NBS (5.89 g, 33 mmol, 1.1 eq.) was added in one portion, the cooling bath removed, and the mixture stirred at ambient tem-perature for 1 h before the organic solvent was removed under reduced pressure. The residue was extracted with Et_2O and the combined organic phases dried over Na_2SO_4, filtered over celite and the solvents removed under reduced pressure. The crude oil was then dissolved in MeOH (90 mL, 3 mL per mmol) and K_2CO_3 (12.5 g, 90 mmol, 3 eq.) added. The resulting suspension was stirred at ambient temperature for 14 h, then the solids were removed by filtration and the solvent removed. This residue was then dissolved in Et_2O and any precipi-tating solids removed by filtration before removing the solvents under reduced pressure. Flash column chromatography (SiO_2, 10%–25% EtOAc in heptane) afforded the compound as clear oil (5.56 g, 15.3 mmol, 51%).

TLC (SiO_2, 25% EtOAc in Heptane) $R_f = 0.33$ TLC (SiO_2, 40% EtOAc in Heptane) $R_f = 0.55$ HRMS (NSI^+) Calculated for $C_{21}H_{30}O_3S$: $[m + H]^+$ $m/z = 363.1988$, found $m/z = 363.1983$ ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.86 (m, 2 H, H-*o*-Ph), 7.63 (m, 1 H, H-*p*-Ph), 7.52 (m, 2 H, H-*m*-Ph), 5.18 (td, 1 H, $J = 7.9$, 1.1 Hz, H-2), 5.09 (m, 1 H, H-6), 3.79 (d, 2 H, $J = 7.9$ Hz, H-1), 2.68 (t, 1 H, $J = 6.2$ Hz, H-10), 2.19 to 2.04 (m, 2 H, H-8), 2.00 (m, 4 H, H-4&5), 1.59 (m, 2 H, H-9), 1.59 (s, 3 H, H-7-Me), 1.30 (d, 3 H, $J = 1.1$ Hz, H-3-Me), 1.28 (s, 3 H, H-*E* −11-Me), 1.25 (s, 3 H, H-*Z* −11-Me).

^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 146.4 (s, C-3), 138.8 (s, C-*q* -Ph), 135.0 (s, C-7), 133.6 (s, C-*p*-Ph), 129.1 (s, C-*m*Ph), 128.6 (s, C-*o*-Ph), 124.1 (s, C-6), 110.5 (s, C-2), 64.2 (s, C-10), 58.4 (s, C-11), 56.2 (s, C-1), 39.7 (s, C-4), 36.4 (s, C-8), 27.5 (s, C-9), 26.3 (s, C-5), 25.0 (s, C-*E* −11-Me), 18.9 (s, C-*Z* −11-Me), 16.3 (s, C-3-Me), 16.1 (s, C-7-Me).

4.2.3. (E,E)-3,7-dimethyl-1-phenylsulfonyl-deca-2,6-diene-10-al **10**

A solution of epoxide **9** (1.10 g, 3 mmol, 1 eq.) in THF (16 mL, 6 mL per mmol) was cooled over ice. Then H_5IO_6 (820 mg, 3.6 mmol, 1.2 eq.) was added in one portion and the mixture stirred and allowed reach ambient temperature. After 2 h, the reaction was diluted with THF (18 mL, 6 mL per mmol) and washed with a mixture of saturated aqueous $NaHCO_3$ (15 mL) and saturated aqueous NaCl (15 mL), then dried over Na_2SO_4, filtered over celite and the solvents removed under reduced pressure. Flash column chromatography (SiO_2, 15%–40% EtOAc in heptane) afforded the compound as clear oil (739 mg, 2.3 mmol, 77%).

TLC (SiO_2, 25% EtOAc in Heptane) $R_f = 0.25$ TLC (SiO_2, 40% EtOAc in Heptane) $R_f = 0.43$.

HRMS (NSI^+) Calculated for $C_{18}H_{24}O_3S$: $[m + H]^+$ $m/z = 321.1519$, found $m/z = 321.1517$.

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 9.73 (t, 1 H, $J = 1.9$ Hz, H-10), 7.86 (m, 2 H, H-*o*-Ph), 7.63 (m, 1 H, H-*p*-Ph), 7.53 (m, 2 H, H-*m*-Ph), 5.17 (dt, 1 H, $J = 8.0$, 1.2 Hz, H-2), 5.08 (m, 1 H, H-6), 3.79 (d, 2 H, $J = 7.9$ Hz, H-1), 2.50 (m, 2 H, H-9), 2.30 (t, 2 H, $J = 7.4$ Hz, H-8), 2.00 (m, 4 H, H-4,5), 1.59 (s, 3 H, H-7-Me), 1.32 (d, 3 H, $J = 1.1$ Hz, H-3-Me).

^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 202.6 (s, C-10), 146.2 (s, C-3), 139.0 (s, C-*q* -Ph), 133.9, 133.7 (s, C-7 & C-*p*-Ph), 129.1 (s, C-*m*-Ph), 128.6 (s, C-*o*- Ph), 124.6 (s, C-6), 110.6 (s, C-2), 56.2 (s, C-1), 42.2 (s, C-9), 39.6 (s, C-4), 31.9 (s, C-8), 26.2 (s, C-5), 16.3 (s, C-3-Me), 16.2 (s, C-7-Me).

4.2.4. (E,E)-10-(N -Methyl-N -tert-butoxycarbonyl-amino)-3,7-dimethyl- 1-phenylsulfonyl-deca-2,6-diene **11**

Aldehyde **10** (464 mg, 1.45 mmol, 1 eq.) was dissolved in a 33% *w/w* solution of NH_2Me in EtOH (5 mL, 3.5 mL per mmol) and

stirred at ambient temperature for 1 h. Then the reaction was cooled over ice and $NaBH_4$ (118 mg, 3 mmol, 2 eq.) added in one portion. After stirring over ice for 75 min, the reaction was diluted with saturated aqueous $NaHCO_3$ (10 mL) and extracted with EtOAc (3 × 10 mL). The organic extracts were joined and dried over Na_2SO_4, filtered over celite and the solvents removed under reduced pressure. The crude mixture was dried *in vacuo*, then dissolved in DCM (7.5 mL, 5 mL per mmol) and cooled over ice. Then Boc_2O (513 mg, 2.3 mmol, 1.5 eq.) was added, the reaction stirred for 16 h and allowed to warm to ambient temperature before the solvents were removed under reduced pressure. Flash column chromatography (SiO_2, 20% EtOAc in heptane) afforded the compound as faintly yellow oil (439 mg, 1.0 mmol, 69%).

TLC (SiO_2, 5% EtOAc in Heptane) R_f = xx HRMS (NSI^+) Calculated for $C_{24}H_{37}NO_4S$: $[m + H]^+$ m/z = 435.2443, found m/z = 458.2322, $[m + H\text{-Boc}]^+$ m/z = 336.1992, found m/z = 336.1983.

^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 7.89 to 7.84 (m, 2 H, H-PG-*o*-Ph), 7.67 to 7.60 (m, 1 H, H-PG-*p*-Ph), 7.57 to 7.49 (m, 2 H, H-PG-*m*-Ph), 5.19 (t, 1 H, J = 7.9 Hz, H-2), 5.06 (m, 1 H, H-6), 3.80 (d, 2 H, J = 7.9 Hz, H-1), 3.15 (t, 2 H, J = 7.0 Hz, H-10), 2.83 (s, 3 H, H—N—Me), 2.00 (m, 4 H, H-4,5), 1.9 (t, 2 H, J = 7.7 Hz, H-8), 1.67 to 1.50 (m, 2 H, H-9), 1.58 (s, 3 H, H-7-Me), 1.45 (s, 9 H, H-PG-Me), 1.31 (s, 3 H, H-3-Me).

^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 155.9 (s, C-Boc-*q*), 146.5 (s, C-3), 138.9 (s, C-*q* -Ph), 135.2 (s, C-7), 133.6 (s, C-*p*-Ph), 129.1 (s, C-*m*-Ph), 128.7 (s, C-*o*-Ph), 123.7 (s, C-6), 110.5 (s, C-2), 79.2 (s, C-Boc-*q* -^{t}Bu), 56.2 (s, C-1), 48.8 (br, C-10), 39.8 (s, C-4), 36.9 (s, C-8), 34.3 (s, C—N—Me), 28.6 (s, C-Boc-^{t}Bu), 26.3 (s, C-5), 16.3 (s, C-3-Me), 16.0 (s, C-7-Me).

