ChemPubSoc Europe

DOI: 10.1002/cbic.201402658

CHEMBIOCHEM Communications

N-Glycosylation with Synthetic Undecaprenyl Pyrophosphate-Linked Oligosaccharide to Oligopeptides by PglB Oligosaccharyltransferase from *Campylobacter jejuni*

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The oligosaccharyltransferase PglB from *Campylobacter jejuni* catalyses the *N*-glycosylation reaction with undecaprenyl-pyrophosphate-linked $Glc_1GalNAc_5Bac_1$ (Und-PP-$Glc_1GalNAc_5Bac_1$). Experiments using chemically synthesized donors coupled to fluorescently tagged peptides confirmed that biosynthetic intermediate Und-PP-Bac_1 and Und-PP-$GalNAc_2Bac_1$ are transferred efficiently to the Asn residue in the consensus sequence (D/E-X′-N-X-T/S, X′,X≠P). The products were analyzed in detail by tandem MS to confirm their chemical structures.

Certain prokaryotes, such as the Gram-negative bacterium *Campylobacter jejuni*,[1] are equipped with an *N*-glycosylation system. The central event in N-glycosylation is the transfer of oligosaccharides preassembled on a lipid carrier, undecaprenyl pyrophosphate (Und-PP), to the asparagine side chain of proteins, catalyzed by PglB oligosaccharyltransferase (OST) in the periplasm.[2] This is strikingly similar to *N*-glycosylation in eukaryotic cells, where the structures of glycans are drastically different.[3] The glycan displayed on the surface of *C. jejuni* cells was shown to play a key role in enteric adhesion to host cells,[4] and is thus of primary importance as a virulence factor.[5] Besides causing gastroenteric disorder, *C. jejuni* infection is suggested to be involved in neuromuscular paralysis, Guillian Barré syndrome (GBS).[6]

In the last decade, the biosynthesis of Und-PP-linked glycans has been studied in some depth (Figure 1). Briefly, cytoplasmic preassembly of bacterial *N*-glycan is catalyzed by consecutive actions of glycosyltransferases PglC, PglA, PglJ, PglH, and PglI. PglC catalyses the transfer of bacillosamine (Bac) from UDP-Bac to undecaprenyl phosphate (Und-P)[7] to afford undecaprenyl-pyrophosphate-linked Bac_1 **1** (Und-PP-Bac_1).[8] Subsequently PglA uses UDP *N*-acetylgalactosamine (UDP-GalNAc) to transfer GalNAc to the lipid-linked Bac.[9] Additional GalNAc residues are incorporated by PglJ and PglH to give trisaccharyl ($GalNAc_2Bac_1$; **2**) and hexasaccharyl ($GalNAc_5Bac_1$) undecaprenyl pyrophosphates, respectively.[2a, 10, 11] Finally, a β-Glc residue is introduced by glucosyltransferase PglI from UDP glucose (UDP-Glc) to complete the construction of the branched heptasaccharide ($Glc_1GalNAc_5Bac_1$; **3**) linked to the lipid carrier.[12] As the catalytic site of the PglB OST is in the periplasm, PglK is proposed to function as a flippase that mediates the transfer of the fully preassembled glycan-linked undecaprenyl pyrophosphate from the cytoplasmic side to the periplasmic side.[13] PglB OST then transfers the heptasaccharide from the lipid carrier to Asn residues of proteins in the periplasm.

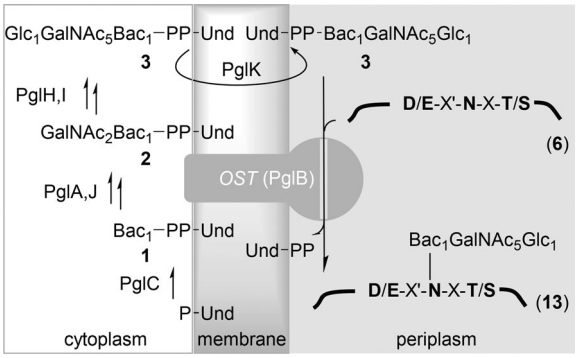

Figure 1. Biosynthesis of N-linked glycoproteins in *C. jejuni*.

Other studies have revealed that PglB OST[2, 11, 14] transfers the glycan on the Und-PP carrier to the consensus sequence (D/E-X′-N-X-T/S; X, X′≠P).[15] PglB OST has been shown to have somewhat relaxed glycan specificity.[16] Chemo-enzymatically assembled Und-PP-linked glycans[9, 10] were used for in vitro glycosylation studies of peptides[17] and proteins.[2a, 18] Structural analysis of PglB has been conducted[19, 20] to understand the catalytic mechanisms of the enzyme, in comparison with eukaryotic OSTs (STT3 subunit).[21–23] In spite of these efforts, the role of the prokaryotic N-glycosylation process in the context of protein quality control have not been clearly demonstrated.

