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11-Phenoxyundecyl phosphate as a 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate acceptor in O-antigen repeating unit assembly of *Salmonella arizonae* O:59 ☆

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ABSTRACT

A synthesis of 11-phenoxyundecyl phosphate and its biochemical transformation (using GlcNAc-P transferase from *Salmonella arizonae* O:59 membranes catalysing transfer of GlcNc-phosphate from UDP-GlcNAc on lipid-phosphate) into P^1-11-phenoxyundecyl, P^2-2-acetamido-2-deoxy-α-D-glucopyranosyl diphosphate are described.

1. Introduction

Most bacteria produce and utilise undecaprenyl phosphate and its glycosyl esters as substrate acceptors for glycosyltransferases involved in the assembly of glycopolymers such as peptidoglycans and lipopolysaccharides.

Low content of polyprenyl phosphate in bacterial cells and high lability make its isolation an extremely labour-consuming procedure and significantly hamper its application in biochemical studies.

It was found[2,3] that bacterial polyprenols can efficiently be substituted in biochemical reactions by structurally close plant analogues, both native and synthetic. The accessibility of plant polyprenols and the development of efficient methods of their chemical phosphorylation[2,3] gave an impetus to systematic investigations of the structure–function relationships within polyprenyl phosphates.

Recently, a new type of lipid phosphate (compound **3**) and lipid diphosphate sugar **6** have been synthesised, which contained an 11-phenoxyundecyl group instead of the polyprenyl chain.[4] Diphosphate **6** was shown to be able to replace a polyprenyl-containing substrate in an assay for β-1,3-galactosyltransferase activity from a series of *Escherichia coli* VW187 strains.[4,5] However, the acceptor properties of 11-phenoxyundecyl phosphate **3** itself remained unknown.

Due to this fact, we decided to investigate acceptor properties of phosphate **3** in biosynthesis of **6** catalysed by the corresponding enzyme from *Salmonella arizonae* O:59.

The required 11-phenoxyundecyl dihydrogen phosphate has recently been synthesised[4] by a multistep procedure.

In the present work, an alternative method for the synthesis of phosphate **3** is reported.

The activity of 11-phenoxyundecyl phosphate **3** as a substrate acceptor was tested with crude GlcNAc-P transferase from *S. arizonae* and the structure of the reaction product prepared by biosynthesis was proved by comparison with a synthetic sample of **6b**.

2. Results and discussion

Synthesis of the target compound **3** is presented in Scheme 1.

11-Phenoxyundecanoic acid **1** was prepared as described.[6] Acid **1** was smoothly reduced with diborane generated in situ to give 11-phenoxyundecan-1-ol **2**, which was phosphorylated with $POCl_3$ in the presence of triethylamine in dry THF affording 11-phenoxyundecyl dihydrogen phosphate **3**. As judged by ^1H NMR spectra, compounds **2** and **3** were identical to the corresponding compounds described earlier.[4]

The transformation of phosphate **3** catalysed by transferase from *S. arizonae* was anticipated to afford diphosphate **6**. We undertook

☆ Preliminary communication see Ref. 1.

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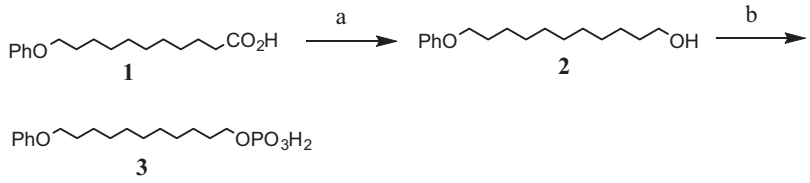


Scheme 1. Reagents and conditions: (a) $BF_3{\cdot}Et_2O/NaBH_4$/THF (anhyd), **2**: yield 83%; (b) (i) $POCl_3$, Et_3N/THF (anhyd), (ii) NaOH (aq), (iii) HCl (aq), **3**: yield 70%.

attempts to synthesise this compound as a reference substance by a procedure different from that described.[4] The idea of our approach[7] was the use of 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate **5** for the coupling with phosphate **3** (Scheme 2).

