European Journal of Medicinal Chemistry 125 (2017) 952–964

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

Synthesis and biological evaluation of chemical tools for the study of Dolichol Linked Oligosaccharide Diphosphatase (DLODP)

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ARTICLE INFO

Article history:
Received 27 July 2016
Received in revised form
19 September 2016
Accepted 7 October 2016
Available online 8 October 2016

Keywords:
CDG
Diphosphatase
Phosphosugars
Disubstituted diphosphates
Biological evaluation
Glycochemistry

ABSTRACT

Citronellyl- and solanesyl-based dolichol linked oligosaccharide (DLO) analogs were synthesized and tested along with undecaprenyl compounds for their ability to inhibit the release of [^{3}H]OSP from [^{3}H] DLO by mammalian liver DLO diphosphatase activity. Solanesyl (C45) and undecaprenyl (C55) compounds were 50–500 fold more potent than their citronellyl (C10)-based counterparts, indicating that the alkyl chain length is important for activity. The relative potency of the compounds within the citronellyl series was different to that of the solanesyl series with citronellyl diphosphate being 2 and 3 fold more potent than citronellyl-PP-Glc*NAc*$_2$ and citronellyl-PP-Glc*NAc*, respectively; whereas solanesyl-PP-Glc*NAc* and solanesyl-PP-Glc*NAc*$_2$ were 4 and 8 fold more potent, respectively, than solanesyl diphosphate. Undecaprenyl-PP-Glc*NAc* and bacterial Lipid II were 8 fold more potent than undecaprenyl diphosphate at inhibiting the DLODP assay. Therefore, at least for the more hydrophobic compounds, diphosphodiesters are more potent inhibitors of the DLODP assay than diphosphomonoesters. These results suggest that DLO rather than dolichyl diphosphate might be a preferred substrate for the DLODP activity.

1. Introduction

N-glycans play crucial roles in cell growth, differentiation and communication [1]. Protein *N*-glycosylation occurs by transfer of Glc$_3$Man$_9$Glc*NAc*$_2$, from dolichol-linked oligosaccharide (DLO, Glc$_3$Man$_9$Glc*NAc*$_2$-PP-dolichol), onto polypeptides containing an Asn residue in the Asn-X-Ser/Thr glycosylation sequon. Dolichyl-diphosphate, the by-product of this reaction, is recycled into DLO. This sequence of reactions constitutes the dolichol cycle, and because dolichol-P (DolP) is rate limiting for protein glycosylation, its interruption leads to hypoglycosylation of glycoproteins. In man, mutations in genes encoding proteins of the dolichol cycle lead to type I congenital disorders of glycosylation (CDG-I) [2], a group of rare inherited diseases, manifesting multisystemic clinical pictures, whose hallmark is the presence of hypoglycosylated serum glycoproteins [3]. Of particular interest for the study of these diseases are DLO regulation and the fate of truncated DLO intermediates often seen in CDG-I. In fact, data show that truncated DLO species are cleaved by a DLO diphosphatase (DLODP), to yield DolP and oligosaccharyl-phosphates (OSP) in cells derived from CDG-I patients [4,5] and it has been hypothesized that such a mechanism may restrict truncated DLO accumulation while at the same time allowing DolP recycling [6]. In order to understand the role of a recently described Co^{2+}-dependent DLODP activity [7,8] (Fig. 1) we initiated a chemistry program aimed at generating chemical tools that are required for DLODP characterization.

In particular, we were interested in defining the structural elements required for molecules to interact with DLODP. Thus, the synthesis of simplified DLO analogs (Fig. 2) has been carried out taking the simplest Glc*NAc*-diphosphoryl-dolichol

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http://dx.doi.org/10.1016/j.ejmech.2016.10.013
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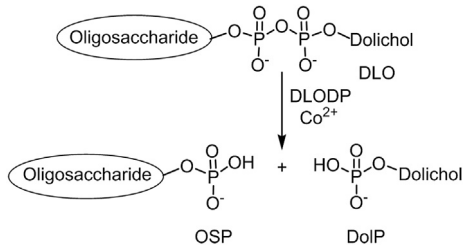


Fig. 1. Role of the DLODP.

(Glc*N*Ac-PP-dolichol, R^1-*O*-P-*O*-P-*O*R^2) as a model. In order to investigate the relative importance of each part of the DLODP substrate, the R^1 position has been substituted with either a Glc*N*Ac residue or a di-*N*-acetylchitobiose moiety, which occur in the natural substrate, and the dolichol residue at the R^2 position has been replaced by the shorter citronellyl or solanesyl moieties. Such R^2 groups were intended to probe the possible requirement of the diphosphatase activity for long polyprenyl chains. Furthermore, to evaluate the importance of the sugar chain for recognition by DLODP, the corresponding citronellyl and solanesyl diphosphates have also been synthesized (R^1 = H).

Additionally, the related monophosphates (R^1-*O*-P or R^2-*O*-P) were also prepared to assist the identification of the reaction products. Finally, the synthesis of citronellyl medronate in which a methylene group replaces the central oxygen atom of the diphosphate moiety has also been achieved as a non-cleavable substrate. Results concerning the biochemical characterization of the Co^{2+}-dependent DLODP have recently been published [7]. Here we report the full results dealing with the efficient synthesis of these complex compounds in pure form and the complete comparison of their inhibition of DLODP activity.

2. Results and discussion

2.1. Chemistry

The preparation of the targeted disubstituted diphosphates **A** has been envisaged through the coupling of the corresponding phosphosugars **B** and phosphodolichol mimics **C** (Fig. 3).

The peracetylated Glc*N*Ac **1** (Scheme 1) was prepared from commercially available Glc*N*Ac by treatment with acetic anhydride in excess in pyridine and the pure α anomer was isolated in 89% yield after flash chromatographic purification. Peracetylated $GlcNAc_2$ **2** was obtained by acetolysis of chitin by H_2SO_4 and acetic

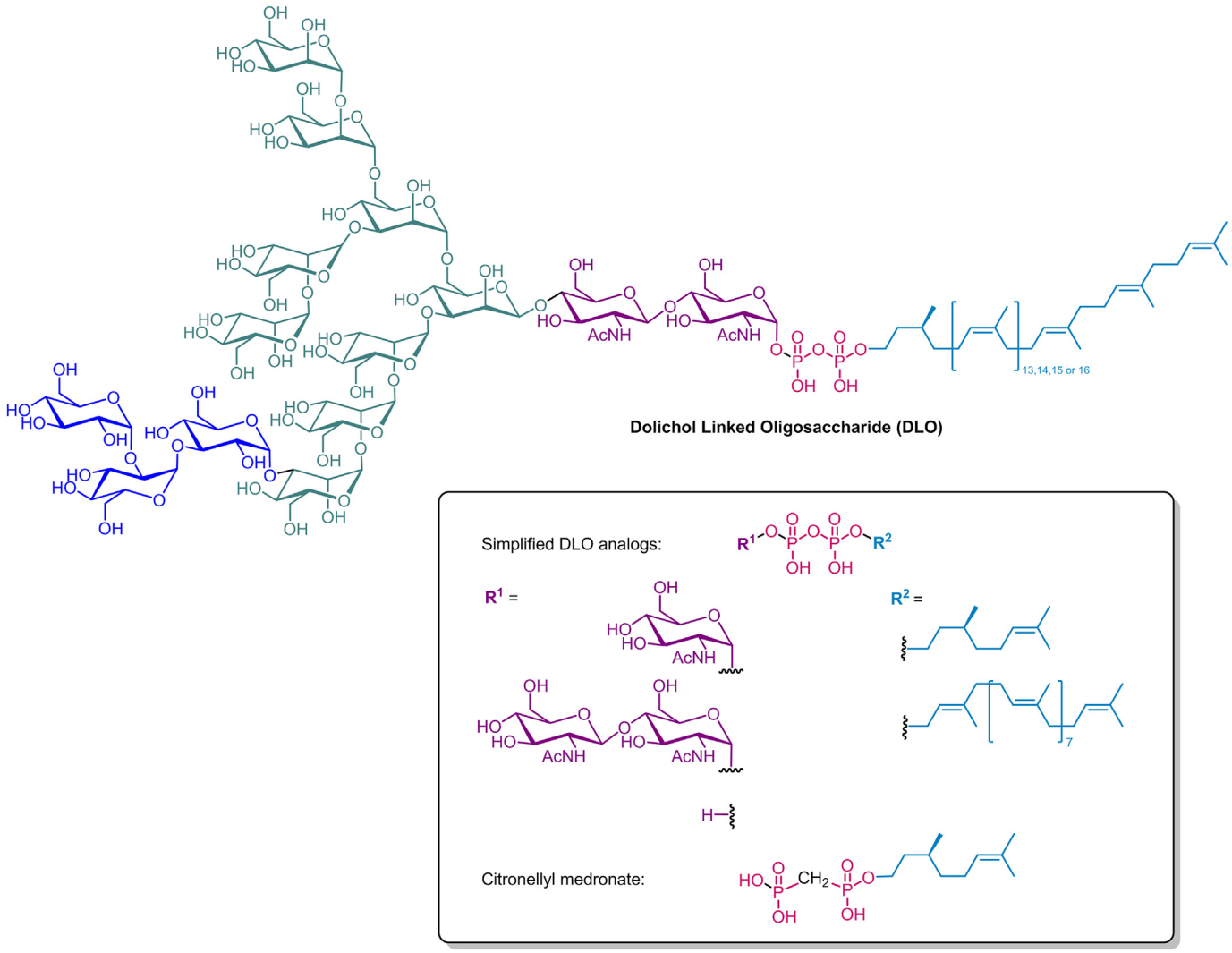


Fig. 2. Structure of DLO and of the targeted compounds.

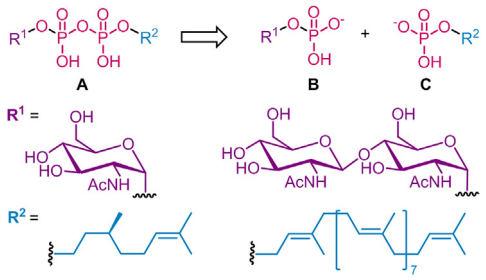
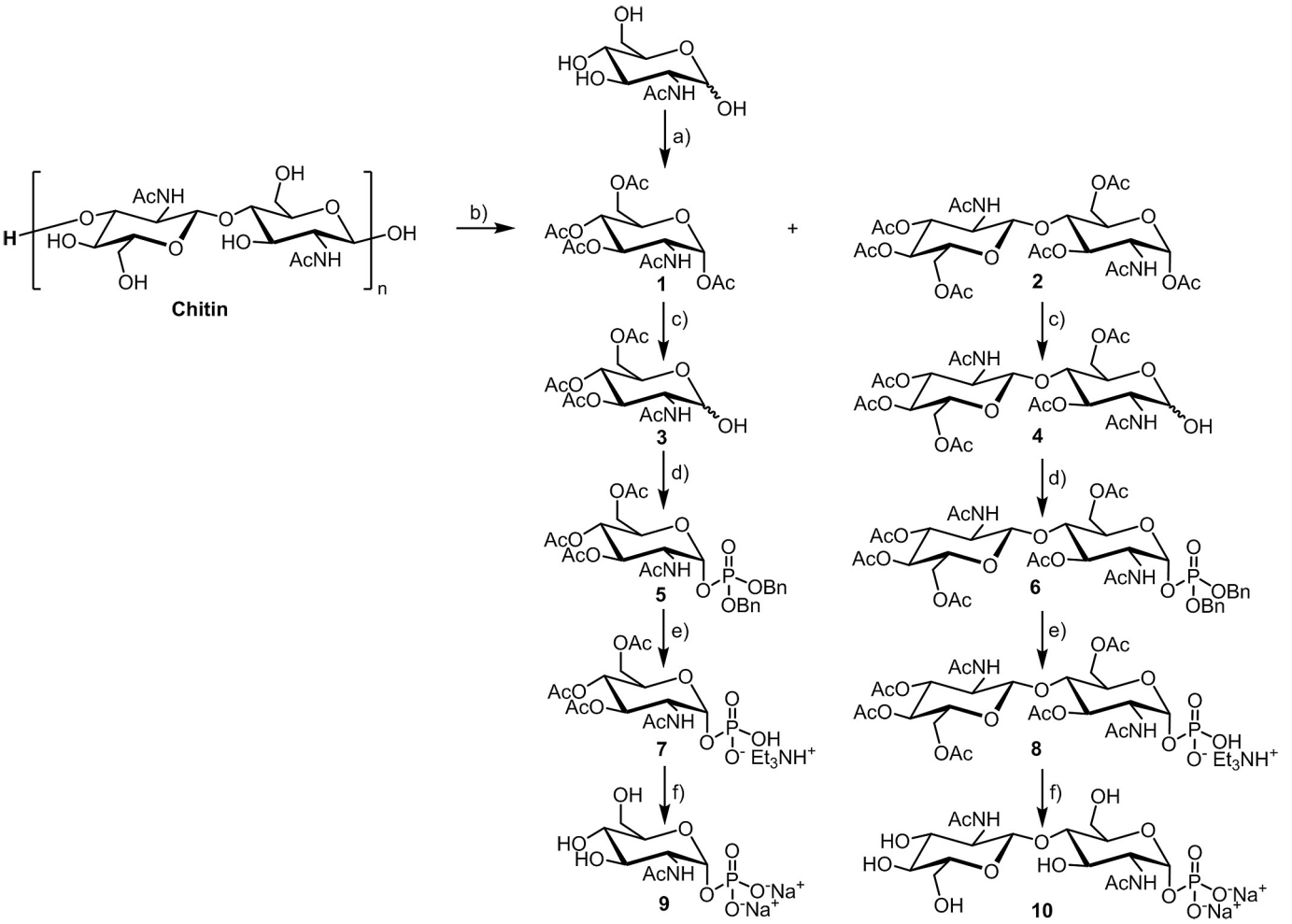


Fig. 3. Retrosynthetic analysis.