In this study, we conducted in vitro N-glycosylation of peptidic substrates with chemically synthesized donors, in order to explore the chemoenzymatic route to glycoproteins. Synthesis of Bac,[24] preparation of Asp-linked glycan,[25] stereoselective

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201402658.

introduction of α-GalNAc moieties,[26] total synthesis of the heptasaccharide ($Glc_1GalNAc_5Bac_1$),[27] synthesis of Und-OH and Und-P,[28] and linkage formation between glycan and lipid moieties to give Und-PP-glycans[29] have been reported.

Und-PP-glycans (Scheme 1) were prepared as previously reported.[24,26–28] For in vitro glycosylation with a peptide acceptor possessing the consensus sequence, we designed the BODIPY-modified D-F-N-V-T substrate **5** (Ac-D-F-N-V-T-$NH(CH_2CH_2O)_2CH_2CH_2NH$-BODIPY), which contains the *N*-glycosylation site found in found in PEB3, one of antigenic glycoprotein of *C. jejuni*.[2a] Imperiali and co-workers[17b,c] investigated the chemoenzymatic synthesis of glycopeptides with PglB,[17] and identified acceptor peptide sequences such as DQNAT, DVNAT, and DFNVT.[17b] As they used Ac-DFNVT-(*p*NF)-NH_2 **8** (*p*NF = *p*-nitrophenylalanine) for radioactivity assays,[17b,c] we designed **5** as the substrate (incorporating a short ether linker to enhance hydrophilicity; Scheme S1). Synthesis of acceptor **5** commenced with the preparation of mono-Boc-protected linker by a known procedure.[29] The peptide consisted of L-threonine, L-valine, L-asparagine, L-phenylalanine, L-aspartic acid, and finally BODIPY.

Full-length PglB OST from *C. jejuni* was expressed in *Escherichia coli*.[19] The membrane fraction was solubilized by Triton X-100 and used without further purification. Using the membrane fraction, transfer of trisaccharyl ($GalNAc_2Bac_1$) Und-PP (**2**) to peptide acceptor **5** (Scheme 2) was tested.[19] Production of the glycopeptides was monitored by SDS-PAGE,[30] which revealed efficient glycosyl transfer (Figure 2A). The K_m value obtained from the saturation curve was calculated to be (17 ± 6) μM (Figure 2B and C). In the case of the reaction of **1** with **5** for the synthesis of **9**, diffuse bands were observed, possibly due to hydrolysis of peptide moiety by a protease in the membrane fraction (Figures S1 and S2 in the Supporting Information). The results clearly indicated that modification with a larger glycan makes the glycopeptide more resistant against the component in the membrane fraction. However, we were not able to identify the BODIPY-linked glycosylated products **9** and **11** by MALDI-[31] or ESI-TOF MS analysis because of the poor formation of charged ions (Figure S2).

To improve the sensitivity, a TAMRA-modified peptide (Ac-A-D-Y-N-V-T-K-R-K(TAMRA)-OH, **6**) was examined as an alternative substrate, in comparison with its congener (Ac-A-A-Y-N-V-T-K-R-K(TAMRA)-OH, **7**; Ala in place of Asp; Figure S3),[32] as a peptide containing the sequence NVT, was previously reported as an efficient substrate of *Pyrococcus furiosus* AglB.[33] Fluorescent labeling with TAMRA was made at the amino group of the C-terminal lysine, instead of the usual N-terminal amino group.

As expected, glycosyltransfer from **1** to **6** was observed (Figure 3A and B), with kinetic parameters $K_m = (21 \pm 3)$ μM and $V_{max} = (42 \pm 1)$ nM min^{-1} (Figure 3E and G). The identity of the glycopeptide product **10** was confirmed by ESI-TOF MS ($[M+3H]^{3+}$; calcd for $C_{84}H_{120}O_{24}N_{19}$ *m/z* 592.9579, found 592.9688; Figure 4A and B, Table S1A). Similarly, **2** transferred $GalNAc_2Bac_1$ to **6** (Figures 2A, 4C and D), as confirmed by ESI-TOF MS (**12**, $[M+3H]^{3+}$; calcd for $C_{100}H_{146}O_{34}N_{21}$ *m/z* 728.3442, found 728.3493) and tandem MS analysis (Figure 4C and D, Table S1B). In this case, the dependence on the initial concentration of substrate **2** was revealed to be different from that of Bac_1-linked substrate **1** (Figure 3F and G). The kinetic parameters of **2** were obtained from the results under low concentration conditions (< 100 μM), as slight inhibition was observed at higher concentrations. Whereas the K_m value (38 ± 5 μM) obtained by fitting to the Michaelis–Menten equation was nearly twice that for **1**, **2** exhibited four times higher V_{max} (Figures 2C, 3F, and S4). Our results, especially in the case of the trisaccharide-linked substrate (and presumably for larger substrates)[17] might be explained by substrate inhibition[34] ($K_m = 60 \pm 67$ μM, $K_i = 65 \pm 93$ μM for **2** with **5**; $K_m = 75 \pm 28$ μM, $K_i = 130 \pm 53$ μM for **2** with **6**) and/or by the formation of un-reactive assembly of donor **2** possibly as a micelle[35–37] because of its amphiphilic nature.[38]