2-Acetamido-2-deoxy-α-D-glucopyranosyl phosphate **5** was prepared by fusion of 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-α-D-glucopyranose with dioxane diphosphate in vacuo followed by deacetylation, it was isolated by anion-exchange chromatography as described.[8]

Triethylammonium phosphate **3** was treated with an excess of 1,1′-carbonyldiimidazole (CDI) giving 11-phenoxyundecyl phosphoroimidazolidate **4**, which reacted with triethylammonium glycosyl phosphate **5**. An attempt to isolate the target compound **6** by anion-exchange chromatography, as described by us earlier,[7,8] was unsuccessful due to almost no separation of the reaction products.

Previously, we have isolated polyprenyl diphosphate sugars in a pure state by ion-exchange chromatography. The failure to isolate 11-phenoxyundecyl-containing diphosphate **6** by ion-exchange chromatography as individual compound is presumably due to aggregation of the *n*-alkyl part of the molecule.

Therefore, compound **6** (as ammonium salt **6a**) was isolated by reversed-phase chromatography on C18 Sep-Pak cartridge with a yield of 25% using a procedure similar to that employed for purification of a decyl analogue of **6**.[9]

According to our knowledge sodium salts of diphosphate compounds are much more stable than ammonium salts. Therefore, we synthesised **6b** by a procedure described in Ref. 4 and obtained **6** by deacetylation of compound **7**[4] with sodium methoxide in methanol followed by neutralisation with AcOH and isolation of sodium salt **6b** by reversed-phase chromatography on C18 Sep-Pak cartridge. Structures of **6a** and **6b** were confirmed by ^1H and ^{31}P NMR spectra, which were similar to those for pyridinium salt of **6** reported earlier.[4] The structure of **6b** was confirmed by high-resolution ESI mass-spectra (HRESIMS).

It is necessary to underline that sodium salt **6b** could be stored for at least 6 months at +20 °C and is much more stable than pyridinium salt of **6** (cf. Ref. 4).

The O-antigen of *S. arizonae* O:59 has a trisaccharide repeating unit with the following structure:[10] →β-D-Gal*p*-(1→3)-α-L-Fuc*p*NAc-(1→3)-β-D-Glc*p*NAc-(1→.

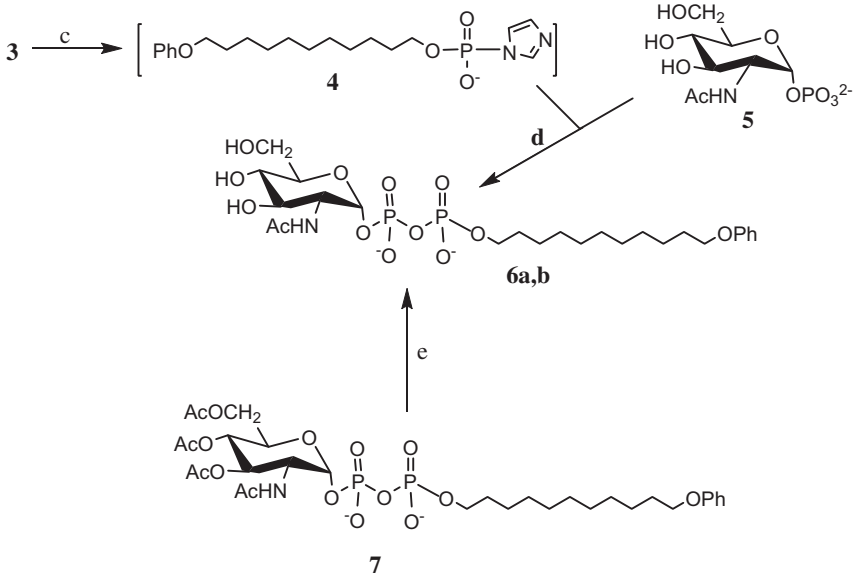
It is known that biochemical synthesis of the O-antigen repeating unit starts with formation of GlcNAcPP Pre from UDP-GlcNAc and polyprenyl phosphate **8** (reaction A). So far, the possibility of substitution of **3** for **8** has not been investigated. Therefore, we assayed 11-phenoxyundecyl phosphate (PhU-P) **3** as a putative acceptor in the initiation reaction of the O-antigen repeating unit assembly catalysed by UDP-GlcNAc:polyprenyl phosphate GlcNAc-phosphotransferase from *S. arizonae* O:59 (Scheme 2; reaction B).