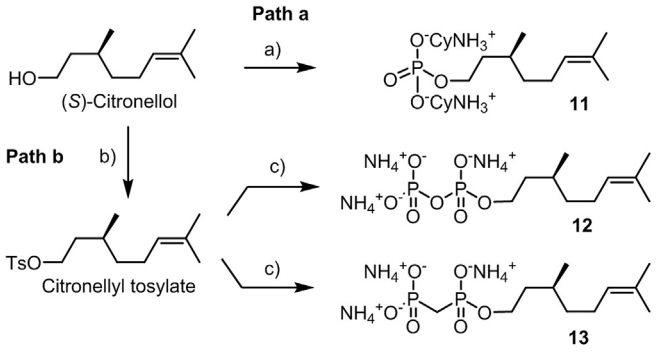
anhydride under sonication conditions [9]. Flash chromatographic purification of the crude permitted us to isolate pure compound **2** in 5% yield and also to recover a small amount of compound **1** (1% isolated yield). The selective deprotection of the anomeric acetate of compound **1** and **2** was performed by treatment with ammonium acetate in the presence of diethylamine in DMF [10] leading to the hemiacetals **3** and **4** in 81% and 47% yield, respectively. The phosphoramidite-oxidation strategy [11] was chosen for the glycosylphosphates **5** and **6** synthesis. The preparation of the intermediate phosphites by reaction of compounds **3** and **4** with dibenzyl *N*,*N*-diethylphosphoramidate and 1*H*-tetrazole in a mixture of THF and CH_3CN was followed by oxidation with hydrogen peroxide to give **5** [12] and **6** [13] in 83% and 78% yield, respectively. Then, benzyl esters cleavage by hydrogenolysis in the presence of 10% Pd/C in EtOH for **5** and in a mixture of $EtOH/CH_2Cl_2$ for **6** gave the corresponding phosphates that were quantitatively isolated as their mono-triethylammonium salts **7** and **8**. Methanolysis of the acetates on the saccharidic units by sodium methylate in MeOH afforded the sodium salts of Glc*N*Ac and Glc*N*Ac_2 phosphates **9** and **10** in 94% and 97% yields.

We next turned to the preparation of citronellyl phosphate **11**, diphosphate **12** and medronate **13**, starting from (*S*)-citronellol (Scheme 2). Phosphorylation of citronellol was carried out according to L. L. Danilov et al. [14] by an excess of $POCl_3$ and Et_3N in CH_2Cl_2 (path a). After dissolution of the crude in a 9/1 acetone/water mixture at 0 °C, cyclohexylamine addition afforded citronellyl phosphate as its dicyclohexylammonium salt **11** in 33% yield after recrystallization in acetone/water (9/1). On another hand (path b), activation of citronellol by tosyl chloride in the presence of DMAP in CH_2Cl_2 furnished the intermediate tosylate which was reacted with either tris(tetrabutylammonium)hydrogen diphosphate or medronate in CH_3CN to afford, after reverse phase HPLC purification, the triammonium salts of citronellyl diphosphate **12** and medronate **13**.

The solanesol was phosphorylated with smoother conditions



Scheme 1. a) Ac_2O, pyridine, 89% b) Ac_2O, H_2SO_4, 35 °C, 14 h, 1% for **1**, 5% for **2**. c) CH_3COONH_4, DIEA, DMF, RT, 3 days, 81% for **3**, 47% for **4**, d) *i.* dibenzyl *N*,*N*-diethylphosphoramidate, 1*H*-tetrazole, THF, CH_3CN, RT, 14h; *ii.* H_2O_2, -78 °C to RT, 1 h, 83% for **5**, 78% for **6**. e) H_2, Pd/C 10%, EtOH, then triethyamine, quant. for **7**, H_2, Pd/C 10%, EtOH, CH_2Cl_2, then triethylamine quant. for **8**. f) MeONa, MeOH, RT, 1 h, 94% for **9**, 14 h, 97% for **10**.



Scheme 2. a) $POCl_3$, Et_3N, CH_2Cl_2, RT, 1 h, then acetone/Et_3N/water, RT, 14 h, recrystallization with cyclohexylamine, 33%. b) TsCl, DMAP, CH_2Cl_2, RT, 14 h, 65%. c) tris(tetrabutylammonium) hydrogen diphosphate or medronate, CH_3CN, RT, 18 h, then reverse phase HPLC, **12**: 38%, **13**: 37%.

(Scheme 3) because allylic phosphates are known to easily undergo elimination side-reactions. Thus, the treatment of solanesol with Cl_3CCN and tetrabutyl ammonium dihydrogen phosphate in excess in CH_2Cl_2 afforded a one pot activation – phosphorylation process [15] that leads to either the monophosphate **14** or diphosphate **15** depending on the reaction duration (Scheme 3). Accordingly, after one hour of reaction, the solanesyl phosphate **14** could be obtained in 33% yield after purification by flash chromatography in dichloromethane/methanol/14% aqueous ammonia 80/18/2 (v/v/v), while the diphosphate synthesis was achieved in 14 h of reaction and its purification required DEAE anion exchange chromatography to give the expected compound **15** in 27% yield.

The synthesis of diphosphates **16**–**19** was achieved by the coupling of phosphosugars **7** and **8** with the phosphorimidazolides intermediates [16] *in situ* generated from the phosphodolichol mimics **11** and **14** (Scheme 4). Typical conditions involved careful drying the partners separately by co-evaporation with toluene and triethylamine followed by treatment of phospholipid **11** or **14** with 1,1′-carbonyldiimidazole in CH_2Cl_2 to give the phosphorimidazolides [17]. Condensation of the resulting crude phosphorimidazolides onto phosphosugar **7** or **8** followed by acetates methanolysis afforded the corresponding diphosphates that were purified by flash chromatography on silica gel or by reverse phase HPLC to afford the pure targeted diphosphates **16**–**19** in yield varying between 12 and 30% over three steps from the dolichol phosphate mimics **11** or **14**.

2.2. Biological studies

2.2.1. GlcNAc-1P blocks phosphomonoesterase activity in the DLODP assay

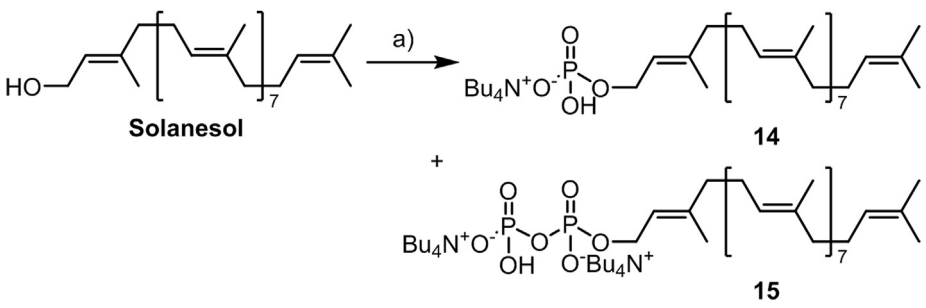
Previously, DLODP has been assayed by incubating detergent suspended $Glc_{3\text{-}0}[^3H]Man_{9\text{-}5}GlcNAc_2$-PP-dolichol with hepatocyte microsomes in the presence of Co^{2+} at pH 5.5. Enzyme activity is quantitated by expressing the radioactivity associated with the [^{3}H]OSP generated as a fraction of the total radioactivity recovered from the incubations (non-hydrolysed [^{3}H]DLO, [^{3}H]OSP and neutral [^{3}H]oligosaccharides and [^{3}H]mannose). In order to gain insight into the physiological susbstrates for this activity, we screened compounds for their ability to interfere with the above-described assay. First, commonly occurring water soluble phospho-compounds were tested (AMP, ADP, ATP, NADP, GDP-Man, UDP-Glc*N*Ac, UDP-Glc), and it was found that such structures had IC_{50} concentrations in the low millimolar range, which is over 1000 fold higher than the substrate concentration. An example of such a compound is Glc*N*Ac-1P **9** that corresponds to the simplest OSP that could be hydrolysed from a DLO (Glc*N*Ac-PP-dolichol) by DLODP, which has an IC_{50} of greater than 3 mM in the DLODP assay (Table 1). Nevertheless, this compound proved useful for examining DLODP activity in microsomes generated from different sources.

As shown in the upper panels A of Fig. 4, microsomes generated from the human hepatocellular carcinoma HepG2 cell line, after 3, 8 and 16 days in culture, generate uniquely OSP from DLO. By contrast (upper panels B), microsomes derived from 3, 8 and 16 day cultures of the TC7 clone of the human colon carcinoma Caco-2 cell line yield both neutral oligosaccharides (nfOS) and OSP as a function of cell growth. Microsomes derived from early growth cells (day 3) generate predominantly OSP whereas at day 16, the microsomes yield predominantly nfOS. At the intermediate growth time (day 8), cells yield microsomes whose capacity to hydrolyse DLO is almost two fold higher than those generated from early or late growth cells.

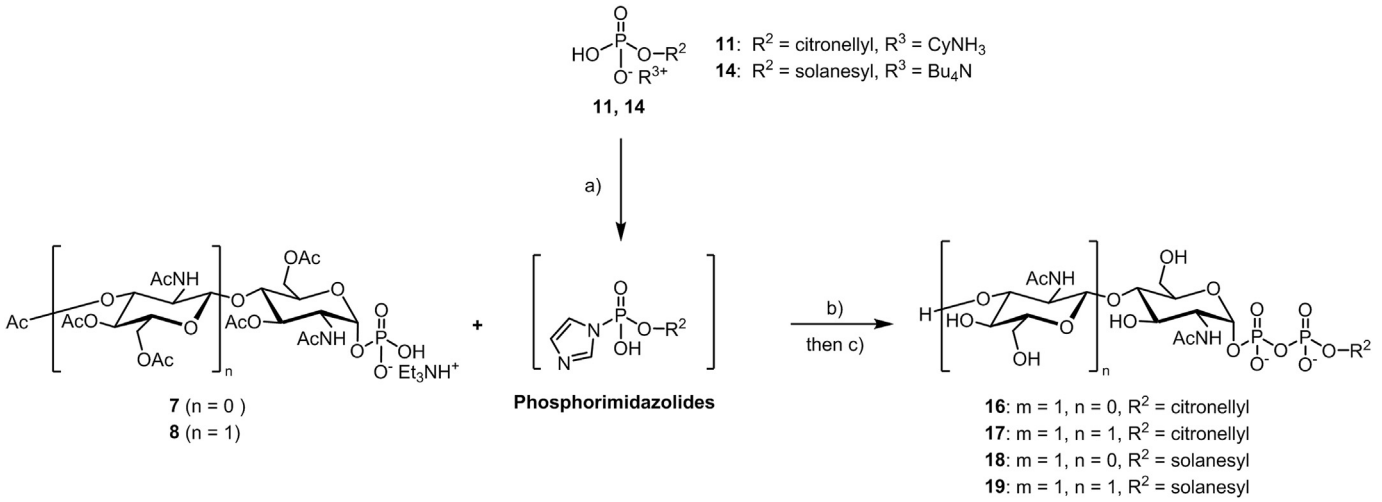
Because the increase in nfOS is at the expense of OSP between the 15 and 20 min incubation time points, it would appear that nfOS are not being liberated directly from DLO, but rather by dephosphorylation of DLODP-generated OSP. This was underlined when DLODP assays were conducted in the presence of increasing concentrations of Glc*N*Ac-1P (**9**). As shown in the middle panel of Fig. 4B, at low concentrations this compound inhibits nfOS production while increasing OSP production and at higher concentrations begins to inhibit OSP production as well. The DLODP activity found in microsomes from HepG2 cells is also inhibited at higher Glc*N*Ac-1P (**9**) concentrations (middle panel of Fig. 4A). These data are consistent with OSP dephosphorylation during DLODP assays using Caco-2 cell microsomes. This hypothesis is underlined by the fact that during Caco-2, but not HepG2, cell growth (lower panels in Fig. 4A and B), there is a differentiation-dependent expression of cell surface alkaline phosphatase, which is known to be able to dephosphorylate sugar 1 phosphates.

2.2.2. Biological testing of water-soluble citronellyl compounds and solanesyl compounds in the DLODP assay

Next, the citronellyl- and solanesyl-based compounds were tested for their ability to interfere with the standard DLODP



Scheme 3. a) Tetrabutylammonium dihydrogen phosphate, CCl_3CN, CH_2Cl_2, 1 h, RT, 33% for **14** or 14 h, RT, 27% for **15**.