4.2.5. (S,Z,E,E)-1-((tert-butyldiphenylsilyl)oxy)-18-(N -methyl-N -tert- butoxycarbonyl-amino)-3,7,11,15-tetramethyl-octadeca-6,10,14- trien ***14*** *(via* ***13****)*

A solution of phenylsulfone **11** (300 mg, 0.7 mmol, 1 eq.) and DMPU (0.4 mL, 3.3 mmol, 5 eq.) in dry THF (1.5 mL, 2 mL per mmol, 1 vol) was cooled over a cooling bath of solid CO_2 and a 1:1 mixture of acetone and ethanol. Then BuLi (2.5 M in hexane, 0.3 mL, 0.75 mmol, 1.1 eq.) was added dropwise and the deep orange solution stirred over the cooling bath. After 2 h at that temperature, a solution of chloride **12**(360 mg, 1.2 eq.) [17] in dry THF (1.5 mL, 1 vol) was added over 5 min, then all solid CO_2 removed from the cooling bath to allow a slow warming up to ambient temperature. After 2 h, the reaction mixture was poured onto saturated aqueous NH_4CL (10 mL) and extracted with heptane (3 × 10 mL). The combined organic extractes were dried over Na_2SO_4, filtered over celite and the solvents removed under reduced pressure. Flash column chro-matography (SiO_2, 25% EtOAc in heptane) afforded the intermediate lipid **13** mixed with an inseparable impurity as clear oil.

TLC (SiO_2, 25% EtOAc in Heptane) R_f = 0.45 HRMS (NSI^+) Calculated for $C_{50}H_{73}NO_5SSi$: $[m + Na]^+$ m/z = 850.4876, found m/z = 850.4864.

A solution of the intermediate lipid (assumed quantitative yield) and $(dppp)PdCl_2$ (40 mg, 0.07 mmol, 0.1 eq.) in dry THF (14 mL, 20 mL per mmol) was cooled over ice, then $LiEt_3BH$ (1.0 M in THF, 3.5 mL, 5 eq.) was added slowly to give a deep red solution which was stirred fro 16 h and allowed to warm to ambient temperature. Then the reaction mixture was poured onto saturated aqueous NH_4Cl (3 mL) and extracted with heptane (2 × 50 mL). The combined organic extracts where dried over Na_2SO_4, filtered over celite and the solvents removed under reduced pressure. Flash column chromatography (SiO_2, 10% EtOAc in heptane) afforded the title compound as faintly yellow oil (386 mg, 0.56 mmol, 80% over two steps).

TLC (SiO_2, 10% EtOAc in Heptane) R_f = 0.42 HRMS (NSI^+) Calculated for $C_{44}H_{69}NO_3Si$: $[M+Na]^+$ m/z = 710.4944, found m/z = 710.4922 ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.68 to 7.66 (m 4 H, H-TBDPS-*o*-Ph), 7.44 to 7.35 (m,6 H, H-TBDPS-*m*&*p*-Ph), 5.12 (m, 3 H, H-6,10,14), 3.69 (m, 2 H, J = 6.0 Hz, H-1), 3.15 (s, br, 2 H, H-18), 2.83 (s, 3 H, H—N—Me), 2.12 to 2.02 (m, 6 H, H— CH_2), 2.02 to 1.88 (m, 6 H, H—CH_2), 1.71 (s, 3 H, H-7-Me), 1.67 to 1.54 (s, 10 H, H-3 & CH_2), 1.45 (s, 9 H, H-Boc-^{t}Bu), 1.41 to 1.20 (m, 2 H, H—CH_2), 1.20 to 1.09 (m, 1 H, H—CH_2), 1.05 (s, 9 H, H-TBDPS-^{t}Bu), 0.83 (d, 3 H, J = 6.3 Hz, H-3-Me).

^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 155.9 (s, C-Boc-*q* -C=O), 135.7 (s, C-TBDPS-*o*-Ph), 135.2, 135.0, 134.3 (s, C-7,11,15), 134.3 (s, C-TBDPS-*q* -Ph), 129.6 (s, C-TBDPS-*p*-Ph), 127.7 (S, C-TBDPS-*m*-Ph), 125.7, 124.7, 124.4 (s, C-6,10,14), 79.2 (s, C-Boc-*q* -^{t}Bu), 62.3 (S, C-1), 48.8 (s, C-18), 39.9, 39.8, 37.6, 36.9 (s, C—CH_2), 34.3 (s, C—N—Me), 32.1, 32.0 (s, C—CH_2) 29.3 (s, C-3), 28.6 (s, C- Boc-^{t}Bu), 27.0 (s, C-TBDPS-^{t}Bu), 26.8, 26.7, 25.4 (s, C—CH_2), 23.6 (s, C-7-Me), 19.7 (s, C-3-Me), 19.4 (s, C-TBDPS-*q* -^{t}Bu), 16.1, 16.0 (s, $C_{-11,15}$ Me).

4.2.6. (S,Z,E,E)-1-((tert-butyldiphenylsilyl)oxy)-18-(N -methyl-N -(5- dimethylamino-1-naphtyl)-sulfonyl)-amino-3,7,11,15-tetramethyl- octadeca-6,10,14-trien ***15***

To a solution of protected amine **14** (35 mg, 0.05 mmol, 1 eq.) in dry DCM (2.5 mL, 50 mL per mmol) was added dry $ZnBr_2$[1] (45 mg, 0.6 mmol, 1.2 eq.) and the suspension stirred at ambient temperature for 16 h. Then the reac-tion mixture was poured onto saturated aqueous $NaHCO_3$ (5 mL), the phases separated, and the aqueous layer extracted with DCM (2 × 5 mL). The combined organic phases were then dried over Na_2SO_4, filtered over celite and the solvents removed under reduced pressure. The so-obtained clear oil was dissolved in dry DCM (2 mL, 40 mL per mmol) and the solution cooled over ice. Then, dry pyridine (0.05 mL, 0.6 mmol, 12 eq.) and DNSCl (13 mg, 0.05 mmol, 1 eq.) were added and the reaction stirred and allowed to warm to ambient temper-ature over 2 h. After removing the solvents under reduced pressure and flash column chromatography (SiO_2, 25% EtOAc in heptane) the title compound as obtained as yellow oil (47 mg, 0.05 mmol, 95% over two steps).

TLC (SiO_2, 25% EtOAc in Heptane) R_f = 0.63 HRMS (NSI^+) Calculated for $C_{51}H_{72}N_2O_3SSi$: $[M+H]^+$ m/z = 821.5106, found m/z = 821.5107.

^{1}H NMR (400 MHz, C_6D_6): δ (ppm) 8.84 (d, 1 H, J = 8.7 Hz, H-DNS-2), 8.43 (d, 1 H, J = 8.5 Hz, H-DNS-6), 8.26 (d, 1 H, J = 7.2 Hz, H-DNS-8), 7.8 (m, 4 H, H-PG-*o*-Ph), 7.34 (t, 1 H, J = 8.1 Hz, H-DNS-3), 7.24 (m, 6 H, H-PG-*m*&*p*-Ph) 7.12 (t, 1 H, J = 7.9 Hz, H-DNS-7), 6.83 (d, 4 H, J = 7.4 Hz, H-DNS-4), 5.31 (m, 1 H, H-10), 5.24 (t, 1 H, J = 6.8 Hz, H-6), 5.14 (t, 1 H, J = 6.4 Hz, H-14), 3.75 (q, 2 H, J = 6.1 Hz, H-1), 3.05 (t, 2 H, J = 7.4 Hz, H-18), 2.54 (s, 3 H, H—N—Me), 2.49 (s, 6 H, H-DNS-NMe_2), 2.21 to 2.04 (m, 10 H, H—CH_2), 1.82 (t, 2 H, J = 7.5 Hz, H-16), 1.74 (s, 3 H, H-7-Me), 1.72 to 1.65 (m, 2 H, H-2a & 3), 1.63 (s, 3 H, H-11-Me), 1.47 (m, 2 H, H-17), 1.45 (m, 3 H, H-15-Me), 1.43 to 1.32 (m, 2 H, H-2b& 4a), 1.19 (s, 10 H, H-PG-^{t}Bu & 4b 0.85 (d, 3 H, J = 6.3 Hz, H-3-Me).