The reaction of compound **3** with UDP-α-D-[^{14}C]-GlcNAc is presented in Scheme 3 (reaction B).

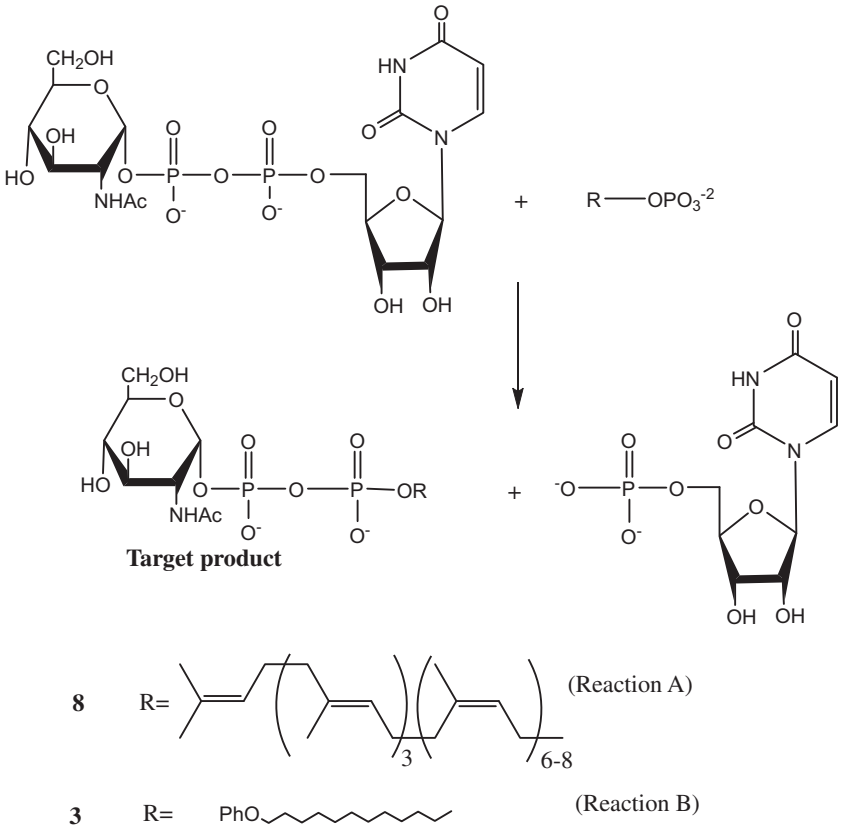
After incubation of **3** with UDP-[^{14}C]GlcNAc and an membrane preparation containing glycosyl transferases (cytoplasmic membranes prepared according procedure described in Ref. 11) from *S. arizonae* O:59, the reaction mixture was deproteinised and applied to a C18 Sep-Pack cartridge. Unconsumed UDP-[^{14}C]GlcNAc was eluted with water and lipid-linked products were eluted with methanol. The eluates were analysed by TLC (Fig. 1).

The UV-absorbing, radioactive zone 1 (Fig. 1) with mobility of the synthetic sample of **6b** was eluted with MeOH and the eluate was analysed by mass spectrometry. High-resolution ESI mass-spectra (HRESIMS): *m/z* 626.2137 (calcd for $C_{25}H_{43}NO_{13}P_2$ $[M-H]^-$ 626.2137).

It has previously been demonstrated that moraprenyl phosphate **8**, the phosphate of C_{50}-C_{60}-polyprenols from mulberry



Scheme 2. Reagents and conditions: (c) (i) Et_3N, (ii) 1,1-CDI/THF, (iii) MeOH; (d) (i) THF/DMSO, (ii) DE-52 (OAc^-)/MeOH–NH_4OAc, (iii) C18 Sep-Pak/MeOH-water, ammonium salt **6a**: yield **25**%; (e) (i) MeONa/MeOH, (ii) AcOH, (iii) C18 Sep-Pak/MeOH–water, sodium salt **6b**: yield 96%.



Scheme 3. Enzymatic synthesis of lipid diphosphate α-D-[^{14}C]GlcNAc from natural (reaction A) and synthetic (reaction B) acceptors.