Scheme 4. a) *i.* Co-evaporation with Et_3N and toluene; *ii.* CDI, THF, 1.5 h from **11** or CDI, CH_2Cl_2, 2 h from **14**. b) RT, THF, 12 h for **16**, RT, DMF, 12 h for **17**, RT, DMF, 6 days for **18**, RT, pyridine, 7 days for **19**. c) MeONa, MeOH, RT then DOWEX 50WX8, H^+ form.

Table 1
Effects of compounds on DLODP activity in HepG2 membranes.

Compound	Number	IC_{50} (mM)[a]
Monophosphates		
Propyl phosphate		*ni* at 3.0 mM
Glc*N*Ac-1-phosphate	**9**	*nd* > 3.0 mM
p-nitrophenyl phosphate		4.2
Citronellyl phosphate	**11**	0.3
Solanesyl phosphate	**14**	0.0078
Undecaprenyl phosphate[b]		~0.010
Diphosphates		
Diphosphate		*nd* > 3.0 mM
Medronate		*ni* at 3.0 mM
Citronellyl diphosphate	**12**	0.1
Citronellyl medronate	**13**	0.7
Solanesyl diphosphate	**15**	0.0047
Undecaprenyl diphosphate[b]		0.0040
Phosphodiesters		
Bis(*p*-nitrophenyl) phosphate		1.2
Uridinyl-PP-Glc*N*Ac		*ni* at 3.0 mM
Citronellyl-PP-Glc*N*Ac	**16**	0.2
Citronellyl-PP-GlcNAc_2	**17**	0.3
Solanesyl-PP-Glc*N*Ac	**18**	0.0012
Solanesyl-PP-GlcNAc_2	**19**	0.0005
Undecaprenyl-PP-Glc*N*Ac[c]		0.0005
Undecaprenyl-PP-Mur*N*Ac (pentapeptide)-Glc*N*Ac (Lipid II)[d]	**9**	0.0005

[a] OSP release from DLO was measured as described in Experimental Procedures. Where possible, the concentration of inhibitor causing 50% inhibition of OSP generation was determined (IC_{50}). In some cases, no inhibition was noted at the concentration indicated (*ni*). In other cases, inhibition was observed, but the maximum concentrations used did not allow determination of IC_{50} values (*nd*).

[b] Undecaprenyl phosphate and undecaprenyl diphosphate were provided by the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences, Warsaw, Poland.

[c] Undecaprenyl-PP-Glc*N*Ac was produced by enzymatic synthesis, using the WecA enzyme [18,19].

[d] Lipid II (Undecaprenyl-PP-Mur*N*Ac(-pentapeptide)-Glc*N*Ac), was produced by enzymatic synthesis, using the purified MraY and MurG enzymes as previously described [20,21].

assay. Data shown in Fig. 5 and Table 1 demonstrate that the former compounds were 50–500 fold less potent than their solanesyl counterparts. Furthermore, the relative potency of the compounds within each series was different. For the citronellyl-based products (citronellyl diphosphate **12** > citronellyl-diphospho-Glc*N*Ac **16** > citronellyl-diphospho-GlcNAc_2 **17**/citronellyl phosphate **11** > citronellyl medronate **13**), the diphosphate is 2 and 3 fold more potent than the diphosphodiesters citronellyl-PP-GlcNAc_2 **17** and citronellyl-PP-GlcNAc **16**, respectively. It is noteworthy that citronellyl medronate **13** is 7 fold less active than the diphosphate **12**, suggesting that the observed interference of the DLODP assay by the latter compound is not entirely related to its physicochemical properties. The decreased potency of the diphosphodiesters **16**/**17** compared to that of the diphosphomonoester **12**, indicates that the physiological susbstrate of the DLODP activity may be a diphosphomonoester, like dolichyl-diphosphate rather than a diphosphodiester like DLO. However, this was not the case for the solanesyl-based compounds. Here (solanesyl-diphospho-GlcNAc_2 **19** > solanesyl-diphospho-Glc*N*Ac **18** > solanesyl-diphosphate **15** > solanesyl-phosphate **14**), the diphosphodiesters **18**/**19** were 4 and 8 fold more potent, respectively, than the diphosphate **15**. In order to get further information on this point, a series of undecaprenyl (C55)-based compounds was tested. Results shown in the lower panel of Fig. 5 and Table 1 demonstrate that the ability of these compounds to interfere with the DLODP assay is similar to that of the solanesyl-based compounds. In addition, the relative potency of these compounds (undecaprenyl-diphospho-Mur*N*Ac(-pentapeptide)Glc*N*Ac/undecaprenyl-diphospho-Glc*N*Ac > undecaprenyl-diphosphate > undecaprenyl-monophosphate), again shows that the diphosphodiesters interfere better with the assay than the diphosphomonoester.

2.2.3. *Further examination of the importance of the R^1 moiety for inhibition of the DLODP assay*

Inhibition of the DLODP assay by the solanesyl and undecaprenyl compounds indicates that the R^2 moiety has a role to play. There are several possibilities for this phenomenon. First, a hydrophilic headgroup may simply help physically orientate the diphosphate moiety of these amphiphilic compounds in their lipid/detergent milieu for access to the enzyme. Second, the monosaccharides in close proximity to the diphosphate moiety may directly interact with the enzyme and therefore promote substrate binding. The potential role of mannose and Glc*N*Ac residues of DLO in recognition by DLODP was addressed by evaluating the capacity of various monosccharides/disaccharides to interfere with the DLODP assay. As shown in Fig. 6, at 50 mM, mannose, α-methyl mannoside and *N*- acetylglucosamine did not inhibit OSP generation.

Although 50 mM β-methyl mannoside isopropylate caused a

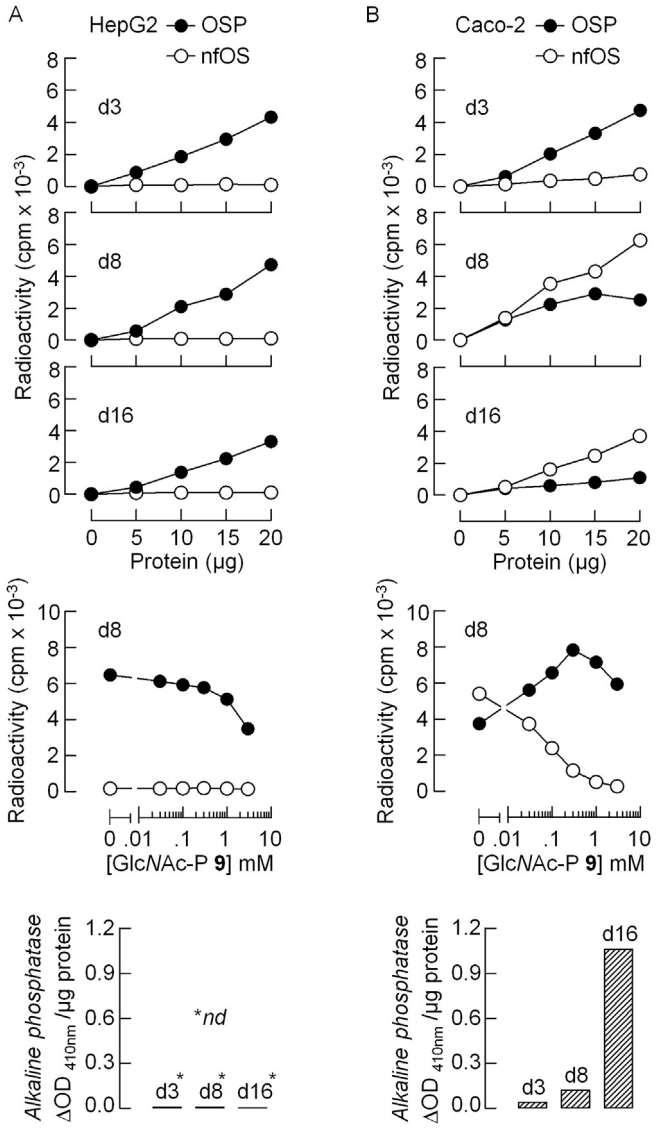


Fig. 4. DLODP activity as a function of cell growth and differentiation. HepG2 (A) and Caco-2 (B) cells were cultivated for 3, 8 and 16 days and total cell membranes were prepared as described in Experimental Procedures. Upper panels. DLODP assays were performed under standard conditions using the indicated amounts of membrane protein, and radioactivity associated with OSP and nfOS was quantitated by scintillation counting. Middle panels. DLODP assays using 20 µg membrane protein derived from cultivated for 8 days were performed in the standard incubation mixture supplemented with the indicated amounts of GlcNAc-1P (**9**), and radioactivity associated with OSP and nfOS was quantitated. Lower panels. Alkaline phosphatase was measured in the membrane preparations described above.

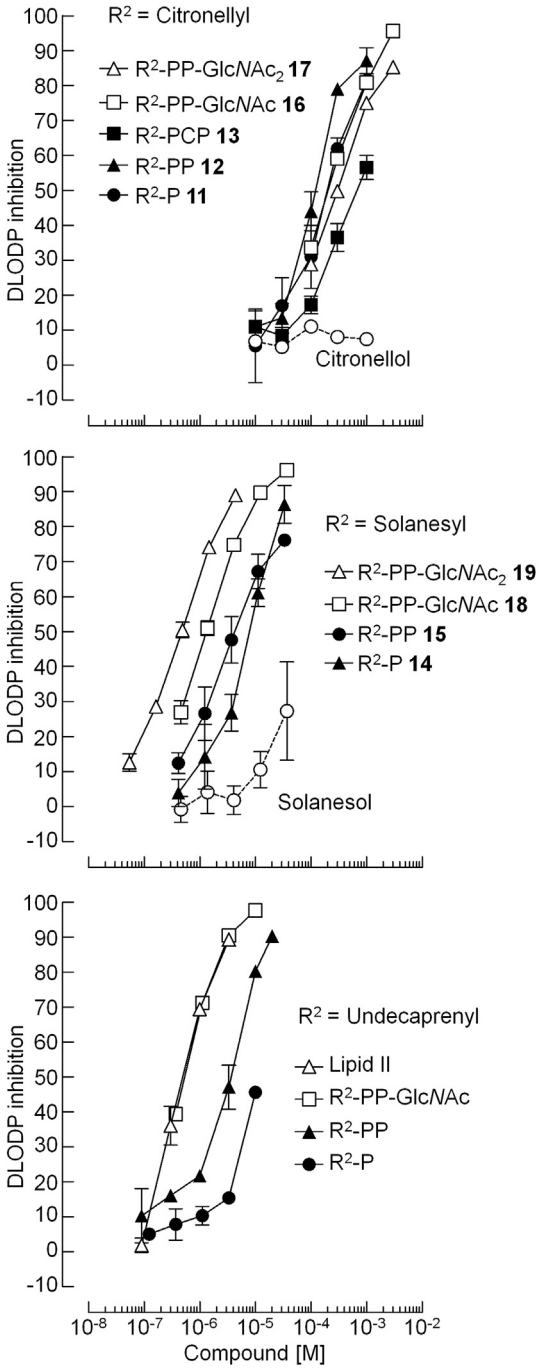


Fig. 5. Inhibition of DLODP assay by different citronellyl and solanesyl compounds. Using 40 nM $Glc_{3-0}[^3H]Man_5GlcNAc_2$-PP-dolichol as substrate, standard DLODP assays were conducted in the presence of different concentrations of the indicated compounds. Dose response curves were generated and the IC_{50} values are reported in Table 1. Concerning the biological activity of solanesyl compounds (shown in the middle panel), this research was originally published in the Journal of Lipid Research [7] A. Massarweh, M. Bosco, S. Iatmanen-Harbi, C. Tessier, N. Auberger, P. Busca, I. Chantret, C. Gravier-Pelletier, S. E. H. Moore, Demonstration of an oligosaccharide-diphosphodolichol diphosphatase activity whose subcellular localization is different than those of dolichyl-phosphate-dependent enzymes of the dolichol cycle, Journal Lipid Res. 57 (2016) 1029–1042 © the American Society for Biochemistry and Molecular Biology.

10% inhibition of OSP generation, the same effect was obtained with 50 mM isopropanol alone, suggesting that β-methyl mannoside itself has no effect on OSP production at this concentration. GlcNAc(β1,4)GlcNAc caused < 10% inhibition of the reaction, whereas at the same concentration Man(β1,4)GlcNAc provoked a 30% inhibition of OSP release from DLO. By contrast, the Gal(β1,4) GlcNAc disaccharide, which does not occur in DLO, did not provoke a statistically significant reduction in DLDOP action. Accordingly the ensemble of this data indicates that the Man(β1,4)GlcNAc motif and to a lesser extent the di-*N*-acetylchitobiose motif interfere with the DLODP assay. These observations suggest that the sugar moiety of DLO may have a role to play in substrate recognition by the OSP-generating activity.

