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Synthesis of Dolichyl Phosphate Derivatives with Fluorescent Label at the ω-End of the Chain, New Tools to Study Protein Glycosylation

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Abstract—Derivatives of dolichyl phosphate (Dol-P) with 2-aminopyridine or 1-aminonaphtalene fluorophore groups at the ω-end of the chain were synthesized. These products serve as substrates for recombinant yeast Dol-P-mannose synthase. Fluorescence resonance energy transfer between a Trp residue of the enzyme and the 1-aminonaphtalene group of the Dol-P analogue was demonstrated.

Dolichyl phosphate (Dol-P) is involved as an obligatory participant in the biosynthesis of Asn-linked oligosaccharides on eukaryotic glycoproteins, *O*-glycosylation of fungal glycoproteins and the formation of glycosylphosphatidylinositol anchors present in numerous membrane proteins.[1,2] All these pathways include conversion of Dol-P into glycosylated derivatives followed by transfer of mono- or oligosaccharide residues onto protein molecules. The lipophilic dolichyl moiety of Dol-P and its derivatives is thought to be localized in the interior of the endoplasmic reticulum (ER) membrane bilayer whereas the polar groups are oriented either to the cytosolic face of the ER membrane for initial reactions of Dol-P glycosylation or to the luminal face for the transfer of mono- or oligosaccharides to the acceptors. The exact topology of the glycosyltransferases participating in these processes and the molecular mechanisms of translocation of the lipid-linked sugars through the ER membrane are still not completely clear. The use of fluorescent methodology will help to characterize interactions of amphipathic Dol-P or its glycosylated derivatives with enzymes which utilize these substrates or proteins participating in sugar transport across the membrane. This is the first report on the synthesis of Dol-P analogues with a fluorescent label at the ω-end of the isoprene chain.

Our strategy for the preparation of these derivatives (Scheme 1) was based on the incorporation of a carbonyl group at the ω-end of the dolichol chain followed by reductive amination of the resulting aldehyde with fluorescent amines and phosphorylation of the resulting amino alcohols. The first stages of the synthetic scheme include selective epoxidation of the ω-terminal isoprene unit as first described by van Tamelen et al.[3,4] followed by periodic acid oxidation.[5] This partially modified procedure was successfully employed[6] for preparation of protected hydroxy aldehyde from (*S*)-citronellol in previous work on synthesis of dolichols (for reviews see[7,8]).

In a model synthesis, 2,3-dihydro-6*Z*-farnesyl acetate (**3a**) was used as a starting material. It was prepared from ethyl 2*E*,6*Z*-farnesoate (**1**)[9] by Li/NH_3 reduction (cf.[10]) and acetylation (total yield 56%). Treatment of **3a** with NBS in aqueous THF (1.5 h, rt) followed by reaction with K_2CO_3 in MeOH and *O*-acetylation of partially saponified product gave the epoxide **4a** (50%) which after reaction with periodic acid dihydrate in THF-Et_2O (1 h, rt) was converted into aldehyde **5a** (85%).

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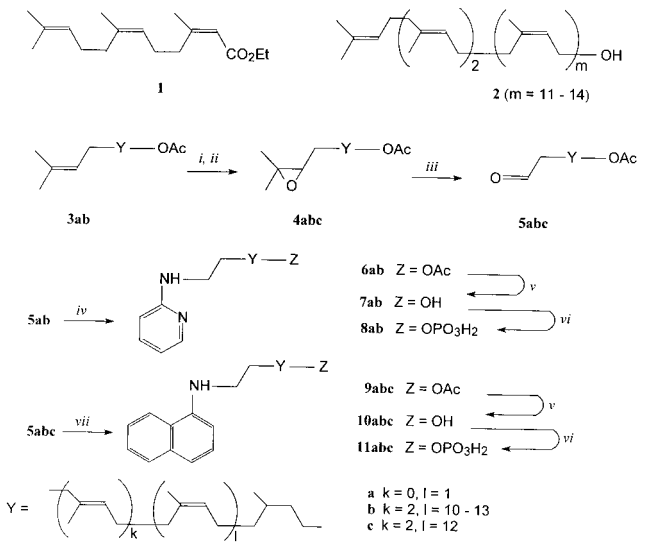

Scheme 1. Reagents: (i) NBS/THF-H_2O; (ii) K_2CO_3/MeOH (series **a**) or K_2CO_3/MeOH-PhH (series **b**), then Ac_2O/Py; (iii) H_5IO_6/THF-Et_2O; (iv) 2-aminopyridine, $NaBH(OAc)_3$/AcOH-$(CH_2Cl)_2$; (v) K_2CO_3/MeOH; (vi) CCl_3CN, $Bu_4N{\cdot}H_2PO_4/CH_2Cl_2$; (vii) 1-aminonaphtalene/Et_2O, then $NaBH_4$/MeOH.