(*Morus alba*) leaves containing C_{55}-polyprenol as the main component,[3] and plant C_{55}-polyprenyl phosphate **9** can substitute efficiently bacterial undecaprenyl phosphate **10** (Chart 1) in biochemical assays with a number of *Salmonella* strains[3] and other bacteria.[12–14]. The acceptor efficiency of compound **3** was compared with that of compound **8**.

Judging from the radioactivity of the main product (reactions A and B, Scheme 2), the relative efficiency of phosphate **3** as the acceptor of 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate was estimated as ~30% compared with that of moraprenyl phosphate **8** (Scheme 2).

The data obtained show that 11-phenoxyundecyl phosphate **3** can substitute moraprenyl derivative (**8**, Chart 1) as a glycosyl phosphate acceptor in biochemical reactions and is suitable for testing enzymes involved in the assembly of O-antigen repeating units. Sodium salt of **6** (Scheme 1) is much more storage-stable than the pyridinium salt (storage without decomposition controlled by NMR for more than one year at −20 °C and at least 3 months at +20 °C).

3. Experimental

3.1. General methods

^{1}H and ^{31}P NMR spectra were recorded on Bruker AC-200 and Bruker AMX-600 spectrometers. High-resolution ESI mass-spectra (HRESIMS) were recorded on a micrOTOF II (Bruker Daltonics) spectrometer at a capillary potential of +3200 V (negative ion

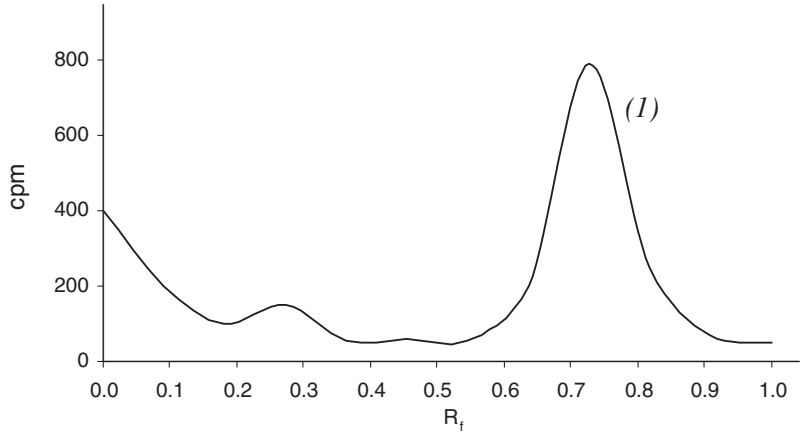
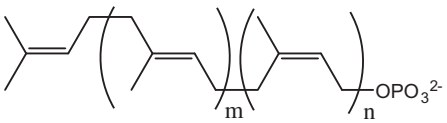


Figure 1. Analysis of products of the enzymatic reaction of **3** with UDP-α-D-GlcNAc by TLC in $CHCl_3$–MeOH–H_2O, 10:10:3 (system B). The mobility of the main radioactive product (1) coincides with that of an authentic synthetic sample of **6a** (R_f 0.73).



8; m=3, n=6-8
9; m=3, n=7
10; m=2, n=8

Chart 1. Chemical structure of polyprenyl phosphates: **8**: moraprenyl phosphate; **9**: plant undecaprenyl phosphate; **10**: bacterial undecaprenyl phosphate.

mode) and −4500 V (positive ion mode) with syringe injection of a methanolic solution of a sample (3 mL/min). Analytical TLC was performed on glass plates with Silica Gel 60 G, Silica Gel 60 F_{254} (E. Merck) or on Silica Gel/TLC cards DC-Alufolien-Kieselgel F_{254} (Fluka) in $CHCl_3$–CH_3OH–H_2O, 60:25:4 (system A), 10:10:3 (system B) or 60:40:5 (system C). Compounds containing PhO-group were visualised on TLC plates in UV light. Phosphoric esters were detected by spraying the plate with a universal reagent for phospholipids[15] followed by heating on a hot plate.

UDP-α-D-[^{14}C]-GlcNAc (261 μCi/μmol) was purchased from Amersham.