3. Conclusion

Two series of citronellyl- and solanesyl-based DLO analogs containing, or not, a mono- or di-saccharidic GlcNAc moiety were

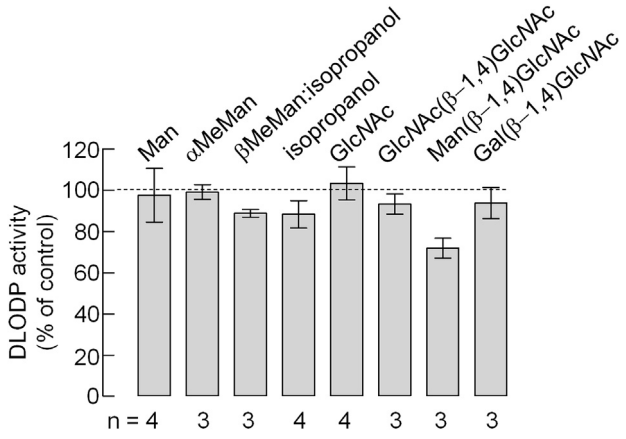


Fig. 6. Inhibition of DLODP assay by different mono- or disaccharides. Using $Glc_{3\text{-}0}$[^{3}H] $Man_5GlcNAc_2$-PP-dolichol as substrate, standard DLODP assays were conducted in the presence of 50 mM of the indicated compounds. The abbreviations are: Gal; galactose, Glc*N*Ac; *N*-acetylglucosamine, Man; mannose, αMeMan; α-methyl mannoside, βMeMan; β-methyl mannoside. For each compound, inhibition values were determined at least in triplicate (n) and the mean and standard error of the mean are indicated. The dotted line indicates the DLODP activity observed in the absence of inhibitors.

synthesized according to appropriate routes. Glycosyl phosphates were obtained by the phosphoramidite-oxidation strategy from peracetylated Glc*N*Ac or Glc*N*Ac_2 derivatives, while citronellyl phosphate was efficiently obtained by treatment of citronellol with phosphorus oxychloride. The corresponding diphosphate resulted from displacement of the tosylate with hydrogen diphosphate salt. The allylic solanesyl mono and diphosphate were advantageously prepared in milder conditions involving a one pot activation − phosphorylation process. Finally, the coupling of glycosyl phosphates with the phosphorimidazolides derived from citronelly and solanesyl phosphates afforded the DLO mimics. The resulting compounds were tested for their ability to inhibit the release of [^{3}H] OSP from [^{3}H]DLO. The solanesyl compounds were 50–500 fold more potent than their water-soluble citronellyl-based counterparts. These data demonstrate the importance of the alkyl chain (R^2) length of the compounds for interfereing with the DLODP assay. The role of the glycan (R^1) moiety is less clear, but at least for compounds containing the long chain R^2 moieties, the diphosphodiesters inhibit the reaction more efficiently than the diphosphomonoesters. How the R^1 substituents promote better inhibition of the DLODP assay will have to await further investigation. Nevertheless these compounds are being used for the characterization of OSP-generating systems from various sources.

4. Experimental

4.1. Chemical synthesis

MS and/or analytical data were obtained using chromatographically homogeneous samples.^{1}H NMR (500 MHz), ^{13}C NMR (126 MHz) and ^{31}P (202 MHz) spectra were recorded a Bruker Avance or Avance II in the given solvents unless otherwise indicated. Chemical shifts (δ) are reported in ppm and coupling constants are given in Hz. To facilitate the understanding of NMR spectroscopic data, the numbering of atoms for the following representative compound **19** is as indicated (Fig. 7).

Optical rotations were measured on a Perkin-Elmer 341 polarimeter with sodium (589 nm) lamp at 20 °C. Low resolution mass spectra (LRMS) were recorded with an ion trap mass analyzer under electrospray ionization (ESI) in positive and negative ionization mode detection. High resolution mass sprectra (HRMS) were recorded with a TOF mass analyzer under electrospray ionization (ESI) in positive or negative ionization mode detection, atmospheric pressure chemical ionization. All reactions were carried out under a argon atmosphere, and were monitored by thin-layer chromatography with Merck 60F-254 precoated silica (0.2 mm) on glass. Flash chromatography was performed with Merck Kieselgel 60 (200–500 μm); the solvent systems were given v/v HPLC reverse phase purification were done on a STABILITY C18 100 Å column; 5 μm; 250 × 20 mm at a flow rate 4 mL/min in the indicated solvents.

4.1.1. 2-Deoxy-2-acetamido-4-O-(2-deoxy-2-acetamido-3′,4′,6′-tri-O-acetyl-β-D-glucopyranosyl)-1,3,6-tri-O-acetyl-α-D-glucopyranose 2

To a suspension of chitin (15 g) in acetic anhydride (150 mL, 1.59 mol) was added dropwise concentrated sulfuric acid (15 mL, 4.13 mmol). The reaction mixture was sonicated for 3 h. The resulting suspension was warmed at 35 °C for 14 h then poured in ice (200 g). The pH of the solution was adjusted at 7 with sodium acetate (120 g). The precipitate was separated by centrifugation and the supernatant was extracted four times with dichloromethane (100 mL). The combined extracts were washed with a saturated aqueous solution of $NaHCO_3$ (400 mL), water (400 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification of the residue by column chromatography (silica gel 300 g, EtOAc) afforded peracetate **1** (347 mg, 0.89 mmol, 1%) as a white solid and peracetate **2** (1.2 g, 1.77 mmol, 5%) as a white solid. Compound **2** [22]: [α]D + 29.5 (*c* 1.0, $CHCl_3$); [α]D + 29.2 (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 6.09 (d, J = 3.6 Hz, 1H, H-1), 5.96 (d, J = 9.1 Hz, 1H, N*H*Ac*), 5.65 (d, J = 9.1 Hz, 1H, N*H*Ac), 5.22 (dd, J = 11.0, 9.0 Hz, 1H, H-3), 5.13 (dd, J = 10, 9.1 Hz, 1H, H-3*), 5.05 (t, J = 10 Hz, 1H, H-4*), 4.48 (d, J = 8.4 Hz, 1H, H-1*), 4.44 (dd, J = 12.2, 3.3 Hz, 1H, H-6), 4.40–4.32 (m, 2H, H-2, H-6*), 4.18 (dd, J = 12.2, 2.4 Hz, 1H, H-6′*), 4.02 (dd, J = 12.2, 2.2 Hz, 1H, H-6′), 3.95 (dt, J = 9.1, 8.4 Hz, 1H, H-2*), 3.89 (ddd, J = 10.0, 3.3, 2.2 Hz, 1H, H-5), 3.74 (broad dd, J = 10.0, 9.0 Hz, 1H, H-4), 3.62 (ddd, J = 10, 4.1, 2.4 Hz, 1H, H-5*), 2.18 (s, 3H, C*H*$_3$CO), 2.14 (s, 3H, C*H*$_3$CO), 2.08 (s, 3H, C*H*$_3$CO), 2.05 (s, 3H, C*H*$_3$CO), 2.01 (s, 3H, C*H*$_3$CO), 2.00 (s, 3H, C*H*$_3$CO), 1.95 (s, 3H, C*H*$_3$CO), 1.92 (s, 3H, C*H*$_3$CO); ^{13}C NMR ($CDCl_3$) δ 171.6, 171.4, 170.9, 170.6, 170.5, 170.2, 169.4, 169.0 (8C, *C*OCH$_3$), 101.9 (C-1*), 90.7 (C-1), 76.1 (C-4), 72.7 (C-3*), 72.1 (C-5*), 70.9 (C-5), 70.8 (C-3), 68.1 (C-4*), 61.9 (C-6*), 61.6 (C-6), 54.7 (C-2*), 51.3 (C-2), 23.3, 23.2, 21.1, 21.1, 20.8, 20.7, 20.7, 20.7 (8C, *C*H$_3$CO); ESI-MS: Calcd for $C_{28}H_{41}N_2O_{17}$: 677.2. $[M+H]^+$ Found: 677.2; ESI-HRMS: Calcd for $C_{28}H_{41}N_2O_{17}$: 677.2400. $[M+H]^+$ Found: 677.2393.

4.1.2. 2-Deoxy-2-acetamido-3,4,6-tri-O-acetyl-α-D-glucopyranose 3 and 2-deoxy-2-acetamido-4-O-(2-deoxy-2-acetamido-3′,4′,6′-tri-O-acetyl-β-D-glucopyranosyl)-3,6-di-O-acetyl-α-D-glucopyranose 4

A solution of peracetate **1** (5 g, 12.8 mmol) in a mixture of dry DMF (50 mL) and *N*,*N*–diisopropylethylamine (19.7 mL, 119.4 mmol) was stirred with ammonium acetate crystals (3.96 g, 51.4 mmol) under argon atmosphere. After 3 days, the reaction mixture was decanted from the undissolved crystals of ammonium acetate, diluted with dichloromethane (700 mL) and washed with a saturated aqueous solution of sodium bicarbonate (200 mL), then water (200 mL), then brine (150 mL), dried over sodium sulfate, filtered, and concentrated *in vacuo*. Purification of the residue by column chromatography (silica gel 200 mL, cyclohexane/EtOAc, v/v, 2/8, 1/9, 0/1) afforded a 98/2 α/β mixture of lactol acetates **3** (3.62 g, 10.4 mmol, 81%) as a colourless oil. For the α anomer [23]: ^{1}H NMR ($CDCl_3$) δ 6.00 (d, J = 9.5 Hz, 1H, N*H*Ac), 5.28 (dd, J = 10.7, 9.5 Hz, 1H, H-3), 5.23 (d, J = 3.5 Hz, 1H, H-1), 5.11 (t, J = 9.5 Hz, 1H, H-4), 4.26 (ddd, J = 10.7, 9.5, 3.5 Hz, 1H, H-2), 4.22–4.17 (m, 2H, H-5, H-6),

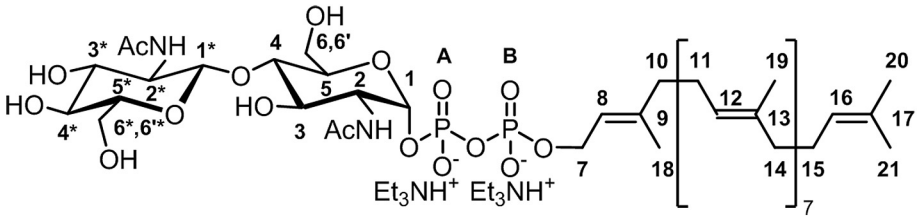


Fig. 7. Numbering of compound **19**.

4.14–4.07 (m, 1H, H-6′), 2.07 (s, 3H, C*H*$_3$CO), 2.01 (s, 3H, C*H*$_3$CO), 2.01 (s, 3H, C*H*$_3$CO), 1.95 (s, 3H, C*H*$_3$CO); ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.5, 171.1, 170.7, 169.6 (4C, CH_3CO), 91.6 (C-1), 71.1 (C-3), 68.5 (C-4), 67.6 (C-5), 62.3 (C-6), 52.5 (C-2), 23.2, 20.9, 20.8, 20.7 (4C, *C*H$_3$CO); ESI-MS: Calcd for $C_{14}H_{22}NO_9$: 348.1. $[M+H]^+$ Found: 348.1; ESI-HRMS: Calcd for $C_{14}H_{22}NO_9$: 348.1289. $[M+H]^+$ Found: 348.1281.

A solution of peracetate **2** (1.02 g, 1.51 mmol) in a mixture of dry DMF (15 mL) and *N*,*N*–diisopropylethylamine (1 mL, 6.06 mmol) was stirred with ammonium acetate crystals (1 g, 13.0 mmol) under argon atmosphere for 3 days. Then, the same treatment as for compound **3** was carried out. Recrystallization of the residue using a mixture of dichloromethane and cyclohexane afforded a 96/4 α/β mixture of lactol acetates **4** (452 mg, 0.71 mmol, 47%) as a white solid.: ^{1}H NMR ($CDCl_3$) for the major α anomer [22,24] δ 7.21 (d, J = 10.0 Hz, 1H, N*H*Ac), 6.20 (d, J = 8.5 Hz, 1H, N*H*Ac*), 5.67 (dd, J = 10.8, 9.3 Hz, 1H, H-3), 5.21 (d, J = 3.3 Hz, 1H, H-1), 5.08 (t, J = 9.6 Hz, 1H, H-4*), 4.97 (t, J = 9.6 Hz, 1H, H-3*), 4.42 (dd, J = 12.5, 4.1 Hz, 1H, H-6*), 4.32–4.27 (m, 2H, H-6, H-2), 4.18–4.11 (m, 3H, H-1*, H-6′, H-2*), 4.07 (ddd, J = 10.0, 3.8, 1.8 Hz, 1H, H-5), 4.03 (dd, J = 12.5, 2.0 Hz, 1H, H-6′*), 3.64 (t, J = 9.3 Hz, 1H, H-4), 3.57 (ddd, J = 9.6, 4.1, 2.0 Hz, 1H, H-5*), 2.13 (s, 3H, C*H*$_3$CO), 2.08 (s, 3H, C*H*$_3$CO), 2.06 (s, 3H, C*H*$_3$CO), 2.01 (s, 3H, C*H*$_3$CO), 2.01 (s, 3H, C*H*$_3$CO), 2.01 (s, 3H, C*H*$_3$CO), 1.94 (s, 3H, C*H*$_3$CO); ^{13}C NMR ($CDCl_3$) δ 172.2, 171.5, 171.3, 171.0, 170.7, 169.3 (7C, *C*OCH$_3$), 102.5 (C-1*), 91.9 (C-1), 76.7 (C-4), 72.2, 72.1 (2C, C-5*, C-3*), 71.1 (C-3), 68.3 (C-5), 67.9 (C-4*), 62.5 (C-6), 61.8 (C-6*), 54.3 (C-2*), 52.01 (C-2), 23.3, 23.0, 21.1, 20.9, 20.8, 20.7, 20.7 (7C, *C*H$_3$CO). ESI-MS: Calcd for $C_{26}H_{39}N_2O_{16}$: 635.2. $[M+H]^+$ Found: 635.3; ESI-HRMS: Calcd for $C_{26}H_{39}N_2O_{16}$: 635.2294. $[M+H]^+$ Found: 635.2286.