^{13}C NMR (100 MHz, C_6D_6): δ (ppm) 151.9 (s, C-DNS-5), 136.0 (s, C-PG-*o*-Ph), 135.2 (s, C-11), 134.9 (S, C-7), 134.5 (s, C-PG-*q* -Ph), 134.1 (s, C-15), 131.1, 130.7 (s, C-DNS-*q*), 128.0 (C-PG-Ph & C-DNS-3)[2] 126.2 (s, C-6), 125.1 (s, C-14), 124.8 (s, C-10), 123.4 (s, C-DNS-7), 121.0 (s, C-DNS-2), 115.5 (s, C-DNS-4), 62.5 (s, C-1), 49.6 (s, C-18), 45.2 (s, C-DNS-NMe_2), 40.2, 40.1 (s, C-2 & C—CH_2), 37.9 (s, C-4), 36.8 (s, C-16), 34.0 (s, C—N—Me), 32.4 (s, C—CH_2), 29.5 (s, C-3), 27.2 (s, C-PG-^{t}Bu), 27.1 (s, C—CH_2) 26.2 (s, C-17), 25.8 (s, C—CH_2), 23.7 (s, C-7-Me), 19.8 (s, C-3-Me) 19.5 (s, C-PG-*q* -^{t}Bu), 16.2 (s, C-11-Me), 15.9 (s, C-15-Me).

[1] Dried *in vacuo* over a 160 °C oil bath for 12 h, then cooled and stored over P_2O_{10}.
[2] Buried under C_6D_6 solvent peak. Revealed in ^{1}H—^{13}C-HSQC NMR.

4.2.7. Ammonium (S,Z,E,E)-18-(N -methyl-N -(5-dimethylamino-1-naphtyl)- sulfonyl)-amino-3,7,11,15-tetramethyl-octadeca-6,10,14-trien-1- phosphate ***16***

A solution of fluorescent **15** (113 mg, 0.14 mmol, 1 eq.) in dry THF (2 mL, 14 mL per mmol) was cooled over ice before TBAF (1 M in THF, 0.4 mL, 0.4 mmol, 3 eq.) was added dropwise and the reaction stirred for 16 h and allowed to reach ambient temperature. Then the reaction mixture was diluted with DCM, washed with saturated aqueous NH_4Cl and the aqueous layer ex-tracted with DCM until the organic extracts were free of fluorescence. The combined organic extracts were then dried over Na_2SO_4, filtered over celite and the solvents removed under reduced pressure. Flash column chromatogra-phy (SiO_2, 25−40% EtOAc in heptane) furnished the free alcohol as yellow oil (66 mg, 0.11 mmol, 84%).

TLC (SiO_2, 25% EtOAc in Heptane) $R_f = 0.2$ TLC (SiO_2, 40% EtOAc in Heptane) $R_f = 0.45$.

To a solution of the free alcohol (66 mg, 0.11 mmol, 1 eq.) in dry DCM (1.5 mL, 13 mL per mmol) was added $TBAH_2PO_4$ (70 mg, 0.21 mmol, 2 eq.) and stirred until a clear solution was obtained. Then CCl_3CN (70 μl, 0.7 mmol, 6.5 eq.) was added quickly and the reaction stirred at ambient temperature for 8 min before being frozen in liquid N_2 and allowed to thaw *in vacuo* to remove all volatiles. The residue was then dissolved in THF (1 mL, 10 mL per mmol) and an aqueous NH_3 (25%, 0.1 mL, 1 mL per mmol) added. After stirring at ambient temperature for 45 min, MeOH (2 mL) and PhMe (2 mL) were added and the mixture stirred for another 20 min at ambient temperature before filtration over a cotton plug and removal of the solvents under reduced pressure. Gravity column chromatography (SiO_2, EtOAc 4: ^{i}PrOH 2: miliQ water 1) and subsequent cation exchange chromatography (Dowex 50 W X8, NH + form in MeOH) afforded the title compound as yellow oil (50 mg, 0.07 mmol, 63%). HRMS (NSI^-) Calculated for $C_{35}H_{53}N_2O_6PS^-$: $[M+H]^-$ $m/z = 661.3446$, found $m/z = 661.3429$.

^{1}H NMR (400 MHz, MeOD): δ (ppm) 8.58 (d, 1 H, $J = 8.5$ Hz, H-DNS-4), 8.25 (d, 1 H, $J = 8.7$ Hz, H-DNS-8), 8.14 (dd, 1 H, $J = 7.3$, 1.2 Hz, H-DNS-2), 7.59 (t, 1 H, $J = 8.5$ Hz, H-DNS-3), 7.57 (t, 1 H, $J = 8.6$ Hz, H-DBS-7),[3] 7.27 (dd, 1 H, $J = 7.5$, 0.5 Hz, H-DNS-6), 5.11 (m, 2 H, H-6&8), 4.99 (td, 1 H, $J = 6.9$, 1.1 Hz, H-14), 3.93 (qi, 2 H, $J = 6.2$ Hz, H-1), 3.11 (t, 2 H, $J = 7.4$ Hz, H-18), 2.88 (s, 6 H, H-DNS-NMe_2, 2.85 (s, 3 H, H−N−Me), 2.07 to 1.98 (m, 6 H, H-3xCH_2), 1.94 (t, 2 H, $J = 7.4$ Hz, H-12), 1.85 (t, 2 H, $J = 7.3$ Hz, H-16), 1.74 to 1.67 1 H, H-2a), 1.65 (d, 3 H, $J = 1.1$ Hz, H-7-Me), 1.62 (m, 1 H, H-3), 1.59 (m, 3 H, H-11-Me), 1.58 to 1.52 (m, 2 H, H-17), 1.46 (s, 3 H, H-15-Me), 1.45 to 1.31 (m, 2 H, H-2b, 4a), 1.22 to 1.10 (m, 1 H, H-4b), 0.91 (d, 3 H, $J = 6.6$ Hz, H-3-Me).

^{13}C NMR (100 MHz, MeOD): δ (ppm) 153.1 (s, C-DNS-5), 136.0 (s, C-7), 135.9 (s, C-11), 135.7 (s, C-DNS-*q*), 134.8 (s, C-15), 131.5 (s, C-DNS-*q*), 131.4 (s, C-DNS-4), 130.8 (s, C-DNS-2), 129.0 (s, C-DNS-7), 126.6 (s, C-7), 126.0 (s, C-14), 125.5 (s, C-11), 124.4 (s, C-DNS-3), 121.0 (s, C-DNS-8), 116.5 (s, C-DNS-6), 64.9 (d, $^2J_{(P)} = 5.5$ Hz, C-1), 50.4 (s, C-18), 45.8 (s, C-DNS-NMe_2), 40.8 (s, C-12), 38.9 (d, $J = 7.7$ Hz C-2), 38.7 (s, C-4), 37.4 (s, C-16), 34.5 (s, C−N−Me), 32.9 (s, C−CH_2), 30.4 (s, C-3), 27.5, 27.5 (s, C-2xCH_2), 26.7 (s, C-17), 26.4 (s, C−CH_2), 23.7 (s, C-7-Me), 19.8 (s, C-3-Me), 16.1 (S, C-11-Me), 15.9 (s, C-15-Me).

^{31}P NMR (162 MHz, MeOD): δ (ppm) 0.8 (s).

4.2.8. Ammonium P ^{1}-α-chitobiosyl-pentaacetate-P ^{2}-(S,Z,E,E)-18-(N - methyl-N -(5-dimethylamino-1-naphtyl)-sulfonyl)-amino-3,7,11,15- tetramethyl-octadeca-6,10,14-trien-1-yl pyrophosphate ***18***

Phosphate **16** (37 mg, 0.053 mmol, 1 eq.) was dissolved in DMF (0.6 mL, 10 mL per mmol, 1 vol) previously dried over flame-dried 4 °Amolecular sieves. Then CDI (43 mg, 0.265 mmol, 5 eq.) was added and the reaction stirred under light exclusion and at ambient temperature. After 2 h, MeOH (0.1 mL, 2.5 mmol, 50 eq.) was added and the reaction stirred at ambient temperature for an additional 45 min before the solvents were removed *in vacuo*. Then a solution of chitobiose-pentaacetate-α-phosphoric acid **17** (74 mg, 0.1 mmol, 2 eq.) [17] in DMF (0.6 mL, 1 vol) previously dried over flame-dried 4 °Amolecular sieves was added the mixture stirred at ambient temperature and under light exclusion for 4 days. After removing the solvents *n vacuo*, purification by column chromatography (basified SiO_2, EtOAc 4: ^{i}PrOH 2: miliQ water 1) and selection of pure fractions by NSI^--HRMS afforded the title compound as yellow lyophilisate (18 mg, 0.013 mmol, 25% over 2 steps).

TLC (SiO_2, EtOAc 4: ^{i}PrOH 2: miliQ water 1) $R_f = 0.48$.

HRMS (NSI^-) Calculated for $C_{61}H_{90}N_4O$ P S $^-$: $[M+H]^-$ $m/z = 1357.5225$, found $m/z = 1357.5184$, $[M]^{2-}$ $m/z = 678.2576$, found $m/z = 678.2555$.