The TLC cards were cut in pieces of (0.5 × 1 cm) and the radioactivity was counted with a dioxane scintillator ZhS-50 (Russian Federation) on a Delta-300 liquid scintillation counter (Tracor Analytic). Mini-column reversed-phase chromatographic separations were performed on C18 Sep-Pak cartridges (Waters). Anion-exchange chromatography was performed on a column (1.2 × 12 cm) with DEAE-cellulose DE-52 (OAc^-, Whatman). Reagent grade solvents were dried and distilled before use according to standard procedures.

3.2. Synthesis of 11-phenoxyundecan-1-ol (2)

A solution of $BF_3{\cdot}Et_2O$ (1.31 g, 9.2 mmol) in dry THF (5 mL) was added slowly to a stirred suspension of $NaBH_4$ (0.38 g, 10 mmol) in dry THF (20 mL) at 10 °C in an atmosphere of argon. After 10 min, acid **1**[6] (1.66 g, 6 mmol) was added in portions (of about 0.2 g) over 10 min. The resulting mixture was stirred for 4 h and kept overnight at room temperature, diluted with $MeOBu^t$ (50 mL), washed with water (5 × 10 mL), saturated aqueous $NaHCO_3$ (10 mL), brine (2 × 10 mL), dried with anhydrous Na_2SO_4, and the filtrate was concentrated to dryness. The residue was crystallised from petroleum ether affording alcohol **2** (1.32 g, 83%); white solid; mp 55–57 °C. ^{1}H NMR (200,13 MHz, $CDCl_3$) δ: 1.22–1.88 (m, 18H, $9CH_2$), 3.65 (t, 2H, $J_{1,2}$ 6.5 Hz, H_2C-1), 3.97 (t, 2H, $J_{10,11}$ 6.5 Hz, H_2C-11), 6.86–6.98 (m, 3H, aromatic protons), 7.22–7.35 (m, 2H, aromatic protons) (cf. Ref. 4).

3.3. Synthesis of 11-phenoxyundecyl dihydrogen phosphate (3)

A solution of 11-phenoxyundecan-1-ol (**2**) (0.27 g, 1.02 mmol) in dry THF (5 mL) was added dropwise to a stirred solution of $POCl_3$ (0.34 g, 2.2 mmol) and Et_3N (0.22 g, 2.2 mmol) in dry THF (5 mL) at room temperature under argon. The mixture was stirred for 1 h and added to an intensively agitated mixture of 10% aqueous NaOH (6 mL) and THF (20 mL). The heterogeneous system was stirred vigorously for 3 h at room temperature and THF was evaporated. Pr^iOH (10 mL) was added to the residual aqueous solution and after agitation the system was concentrated in vacuum. The residue was solubilised in H_2O (10 mL) and 10% aqueous HCl (7 mL) was added. The emulsion obtained was extracted with $MeOBu^t$ (30 mL), the organic phase was washed with brine, dried with anhydrous Na_2SO_4 and the filtrate was concentrated to dryness. The residue was crystallised from MeCN affording phosphate **3** (0.24 g, 70%). R_f 0.20 (system A), 0.85 (system B) on glass plates. ^{1}H NMR (200,13 MHz, $CDCl_3$–CD_3OD, 5:1) δ: 1.05–1.75 (m, 18H, $9CH_2$), 3.79–3.90 (m, 4H, CH_2-1, CH_2-11), 6.86–6.98 (m, 2H, aromatic protons), 7.22–7.35 (m, 2H, aromatic protons); ^{31}P NMR (242.94 MHz, CD_3OD) δ: 0.5 (s) (cf. Ref. 4).

3.4. Synthesis of P^1-11-phenoxyundecyl, P^2-2-acetamido-2-deoxy-α-D-glucopyranosyl diphosphate (6)

3.4.1. Ammonium salt (6a)

To phosphate **3** (26 mg, 0.075 mmol), an excess of triethylamine (0.5 mL) and dry toluene (3 mL) were added and the mixture was concentrated to dryness. The residue of triethylammonium salt was dissolved in MeOH (0.1 mL), dry benzene (3 mL) was added and the mixture was lyophilised. Then CDI (98 mg, 0.73 mmol) and dry THF (2 mL) were added under argon and the solution was stirred at room temperature for 2 h. Analysis by TLC (system B) showed the conversion of **3** (R_f 0.85, system B) into 11-phenoxyundecyl phosphoroimidazolidate **4** (R_f 0.90, system B).