4.1.3. 2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyranose 1-dibenzylphosphate 5 and 2-deoxy-2-acetamido-4-O-(2-deoxy-2-acetamido-3′,4′,6′-tri-O-acetyl-β-D-gluco pyranosyl)-3,6-di-O-acetyl-α-D-glucopyranose 1-dibenzylphosphate 6

To a solution of lactol **3** (1 g, 2.89 mmol) in dry THF (32 mL) were successively added a solution of *1H*-tetrazole in acetonitrile (25.6 mL, 0.45 M, 11.5 mmol) and dibenzyl *N*,*N*-diethylphosphoramidite (1.9 mL, 5.76 mmol). The reaction mixture was stirred 14 h at room temperature. Then the solution was cooled by a dry ice/acetone bath and an aqueous solution of hydrogen peroxide (0.88 mL, 30%, 8.64 mmol) was added dropwise. The reaction mixture was stirred at −78 °C during 1 h then it was allowed to warm at room temperature. The solution was diluted with diethylether (300 mL). The organic layer was washed with an aqueous solution of $NaHSO_3$ (10% in weight, 100 mL), brine (100 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification of the residue by column chromatography (silica gel 200 mL, cyclohexane/EtOAc with 1% of Et_3N, v/v, 2/3, 1/3, 0/1) afforded the phosphate **5** (1.46 g, 2.40 mmol, 83%) as a white solid [12,25]. [α]D + 59.7 (*c* 1.0, $CHCl_3$); [α]D + 29.0 (*c* 0.17, MeOH) [25]; [α]D + 4.8 (*c* 1.0, $CHCl_3$) [26]; ^{1}H NMR ($CDCl_3$) δ 7.41–7.31 (m, 10H, H aromatic), 5.76 (d, J = 9.2 Hz, 1H, N*H*Ac), 5.66 (dd, J = 6.0, 3.3 Hz, 1H, H-1), 5.18–5.01 (m, 6H, H benzylic × 4, H-3, H-4), 4.37 (ddt, J = 10.7, 9.2, 3.3 Hz, 1H, H-2), 4.13 (dd, J = 12.5, 4.0 Hz, 1H, H-6), 4.00 (ddd, J = 9.7, 4.0, 2.2 Hz, 1H, H-5), 3.92 (dd, J = 12.5, 2.2 Hz, 1H, H-6′), 2.02 (s, 3H, C*H*$_3$CO), 2.00 (s, 3H, C*H*$_3$CO), 2.00 (s, 3H, C*H*$_3$CO), 1.71 (s, 3H, C*H*$_3$CO); ^{13}C NMR ($CDCl_3$) δ 171.2, 170.6, 170.3, 169.2 (4C, CH_3CO), 135.5 (d, J = 6.3 Hz, *C*q aromatic), 135.3 (d, J = 6.5 Hz, *C*q aromatic), 129.1, 128.9, 128.9, 128.2, 128.2 (10 C, *C* aromatic), 96.4 (d, J = 6.6 Hz, C-1), 70.2 (C-3), 70.09 (d, J = 5.8 Hz, *C* benzylic), 70.04 (d, J = 5.8 Hz, C benzylic), 69.8 (C-5), 67.5 (C-4), 61.4 (C-6), 51.9 (d, J = 7.7 Hz, C-2), 22.8, 20.7, 20.7, 20.6 (4C, *C*H$_3$CO); ^{31}P NMR ($CDCl_3$) δ −2.48; ESI-MS: Calcd for $C_{28}H_{35}NO_{12}P$: 608.2. $[M+H]^+$ Found: 608.2; ESI-HRMS: Calcd for $C_{28}H_{35}NO_{12}P$: 608.1891. $[M+H]^+$ Found: 608.1883.

To a solution of lactol **4** (420 mg, 662 μmol) in dry THF (12 mL) were successively added a solution of *1H*-tetrazole in acetonitrile (5.9 mL, 0.45 M, 1.65 mmol) and dibenzyl *N*,*N*-diethylphosphoramidite (1.9 mL, 5.76 mmol). The reaction mixture was stirred 14 h at room temperature. Then, the same treatment as for compound **5** was carried out. Purification of the crude by column chromatography (silica gel 150 mL, cyclohexane/EtOAc with 1% of Et_3N, v/v, 2/3, 1/3) afforded the phosphate **6** (464 mg, 518 μmol, 78%) as a white solid. [α]D + 21.3 (*c* 1.0, $CHCl_3$); $[\alpha]_D$ −15 (*c* 1.1, $CHCl_3$) [24]; ^{1}H NMR ($CDCl_3$) in agreement with reported data [24,27] δ 7.42–7.30 (m, 10H, H aromatic), 5.85 (d, J = 8.9 Hz, 1H, N*H*Ac*), 5.62 (dd, J = 5.8, 3.3 Hz, 1H, H-1), 5.58 (d, J = 9.3 Hz, 1H, N*H*Ac), 5.20 (dd, J = 10.4, 9.4 Hz, 1H, H-3*), 5.14 (dd, J = 10.9, 9.1 Hz, 1H, H-3), 5.13–5.00 (m, 5H, H benzylic, H-4*), 4.55 (d, J = 8.3 Hz, 1H, H-1*), 4.37 (dd, J = 12.4, 4.2 Hz, 1H, H-6*), 4.31–4.22 (m, 2H, H-6, H-2), 4.06 (dd, J = 12.3, 2.2 Hz, 1H, H-6), 4.00 (dd, J = 12.4, 2.4 Hz, 1H, H-6′*), 3.94 (ddd, J = 10.2, 3.1, 2.2 Hz, 1H, H-5), 3.82 (dt, J = 10.4, 8.3 Hz, 1H, H-2*), 3.71 (dd, J = 10.2, 9.1 Hz, 1H, H-4), 3.61 (ddd, J = 10.0, 4.2, 2.4 Hz, 1H, H-5*), 2.07 (s, 6H, C*H*$_3$CO), 2.02 (s, 3H, C*H*$_3$CO), 2.01 (s, 3H, C*H*$_3$CO), 2.00 (s, 3H, C*H*$_3$CO), 1.93 (s, 3H, C*H*$_3$CO), 1.69 (s, 3H, C*H*$_3$CO); ^{13}C NMR ($CDCl_3$) in agreement with reported data [27] δ 171.2, 171.0, 170.8, 170.7, 170.4, 170.3, 169.5 (7C, $COCH_3$), 135.5 (d, J = 6.2 Hz, *C*q aromatic), 135.4 (d, J = 6.6 Hz, *C*q aromatic), 129.1, 129.0, 128.9, 128.3, 128.2 (10C, *C*H aromatic), 101.3 (C-1*), 96.2 (d, J = 6.2 Hz, C-1), 75.7 (C-4), 72.6 (C-3*), 72.1 (C-5*), 70.8 (C-5), 70.3 (C-3), 70.1 (d, J = 5.6 Hz, *C*H benzylic), 70.0 (d, J = 5.4 Hz, *C*H benzylic), 68.2 (C-4*), 61.9 (C-6*), 61.4 (C-6), 55.1 (C-2*), 52.1 (d, J = 7.7 Hz, C-2), 23.3, 22.9, 21.0, 20.8, 20.7 (7C, *C*H$_3$CO); ^{31}P NMR ($CDCl_3$) in agreement with reported data [22] δ −2.23; ESI-MS: Calcd for $C_{40}H_{51}N_2O_{19}P$: 895.3. $[M+H]^+$ Found: 895.2; ESI-HRMS: Calcd for $C_{40}H_{51}N_2O_{19}P$: 895.2896. $[M+H]^+$ Found: 895.2882.

4.1.4. 2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyranose 1-phosphate 7 and 2-deoxy-2-acetamido-4-O-(2-deoxy-2-acetamido-3′,4′,6′-tri-O-acetyl-β-D-glucopyranosyl)-3,6-di-O-acetyl-α-D-glucopyranose 1-phosphate 8

To a solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranose 1-dibenzylphosphate **5** (100 mg, 164 μmol) in ethanol (10 mL) under argon atmosphere was added a catalytic amount of palladium on charcoal (10 mg, 9 μmol). Then, the reaction mixture was purged with hydrogen gas and stirred during

30 min under hydrogen atmosphere. The resulting solution was degassed with argon atmosphere and it was filtered through a celite© patch. To the filtrate was added triethylamine (50 μL) and concentration under vacuum furnished the product **7** (86 mg, 162 μmol, quant.) as a white solid [25]. ^{1}H NMR (CD_3OD) δ 5.49 (dd, J = 7.0, 3.4 Hz, 1H, H-1), 5.31 (dd, J = 10.6, 9.5 Hz, 1H, H-3), 5.09 (t, J = 9.8 Hz, 1H, H-4), 4.30 (ddd, J = 9.8, 3.5, 2.1 Hz, 1H, H-5), 4.30–4.25 (m, 1H, H-6), 4.30 (ddd, J = 10.6, 3.4, 2.2 Hz, 1H, H-2), 4.17–4.11 (m, 1H, H-6′), 3.18 (q, J = 7.3 Hz, 8H, $(CH_3CH_2)_3NH^+$), 2.05 (s, 3H, CH_3CO), 2.01 (s, 3H, CH_3CO), 1.97 (s, 3H, CH_3CO), 1.94 (s, 3H, CH_3CO), 1.31 (t, J = 7.3 Hz, 12H, $(CH_3CH_2)_3NH^+$); ^{13}C NMR (CD_3OD) δ 173.5, 172.4, 172.0, 171.3 (4C, CH_3CO), 95.06 (d, J = 5.9 Hz, C-1), 72.8 (C-3), 69.9 (C-4), 69.6 (C-5), 63.0 (C-6), 53.5 (d, J = 7.8 Hz, C-2), 47.5 (4C, $(CH_3CH_2)_3NH^+$), 22.6, 20.7, 20.6 (4C, CH_3CO), 9.2 (4C, $(CH_3CH_2)_3NH^+$); ^{31}P NMR (CD_3OD) δ −1.11; ^{31}P NMR non decoupled (CD_3OD) δ −1.11 (d, J = 6.3 Hz); ESI-MS: Calcd for $C_{28}H_{45}N_2O_{24}P_2^-$: 853.2. $[2M+H]^-$ Found: 853.3; ESI-HRMS: Calcd for $C_{14}H_{21}NO_{12}P^-$: 426.0807. $[M]^-$ Found: 426.0791.

To a solution of 2-deoxy-2-acetamido-4-*O*-(2-deoxy-2-acetamido-3′,4′,6′-tri-*O*-acetyl-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-α-D-glucopyranose 1-dibenzylphosphate **6** (55 mg, 74 μmol) in a mixture of ethanol (8 mL) and dichloromethane (5.5 mL) under argon atmosphere was added a catalytic amount of palladium on charcoal (11 mg, 10 μmol). Then, the same procedure as for compound **5** was carried out to furnish the product **8** (60 mg, 74 μmol, quant.) as a white solid [13]. ^{1}H NMR (CD_3OD) δ 5.43 (dd, J = 6.9, 3.4 Hz, 1H, H-1), 5.37 (dd, J = 10.4, 9.3 Hz, 1H, H-3*), 5.25 (dd, J = 10.6, 9.1 Hz, 1H, H-3), 4.95 (dd, J = 9.9, 9.3 Hz, 1H, H-4*), 4.80 (d, J = 8.4 Hz, 1H, H-1*), 4.54 (dd, J = 12.1, 1.9 Hz, 1H, H-6), 4.43 (dd, J = 12.4, 4.1 Hz, 1H, H-6*), 4.21–4.14 (m, 2H, H-2, H-5), 4.06 (dd, J = 12.1, 4.2 Hz, 1H, H-6′), 4.02 (dd, J = 12.4, 2.3 Hz, 1H, H-6′*), 3.89 (dd, J = 9.9, 9.1 Hz, 1H, H-4), 3.79 (ddd, J = 10.1, 4.1, 2.3 Hz, 1H, H-5*), 3.57 (dd, J = 10.4, 8.4 Hz, 1H, H-2*), 3.12 (q, J = 7.3 Hz, 8H, $(CH_3CH_2)_3NH^+$), 2.10 (s, 3H, CH_3CO), 2.06 (s, 3H, CH_3CO), 2.03 (s, 3H, CH_3CO), 1.98 (s, 3H, CH_3CO), 1.97 (s, 3H, CH_3CO), 1.93 (s, 3H, CH_3CO), 1.89 (s, 3H, CH_3CO), 1.28 (t, J = 7.3 Hz, 12H, $(CH_3CH_2)_3NH^+$); ^{13}C NMR (CD_3OD) δ 173.6, 173.5, 172.6, 172.3, 172.2, 171.8, 171.3 (7C, $COCH_3$), 101.6 (C-1*), 95.0 (d, J = 5.9 Hz, C-1), 77.0 (C-4), 73.5 (C-3*), 73.3 (C-3), 72.8 (C-5*), 70.5 (C-5), 70.0 (C-4*), 63.4 (C-6), 63.1 (C-6*), 56.7 (C-2*), 53.6 (d, J = 8.1 Hz, C-2), 47.7 (4C, $(CH_3CH_2)_3NH^+$), 22.9, 22.6, 21.1, 20.9, 20.7, 20.6, 20.5 (7C, CH_3CO), 9.4 (4C, $(CH_3CH_2)_3NH^+$); ^{31}P NMR (CD_3OD) δ −1.12; ^{31}P NMR non decoupled (202 MHz, CD_3OD) δ −1.09 (d, J = 6.1 Hz); ESI-MS: Calcd for $C_{26}H_{38}N_2O_{19}P^-$: 713.2. $[M]^-$ Found: 713.6; ESI-HRMS: Calcd for $C_{26}H_{38}N_2O_{19}P^-$: 713.1812. $[M]^-$ Found: 713.1802.