We also confirmed that our system was able to transfer the natural heptasaccharide from heptasaccharyl undecaprenyl pyrophosphate **3** to **6**, although the conversion was low. Formation of the heptasaccharyl peptide **13** (Figures S3, S5A and B) was confirmed by ESI-TOF MS ($[M+3H]^{3+}$; calcd for $C_{130}H_{195}O_{54}N_{24}$ *m/z* 985.4411, found 985.4308) and tandem MS analysis (Figure 4E and F, Table S1C).

That PglB is able to transfer a mono- as well as a tri-saccharide indicates relaxed specificity. To explore the possibility of constructing a GlcNAc-Asn linkage,[15b,c,39] chemically synthesized chitotetraosyl undecaprenyl pyrophosphate **4** was tested as the glycan donor. However, the corresponding glycopeptide was not detected, even when the reaction was carried out under very high substrate concentrations (Figures S3, S5C, and D).

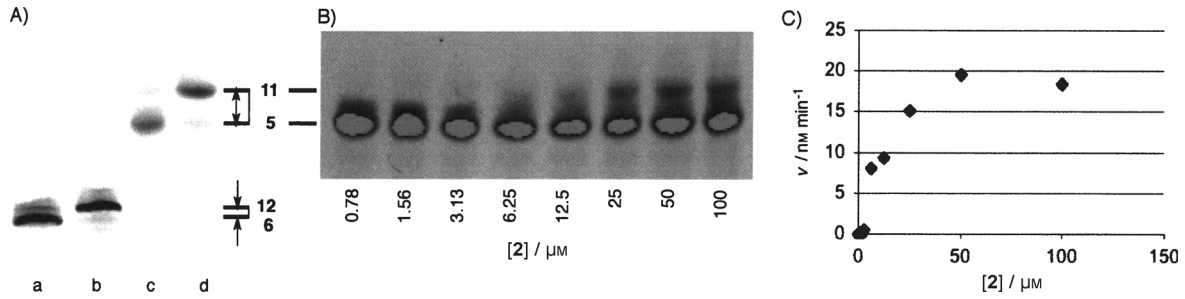
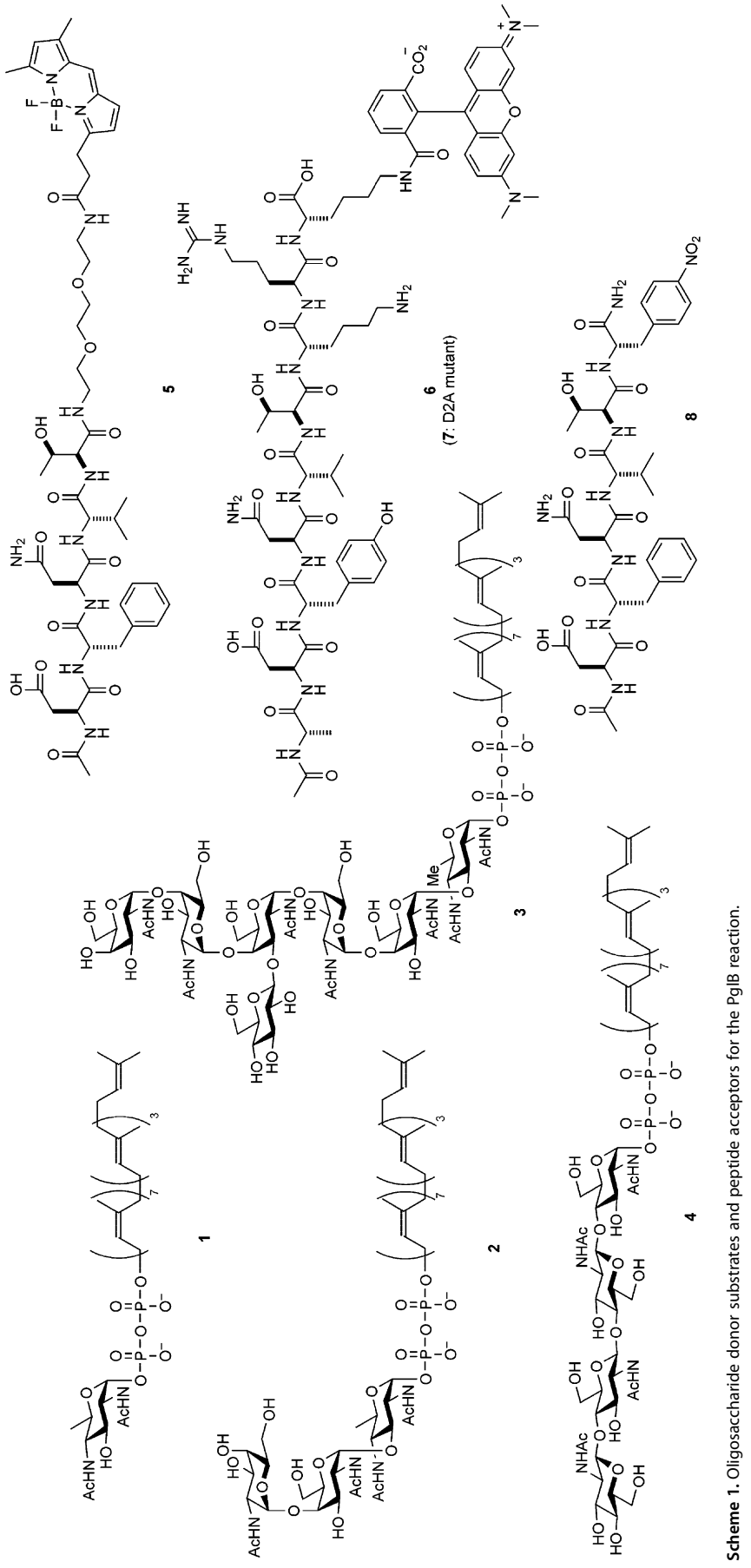
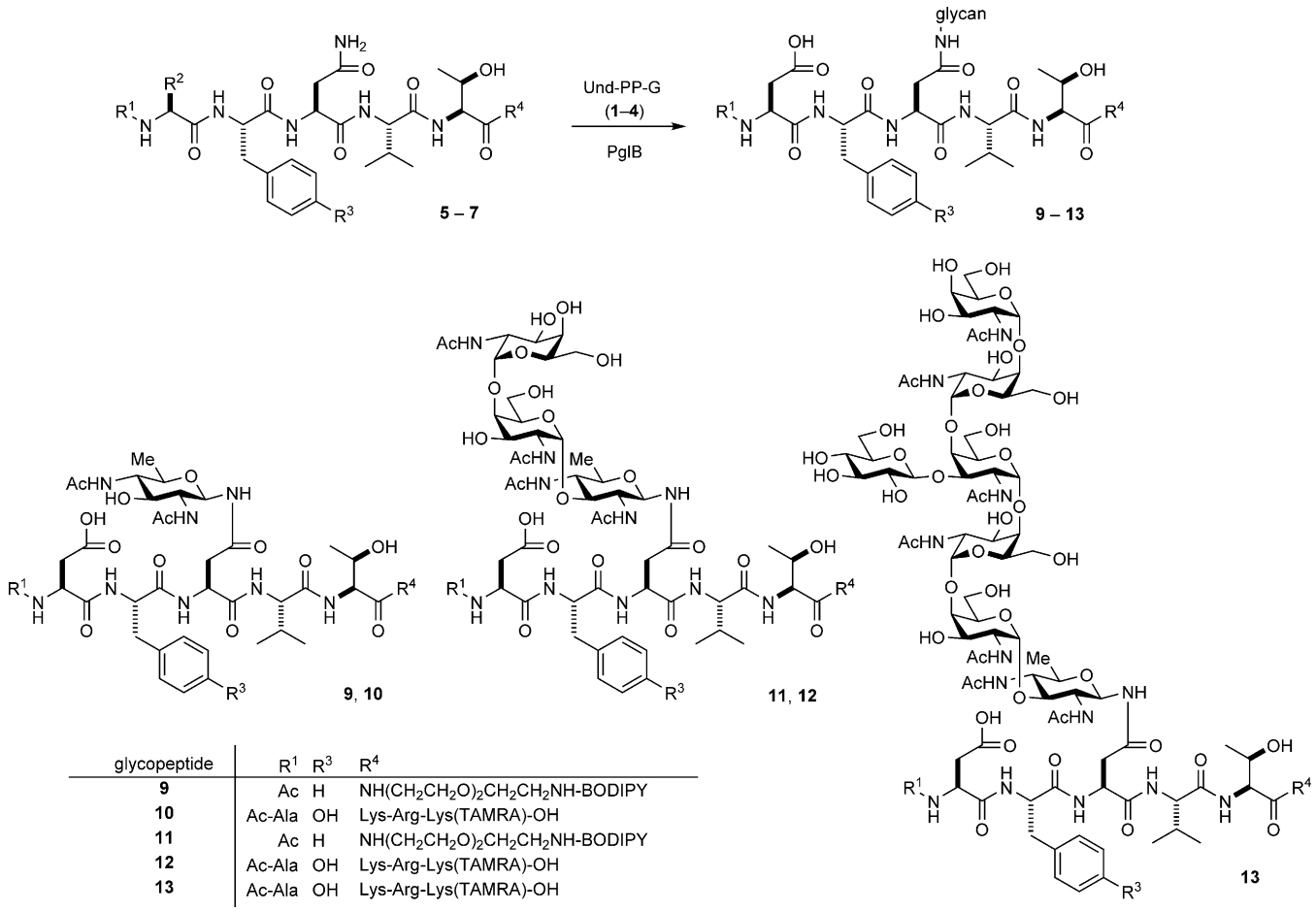