^{1}H NMR (400 MHz, MeOD): δ (ppm) 8.58 (d, 1 H, $J = 8.5$ Hz, H-DNS-6), 8.32 (d, 1 H, $J = 8.7$ Hz, H-DNS-2), 8.14 (dd, 1 H, $J = 7.3$, 1.2 Hz, H-DNS-8), 7.58 (m, 2 H, H-DNS-7&3), 7.21 (d, 1 H, $J = 7.4$ Hz, H-DNS-4), 5.51 (dd, 1 H, $J = 7.3$, 3.2 Hz, H-Glc1-1), 5.34 (t, 1 H, $J = 9.9$ Hz, H-Glc2-3), 5.25 (t, 1 H, $J = 9.9$ Hz, H-Glc1-3), 5.1 (m, 2 H, H-6/10/14), 5.0 (m, 1 H, H-6/10/14),[4] 4.96 (m, 1 H, H-Glc2-4),[4] 4.82 (m, 1 H, H-Glc2-1),[4] 4.58 (d, 1 H, $J = 11.3$ Hz, H-Glc1-6a), 4.43 (dd, 1 H, $J = 12.5$, 4.0 Hz, H-Glc2-6a), 4.20 (m, 2 H, H-Glc1-2, H-Glc1-5), 4.07 (dd, 1 H, $J = 12.2$, 3.8 Hz, H-Glc1-6b), 4.04 to 3.98 (m, 3 H, H-1, H-Glc2-6b), 3.92 (t, 1 H, $J = 9.5$ Hz, H-Glc1-4), 3.81 (m, 1 H, H-Glc2-5), 3.64 (m, 1 H, H-Glc2-2), 3.11 (t, 2 H, $J = 7.4$ Hz, H-18), 2.88 (s, 6 H, H-DNS-NMe_2), 2.86 (s, 3 H, H−N−Me), 2.11, 2.05, 2.04, 1.98, 1.97, 1.96, 1.90 (s, 7 × 3 H, H−NH/OAc), 2.10 to 1.89 (m, 10 H, H−CH_2), 1.85 (t, 2 H, $J = 7.3$ Hz, H-16), 1.70 (m, 1 H, H-2a), 1.66 (s, 3 H, H-7-Me), 1.59 (d, 3 H, $J = 0.8$ Hz, H-11-Me), 1.56 (m, 3 H, H-3, 17), 1.46 (s, 3 H, H-15-Me), 1.39 to 1.32 (m, 2 H, H-2b, 4a), 1.16 (m, 1 H, H-4b), 0.91 (d, 3 H, $J = 6.6$ Hz, H-3-Me).

^{13}C NMR (100 MHz, MeOD): δ (ppm) 173.9, 173.6, 172.6, 172.2, 172.1, 171.8, 171.3 (s, C−NH/OAc-*q*), 153.2 (s, C-DNS-5), 136.0, 135.9, 135.7, 134.9 (s, C-*q*), 131.5 (s, C-DNS-6), 130.8 (s, C-DNS-8), 129.0 (s, C-DNS-3), 126.6 (s, C-6), 126.0 (s, C-14), 125.5 (s, C-10), 124.4 (s, C-DNS-7), 120.9 (s, C-DNS-2), 116.5 (s, C-DNS-4), 101.8 (s, C-Glc1-1), 95.8 (s, C-Glc2-1), 76.9 (s, C-Glc1-4), 73.6 (s, C-Glc2-3), 72.9 (s, C-Glc1-3), 72.7 (s,C-Glc2-5), 71.0 (s, C-Glc1-5), 69.9 (s, C-Glc2-4), 65.8 (s, C-1), 63.2 (s, C-Glc1-6), 63.0 (s, C-Glc2-6), 56.4 (s, C-Glc2-2), 53.5 (s, C-Glc1-2), 50.4 (s, 18), 45.8 (s, C-DNS-NMe_2), 40.8 (s, C−CH_2), 38.7 (s, C-2), 37.4 (s, C-16), 34.5 (s, C−N−Me), 32.9 (s, C−CH_2), 30.5 (s, C-3), 27.6, 27.5 (s, C−CH_2), 26.7 (s, C-17), 26.4 (s, C−CH_2), 23.8 (s, C-7-Me), 23.0, 22.8, 21.1, 21.0, 20.7, 20.6, 20.5, (s, C−NH/OAc), 19.8 (s, C-3-Me), 16.1 (s, C-11-Me), 15.9 (s, C-15-Me).

^{31}P NMR (162 MHz, MeOD): δ (ppm) −10.4 (d, $J = 16.4$ Hz, P-2), −13.3 (d, $J = 15.7$ Hz, P-1).

4.2.9. Ammonium P ^{1}-α-chitobiosyl-P ^{2}-(S,Z,E,E)-18-(N -methyl-N -(5- dimethylamino-1-naphtyl)-sulfonyl)-amino-3,7,11,15-tetramethyl- octadeca-6,10,14-trien-1-yl pyrophosphate ***5***

To a solution of protected LLO **18** (22 mg, 0.016 mmol, 1 eq.) in MeOH (5 mL) was added aqueous NH_3 (25%, 1.7 mL, 1000 eq., 200

[3] Both peaks are very close and overlap to give the impression of a quartet. With sufficient resolution, the two triplets can be separated.

[4] Hidden under MeOD solvent peak. Identified in ^{1}H−^{13}C HSQC NMR.

eq. per OAc) dropwise and the reaction stirred at ambient temperature and under light exclusion for 16 h before being lyophilised. Purification over a C18 cartridge (20–100% MeOH in 0.1 M NH_4HCO_3) afforded the title compound as yellow oil (5 mg, 0.008 mmol, 48%).

TLC (SiO_2, EtOAc 4: ^{i}PrOH 2: miliQ water 2) $R_f = 0.4$.

HRMS (NSI$^-$) Calculated for $C_{51}H_{80}N_4O$ P S $^-$: $[M+H]^-$ $m/z = 1147.4696$, found $m/z = 1147.4679$, $[M]^{2-}$ $m/z = 573.2303$, found $m/z = 573.2312$.

^{1}H NMR (400 MHz, MeOD): δ (ppm) 8.58 (d, 1 H, $J = 8.5$ Hz, H-DNS-6), 8.32 (d, 1 H, $J = 8.7$ Hz, H-DNS-2), 8.14 (d, 1 H, $J = 7.3$ Hz, H-DNS-8), 7.58 (m, 2 H, H-DNS-7 & 3), 7.27 (d, 1 H, $J = 7.5$ Hz, H-DNS-4), 5.53 (m, 1 H, H-Glc1-1), 5.11 (m, 2 H, H-6 & 10), 4.99 (t, 1 H, $J = 7.1$ Hz, H-14), 4.57 (m, 1 H, H-Glc2-1), 4.00 (m, 4 H, H-Glc1-2 & Glc & H-1), 3.88 (m, 2 H, H-Glc1-3 & Glc-6a), 3.65 (m, 4 H, H-Glc & 3x Glc-6), 3.57, 3.49 (m, 1 H, H-Glc), 3.31 (m, 2 H, H-Glc),[5] 3.11 (t, 2 H, $J = 7.4$ Hz, H-18), 2.88 (s, 6 H, H-DNS-NMe_2), 2.86 (s, 3 H, H–N–Me), 2.09 to 1.97 (m, 8 H, H–CH_2), 2.04, 2.01 (s, 3 H, H-NHAc), 1.95 (m, 2 H, H-12), 1.84 (t, 2 H, $J = 7.3$ Hz, H-16), 1.73 to 1.64 (m, 2 H, H-2a & 3), 1.65 (s, 3 H, H-7-Me), 1.59 (s, 3 H, H-11-Me), 1.55 (t, 2 H, $J = 7.4$ Hz, H-17), 1.42 to 1.32 (m, 2 H, H-2b & 4a), 1.45 (s, 3 H, H-15-Me), 1.29 (m, 2 H, H–CH_2), 1.16 (m, 1 H, H-4b), 0.91 (d, 3 H, $J = 6.5$ Hz, H-3-Me).