4.1.5. 2-Acetamido-2-deoxy-α-D-glucopyranose 1-phosphate 9 and 2-deoxy-2-acetamido-4-O-(2-deoxy-2-acetamido-β-D-glucopyranosyl)-α-D-glucopyranose 1-phosphate 10

To a solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranose 1-phosphate **7** (72 mg, 169 μmol, 1 eq.) in methanol (20 mL) was added dropwise a solution of sodium methoxide (1.6 mL, 0.53 M, 5 eq.). The reaction mixture was stirred at room temperature for 1 h. The reaction mixture was neutralized with excess of cation exchange resin (Dowex 50Wx8, H^+ form). The resin was filtered and washed with methanol. The combined phases were evaporated. The obtained white solid was dissolved in water and was freeze dried to furnish the product **9** (55 mg, 159 μmol, 94%) as a white solid [11]. ^{1}H NMR (D_2O) δ 5.38 (dd, J = 7.5, 3.2 Hz, 1H, H-1), 4.00 (ddd, J = 9.6, 5.2, 2.1 Hz, 1H, H-5), 3.95 (dd, J = 10.4, 3.2, 1.8 Hz, 1H, H-2), 3.92 (d, J = 12.3, 2.1 Hz, 1H, H-6), 3.83 (broad dd, J = 10.4, 9.6 Hz, 1H, H-3), 3.80 (dd, J = 12.3, 5.2 Hz, 1H, H-6′), 3.51 (t, J = 9.6 Hz, 1H, H-4), 2.10 (s, 3H, CH_3CO); ^{13}C NMR (D_2O) δ 174.7 (s, CH_3CO), 92.7 (d, J = 5.5 Hz, C-1), 72.1 (s, C-5), 71.6 (s, C-3), 70.2 (s, C-4), 60.8 (s, C-6), 54.2 (d, J = 7.3 Hz, C-2), 22.2 (s, CH_3CO); ^{31}P NMR (D_2O) δ 2.22 (s); ^{31}P NMR non decoupled (D_2O) δ 2.22 (d, J = 7.5 Hz); ESI-MS: Calcd for $C_8H_{15}NO_9P^-$: 300.0. $[M]^-$ Found: 300.0; ESI-HRMS: Calcd for $C_8H_{15}NO_9P^-$: 300.0479. $[M]^-$ Found: 300.0471.

To a solution of 2-deoxy-2-acetamido-4-*O*-(2-deoxy-2-acetamido-3′,4′,6′-tri-*O*-acetyl-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-α-D-glucopyranose 1-phosphate **8** (43 mg, 60 μmol, 1 eq.) in methanol (20 mL) was added dropwise a solution of sodium methoxide (1.3 mL, 0.325 M, 7 eq.). The reaction mixture was stirred at room temperature for 14 h. Then the same procedure as for compound **9** was carried out to furnish the product **10** (32 mg, 58 μmol, 97%) as a white solid [28]. ^{1}H NMR (D_2O) δ 5.37 (dd, J = 7.6, 2.9 Hz, 1H, H-1), 4.66 (d, J = 8.5 Hz, 1H, H-1*), 4.05–4.01 (m, 1H, H-5), 4.00–3.92 (m, 3H, H-2, H-5*, H-6), 3.88 (dd, J = 12.1, 1.6 Hz, 1H, H-6*), 3.83–3.75 (m, 2H, H-2*,H-6), 3.73–3.66 (m, 2H, H-6*, H-4), 3.65–3.60 (m, 1H, H-3*), 3.57–3.49 (m, 2H, H-3, H-4*), 2.11 (s, 3H, CH_3CO), 2.09 (s, 3H, CH_3CO); ^{13}C NMR (D_2O) δ 174.7 (s, CH_3CO), 174.6 (s, CH_3CO), 101.3 (s, C-1*), 92.4 (d, J = 5.4 Hz, C-1), 79.7 (s, C-4), 76.0 (s, C-3), 73.6 (s, C-3*), 70.5 (s, C-5), 70.1 (s, C-5*), 69.8 (s, C-4*), 60.6 (s, C-6), 60.2 (s, C-6*), 55.7 (s, C-2*), 53.7 (d, J = 7.4 Hz, C-2), 22.2 (s, CH_3CO), 22.2 (s, CH_3CO); ^{31}P NMR (D_2O) δ 2.21 (s); ^{31}P NMR non decoupled (D_2O) δ 2.19 (d, J = 7.2 Hz); ESI-MS: Calcd for $C_{17}H_{31}N_2O_{14}P^-$: 518.2. $[M]^-$ Found: 518.4; ESI-HRMS: Calcd for $C_{16}H_{28}N_2O_{14}P^-$: 503.1273. $[M]^-$ Found: 503.1275.

4.1.6. Dicyclohexylammonium (S)-citronellyl phosphate 11

To dichloromethane (60 mL) under argon atmosphere at room temperature were added successively $POCl_3$ (4.2 mL, 45.2 mmol, 3 eq.), triethylamine (6.3 mL, 45.2 mmol, 3eq.) and (S)-(−)-β-citronellol (3 mL, 15.1 mmol, 1 eq.). The reaction mixture was kept under agitation at room temperature then 90 mL of an acetone, triethylamine and water (88/10/2, v/v/v) solution was added. After 14 h stirring, solvents were removed. The crude product was diluted with water (15 mL). The aqueous layer was extracted with dichloromethane (3 × 75 mL). The combined organic layers were washed with water (45 mL), brine (50 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was dissolved in a mixture of acetone and water (120 mL, 9/1, v/v) and cyclohexylamine (3 mL) was added. The mixture was kept at 0 °C for 1 h. The formed crystals were collected by filtration and they were submitted to recrystallization in 45 mL of a mixture of acetone and water (9/1, v/v) to furnish the product **11** (2.22 g, 4.94 mmol, 33%) as white crystals. A part of this product was purified by reverse phase HPLC (0% MeCN 100% aq. NH_4HCO_3 25 mM t_0, 100% MeCN 0% aq. NH_4HCO_3 25 mM over 50 min; t_R 28 min) to furnish the diammonium salt of (S)-citronellyl phosphate **11**. ^{1}H NMR (D_2O) δ 5.29 (tq, J = 7.2, 1.4 Hz, 1H, H-6), 4.02–3.88 (m, 2H, H-1), 2.15–1.99 (m, 2H, H-5), 1.77–1.69 (m, 4H, H-2, CH_3-9), 1.68 (s, 3H, CH_3-10), 1.67–1.58 (m, 1H, H-3), 1.53–1.45 (m, 1H, H-2′), 1.44–1.36 (m, 1H, H-4), 1.24 (dddd, J = 13.5, 9.2, 7.7, 6.2 Hz, 1H, H-4′), 0.95 (d, J = 6.7 Hz, 3H, CH_3-8); ^{13}C NMR (D_2O) δ 133.2 (s, C-7), 125.3 (s, C-6), 64.4 (d, J = 5.5 Hz, C-1), 36.8 (d, J = 6.8 Hz, C-2), 36.3 (s, C-4), 28.6 (s, C-3), 24.8 (s, C-5), 24.8 (s, C-9), 18.6 (s, C-8), 16.9 (s, C-10); ^{31}P NMR non decoupled (D_2O) δ 0.53 (t, J = 6.6 Hz); ^{31}P NMR (D_2O) δ 0.54 (s); ESI-MS: Calcd. For $C_{10}H_{20}O_4P^-$: 235.1. $[M+H]^-$ Found 235.3; ESI-HRMS: Calcd. For $C_{10}H_{20}O_4P^-$: 235.1105. $[M+H]^-$ Found 235.1094.

4.1.7. (S)-Citronellyl diphosphate 12

To a solution of (*S*)-(−)-β-citronellol (0.5 mL, 2.51 mmol) in dichloromethane (10 mL) under argon atmosphere were added triethylamine (0.38 mL, 2.76 mmol), tosyl chloride (280 mg, 2.76 mmol) and 4-(dimethylamino)pyridine (30 mg, 0.25 mmol). The reaction mixture was stirred during 14 h at room temperature, and then diluted in diethyl ether (50 mL). The resulting precipitate was removed by filtration through a celite pad. The filtrate was concentrated *in vacuo* and the crude product was purified by flash

chromatography on silica gel column (50 g, EtOAc/cyclohexane, 1/9, v/v) to yield the citronellyl tosylate as a colourless oil (532 mg, 1.64 mmol, 65%).

To a suspension of tris(tetrabutylammonium) hydrogen diphosphate (668 mg, 0.74 mmol) in distilled acetonitrile (10 mL) under argon atmosphere was added the previous citronellyl tosylate (100 mg, 0.31 mmol). The reaction mixture was stirred during 18 h at room temperature and concentrated *in vacuo*. It was then dissolved in water (10 mL) and DOWEX 50WX8 (NH_4^+ form) was added to exchange tetrabutylammonium ions. Resin was removed by filtration and the filtrate was freeze dried. The lyophilisate was dissolved in 100 mM aq. NH_4HCO_3 (3 mL) and a mixture of acetonitrile/isopropanol (1/1, v/v, 3 mL) was added. After centrifugation of the suspension for 5 min at 2000 rpm, the supernatant was removed and the process was repeated three times. The combined supernatants were concentrated *in vacuo* and freeze dried. The lyophilisate was purified by reverse phase HPLC (0% MeCN 100% aq. NH_4HCO_3 25 mM t_0, 0–15% MeCN over 20 min, 15–25% over 30 min; t_R 37.1 min) to afford the desired citronellyl diphosphate **12** as a white powder (39 mg, 0.12 mmol, 38%). ^{1}H NMR (D_2O) δ 5.34–5.24 (m, 1H, H-6), 4.09–3.91 (m, 2H, H-1), 2.13–1.97 (m, 2H, H-5), 1.78–1.69 (m, 4H, H-2, CH_3-9), 1.67 (s, 3H, CH_3-10), 1.65–1.58 (m, 1H, H-3), 1.55–1.46 (m, 1H, H-2′), 1.45–1.37 (m, 1H, H-4), 1.29–1.18 (m, 1H, H-4′), 0.95 (d, J = 6.6 Hz, 3H, CH_3-8); ^{13}C NMR (D_2O) δ 133.1 (s, C-7), 125.4 (s, C-6), 64.7 (d, J = 5.7 Hz, C-1), 37.0 (d, J = 7.3 Hz, C-2), 36.5 (s, C-4), 28.7 (s, C-3), 24.9 (s, C-5), 24.8 (s, C-9), 18.7 (s, C-8), 16.9 (s, C-10).

^{31}P NMR non decoupled (202 MHz, D_2O) δ −6.03 (d, J = 21.7 Hz, P-A), −10.08 (dt, J = 21.7, 6.4 Hz, P–B). ^{31}P NMR (202 MHz, D_2O) δ −6.02 (d, J = 21.7 Hz, P-A), −10.08 (d, J = 21.7 Hz, P–B); ESI −MS Calcd. For $C_{10}H_{21}O_7P_2^-$: 315.1. [M + 2H]$^-$ Found 315.3; ESI-HRMS Calcd. For $C_{10}H_{21}O_7P_2^-$: 315.0768. [M + 2H]$^-$ Found 315.0773.

4.1.8. (S)-Citronellyl medronate 13

The medronate was prepared by the same methodology as the citronellyl diphosphate **12** starting from tris(tetrabutylammonium) hydrogen medronate (800 mg, 0.89 mmol) and citronellyl tosylate (170 mg, 0.55 mmol) in distilled acetonitrile (10 mL) to afford after reverse phase HPLC purification (0% MeCN 100% aq. NH_4HCO_3 25 mM t_0, 0–20% MeCN over 20 min, 20–50% over 30 min; t_R 36.0 min) the desired citronellyl medronate **13** as a white powder (75 mg, 0.21 mmol, 37%). ^{1}H NMR (D_2O) δ 5.33–5.22 (m, 1H, H-6), 4.09–3.91 (m, 2H, H-1), 2.30 (t, J = 20.1 Hz, 2H, H-11), 2.14–1.98 (m, 2H, H-5), 1.78–1.69 (m, 4H, H-2, CH_3-9), 1.67 (s, 3H, CH_3-10), 1.65–1.59 (m, 1H, H-3), 1.55–1.46 (m, 1H, H-2′), 1.45–1.36 (m, 1H, H-4), 1.29–1.18 (m, 1H, H-4′), 0.95 (d, J = 6.6 Hz, 3H, CH_3-8); ^{13}C NMR (D_2O) δ 133.2 (s, C-7), 125.3 (s, C-6), 64.0 (d, J = 5.9 Hz, C-1), 37.0 (d, J = 6.1 Hz, C-2), 36.3 (s, C-4), 28.6 (s, C-3), 26.7 (t, J = 125 Hz, C-11), 24.9 (s, C-5), 24.8 (s, C-9), 18.7 (s, C-8), 16.9 (s, C-10); ^{31}P NMR (D_2O) δ 17.86 (d, J = 9.2 Hz, P–B), 17.22 (d, J = 9.2 Hz, P-A); ^{31}P NMR non decoupled (D_2O) δ 18.05–17.65 (m, P–B), 17.22 (td, J = 20.1, 9.2 Hz, P-A).