Figure 2. A) PglB OST reaction using synthetic undecaprenyl pyrophosphate-linked oligosaccharide **2** and peptides **5** and **6** at 37 °C: a) **6**, 0 h; b) **6**, 1 h; c) **5**, 0 h; d) **5**, 1 h. B) PglB OST reaction with BODIPY-modified peptide **5** (3 μL, 10 μM) with various concentrations of **2**. C) Saturation curve for the PglB OST reaction of **2** with **5** (data from above). K_m was estimated at 17 μM ± 6 μM by using the Michaelis–Menten fitting equation and 60 μM ± 67 μM by using the substrate inhibition fitting equation. K_i was estimated at 65 μM ± 93 μM.



Scheme 1. Oligosaccharide donor substrates and peptide acceptors for the PglB reaction.



glycopeptide	R^1	R^3	R^4
9	Ac	H	$NH(CH_2CH_2O)_2CH_2CH_2NH$-BODIPY
10	Ac-Ala	OH	Lys-Arg-Lys(TAMRA)-OH
11	Ac	H	$NH(CH_2CH_2O)_2CH_2CH_2NH$-BODIPY
12	Ac-Ala	OH	Lys-Arg-Lys(TAMRA)-OH
13	Ac-Ala	OH	Lys-Arg-Lys(TAMRA)-OH

Scheme 2. Oligosaccharyl transfer reaction by PglB.

In summary, the synthetic monosaccharyl (Bac_1) and trisaccharyl ($GalNAc_2Bac_1$) undecaprenyl pyrophosphates (**1** and **2**) as well as the endogenous full-length heptasaccharyl ($Glc_1GalNAc_5Bac_1$) undecaprenyl pyrophosphate **3** served as donor substrates for the PglB OST from *C. jejuni*, as unambiguously verified by MS analysis of the reaction products of fluorescent-modified peptides containing the consensus sequence (D/E-X′-N-X-T/S). Although the biosynthetic intermediates **1** and **2** (as for **3**) should localize to the cytoplasmic side of the membranes (opposite the side where the OST catalytic site faces in vivo), PglB accepted these intermediate donors with relaxed glycan specificity. Non-natural chitotetraosyl substrate **4**, however, was shown not to be a substrate for PglB. Our results as well as previously reported glycan specificity for the PglB OST suggest that membrane compartmentalization and PglK flippase[13b] are essential for the precise heptasaccharide modification of glycoproteins, by physical separation between the preassembly and transfer stages of *N*-glycan biosynthesis.

Experimental Section

Preparation of *E. coli* membrane fractions containing full-length PglB: The codon-optimized DNA sequence of *C. jejuni* PglB was subcloned into pET-41b (+).[19] Transformed *E. coli* BL21-Gold (DE3) cells (Novagen) were grown at 37 °C in LB medium supplemented with kanamycin (30 μg mL^{-1}). When A_{600} reached 0.7, protein expression was induced by the addition of isopropyl-1-β-D-thiogalactopyranoside (0.5 mM). After 4 h at 37 °C, the cells were harvested by centrifugation. The cell pellet (from 1 L LB culture) was resuspended in Tris·HCl (30 mL, 50 mM, pH 7.5) with NaCl (150 mM) and EDTA-free protease inhibitor cocktail (Roche). After cell disruption by sonication, the lysate was centrifuged (9000 *g*, 10 min, 4 °C), and the supernatant was ultracentrifuged (100000 *g*, 1 h, 4 °C). The pellet was dissolved in Triton buffer (Tris·HCl (20 mL, 50 mM, pH 7.5), NaCl (150 mM), Triton X-100 (1%)). After incubation on ice for 1 h, the solution was ultracentrifuged again. The supernatant was recovered as the detergent-solubilized membrane fraction, and stored at 4 °C.