MeOH (0.2 mL) was added to the mixture and stirring was continued for 1 h. The solution was concentrated in vacuum and the residue was dissolved in dry THF (0.3 mL) under argon. 2-Acetamido-2-deoxy-α-D-glucopyranosyl phosphate **5**[8] (triethylammonium salt, 40 mg, 0.094 mmol) was dissolved in MeOH (0.1 mL), mixed with dry benzene (3 mL) and lyophilised. The residue was dissolved in dry DMSO (0.4 mL) and the solution was added to the activated lipid phosphate **4**. The reaction mixture was stirred under argon for 48 h at room temperature, diluted with MeOH (25 mL) and applied onto a column with DEAE-cellulose DE-52 (OAc^-). The column was eluted with MeOH (25 mL), 0.05 M NH_4OAc in MeOH (100 mL), 0.08 M NH_4OAc in MeOH (100 mL) and 0.2 M NH_4OAc in MeOH (50 mL) and fractions (6 mL) were collected. Fractions containing compound **6** (TLC: on glass plates R_f 0.10, system A; 0.65, system B; on aluminium cards R_f 0,74, system B) (with admixture of nonseparated by-products) were pooled, concentrated to dryness, MeOH was repeatedly added to, and distilled from, the residue (10 mL × 5) and the residue was dissolved in water (15 mL). Aliquots of the solution (5 mL) were applied on preactivated C18 Sep-Pak cartridge and elution was carried out with water (10 mL), 5% MeOH in water (10 mL) and 10% MeOH in water (10 mL). The latter eluate contained **6** (TLC). Portions of the eluate containing chromatographically homogeneous **6** were collected and concentrated in vacuum to dryness. The residue was dissolved in dry benzene (5 mL) and lyophilised affording ammonium salt **6a** as a white amorphous solid (11 mg, 25%); R_f 0.10 (system A), 0.65 (system B) on glass plates or 0.74 (system B) on aluminium cards. ^{1}H NMR (600.13 MHz, CD_3OD) δ: 1.30–1.45 (m, 12H, CH_2), 1.51 (m, 2H, CH_2), 1.68 (m, 2H, CH_2), 1.78 (m, 2H, CH_2), 2.07 (s, 3H, CH_3CO), 4.14–3.66 (m, 10 H, H-2, H-3, H-4, H-5, H-6, H-6′, CH_2OP, CH_2OPh), 5.55 (dd, 1H, $J_{1,2}$ 3.0 Hz, $J_{1,P}$ 7.0 Hz, H-1), 6.90 (m, 3H, aromatic protons), 7.26 (m, 2H, aromatic protons); ^{31}P NMR (242.94 MHz, CD_3OD) δ: −10.1 (br, P-2), −12.3 (br, P-1).

3.4.2. Sodium salt (6b)

Compound **7** was synthesised from 11-phenoxyundecyl phosphate **3** (obtained as described in Section 3.3) and 2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-glucopyranosyl phosphate as described previously.[4]

Methanolic MeONa (2 M) was added to a stirred solution of **7**, ammonium salt (0.025 g, 0.032 mmol), in MeOH (5 mL) to pH 12. The reaction mixture was agitated until O-deacetylation was complete (control by TLC, system C), neutralised with AcOH and concentrated in vacuum. The residue was dissolved in H_2O (20 mL) and passed through a preactivated C18 Sep-Pac cartridge, which was washed with H_2O (100 mL) and MeOH (20 mL). The methanolic eluate was concentrated, the residue was dissolved in MeOH