ESI-MS: Calcd. For $C_{11}H_{23}O_7P_2^-$: 313.1. [M + 2H]$^-$ Found 313.3; ESI-HRMS Calcd. For $C_{11}H_{23}O_7P_2^-$: 313.0975. [M + 2H]$^-$ Found 313.0979.

4.1.9. Tetrabutylammonium solanesyl phosphate 14

To a solution of solanesol (50 mg, 79 μmol, 1 eq.) in dichloromethane (1 mL) was added tetrabutylammonium phosphate (87 mg, 256 μmol, 3.2 eq.). The reaction mixture was placed in a dark place. Then, trichloroacetonitrile (39 μL, 390 μmol, 5 éq.) was added to the mixture. After 1 h stirring at room temperature, the solvent was evaporated. To the residue was added THF (0.75 mL) and a concentrated solution of aqueous ammonium hydroxide (28%, 0.15 mL). After 14 h stirring at room temperature, a mixture of toluene and methanol (3.25 mL, 1/1, v/v) was added. After 30 min, the precipitate was removed by filtration and the filtrate was concentrated *in vacuo*. The crude product was purified by flash chromatography on silica gel column (5 g, dichloromethane/methanol/ammonia 14%, 80/18/2, v/v/v) to yield the tetrabutylammonium solanesyl phosphate **14** as a colourless oil (21 mg, 28 μmol, 33%). ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.39 (t, J = 5.9 Hz, 1H, H-2), 5.11 (t, J = 6.2 Hz, 8H, 8 × H-7), 4.45 (t, J = 5.7 Hz, 2H, 2 × H-1), 3.42–3.18 (m, 8H, ($CH_3CH_2CH_2CH_2$)$_4N^+$), 2.17–2.02 (m, 16H, 14 × H-6, 2 × H-11), 2.01–1.86 (m, 16H, 14 × H-9, 2 × H-4), 1.70–1.55 (m, 8H, ($CH_3CH_2CH_2CH_2$)$_4N^+$), 1.67 (s, 6H, CH_3-5, CH_3-15), 1.62 (s, 3H, CH_3-14), 1.59 (s, 21H, 7 × CH_3-10), 1.52–1.36 (m, 8H, ($CH_3CH_2CH_2CH_2$)$_4N^+$), 0.98 (t, J = 7.2 Hz, 12H, ($CH_3CH_2CH_2CH_2$)$_4N^+$); ^{31}P NMR (101 MHz, $CDCl_3$) δ 1.62 (s); ESI-MS Calcd. For $C_{45}H_{74}O_4P^-$: 709.6. [M+H]$^-$ Found 708.7; ESI-HRMS Calcd. For $C_{45}H_{74}O_4P^-$: 709.5530. [M+H]$^-$ Found 709.5329.

4.1.10. Ammonium solanesyl diphosphate 15

To a solution of solanesol (95 mg, 150 μmol, 1 eq.) in dichloromethane (2 mL) was added tetrabutylammonium phosphate (204 mg, 600 μmol, 4 eq.). The reaction mixture was placed in a dark place. Then trichloroacetonitrile (75 μL, 750 μmol, 5 eq.) was added to the mixture. After 14 h stirring at room temperature, the solvent was evaporated. To the residue was added THF (1.5 mL) and a concentrated solution of aqueous ammonium hydroxide (28%, 0.3 mL). After 30 min stirring at room temperature, a mixture of toluene and methanol (8 mL, 1/1, v/v) was added. After 30 min, the precipitate was removed by filtration and the filtrate was concentrated *in vacuo*. The crude product was purified by DEAE anion exchange chromatography. The product was eluted with a mixture of chloroform/methanol/water (10/10/3, v/v/v), then a mixture of chloroform/methanol/5 mM aqueous ammonium acetate (10/10/3, v/v/v), then a mixture of chloroform/methanol/100 mM aqueous ammonium acetate (10/10/3, v/v/v). Fractions containing the desired product were pooled and lyophilized to furnish the product **15** (34 mg, 40 μmol, 27%) as a white solid. ^{1}H NMR (250 MHz, 78% $CDCl_3$, 19.5% CD_3OD, 2.5% D_2O) δ 5.33–5.19 (m, 1H, H-2), 5.11–4.90 (m, 8H, 7 × H-7, H-12), 4.41–4.24 (m, 2H, 2 × H-1), 2.07–1.90 (m, 16H, 14 × H-6, 2 × H-11), 1.90–1.75 (m, 16H, 14 × H-9, 2 × H-4), 1.54, 1.53 (2 × s, 6H, CH_3-5, CH_3-15), 1.46 (s, 24H, 7 × CH_3-10, CH_3-14); ^{31}P NMR (101 MHz, 78% $CDCl_3$, 19.5% CD_3OD, 2.5% D_2O) δ −5.04 (d, J = 17.1 Hz, P-A), -6.05 (d, J = 17.1 Hz, P–B); ESI-HRMS Calcd. For $C_{45}H_{74}O_7P_2^-$: 789.4994. [M+H]$^-$ Found 789.4999.

4.1.11. P^1-(2-Acetamido-2-deoxy-α-D-glucopyranosyl) P^2-(S)-(−)-β-(S)-citronellyl diphosphate 16

To a solution of (S)-citronellyl phosphate **11** [264 mg, 607 μmol, 1.3 eq., previously co-evaporated with dry toluene (5 mL) and triethylamine (50 μL) three times] in dry THF (15 mL) was added 1,1′-carbonyldiimidazole CDI (325 mg, 2 mmol, 4.3 eq.) under argon atmosphere. After 1.5 h stirring at room temperature, dry methanol (210 μmol) was added and the mixture was stirred for 1 h to destroy the excess of CDI. Then, solvents were removed. The resulting crude activated phosphate was solubilized in dry THF (15 mL) and was transferred to a flask containing the phosphate **7** [225 mg, 427 μmol, 1 eq., previously co-evaporated with dry toluene (5 mL) and triethylamine (50 μL) three times]. The reaction mixture was stirred overnight at room temperature and then was concentrated *in vacuo*. The crude product was quickly purified by column chromatography on silica gel (50 g, CH_2Cl_2/MeOH/H_2O/Et_3N, 90/10/0/0.2 to 80/19/1/0.2, v/v/v/v) to furnish a mixture of partially deacetylated diphosphate (120 mg).

At room temperature, the previous mixture (120 mg) was dissolved in methanol (12 mL) then a solution of sodium methoxide (1 mL, 0.43 M, 430 μmol) was added to the reaction mixture. After

30 min stirring, the reaction was stopped with addition of a cation exchange resin (DOWEX 50WX8, H^+ form). After 30 min stirring, the resin was removed by filtration and the resin was washed with methanol. The filtrate was concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (12 g, CH_2Cl_2/MeOH/H_2O/Et_3N, 90/10/0/0.2 to 75/25/1/0.2, v/v/v/v) to furnish the product **16** (80 mg, 124 μmol, 29% over two steps) as a monotriethyl-ammonium salt [23]. ^{1}H NMR (D_2O) δ 5.53 (dd, J = 7.2, 3.3 Hz, 1H, H-1), 5.32–5.23 (m, 1H, H-12), 4.09–4.00 (m, 3H, H-2, 2 × H-7), 3.98 (ddd, J = 10.2, 4.6, 2.3 Hz, 1H, H-5), 3.92 (dd, J = 12.4, 2.3 Hz, 1H, H-6), 3.85 (t, J = 9.8 Hz, H-3), 3.85 (dd, J = 12.4, 4.6 Hz, 1H, H-6′), 3.58 (dd, J = 10.2, 9.8 Hz, 1H, H-4), 3.24 (q, J = 7.3 Hz, 6H, $(CH_3CH_2)_3NH^+$), 2.12 (s, 3H, CH_3CONH), 2.14–1.99 (m, 2H, 2 × H-11), 1.80–1.69 (m, 1H, H-8), 1.74 (s, 3H, CH_3-15), 1.67 (s, 3H, CH_3-16), 1.63 (td, J = 12.0, 7.1 Hz, 1H, H-9), 1.50 (ddd, J = 16.5, 12.0, 5.0 Hz, 1H, H-8′), 1.46–1.35 (m, 1H, H-10), 1.32 (t, J = 7.3 Hz, 9H, $(CH_3CH_2)_3NH^+$), 1.29–1.17 (m, 1H, H-10′), 0.95 (d, J = 6.6 Hz, 3H, CH_3-14); ^{13}C NMR (D_2O) δ 174.8 (s, CH_3CONH), 133.2 (s, C-13), 125.3 (s, C-12), 94.5 (d, J = 6.3 Hz, C-1), 73.0 (s, C-5), 71.0 (s, C-3), 69.6 (s, C-4), 65.3 (d, J = 6.1 Hz, C-7), 60.4 (s, C-6), 53.7 (d, J = 8.6 Hz, C-2), 46.7 (s, 3C, $(CH_3CH_2)_3NH^+$), 36.8 (d, J = 7.3 Hz, C-8), 36.4 (s, C-10), 28.6 (s, C-9), 24.9 (s, C-11), 24.8 (s, C-15), 22.1 (s, CH_3CONH), 18.6 (s, C-14), 16.9 (s, C-16), 8.25 (s, 3C, $(CH_3CH_2)_3NH^+$); ^{31}P NMR (D_2O) δ −10.70 (d, J = 20.7 Hz, P–B), -13.22 (d, J = 20.7 Hz, P-A); ^{31}P NMR non decoupled (D_2O) δ −10.71 (dt, J = 20.7, 7.2 Hz, 1H, P–B), -13.23 (dd, J = 20.7, 7.8 Hz, 1H, P-A); ESI-MS Calcd. For $C_{18}H_{34}O_{12}P_2^-$: 518.2. $[M+H]^-$ Found 518.4; HRMS (ESI-) Calcd. For $C_{18}H_{34}O_{12}P_2^-$: 518.1562. $[M+H]^-$ Found 518.1556.

4.1.12. P^1-[2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2-Acetamido-2-deoxy-α-D-glucopyranosyl] P^2-(S)-(−)-β -citronellyl diphosphate 17

To a solution of (S)-citronellyl phosphate **11** [236 mg, 545 μmol, 3.3 eq., previously co-evaporated with dry toluene (5 mL) and triethylamine (50 μL) three times] in dry THF (15 mL) was added 1,1′-carbonyldiimidazole CDI (380 mg, 2.34 mmol, 14.2 eq.) under argon atmosphere. After 1.5 h stirring at room temperature, dry methanol (240 μmol) was added and the mixture was stirred for 1 h to destroy the excess of CDI. Then, solvents were removed. The resulting crude activated phosphate was solubilized in dry DMF (10 mL) and was transferred to a flask containing the phosphate **8** [135 mg, 165 μmol, 1 eq., previously co-evaporated with dry toluene (5 mL) and triethylamine (50 μL) three times]. The reaction mixture was stirred overnight at room temperature. The reaction mixture was concentrated *in vacuo*. The crude product was purified by column chromatography on silica gel (40 g, CH_2Cl_2/MeOH/H_2O/Et_3N, 90/10/0/0.2 to 75/22/3/0.2, v/v/v/v) to furnish a mixture of partially deacetylated diphosphate (105 mg).