Preparation of undecaprenyl pyrophosphate-linked glycans 1–4 and peptide substrates 5–7: Undecaprenyl pyrophosphate-linked glycans were obtained by the previously reported chemical synthesis for **1**, **2** and **4**[28] and extraction for **3** reported previously.[19] BODIPY-modified peptide substrate **5** was synthesized as shown in

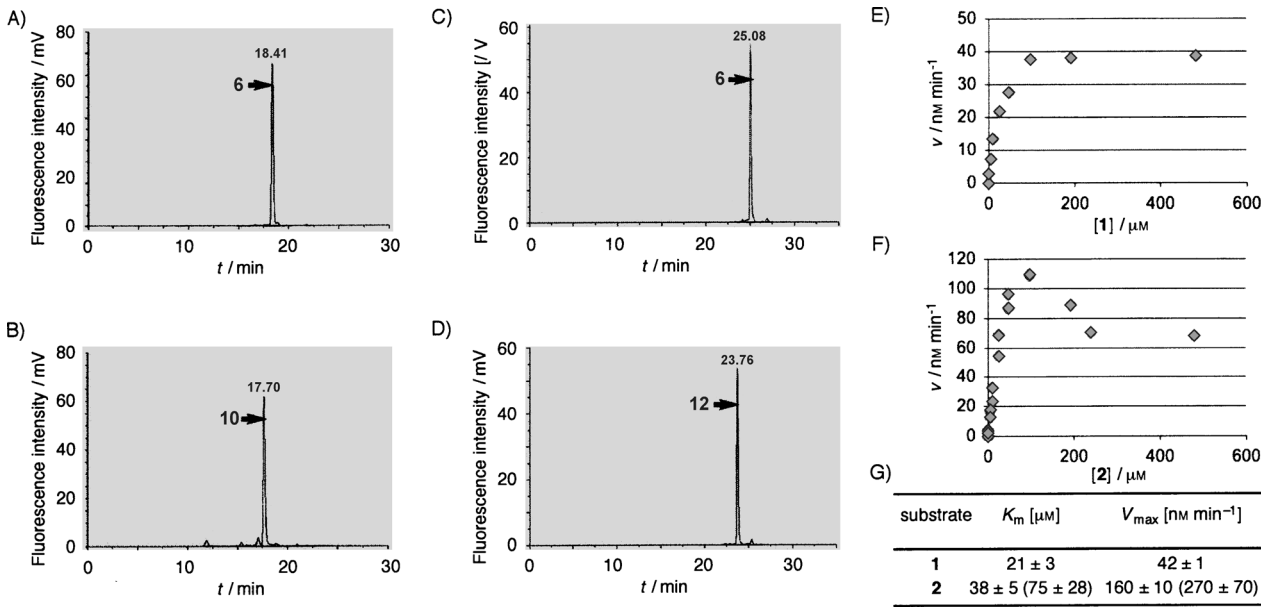


Figure 3. OST reaction using undecaprenyl pyrophosphate-linked oligosaccharides (**1**, **2**) and TAMRA-modified peptide **6**. HPLC profiles: A) **6** only (t_R = 18.41 min); B) **1** with **6** (peak at t_R = 17.70 min is for **10**); C) **6** only (t_R = 25.08 min); D) **2** with **6** (peak at t_R = 23.76 min is for **12**). Saturation curves for *N*-glycosylation of E) **1** and F) **2**. Detailed reaction conditions are shown in general procedure for OST reaction for **1**–**4** with modifications as follows: buffer/PglB, peptide = (B) 40 μL/15 μL, 15 μL of 10 μM, (D) 24 μL/9 μL, 9 μL of 10 μM, and (E,F) 8 μL/1 μL, 3 μL of 100 μM with 0.5 μL of protease inhibiter cocktail (1×). Chromatographic separations were performed using linear gradients: for (A) and (B) 25–42.5% solvent B in solvent A over 25 min, after 5 min of initial isocratic phase of 25% solvent B in solvent A. (C)–(D) 15–50% of solvent B in solvent A over 30 min, after 5 min of initial isocratic phase of 15% solvent B in solvent A. Solvent A: 0.1% (*v*/*v*) TFA in water; solvent B: 0.1% (*v*/*v*) TFA in 80% acetonitrile. G) Kinetic values obtained from (E) and (F) with the Michaelis–Menten fitting equation (values in parentheses obtained from the substrate inhibition fitting equation). For **2** below 200 μM, data were analyzed with the Michaelis–Menten fitting equation. K_i value of **2** were estimated as 130 μM ± 53 μM from substrate inhibition fitting.