For incorporation of 2-aminopyridine fluorophore, interaction of **5a** and 2-aminopyridine (molar ratio 1:1) was performed in the presence of AcOH and sodium triacetoxyborohydride (2.0 and 1.5 mol per mol of the substrates) in 1,2-dichloroethane (1 h, rt, cf.[11]). After flash chromatography on SiO_2, **6a** (72%) was isolated which after treatment with K_2CO_3/MeOH (2 mol/mol of **6a**, 40 min, rt) produced amino alcohol **7a** (95%).

For reductive amination of **5a** with 1-aminonaphtalene, the best results were obtained using two-step, one-pot procedure. The aldehyde was treated initially with the amine in the presence of molecular sieves 3A (Et_2O, 4 h, rt) followed by reaction with $NaBH_4$ in MeOH (30 min, rt). After purification with flash chromatography, the resulting **9a** was deacetylated (K_2CO_3/MeOH) to give **10a** (total yield of 67% from **5a**).[12]

The same reaction sequence was successfully employed for fluorescent labelling of long-chain dolichols. For these experiments, C_{70}–C_{85} polyprenols from pine needles[13] (**2,** the ratio of components with $m = 11$, 12, 13, and 14 was determined by HPLC as 6:13:14:7) were converted into racemic dolichols through selective reduction of α-terminal isoprene unit with the use of recently described[14] simple procedure (oxidation of polyprenols with MnO_2, followed by reduction of the aldehydes with $Na_2S_2O_4$ and then with $NaBH_4$), and the dolichols were *O*-acetylated to give (±)-**3b**. Selectivity of the ω-terminal epoxidation in **3b** under conditions similar to those used for the short-chain analogue was found to be quite satisfactory.[15] Reaction of **3b** with NBS in THF/H_2O was completed in 3 h at rt. The bromohydrine formed was purified with SiO_2 chromatography prior to treatment (30 min, rt) with K_2CO_3 in benzene:MeOH mixture (2:1) and *O*-acetylation which resulted in **4b** (35% from **3b**). Further conversions **4b**→**5b** (85%), **5b**→**6b** (70%), **6b**→**7b** (96%), **5b**→**9b** (74%), and **9b**→**10b** (97%) were performed essentially under the same conditions as in the **a** series. The mixture of isoprenologues **4b** was effectively separated into components with HPLC (10 μ Silasorb C-8, 240/24 mm column, Pr^{i}OH:MeCN, 1:1 as a solvent, 7 mL/min) and the conversion of the C_{80}-epoxide **4c** into **5c**, **9c** and **10c** was performed.[16]

Phosphorylation with $Bu_4N{\cdot}H_2PO_4/CCl_3CN$[17,18] was used to prepare fluorescent dolichyl phosphate analogues **8a**, **8b**, **11a**, **11b** and **11c** from modified dolichols. In the present series, phosphorylation was found to proceed more slowly and to require higher excess of the reagents than for previously described examples of polyprenols.[8,18] Thus, for the synthesis of **11c** (with CH_2Cl_2 as a solvent) it was necessary to use the ratio alcohol:phosphate:CCl_3CN equal to 1:2.4:2.7, the reaction was completed in 18 h at rt. The standard procedure for the isolation of modified dolichyl phosphates as ammonium salts included distribution of the reaction products between Bu^{n}OH and water, the conversion of the phosphate (present in the organic phase) into NH_4-salt by treatment with a cation exchange resin, its purification by anion exchange chromatography (DE-52, AcO^-, elution with $AcONH_4$ in $CHCl_3$:MeOH 2:1 or MeOH) and removal of salts by extraction of the product from a residue after solvent evaporation with toluene. Using this technique, good yields of the phosphates with spectral properties corresponding to their structure were obtained (Table 1). Particularly, they showed UV-absorption characteristic for the incorporated chromophores and intensive fluorescence with excitation/emission maxima at 315/360 nm for **8ab** and at 340/410 nm for **11bc** (in *n*-heptane:2-propanol, 4:1).