(0.5 mL), benzene (5 mL) was added and solution was lyophilised affording sodium salt **6b** as a white amorphous solid (0.020 g, 96%); R_f 0.65 (system B), 0.25 (system C) on glass plates or 0.75 (system B) on TLC cards. ^{1}H NMR (600.13 MHz, CD_3OD) δ: 1.30–1.45 (m, 12H, $6CH_2$), 1.50 (m, 2H, CH_2), 1.65 (m, 2H, CH_2), 1.76 (m, 2H, CH_2), 2.05 (s, 3H, CH_3CO), 3.35 (t, 1H, $J_{3,4}$ 10.0 Hz, $J_{3,2}$ 10.0 Hz, H-3), 3.65 (dd, 1H, $J_{5,6}$ 6.0 Hz, $J_{6,6'}$ 12.0 Hz, H-6′), 3.75 (t, 1H, $J_{4,3}$ 10.0 Hz, $J_{4,5}$ 10.0 Hz, H-4), 3.92 (dd, 1H, $J_{6,6'}$ 12.0 Hz, $J_{6,5}$ 2.0 Hz, H-6), 3.94 (t, 2H, $J_{H,H'}$ 6.5 Hz, CH_2OPh), 3.97(m, 2H, CH_2OP), 3.98 (m, 2H, H-2, H-5), 5.55 (dd, 1H, $J_{1,2}$ 2.9 Hz, $J_{1,P}$ 6.8 Hz, H-1), 6.88 (m, 3H, aromatic protons), 7.23 (m, 2H, aromatic protons); ^{13}C NMR (150.90 MHz, CD_3OD) δ: 174.5, 160.7, 130.5, 121.6, 115.7 (aromatic carbons), 96.3 (d, $J_{1,P1}$ 3.8 Hz, C-1), 75.1 (C-5), 77.1 (C-4), 72.5 (C-3), 69.0 (CH_2OPh), 67.5 (d, $J_{C,P2}$ 3.3 Hz, CH_2OP), 63.1 (C-6). 55.5 (d, $J_{2,P1}$ 7.7 Hz, C-2), 32.0(d, $J_{C,P2}$ 5.8 Hz, CH_2CH_2OP), 31.9, 30.9, 30.8, 30.7, 30.6, 30.5, 27.3, 27. 1, 23.2, 19.6 (aliphatic carbons) ; ^{31}P NMR (242.94 MHz, CD_3OD) δ: −10.7 (d, $J_{P2,P1}$18.0 Hz, P-2), −12.2 (d, $J_{P1,P2}$18.0 Hz, P-1); HRESIMS: calculated for $C_{25}H_{43}NO_{13}P_2Na$ $[M+H]^+$: *m/z* 650.2102; found: *m/z* 650.2069.

3.5. Biochemical assays

The incubation mixtures for testing compound **3** (P-PhU, ammonium salt) contained (in a total volume of 80 μL) 40 nmol of phosphate **3** or moraprenyl phosphate **8** (ammonium salt) (final concentration 0.5 mM); 30 μL of 0.1 M Tris–HCl buffer, pH 8.0 (final concentration 35 mM); 4 μL of 0.2 M $MgCl_2$ (final concentration 10 mM); 15 μL of 0.5 % aqueous Tween 85 and 10 μL of MeOH. The mixture was Vortexed for 60 s and UDP-α-D-[^{14}C]-GlcNAc was added (1.5 nmol, 100,000 cpm for radioactive substrate).

Membrane preparation (prepared according to procedure described in Ref. 11) had been added at the last moment (20 μL, ~20 γ of protein). After incubation of probes at 35 °C for 45 min with gentle mixing, cold distilled water was added up to the final volume of 400–500 μL and the test tubes were kept at 0–4 °C for 12–20 h. Coagulated protein was removed by centrifugation (8000 rpm, 10 min), the supernatant was applied on a C18 Sep-Pak cartridge, the excess of nucleotide sugar was eluted with water (1 mL × 5), completeness of its removal was monitored by measuring radioactivity. The reaction product was eluted with MeOH (1 mL × 5), methanolic eluate was concentrated and analysed on TLC cards (system B) with reference to diphosphate **6b**. The radioactive UV-absorbing zone from the TLC plate that contained the biochemically synthesised α-D-[^{14}C]GlcNAc-PP-PhU, which coincided in mobility with that of the authentic sample of **6b**, was eluted with MeOH and HRESIMS of the eluate confirmed the molecular mass of the reaction product (calcd for $C_{25}H_{43}NO_{13}P_2$ $[M-H]^-$ 626.2137; found *m/z* 626.2137).

Acknowledgement

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