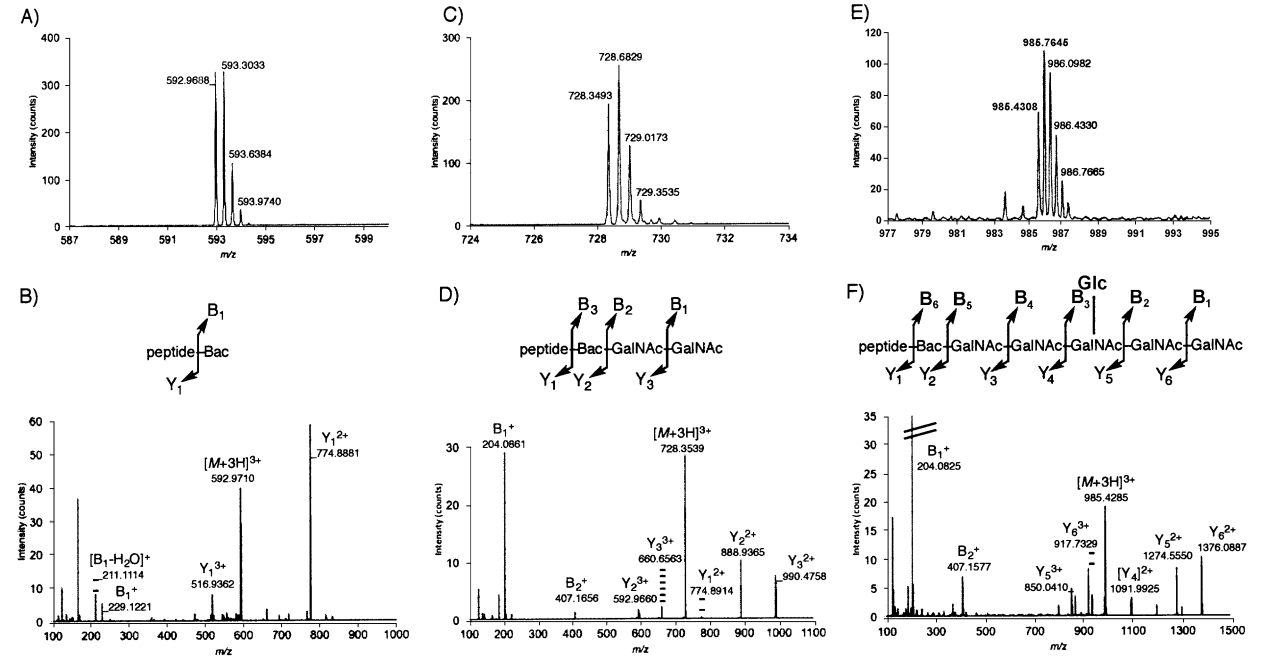


Figure 4. Product identification by MS. A) Detail of trace of HRMS for Bac_1-peptide **10**; B) Tandem MS analysis of **10**; C) Detail of trace of HRMS for $GalNAc_2Bac_1$-peptide **12**; D) Tandem MS analysis of **12**; E) Detail trace of HRMS for $Glc_1GalNAc_5Bac_1$-peptide **13**; F) Tandem MS analysis of **13**. Detailed values are listed in Table S1.

Supporting Information. TAMRA-modified peptide substrates (**6** and its mutant **7**) were custom-synthesized (Hayashi Kasei, Osaka, Japan).[33] 5/6-Carboxytetramethylrhodamine (TAMRA) dye was attached to the side-chain amino group of the C-terminal lysine residue for fluorescent detection (**6**: ESI-TOF MS calcd for $[M+3H]^{3+}$, $C_{74}H_{104}N_{17}O_{20}$, *m*/*z* 516.9209, found 516.9405). The peptide con-

centrations were determined by the absorbance (**5**: ε_{505} = 80000 $M^{-1}cm^{-1}$; **6** and **7**: ε_{555} = 90000 $M^{-1}cm^{-1}$).

General procedure for oligosaccharyltransferase reaction for the synthetic undecaprenyl pyrophosphate-linked glycans (1, 2, and 4) and endogenous 3. Glycan **2** (1.2 mM) in $CHCl_3/CH_3OH/H_2O$ (10:10–20:3, 1.0 μL) was transferred into a 1.5 mL plastic tube and dried with a SpeedVac concentrator (Thermo Scientific). Buffer solution (8 μL, Tris·HCl (50 mM, pH 7.5), dithiothreitol (1 mM), $MnCl_2$ (10 mM), Tween-20 (0.02%)) was added to the tube, and the mixture was sonicated in a bath-type sonicator for 5 min. *E. coli* membrane fractions (1 μL) containing the full-length PglB and accepter peptide substrate **5** (3 μL, 10 μM) were then added, and the reaction solution was incubated for 2 h at 37 or 30 °C. For SDS-PAGE, the reaction was stopped by the addition of SDS sample buffer (5×, 2.4 μL) and heated at 90 °C for 5 min.