Preliminary results of biochemical and biophysical studies of the fluorescent dolichyl phosphate derivatives revealed their usefulness for investigation of enzyme-substrate interactions. As a model enzyme for these studies, Dol-P-Man synthase (EC 2.4.1.83), an ER membrane glycosyltransferase, was used. The enzyme is known[8] to show rather wide specificity towards structure of the dolichyl phosphate particularly accepting as substrates the derivatives of both (*S*)- and (*R*)-dolichol. Accordingly, the long-chain, racemic phosphates **8b**, **11b** and **11c** were found to serve as efficient substrates for mannosyl transfer catalysed by the highly purified[19] recombinant yeast enzyme from *E. coli* (Table 2).

The reaction with **8a** was not detected under standard assay conditions[20,21] for Dol-P but a radioactive, water-soluble product was identified by paper chromatographic analysis of the incubation mixture as described for phenyl phosphate.[20,21] Chromatographic mobility of the product was slightly lower than that for phenyl phosphate mannose.

For biophysical studies, the DPMΔ3 mutant form[19] of Dol-P-Man synthase was chosen which is fully active in the mannosyl transfer reaction. It differs from the wild type enzyme only by truncation of the very hydrophobic 28 amino acids at the C-terminus and contains only one tryptophan residue (Trp133; the other tryptophan

Table 1. Synthesis and selected spectral properties of ammonium dolichyl phosphate derivatives

	8a	**8b**	**11a**	**11b** [a]	**11c**
Yield, %	58[b]	93[c]	70	48	61
ES-MS data, *m/z* for [M(free acid)-H]$^-$	355	1105, 1173, 1241, 1309	n.d.	1154, 1222, 1290	1290
NMR ^{1}H data[d] ($CDCl_3$), δ	3.93 (m, H1)	3.91 (m, H1)	3.90 (m, H1)	3.91 (m, H1)	3.92 (m, H1)
NMR ^{13}C data[d] ($CDCl_3$), δ	63.3 (br, C1)	63.4 (br, C1)	64.4 (br, C1)	64.6 (br, C1)	64.7 (br, C1)
NMR ^{31}P data ($CDCl_3$), δ	3.06	3.94	1.82	6.66	1.93

[a]Isolated as disodium salt.
[b]After ion exchange chromatography, additional purification was performed with SiO_2 chromatography.
[c]Purification of the product was achieved without the use of ion exchange chromatography by distribution between *n*-octane and MeOH, the phosphate was isolated from the methanolic layer.
[d]Only signals confirming formation of the phosphates (cf. [17,18]) are shown, other signals are close to those described in Notes 12 and 16.

Table 2. Substrate properties of Dol-P and its derivatives for Dol-P-Man synthase[a]

	Dol-P[b]	**8b**	**11b**	**11c**
Apparent K_m (μM)	9.8 ± 0.8	11.6 ± 1.0	14.5 ± 0.8	11.7 ± 0.7
Relative k_{cat}	1.00	1.34	1.18	1.27

[a]See [20,21] for assay procedures.
[b]Porcine liver dolichyl phosphate (C_{80}–C_{105}).

residue, Trp251, of the wild-type enzyme was in its deleted C-terminal part). The use of this mutant enzyme allowed for the simplification of the fluorescent measurements and of the analysis of the spectral data. Clear evidence was obtained for fluorescence resonance energy transfer (FRET) between the single tryptophan residue of the mutant enzyme and naphtylamine chromophore groups in **11b** or **11c**. Measurement of fluorescence spectra of the enzyme-substrate mixtures with excitation at 298 nm showed gradual decrease of the observed donor emission at 350 nm with increase of the modified Dol-P derivative concentration and increase of the acceptor emission at 410 nm upon addition of the enzyme. Using a standard procedure for treatment of FRET data,[22] the distance between Trp133 in DPMΔ3 and naphtylamine residue in **11c** within the enzyme-substrate complex was estimated as 18.6 Å (assuming free rotation of both groups).

In conclusion, the developed procedures for chemical synthesis of fluorescent-labeled Dol-P derivatives result in creation of new tools to study enzymes of dolichol pathway of protein glycosylation and related processes. With the use of wider repertoire of these derivatives, it will be possible to gain essential information on structure of enzyme-substrate complexes of Dol-P-Man synthase and other Dol-P-requiring enzymes.