At room temperature, the previous mixture (105 mg) was dissolved in methanol (10 mL) then a solution of sodium methoxide in methanol (5 mL, 0.43 M, 2.15 mmol) was added to the reaction mixture. After overnight stirring, the reaction was stopped with addition of a cation exchange resin (DOWEX 50WX8, H^+ form). After 1 h stirring, the resin was removed by filtration and the resin was washed with methanol. The filtrate was concentrated *in vacuo*. The residue was purified by reverse phase HPLC (0% MeCN 100% aq. NH_4HCO_3 25 mM t_0, 0–20% MeCN over 10 min, 20–30% over 20 min; t_R 13.0 min) to furnish the product **17** (32 mg, 50 μmol, 30% over two steps) as a di-ammonium salt. ^{1}H NMR (D_2O) δ 5.52 (dd, J = 7.3, 3.2 Hz, 1H, H-1), 5.34–5.24 (m, 1H, H-12), 4.67 (d, J = 8.5 Hz, 1H, H-1*), 4.09–3.93 (m, 6H, H-2, 2 × H-7, H-5, H-6*, H-3), 3.89 (dd, J = 12.3, 1.9 Hz, 1H, H-6), 3.83–3.74 (m, 3H, H-2*, H-6*, H-4), 3.72 (dd, J = 12.3, 4.4 Hz, 1H, H-6), 3.62 (dd, J = 10.4, 8.7 Hz, 1H, H-3*), 3.58–3.52 (ddd, J = 9.7, 5.9, 2.1 Hz, 1H, H-5*), 3.50 (dd, J = 9.7, 8.7 Hz, 1H, H-4*), 2.11 (s, 6H, 2 × CH_3CO), 2.19–1.98 (m, 2H, 2 × H-11), 1.80–1.70 (m, 1H, H-8), 1.68 (s, 3H, CH_3-16), 1.66–1.58 (m, 1H, H-9), 1.50 (td, J = 13.6, 7.0 Hz, 1H, H-8′), 1.45–1.33 (m, 1H, H-10), 1.31–1.19 (m, 1H, H-10′), 0.96 (d, J = 6.6 Hz, 3H, CH_3-14); ^{13}C NMR (D_2O) δ 174.7 (s, CH_3CO), 174.5 (s, CH_3CO), 133.1 (s, C-13), 125.4 (s, C-12), 101.3 (s, C-1*), 94.1 (d, J = 6.1 Hz, C-1), 79.2 (s, C-4), 76.0 (s, C-5*), 73.7 (s, C-3*), 71.5 (s, C-5), 69.9 (s, C-3), 69.8 (s, C-4*), 65.3 (d, J = 6.1 Hz, C-7), 60.7 (s, C-6*), 59.9 (s, C-6), 55.7 (s, C-2*), 53.2 (d, J = 8.4 Hz, C-2), 36.8 (d, J = 7.2 Hz, C-8), 36.4 (s, C-10), 28.7 (s, C-9), 24.9 (s, C-11), 24.9 (s, C-15), 22.2 (s, CH_3CO), 22.2 (s, CH_3CO), 18.7 (s, C-14), 17.00 (s, C-16); ^{31}P NMR (D_2O) δ −10.63 (broad s, P–B), -13.15 (broad s, P-A); ESI-MS Calcd. For $C_{26}H_{47}O_{17}P_2^-$: 721.2. $[M+H]^-$ Found 721.5; ESI-HRMS Calcd. For $C_{26}H_{47}O_{17}P_2^-$: 721.2355. $[M+H]^-$ Found 721.2359.

4.1.13. P^1-(2-Acetamido-2-deoxy-α-D-glucopyranosyl) P^2-solanesyl diphosphate 18

To a solution of solanesyl phosphate **14** [50 mg, 52 μmol, 1 eq., previously co-evaporated with dry toluene (5 mL) and triethylamine (50 μL) three times] in dry dichloromethane (5 mL) was added 1,1′-carbonyldiimidazole CDI (43 mg, 262 μmol, 5 eq.) under argon atmosphere. After 2 h stirring at room temperature, dry methanol (100 μmol) was added and the mixture was stirred for 1 h to destroy the excess of CDI. Then solvents were removed. The resulting crude activated phosphate was solubilized in dry DMF (5 mL) and was transferred to a flask containing the phosphate **7** [40 mg, 75 μmol, 1.8 eq., previously co-evaporated with dry toluene (5 mL) and triethylamine (50 μL) three times]. The reaction mixture was stirred 6 days at room temperature. The reaction mixture was concentrated *in vacuo*. The crude product was purified by column chromatography on silica gel (5 g, CH_2Cl_2/MeOH/NH_4OH 14%, 80/18/2 to 70/27/3, v/v/v) to furnish the tri-*O*-acetyl diphosphate (26 mg, 22.5 μmol, 43%).

At room temperature, the previous tri-*O*-acetyl diphosphate (26 mg, 22.5 μmol, 1 eq.) was dissolved in methanol (3 mL) and dichloromethane (3 mL) then a solution of sodium methoxide in methanol (250 μL, 0.43 M, 107 μmol, 4.8 eq.) was added to the reaction mixture. After 1 h 30 stirring, the reaction was stopped by addition of a cation exchange resin (DOWEX 50WX8, H^+ form). After 30 min stirring, the resin was removed by filtration and the resin was washed with methanol. The filtrate was concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (1 g, CH_2Cl_2/MeOH/H_2O/Et_3N, 90/10/0/0.2 to 75/25/1/0.2, v/v/v/v) to furnish the product **18** (14 mg, 13.6 μmol, 60%, 26% over two steps) as a di-ammonium salt. ^{1}H NMR (250 MHz, 78% $CDCl_3$, 19.5% CD_3OD, 2.5% D_2O) δ 5.44–5.32 (m, 1H, H-1), 5.25 (broad t, J = 6.5 Hz, 1H, H-8), 5.00 (broad t, J = 5.9 Hz, 8H, 7 × H-12, H-16), 4.42–4.25 (m, 2H, 2 × H-7), 3.88–3.72 (m, 3H, H-5, H-2, H-6), 3.72–3.50 (m, 2H, H-6′, H-4), 3.30 (broad t, J = 9.3 Hz, 1H, H-4), 2.05–1.77 (m, 35H, 2 × H-10, $NCOCH_3$, 14 × H-11, 14 × H-14, 2 × H-15), 1.56 (s, 6H, CH_3-18, CH_3-21), 1.48 (s, 24H, 7 × CH_3-19, CH_3-20); ^{31}P NMR (101 MHz, 78% $CDCl_3$, 19.5% CD_3OD, 2.5% D_2O) δ −6.16 (broad s, P–B), -8.27 (broad s, P-A); ESI-MS Calcd. For $C_{53}H_{88}O_{12}NP_2^-$: 992.6. $[M+H]^-$ Found 993.1; ESI-HRMS Calcd. For $C_{53}H_{88}O_{12}NP_2^-$: 992.5743. $[M+H]^-$ Found 992.5787.

4.1.14. P^1-[2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-α-D-glucopyranosyl] P^2-solanesyl diphosphate 19

To a solution of a crude mixture containing tetrabutylammonium solanesyl phosphate **14** and tetrabutylammonium solanesyl diphosphate **15** [**14**/**15** ≈ 2/1, 100 mg, 94 μmol, previously co-evaporated with dry toluene (5 mL) and triethylamine (50 μL) three times] in dry dichloromethane (5 mL) was added 1,1′-carbonyldiimidazole CDI (85 mg, 525 μmol) under argon atmosphere. After stirring 2 h at room temperature, methanol (100 μmol) was

added and the mixture was stirred 1 h to destroy the excess of CDI. Then solvents were removed. The resulting crude activated phosphate was solubilized in dry pyridine (2 mL) and was transferred to another flask containing the phosphate **8** [60 mg, 74 μmol, 1 eq., previously co-evaporated with dry toluene (5 mL) and triethylamine (50 μL) three times]. The reaction mixture was stirred for 7 days at room temperature. The reaction mixture was concentrated *in vacuo*. The crude product was purified by column chromatography on silica gel (50 g, CH_2Cl_2/MeOH/NH_4OH 14%, 80/18/2 to 70/27/3, v/v/v) to furnish acetylated diphosphate (25 mg, 15 μmol, 25%) and acetylated triphosphate (17 mg, 9.6 μmol, 30%).

At room temperature, the acetylated diphosphate (25 mg, 15 μmol) was dissolved in a mixture of methanol (3 mL) and dichloromethane (3 mL) then a solution of sodium methoxide (160 μL, 0.43 M, 69 μmol) was added to the reaction mixture. After 1 h 30 stirring, the reaction was stopped by addition of a cation exchange resin (DOWEX 50WX8, H^+ form). After 30 min stirring, the resin was removed by filtration and the resin was washed with methanol. The filtrate was concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (900 mg, CH_2Cl_2/MeOH/14% aqueous NH_4OH, 80/18/2 to 70/27/3, v/v/v then CH_2Cl_2/MeOH/H_2O/Et_3N, 70/27/2/1, v/v/v/v) to furnish the product **19** (10 mg, 7.1 μmol, 47%) as a ditriethyl-ammonium salt. ^{1}H NMR (250 MHz, 78% $CDCl_3$, 19.5% CD_3OD, 2.5% D_2O) δ 5.40–5.30 (m, 1H, H-1), 5.28–5.19 (m, 1H, H-8), 5.00 (t, J = 5.9 Hz, 8H, 7 × H-12, H-16), 4.41 (d, J = 8.3 Hz, 1H, H-1*), 4.38–4.30 (m, 2H, 2 × H-7), 3.83–3.28 (m, 12H, H-2, H-5, H-2*, H-5*, H-6, H-6′, H-6*, H-6'*, H-3, H-3*, H-4, H-4*), 3.01 (q, J = 7.2 Hz, 12H, 2 × $(CH_3CH_2)_3NH^+$), 2.04–1.78 (m, 38H, 2 × H-10, 2 × NCOCH_3, 14 × H-11, 14 × H-14, 2 × H-15), 1.55 (s, 6H, CH_3-18, CH_3-21), 1.48 (s, 24H, 7 xCH_3-19, CH_3-20), 1.18 (t, J = 7.2 Hz, 18H, $(CH_3CH_2)_3NH^+$); ^{31}P NMR (101 MHz, 78% $CDCl_3$, 19.5% CD_3OD, 2.5% D_2O) δ −7.00 (broad s, P–B), -9.16 (broad s, P-A); ESI-MS Calcd. For $C_{61}H_{101}O_{17}N_2P_2^-$: 1195.7. $[M+H]^-$ Found 1196.1; ESI-HRMS Calcd. For $C_{61}H_{101}O_{17}N_2P_2^-$: 1195.6570. $[M+H]^-$ Found 1195.6543.

4.2. Source of undecaprenyl compounds

Undecaprenyl phosphate and undecaprenyl diphosphate were provided by the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences, Warsaw, Poland. Undecaprenyl-PP-GlcNAc was produced by enzymatic synthesis, using the WecA enzyme [18,19]. Lipid II (Undecaprenyl-PP-Mur*NAc*(-pentapeptide)-Glc*NAc*), was produced by enzymatic synthesis, using the purified MraY and MurG enzymes as previously described [20,21].

4.3. Enzymatic assays

Standard DLODP assays – Glc$_{3\text{-}0}$[^{3}H]Man$_5$GlcNAc$_2$-PP-dolichol and lipid soluble solanesyl-based test compounds were dried into assay tubes and resuspended in 5 μL 1% NP-40. Further components were added to give 50 μL of a final reaction mixture containing 100 mM MES, pH 5.5, 1 mM $CoCl_2$, 0.1% NP-40, 0–10 mM water soluble test compounds, and 0–50 μg microsomal proteins. After incubating between 20 and 60 min at 37 °C the tubes were placed on ice before adding 150 μL 10 mM $MgCl_2$ (4 °C), 400 μL MeOH (4 °C) and 600 μL $CHCl_3$. After shaking and centrifugation, two phases were obtained. After removal of organic solvent, radioactivity associated with the lower $CHCl_3$ phase was assayed by scintillation counting (cpm DLO). The upper phase was dried and applied to Dowex 50WX2 (H^+ form) and Dowex 1X2 (acetate form) ion-exchangers in H_2O. Neutral radioactive components were dried and quantitated by scintillation counting. The Dowex 1X2 resin was eluted with 3.0 M formic acid and, after drying, negatively charged material was assayed by scintillation counting (cpm FA). DLODP activity is defined as cpm FA/(cpm DLO + cpm FA). Percent inhibition of DLODP activity is defined as 100 − (100 × DLODP activity$_{+\text{inhibitor}}$/DLODP activity$_{\text{control}}$). Alkaline phosphatase was assayed as previously described. Glc*NAc*(β1,4)Glc*NAc*, Man(β1,4)Glc*NAc* and Gal(β1,4)Glc*NAc* were obtained from Dextra Laboratories (Reading, UK), whereas α-methyl mannoside and β-methyl mannoside were purchased from Toronto Research Chemicals (Toronto, CA).

Cell culture – HepG2 cells (ATCC, Rockville, MD) were cultivated in RPMI 1640 Glutamax™ medium containing 10% fetal calf serum and 1% penicillin/streptomycin. The TC7 clone derived from the colonic adenocarcinoma Caco-2 cell line was a kind gift from Dr. Monique Rousset [29] and was cultivated in RPMI 1640 Glutamax™ medium containing 10% fetal calf serum and 1% penicillin/streptomycin and 1% non essential amino acids. All cells were cultivated at 37 °C under an atmosphere containing 5% CO_2.

Acknowledgments

We gratefully acknowledge la Fondation pour la Recherche Médicale (FRM: DCM20121225751) for the financial support of this work and for a post-doctoral fellowship granted to M.B. This work is supported by the European Union FP6-Coordination Action EUROGLYCANET (LSHM-CT-2005-512131), the E-Rare-2 Joint Transnational Call 2011 (EURO-CDG) and institutional funding from INSERM, CNRS and the Ministère de l'Enseignement Supérieur et de la Recherche. Ahmad Massarweh is supported by a French government/An-Najah National University in Palestine joint doctoral fellowship. Assia Hessani (Université Paris Decartes) is gratefully acknowledged for assistance with low resolution and high resolution mass spectra analyses. The NMR experiments were performed at the Interdisciplinary Center for Chemistry and Biology, Paris.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.10.013.

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