^{13}C NMR (100 MHz, MeOD): δ (ppm) 174.1173.7 (s, C-NHAc-*q*), 153.2 (s, C-DNS-5), 136.0, 135.8 (s, C-7, 11), 135.7 (s, C-DNS-*q*), 134.8 (s, C-15), 131.5, 131.4 (s, C-DNS-*q*), 131.5 (s, C-DNS-6), 130.8 (s, C-DNS-8), 129.0 (S, C-DNS-3), 126.7, 126.0, 125.5 (s, C-6, 10, 14), 124.3 (s, C-DNS-7), 120.9 (s, C-DNS-2), 116.5 (S, C-DNS-4), 102.8 (s, C-Glc2-1), 96.0 (s, C-Glc1-1), 81.3 (s, C-Glc2-2), 78.2, 75.9, 73.2, 72.0, 71.3 (s, C-Glc), 65.5 (s, C-1), 62.6, 61.9 (s, C-Glc-6), 57.6 (s, C-Glc), 54.8 (s, C-Glc1-2), 50.4 (s, C-18), 45.8 (s, C-DNS-NMe_2), 40.8 (s, C-12), 38.8 (s, C-2 & 4), 37.4 (s, C-16), 34.5 (s, C–N–Me), 32.9, 30.8 (s, C–CH_2), 30.6 (s, C-3), 27.6, 27.5, 26.7 (s, C–CH_2), 26.4 (s, C-17), 23.8 (s, C-7-Me), 23.1, 23.0 (s, C-NHAc), 19.8 (s, C-3-Me), 16.1 (s, C-11-Me), 15.9 (S, C-15-Me).

^{31}P NMR (162 MHz, MeOD): δ (ppm) −10.2 (d, $J = 19.2$ Hz, P-2), −12.7 (d, $J = 19.1$ Hz, P-1).

UV/Vis (MeOH) $\lambda_{ex} = 337$ nm, $\lambda_{em} = 525$ nm.

4.2.10. Ammonium β-mannopyranosly-(S,Z,E)-18-(N -methyl-N -(5- dimethylamino-1-naphtyl)-sulfonyl)-amino-3,7,11,15-tetramethyl- octadeca-6,10,14-trien-1-yl phosphate **6**

The fluorescent lipid **16** was deprotected as described in the synthesis of **16** above. Then, to a solution of tetra-*O* -acetyl-D-mannopyranosly-β-phosphoric acid **19** (110 mg, 0.26 mmol, 1 eq.) [7] and this fluorescent lipid (329 mg, 0.56 mmol, 2 eq.) in dry pyridine (5 mL, 20 mL per mmol of sugar) was added CCl_3CN (0.25 mL, 2.5 mmol, 10 eq.) and the mixture heated at 65 °C. After stirring at this temperature and under light exclusion for 17 h, the solvents were removed under reduced pressure. Then the residue was dissolved in MeOH (50 mL, 200 mL per mmol), aqueous NH_3 (25%, 15 mL, 200 eq. per OAc) added and the mixture stirred under light exclusion for 16 h before the organic solvents were removed under reduced pressure and the residue lyophilised. Preparative HPLC (65% MeCN in 0.1 M NH_4HCO_3) afforded the title compound as yellow lyophilisate (34 mg, 0.04 mmol, 16%).

TLC (SiO_2, EtOAc 4: ^{i}PrOH 2: miliQ water 2) $R_f = 0.27$.

HRMS (NSI$^-$) Calculated for $C_{41}H_{64}N_2O_{11}PS^-$: $[M+H]^-$ $m/z = 823.3973$, found $m/z = 823.3990$.

^{1}H NMR (400 MHz, MeOD): δ (ppm) 8.58 (d, 1 H, $J = 8.6$ Hz, H-DNS-4), 8.32 (d, 1 H, $J = 8.7$ Hz, H-DNS-8), 8.14 (dd, 1 H, $J = 7.3$, 1.1 Hz, H-DNS-2), 7.59 (t, 1 H, $J = 8.5$ Hz, H-DNS-3), 7.57 (t, 1 H, $J = 8.7$ Hz, H-DNS-7),[6] 7.27 (d, 1 H, $J = 7.4$ Hz, H-DNS-6), 5.15 to 5.06 (m, 3 H, H-Man-1 & H-6&10), 4.99 (t, 1 H, $J = 6.5$ Hz, H-14), 3.95 (qu, 1 H, $^3J_{(P)} = 6.3$ Hz, H-Man-2), 3.91 (d, 1 H, $J = 2.9$ Hz), 3.86 (d, 1 H, $J = 11.5$ Hz, H-Man-6a), 3.72 (dd, 1 H, $J = 11.8$, 5.8 Hz, H-Man-6b), 3.56 (t, 1 H, $J = 9.4$ Hz, H-Man-4), 3.48 (dd, 1 H, $J = 9.4$, 3.0 Hz, H-Man-3), 3.27 (m, 1 H, H-Man-5), 3.11 (t, 2 H, $J = 7.4$ Hz, H-18), 2.88 (s, 6 H, H-DNS-NMe_2), 2.86 (s, 3 H, H–N–Me), 2.09 to 1.98 (m, 8 H, H–CH_2), 1.85 (t, 2 H, $J = 7.4$ Hz, H-16), 1.70 (m, 1 H, H-2a), 1.66 (d, 3 H, $J = 0.7$ Hz, H-7-Me), 1.59 (s, 3 H, H-11-Me), 1.56 (m, 2 H, H-17), 1.46 (s, 3 H, H-15-Me), 1.45 to 1.31 (m, 2 H, H-2b&4a), 1.17 (m, 1 H, H-4b), 0.91 (d, 2 H, $J = 6.5$ Hz, H-3-Me).

^{13}C NMR (100 MHz, MeOD): δ (ppm) 153.2 (s, C-DNS-5), 136.0, 135.9 (s, C- DNS-4a,8a), 135.8, 134.8 (s, C-7,11,15), 131.5 (s, C-DNS-1), 131.4 (s, C-DNS-4), 130.8 (s, C-DNS-2), 129.0 (s, C-DNS-7), 126.6 (s, C-6), 126.0 (S, C-14), 125.5 (s, C-10), 124.3 (s, C-DNS-3), 120.9 (s, C-DNS-8), 116.5 (s, C-DNS-6), 97.1 (d, $^2J_{(P)} = 4.4$ Hz, C-Man-1), 78.9 (s, C-Man-5), 75.0 (s, C-Man-3), 72.9 (d, $^3J_{(P)} = 6.4$ Hz, C-Man-2), 68.2 (s, C-Man-4), 65.3 (d, $^2J_{(P)} = 5.5$ Hz, C-1), 62.9 (s, C-Man-6), 50.4 (s, C-18), 45.8 (s, C-DNS-NMe_2 40.7 (s, C–CH_2), 38.9 (d, $^3J_{(P)} = 7.9$ Hz, C-2), 38.7 (s, C-4), 37.4 (s, C-16), 34.5 (s, C–N–Me), 32.9 (s, C–CH_2), 30.5 (s, C-3), 27.6, 27.5 (s, C–CH_2), 26.7 (s, C-17), 26.4 (s, C–CH_2), 23.7 (s, C-7-Me), 19.8 (s, C-3-Me), 16.1 (s, C-11-Me), 18.9 (s, C-15-Me).

^{31}P NMR (162 MHz, MeOD): δ (ppm) −1.25 (s, P-1).

4.2.11. (S,Z,E)-1-hydroxy-3,7,11,15-tetramethyl-octadeca-6,10,14-trien-18-al ***21***

To as solution of Dol_{25} lipid **20** (1.4 g, 4 mmol) [1,17] in dry DCM (20 mL, 5 mL per mmol) was added NEt_3 (0.7 mL, 5 mmol, 1.25 eq.), acetic anhydride (0.42 mL, 4.4 mmol, 1.1 eq.) and a few chrystals of DMAP (cat.). After stirring for 16 h at ambient temperature, the mixture was diluted with DCM (40 mL) and washed with HCl 1 M (30 mL) and saturated aqueous $NaHCO_3$ (30 mL). Then the organic phase was dried over Na_2SO_4, filtered over celite and the solvents removed under reduced pressure.

The so obtained oil was dissolved in ^{t}BuOH (40 mL, 10 mL per mmol) and water added (ca. 30 mL) before the mixture was cooled over ice. Then water was added more slowly until slight turbidity was achieved. *Note: this should be approached like a titration, with ^{t}BuOH being added if too much water was added. The mixture should become a clear homogeneous solution upon warming to ambient temperature.* Then NBS (712 mg, 4 mmol, 1.0 eq.) was added, the cooling bath removed, and the mixture stirred at ambient temperature for 90 min. After removing the organic solvent under reduced pressure, the residue was extracted with Et_2O (3 × 30 mL) and the combined organic phases dried over Na_2SO_4, filtered over celite and the solvents removed under reduced pressure. The residue was then dissolved in MeOH (40 mL, 10 mL per mmol) and K_2CO_3 added. This suspension was stirred at ambient temperature for 3 h before the solvent was removed under reduced pressure. Then water was added (30 mL), the mixture extracted with Et_2O (3 × 30 mL) and the combined organic phases dried over Na_2SO_4, filtered over celite and the solvents removed under reduced pressure.