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12. Satisfactory elemental analysis data were obtained for **4a**, **5a**, **6a**, **7a**, **9a** and **10a**. Selected data for characterization: **7a** EI–MS: *m/z* 276 (M^+, 16%); NMR data (δ, atoms of the Y fragment are underlined): ^{1}H ($CDCl_3$): 0.88 (3H, d, J = 6.4 Hz, *Me*CH), 1.09–1.80 (7H, m, H2, H3, H4, and H9), 1.68 (3H, s, MeC=C), 1.91–2.19 (4H, m, CH_2C=), 3.22 (2H, m, CH_2N), 3.64 (2H, m, CH_2OH), 4.74 (1H, s, NH), 5.16 (1H, t, J = 7.5 Hz, HC=), 6.35 (1H, d, J = 8.6 Hz), 6.54 (1H, m), 7.40 (1H, m), 8.04 (1H, d, J = 5.8 Hz, four signals of pyridine ring CH); ($CDCl_3$): 19.6 (*Me*CH), 23.2 (*Me*C=), 25.2 (C5), 27.6, 28.9, and 29.2 (C3, C8, and C9), 37.2 (C4), 39.6 (C2), 41.9 (CH_2N), 60.6 (CH_2O), 106.2 (Py-C3), 112.5 (Py-C5), 126.1 (HC=), 134.0 (MeC=), 137.6 (Py-C4), 147.8 (Py-C6), 158.8 (Py-C2); **10a** EI–MS: *m/z* 325 (M^+, 41%); NMR data: ^{1}H ($CDCl_3$): 0.83 (3H, d, J = 6.1 Hz, *Me*CH), 1.08–1.60 (5H, m, H2, H3, and H4), 1.78 (3H, s, MeC=C), 1.83–2.30 (6H, m, CH_2C= and H9), 3.28 (2H, t, J = 6.7 Hz, CH_2N), 3.55 (2H, m, CH_2OH), 5.20 (1H, t, J = 7.4 Hz, HC=), 6.59 (1H, d, J = 8.3 Hz, Ar-H2), 7.17–7.84 (6H, m, Ar-H). In addition to the signals of the Y fragment atoms, in NMR ^{1}H spectra of the synthetic intermediates characteristic signals of the end groups are

present: Me of dimethyloxirane ring: 1.14 (3H, s) and 1.21 (3H, s) (for **4a**); HC=O: 9.78 (1H, t, J 1.6 Hz) (for **5a**); CH_2OAc: 4.02–4.07 (2H, t, J = 6.8–7.2 Hz) and MeCO 1.96–2.06 (3H, s) (for **4a**, **5a**, **6a**, **9a**).
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16. The derivatives of the **b** and **c** series were homogenous on TLC and characterized by NMR ^1H and ^{13}C spectra. The chemical shifts and multiplicity of the signals of the end groups are similar to those of the corresponding derivatives of the **a** series (see Note 12). In NMR ^1H spectra the signals of the Y fragment, except for the MeC= signals are present mainly in multiplets as illustrated by the spectrum of **10c** ($CDCl_3$): δ 0.94 (3H, d, J = 6.2 Hz, *Me*CH), 1.10–1.65 (5H, m, H2, H3, and H4), 1.63 (6H, s, Me of *E*-isoprene units), 1.71 (36H, s, Me of *Z*-isoprene units), 1.80–2.30 (58H, m, CH_2C=, H60 and H61), 5.05–5.30 (14H, m, HC=). In NMR ^{13}C spectra strong series of signals of internal *Z*-isoprene units may be easily identified, for **10c**: δ 23.5 (MeC=), 26.4 (CH_2CH=), 32.2 (CH_2C(Me)=), 125.1 (HC=), 135.3 (C(Me)=). The corresponding signals of the *E*-isoprene units are at δ 16.0, 26.7, 39.9, 124.3 and 134.8, those of α-terminal dihydroisoprene unit are at δ 19.5 (*Me*CH), 29.3 (C3), 37.4 (C4), 39.7 (C2), and 61.2 (C1). Some of the main signals of internal isoprene units shown are accompanied by groups of more weak signals due to influence of the stereochemistry of neighboring isoprene units. The characteristic signals of naphtalene fragment in **10c** are at δ 104.2, 117.1, 119.8, 128.7 and 143.6, other signals are close to signals of H*C*= and *C*(Me)= atoms of internal isoprene units.
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