Kinetic analysis. The amount of BODIPY-modified glycopeptide product in the reaction solution was measured by SDS-PAGE. PhastSystem HD electrophoresis (high-density gel, GE Healthcare) was carried out the run with a limiting voltage of 250 V, current of 5 mA, and power of 1.5 W was stopped at 180 Vah after 45–50 min, and fluorescent images were taken with an LAS-3000 CCD imaging system (Fujifilm). The amount of TAMRA-modified glycopeptide product in the solution was measured by reversed-phase UPLC (Agilent Technologies) with a ZORBAX RRHD Eclipse Plus C18 threaded column (2.1×50 mm, Agilent Technologies; for Und-PP-glycan **2**) and by reversed-phase HPLC with a COSMOSIL 5C18-AR-II column (4.6×150 mm, Nacalai tesque, Kyoto, Japan; for Und-PP-glycan **1**). Elution was achieved by linear gradients of solvent A (TFA (0.1%)) and solvent B (TFA (0.1%) and acetonitrile (80%, *v/v*)) with a flow rate of 0.8 mL min^{-1} (UPLC) or 1.0 mL min^{-1} (HPLC). The fluorescence intensities of the BODIPY and the TAMRA dyes were measured (excitation 502 and 553 nm, emission 520 and 580 nm, respectively). Acceptor peptide of known concentration was used as an external standard. The Michaelis–Menten constant, K_m, was estimated by nonlinear data fitting in Prism 6 (GraphPad Software) with the equation of substrate inhibition in Equation (1).

$$v = \frac{V_{max} \cdot [S]}{K_m + [S] + [S]^2/K_i} \quad (1)$$

Isolation of the glycopeptide products and identification by MS: For structural elucidation of glycopeptide products, the reaction solution was separated by reversed-phase HPLC with a COSMOSIL 5C18-AR-II column (4.6×150 mm, Nacalai tesque). Elution was achieved by a linear gradient of solvent A and solvent B (above) with a flow rate of 1.0 mL min^{-1} (detailed conditions given in the figure legends). The fractions exhibiting fluorescence peaks were collected and dried with a SpeedVac concentrator. The dried pellets were dissolved in formic acid (0.1%) and methanol (50%) for direct-injection ESI-MS analysis. A QSTAR Elite mass spectrometer (ABSciex, Framingham, MA) was used with MS settings in the positive ion mode (ion spray voltage 5500 V, curtain gas 138 kPa, ion source gas 1 138 kPa, declustering potential 80 V, focusing potential 250 V). Nitrogen was used as the collision gas in MS/MS experiments.

Acknowledgements

We thank Dr. Hiroyuki Koshino (RIKEN Global Research Cluster) and his staff for technical help with ESI-MS. We also thank Akemi Takahashi for her kind technical assistance. This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology (No. 26350966 for AI and No. 24370047 for DK).

Keywords: glycopeptides · oligosaccharides · oligosaccharyltransferases · substrate specificity · undecaprenyl pyrophosphate-linked glycan

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Received: November 13, 2014
Published online on ■■ ■■, 0000

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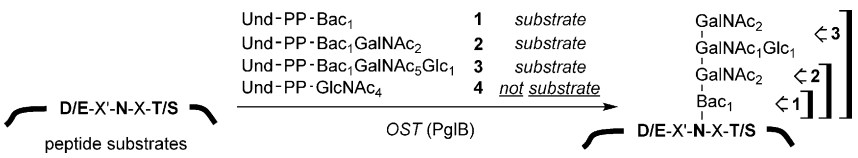
N-Glycosylation with Synthetic Undecaprenyl Pyrophosphate-Linked Oligosaccharide to Oligopeptides by PglB Oligosaccharyltransferase from *Campylobacter jejuni*



Sweetening peptides: The oligosaccharyltransferase PglB from *C. jejuni* can catalyze *N*-glycosylation with chemically synthesized biosynthetic intermediate Bac_1 and $GalNAc_2Bac_1$ derivatives to fluorescent-modified peptide with the consensus sequence (D/E-X′-N-X-T/S, X′,X ≠ P). Although relaxed PglB glycan specificity was shown, a non-natural chitotetraosyl donor substrate was not accepted.