The so obtained oil was dissolved in THF (40 mL, 10 mL per mmol) and the mixture cooled over ice. Then H_5IO_6 (1.35 g, 6 mmol, 1.5 eq.) was added and the mixture stirred for 16 h while being allowed to reach ambient temperature. Then saturated aqueous $NaHCO_3$ was added, the mixture extracted with Et_2O and the combined extracts dried over Na_2SO_4, filtered over celite and the solvents removed under reduced pressure. Flash column

[5] Hidden under MeOD solvent peak. Identified in ^{1}H–^{13}C HSQC NMR.

[6] Both peaks are very close and overlap to give the impression of a quartet. With sufficient resolution, the two triplets can be separated.

chromatography afforded the title compound as yellow oil (430 mg, 1.28 mmol, 32%, 42% brsm over 4 steps) along a portion of the starting material (365 mg, 1 mmol, 25%).

^{1}H NMR (400 MHz, C_6D_6): δ (ppm) 9.33 (t, 1 H, $J = 1.8$ Hz, H-18), 5.31 to 5.21 (m, 2 H, H-10& 6), 5.09 (m, 1 H, H-14), 3.50 to 3.38 (m, 2 H, H-1), 2.22 to 2.14 (m, 4 H, H−CH_2), 2.14 to 2.01 (m 8 H, H−CH_2), 2.01 to 1.94 (m, 2 H, H-17), 1.74 (d, 3 H, $J = 1.2$ Hz, H-7-Me), 1.61 (s, 3 H, H-11-Me), 1.58 to 1.44 (m, 1 H, H-2a), 1.41 (s, 3 H, H-15-Me), 1.38 to 1.33 (m, 1 H, H-4a), 1.27 to 1.13 (m, 2 H, H-2a&4a), 0.86 (d, 3 H, $J = 6.6$ Hz, H-3-Me).

^{13}C NMR (100 MHz, C_6D_6): δ (ppm) 200.7 (s, C-18), 135.0, 134.9 (s, C-7, 11), 133.4 (s, C-15), 126.2 (s, C-6), 125.4 (s, C-14), 125.0 (s, C-10), 60.9 (s, C-1), 42.1 (s, C-17), 40.3 (s, C-2), 40.0 (s, C−CH_2), 38.0 (s, C-4), 32.3 (s, C−CH_2), 32.1 (s, C-16), 29.6 (s, C-3), 27.0, 26.9 (s, C−CH_2), 23.7 (s, C-7-Me), 19.8 (s, C-3-Me), 16.1, 16.0 (s, C-11,15-Me).

4.2.12. (S,Z,E,E)-18-N -(2H -4-trifluoromethylbenzopyran-2-one-7-yl)- amino-3,7,11,15-tetramethyl-octadeca-6,10,14-trienol **22**

To a solution of aldehyde **21** (230 mg, 0.68 mmol, 1 eq.) in MeOH (10 mL, 15 mL per mmol) were added KOAc (600 mmol, 0.2 M) and AcOH (0.24 mL, 0.4 M) and 7-amino-4-trifluoromethyl-Coumarin (229 mg, 1 mmol, 1.5 eq.) and the reaction stirred for 5 min. Then $NaCNBH_3$ (65 mg, 1 mmol, 1.5 eq.) was added and the reaction stirred at ambient temperature for 22 h. The reac-tion mixture was then poured onto saturated aqueous $NaHCO_3$ (20 mL) and extracted with EtOAc until the aqueous phase was colourless. The combined organic phases were then dried over Na_2SO_4, filtered over celite and the solvents removed under reduced pressure. Flash column chromatography (SiO_2, 0−5% acetone in DCM) furnished the title compound as bright yellow oil (122 mg, 0.22 mmol, 33%).

TLC (SiO_2, DCM) $R_f = 0.15$ HRMS (NSI^+) Calculated for $C_{32}H_{44}F_3NO_3$: $[M+H]^+$ $m/z = 548.3346$, found $m/z = 548.3330$.

^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ (ppm) 7.42 (dd, 1 H, $J = 8.9$, 2.1 Hz, H-Coum-5), 6.73 (dd, 1 H, $J = 9.0$, 2.3 Hz, H-Coum-6), 6.52 (d, 1 H, $J = 2.3$ Hz, H-Coum-8), 6.36 (s, 1 H, H-Coum-3), 6.31 (s,br, 1 H, H−NH), 5.22 to 5.14 (m, 2 H, H-10&14), 5.12 (m, 1 H, H-6), 3.57 (qi, 2 H, $J = 6.3$ Hz, H-1), 3.37 (t, 1 H, $J = 5.2$ Hz, H−OH), 3.24 (qu, 2 H, $J = 6.6$ Hz, H-18), 2.16 to 1.94 (m, 8 H, H−CH_2), 1.78 (qi, 2 H, $J = 7.3$ Hz, H-17), 1.65 (d, 3 H, $J = 1.1$ Hz, H-7-Me), 1.64 (s, 3 H, H-15-Me), 1.62 (s, 3 H, H-11-Me), 1.60 to 1.51 (m, 2 H, H-2a&3), 1.38 to 1.26 (m, 4 H, H-2b, 4a & CH_2 1.15 (m, 1 H, H-4b), 0.88 (d, 3 H, $J = 6.6$ Hz, H-3-Me).

^{13}C NMR (100 MHz, $(CD_3)_2CO$): δ (ppm) 160.29 (s, C-Coum-2), 158.48 (s, C-Coum-8a), 154.51 (s, C-Coum-7), 141.99 (qu, $^3J_{(F)} = 31.9$ Hz, C-Coum-4), 135.70 (s, C-11), 135.48 (s, C-7), 134.99 (S, C-15), 126.70 (d, $^4J_{(F)} = 2.2$ Hz, C-Coum-5), 126.55 (s, C-6), 125.85 (s, C-14), 125.30 (s, C-10), 123.33 (d, $^1J_{(F)} = 274.9$ Hz, C-Coum-CF_3), 112.30 (s, C-Coum-6), 108.71 (qu, $^3J_{(F)} = 5.8$ Hz, C-Coum-3), 103.44 (s, C-Coum-4a), 98.14 (s, C-Coum-8), 60.74 (s, C-1), 43.37 (S, C-18), 41.06 (s, C-2), 40.52 (S, C−CH_2), 38.57 (s, C-4), 37.67 (s, C-16), 32.66 (s, C−CH_2)), 30.1 (s, C−CH_2 & 3),[7] 27.93 (s, C-17), 27.35, 27.34, 26.17 (s, C−CH_2), 23.74 (s, C-7-Me), 20.08 (S, C-3-Me), 16.20, 16.18 (s, C-11-Me & 15-Me).

^{19}F NMR (376 MHz, $(CD_3)_2CO$): δ (ppm) −65.10 (s, F-CF_3).

4.2.13. Ammonium (S,Z,E,E)-18-N -(2H -4-trifluoromethylbenzopyran- 2-one-7-yl)-amino-3,7,11,15-tetramethyl-octadeca-6,10,14-trien- 1-phosphate **23**

To a solution of fluorescent alcohol **1** (141 mg, 0.26 mmol, 1 eq.) in dry DCM (2 mL, 7.7 mL per mmol) was added $TBAH_2PO_4$ (170 mg, 0.5 mmol, 2 eq.) and stirred at ambient temperature until a clear yellow solution was obtained. Then CCl_3CN (0.17 mL, 6.5 eq.) was added quickly and the now orange so-lution stirred at ambient temperature for 8 min before being frozen in liquid N_2 and allowed to thaw *in vacuo* to remove all volatiles. The residue was then dissolved in THF (2 mL, 7.7 mL per mmol) and an aqueous NH_3 (25%, 0.2 mL, 0.77 mL per mmol) added. After stirring at ambient temperature for 1 h, the resulting suspension was filtered over a plug of glass wool and the solvents re-moved under reduced pressure. Gravity column chromatography (SiO_2, EtOAc 4: ^{i}PrOH 2: miliQ water 1) followed by a precipitation of inorganic materials from MeOH and filtration over a sintered glass frit furnished the title compound as yellow oil (50 mg, 0.056 mmol, 22%).

HRMS (NSI^-) Calculated for $C_{32}H_{43}F_3NO_6P_2^-$: $[M+H]^-$ $m/z = 626.2864$, found $m/z = 626.2861$.

^{1}H NMR (400 MHz, MeOD): δ (ppm) 7.42 (dd, 1 H, $J = 9.0$, 1.9 Hz, H-COUM-5), 6.67 (dd, 1 H, $J = 9.0$, 2.3 Hz, H-COUM-6), 6.47 (d, 1 H, $J = 2.2$ Hz, H-COUM-8), 6.35 (s, 1 H, H-COUM-3), 5.21 to 5.05 (m, 3 H, H-6,10,14), 3.87 (m, 2 H, H-1), 3.15 (m, 2 H, H-18), 2.11 (m, 2 H, H-16), 2.07 to 1.93 (m, 10 H, H−CH_2), 1.75 (m, 2 H, H-17), 1.70 to 1.57 (m, 11 H, H-7,11,15-Me & H-2a, 3), 1.40 (m, 1 H, H-2b), 1.30 (m, 2 H, H−CH_2), 0.89 (d, 3 H, H-3-Me).

^{13}C NMR (100 MHz, MeOD): δ (ppm) 162.4, C-158.8, C-155.2 (s, C-COUM-*q*), 135.9, 135.8, 135.1 (s, C-7,11,15), 126.9 (s, C-COUM-5), 126.6, 126.2, 126.0 (s, C-6,10,14), 112.7 (s, C-COUM-6), 107.8 (s, C-COUM-3), 103.8 (s, C-COUM-*q*), C-98.0 (s, C-COUM-8), 64.5 (s, C-1), 43.4 (s, C-18), 40.7 (s, C−CH_2), 38.7 (s, C-2), 37.9 (s, C-16), 32.8 (s, C-3 & C−CH_2), 28.0 27.6, 27.4, 26.4 (s, C−CH_2), 24.5 24.4 (s, C−Me) 19.85 C-3-Me), 16.1 (s, C−Me).

^{31}P NMR (162 MHz, MeOD): δ (ppm) −0.2 (s, P-1).

^{19}F NMR (376 MHz, MeOD): δ (ppm) −65.8 (s, F-CF_3).

4.2.14. Ammonium P ^{1}-α-chitobiosyl-pentaacetate-P ^{2}-(S,Z,E,E)-18-N - (2H -4-trifluoro-methylbenzopyran-2-one-7-yl)-amino-3,7,11,15- tetramethyl-octadeca-6,10,14-trien-1-yl pyrophosphate **24**

To a solution of fluorescent lipid phosphate **23** (50 mg, 0.056 mmol, 1 eq.) in dry DMF (5 mL, 1 mL per 10 mg) was added CDI (62 mg, 0.380 mmol, 6.8 eq.) and the mixture stirred at ambient temperature and under light exclusion for 2 h. After the addition of dry MeOH (0.5 mL, 30 eq. respective to CDI) and stirring at ambient temperature and under light exclusion for 1 h, the solvents were removed *in vacuo*. Then a solution of chitobiose-pentaacetate-α-phosphoric acid (75 mg, 0.1 mmol, 1.8 eq.) [17] in dry DMF (5 mL, 100 mL per mmol) was added and the reaction stirred at ambient temperature and under light exclusion for 7 days before removing the solvents *in vacuo*. Purification by column chromatography (basified SiO_2, EtOAc 4: ^{i}PrOH 2: miliQ water 1) and subsequent trituration with MeOH to remove inorganic materials afforded the title compound as yellow oil (16.7 mg, 0.012 mmol, 22% over two steps).

TLC (SiO_2, EtOAc 4: ^{i}PrOH 2: miliQ water 2) $R_f = 0.27$.

HRMS (NSI^-) Calculated for $C_{58}H_{80}F_3N_3OP^-$: $[M+H]^-$ $m/z = 1322.4643$, found $m/z = 1322.4637$, $[M]^{2-}$ $m/z = 660.7285$, found $m/z = 660.7282$.

^{1}H NMR (400 MHz, MeOD): δ (ppm) 7.42 (dd, 1 H, $J = 9.0$, 2.0 Hz, H-Coum-5), 6.67 (dd, 1 H, $J = 9.0$, 2.3 Hz, H-Coum-6), 6.47 (d, 1 H, $J = 2.3$ Hz, H-Coum-8), 6.35 (s, 1 H, H-Coum-3), 5.51 (dd, 1 H, $J = 7.1$, 3.2 Hz), H-Glc1-1), 5.38 (t, 1 H, $J = 9.8$ Hz, H-Glc2-3), 5.28 (t, 1 H, $J = 10.4$, 9.4 Hz, H-Glc1-3), 5.17 (t, 1 H, $J = 7.1$ Hz, H-6/10/14), 5.14 to 5.06 (m, 2 H, H-6/10/14), 4) 83 (m, 1 H, H-Glc2-1,[8] 4.58 (d, 1 H, $J = 10.9$ Hz, H-Glc1-6a), 4.43 (dd, 1 H, $J = 12.4$, 4.0 Hz, H-Glc2-6a), 4.23 (m, 2 H, H-Glc1-2 & Glc1-5), 4.11 to 3.98 (m, 4 H, H-Glc1-6b &

[7] Buried under $(CD_3)_2CO$ solvent peak. Revealed in ^{1}H−^{13}C-HSQC NMR.

[8] Hidden under solvent peak. Identified in ^{1}H−^{13}C HSQC NMR.

Glc2-6b & H-1), 3.92 (t, 1 H, $J = 9.6$ Hz, H-Glc1-4), 3.79 (dqu, 1 H, $J = 10.0$, 2.1 Hz, H-Glc2-5), 3.57 (dd, 1 H, $J = 10.1$, 8.6 Hz, H-Glc2-2), 3.15 (t, 1 H, $J = 7.1$ Hz, H-18), 2.15 to 2.07 (m, 4 H, H-16 & CH_2), 2.07 to 1.93 (m, 6 H, H−CH_2), 2.10, 2.05, 2.03, 1.96, 1.89 (s, 3 H, H-OAc/NHAc), 1.98 (s, 6 H, H-OAc/NHAc), 1.80 to 1.67 (m, 3 H, H-17 & 2a), 1.64, 1.63, 1.61 (s, 3 H, H-7,11,15-Me), 1.60 (m, 1 H, H-3), 1.44 (m, 1 H, H-2b), 1.33 (m, 1 H, H-4a), 1.15 (m, 1 H, H-4b) 0.90 (d, 3 H, $J = 6.6$ Hz, H-3-Me).

^{13}C NMR (100 MHz, MeOD): δ (ppm) 174.0, 143.6, 172.6, 172.2, 172.1, 171.8, 171.3 (s, C-OAc/NHAc-*q*), 162.4 (s, C-Coum-2), 158.8 (s, C-Coum-8a), 155.2 (s, C-Coum-7), 143.3 (d, $^{2}J_{(F)} = 32.1$ Hz, C-Coum-4), 135.9, 135.8, 135.2 (s, C-7, 11, 15), 126.9 (s, C-Coum-5), 126.7, 126.2, 125.6 (s, C-6, 10, 14), 123.5 (d, $^{1}J_{(F)} = 274.8$ Hz, C-Coum-CF_3), 112.7 (s, C-Coum-6), 107.8 (qu, $^{3}J_{(F)} = 5.6$ Hz, C-Coum-3), 103.7 (s, C-Coum-4a), 101.6 (s, C-Glc2-1), 98.0 (s, C-Coum-8), 95.8 (s, $^{2}J_{(P)} = 5.5$ Hz, C-Glc1-1), 76.9 (s, C-Glc1-4), 73.5 (s, C-Glc2-3), 73.2 (s, C-Glc1-3), 72.8 (S, C-Glc2-5), 70.9 (s, C-Glc2-4), 70.0 (s, C-Glc1-5), 65.8 (d, $^{2}J_{(P)} = 6.0$ Hz, C-1), 63.3, 63.0 (s, C-Glc1/2−6), 56.7 (s, C-Glc2-2), 53.4 (d, $J = 8.2$ Hz, C-Glc1-2), 43.4 (s, C-18), 40.7 (S, C−CH_2), 38.8, 38.8 (s, C-2 & 4), 37.9 (s, C-16), 32.9 (s, C−CH_2), 30.6 (s, C-3), 28.0 (s, C-17), 27.6, 27.4, 26.4 (s, C−CH2), 23.7 (s, C-7/11/15-Me), 22.9, 22.8 (s, C-NHAc), 21.1, 20.9, 20.7, 20.6, 20.5 (s, C-OAc), 19.8 (s, C-3-Me), 16.1 (s, 2x C-7/11/15-Me).

^{19}F NMR (376 MHz, MeOD): δ (ppm) −65.8 (s, F-CF_3).

^{31}P NMR (162 MHz, MeOD): δ (ppm) −10.5 (d, $J = 20.2$ Hz, P-2), −13.4 (d, $J = 19.9$ Hz, P-1).

4.2.15. Ammonium P ^{1}-α-chitobiosyl-P ^{2}-(S,Z,E,E)-18-N -(2H -4-trifluoro- methylbenzopyran-2-one-7-yl)-amino-3,7,11,15-tetramethyl-octadeca- 6,10,14-trien-1-yl pyrophosphate **7**

To a solution of protected LLO **24** (18 mg, 0.013 mmol, 1 eq.) in MeOH (5 mL) was added aqueous NH_3 (25%, 1 mL, 1000 eq., 200 eq. per OAc) dropwise and the reaction stirred at ambient temperature and under light exclusion for 32 h before being lyophilised. Purification over a C18 cartridge (20−100% MeOH in 0.1 M NH_4HCO_3) afforded the title compound as yellow oil (1 mg, 0.001 mmol, 8%) along with a slightly impure fraction (6 mg, 0.006 mmol, 48%).

HRMS (NSI$^+$) Calculated for $C_{32}H_{44}F_3NO_3$: $[M+H]^+$ $m/z = 548.3346$, found $m/z = 547.3330$.

^{1}H NMR (400 MHz, MeOD): δ (ppm) 7.42 (m, 1 H, H-Coum-5), 6.67 (dd, 1 H, $J = 9.0$, 2.3 Hz, H-COum-6), 6.47 (d, 1 H, $J = 2.3$ Hz, H-Coum-8), 6.35 (s, 1 H, H-Coum-3), 5.52 (m, 1 H, H-Glc1-1), 5.20 to 5.06 (m, 3 H, H-6, 10, 14), 4.55 (d, 1 H, $J = 8.4$ Hz, H-Glc2-1), 4.08 to 3.95 (m, 4 H, H-Glc1-2 & 5 & H-1), 3.92 to 3.80 (m, 3 H, H-Glc1-3 & H-Glc-6), 3.70 to 3.60 (m, 3 H, H-Glc2-2 & H-Glc-6), 3.60 to 3.52 (m, 1 H, H-Glc1-4), 3.45 to 3.38 (m, 1 H, H-Glc2), 3.38 to 3.26 (m, 2 H, H-Glc2),[9] 3.15 (m, 2 H, H-18), 2.11 (m, 4 H, H-16 & H−CH_2), 2.07 to 1.96 (m, 8 H, H−CH_2), 2.04, 2.00 (s, 3 H, H-NHAc), 1.79 to 1.72 (m, 3 H, H-2a & H-17), 1.64, H-1.63, H-1.61 (s, 3 H, H-7,11,15-Me), 1.47 to 1.37 (m, 1 H, H-2b), 1.37 to 1.31 (m, 1 H, H-4a), 1.31 to 1.27 (m, 2 H, H−CH_2), 1.19 to 1.10 (m, 1 H, H-4b), 0.91 (d, 3 H, $J = 6.4$ Hz, H-3-Me).

^{13}C NMR (100 MHz, MeOD): δ (ppm) 174.2, 173.7 (s, C-NHAc-*q*), 162.4, 158.8, C-155.2 (s, C-Coum-*q*), 143.3 (d, $J = 32.3$ 2; F, C-Coum-4), 135.9, 135.8, 135.2 (s, C-7,11,15-*q*), 126.9 (s, C-Coum-5), 126.7, 126.2, 125.6 (s, C-6, 10, 14), 123.5 (d, $J = 274.9$ 1; F, C-CF_3), 112.7 (s, C-Coum-6), 107.7 (d, $J = 5.5$ 3; F, C-Coum-3), 103.7 (s, C-Coum-*q*), 102.8 (s, C-Glc2-1), 98.0 (s, C-Coum-8), 95.9 (d, $J = 5.8$ 2; P, C-Glc1-1), 81.4 (s, C-Glc1-4), 78.2, C-75.9 (s, C-Glc2), 73.2 (s, C-Glc1-5), 72.0 (s, C-Glc2), 71.5 (s, C-Glc1-3), 65.6 (d, $J = 5.8$ 2; P, C-1), 62.6, 61.9 (s, C-Glc-6), 57.6 (s, C-Glc2-2), 54.8 (s, C-Glc1-2), 43.4 (s, C-18), 40.7 (s, C−CH_2), 38.8 (s, C-2 & 4), 37.9 (s, C-16), 32.9 (s, C−CH_2), 30.6 (s, C-3), 28.0 (s, C-17), 27.6, 27.4, 27.2, C-26.4 (s, C−CH_2), 23.7 (s, C-7-Me), 23.1, 23.0 (s, C-NHAc), 19.8 (s, C-3-Me), 16.1 (s, C-11,15-Me).

^{19}F NMR (376 MHz, MeOD): δ (ppm) −65.8 (s, F-CF_3).

^{31}P NMR (162 MHz, MeOD): δ (ppm) −10.2 (d, $J = 19.7$ Hz, P-2), −12.7 (d, $J = 20.2$ Hz, P-1).

UV/Vis (MeOH) $\lambda_{ex} = 394$ nm, $\lambda_{em} = 499$ nm.

4.3. Biochemical assays

4.3.1. OST transfer assay

Reaction mixtures contained 0.1 μM purified *T. brucei* STT3A or *S. cervisiae* OST, 30 μM LLO analog, 10 mM $MnCl_2$ and 10 μM of the fluorescently labelled peptide 5-TAMRA-YANATS for *Sc*OST or 5CF-GSDANYTYTQ for *Tb*STT3A in a final volume of 20 μL. Reactions were incubated at 30 °C 2 μL samples were taken at different time points, and diluted in 10% ACN, 10 mM phosphate buffer pH 7. Samples were analyzed by reverse-phase chromatography using a UPLC Dionex UltiMate 3000 with an Accucore 150-C18 100 × 2.1 mm 2.6 μm column (Thermo Fisher Scientific) as described previously [30]. Peaks for glycopeptide and peptide were integrated using the Software Chromeleon, and the amount of produced glycopeptide was determined for each data point. Turnover rates were calculated by fitting of the data to linear regression using PRISM software.

4.3.2. LLO elongation assay

Elongation of the chemically synthesized LLO-$GlcNAc_2$ analogs into Dol_{25}- PP-$GlcNAc_2$-Man_9-Glc_1, dansyl-Dol_{22}-PP-$GlcNAc_2$-Man_9-Glc_1 and coumarin- Dol_{22}-PP-$GlcNAc_2$-Man_9-Glc_1 was performed enzymatically, according to pub-lished protocols [7,16]. In brief, LLO-chitobiose analogs were incubated with ALG1 enzyme in a buffer containing 150 mM NaCl, 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 2 mM beta-mercaptoethanol, 0.03% DDM and 0.006% CHS. A sugar donor was added to reach 2:1 M ratio (GDP-mannose:LLO), and the re-action let proceed for 1 h at 37 °C. Addition of ALG2 and ALG11 enzymes was preceded by boiling of the ALG1 reaction mix for 5 min and by supple-ment of GDP-mannose (4:1 M ratio compared to LLO). To obtain complete conversion to LLO-Man_5, reactions were incubated at 25 °C overnight. Manno-sylation reactions by ER-luminal ALGs were performed as follows: ALG3 was incubated overnight with previously generated LLO-Man_5 and with the sugar donor Dol-P-Man, in the same buffer described above. After boiling, the reac-tion mix was supplemented with ALG9, ALG12 and additional DolPMan (6:1 M ratio of DolPMan to LLO-Man_6). Reactions were performed at 25 °C overnight. Using the same reaction buffer and temperature, ALG6 was mixed with LLO Man_9 and DolPGlc to produce LLO-Man_9-Glc_1. The set of ALG reactions' products were analyzed by TbSTT3A-catalysed glycosylation of flu- orescent peptides and by separation of the resulting glycopeptides via tricine gels, as previously reported [7,16]. *Tb*STT3A and all ALG enzymes used in the procedure were expressed and purified as described [7,16].

4.3.3. LLO elongation using a fluorescent Man donor

The acceptor substrate, Dol_{25}-PP-$GlcNAc_2Man_5$, synthesized as previously de-scribed.2 Mannosyltransferase expression and purification as well as synthesis of Dol_{25}-PP-$GlcNAc_2Man_9$ were carried out as previously published [2]. The result-ing lipid-linked oligosaccharides were then transferred to a fluorescent peptide for visualization on a tricine gel using STT3A from Trypanosoma brucei [17].

Declaration of competing interest

The authors declare that they have no known competing

[9] Hidden under solvent peak. Identified in ^{1}H−^{13}C-HSQC and ^{1}H−^{1}H-TOCSY NMR.

financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tet.2023.133361.

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